ANALOGUES OF INSULIN-LIKE GROWTH FACTOR-I

A thesis submitted by

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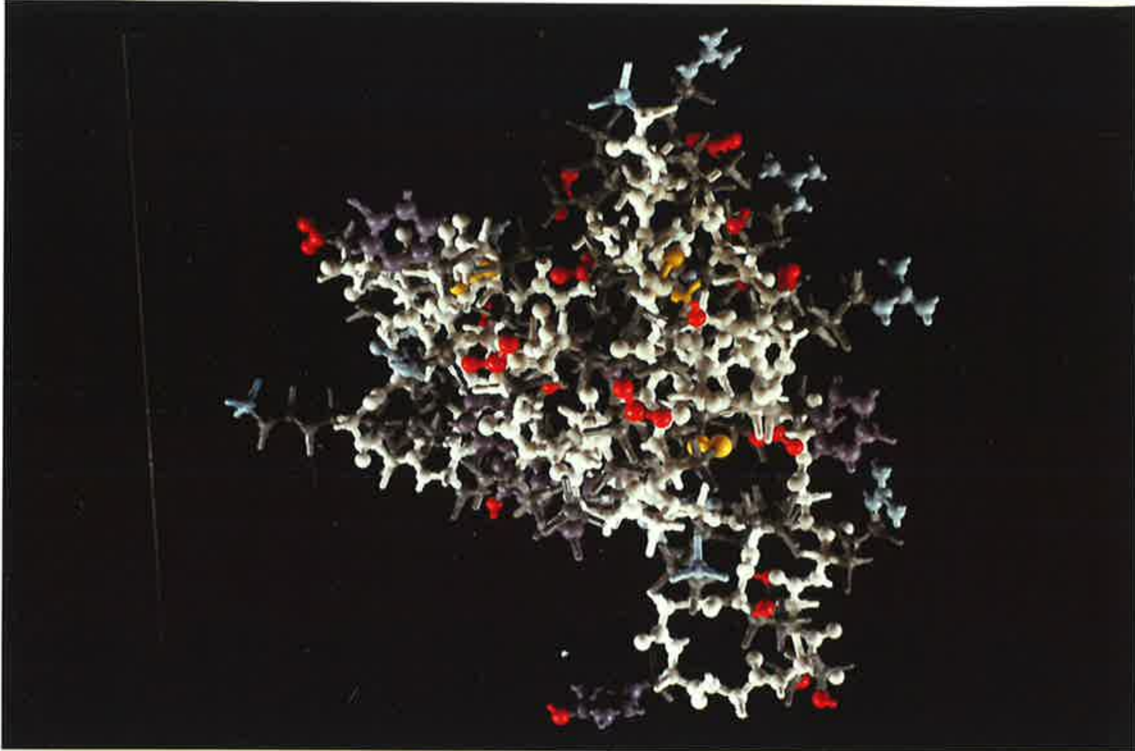
June, 1989

MODEL OF INSULIN-LIKE GROWTH FACTOR-I



A photograph of a model of IGF-I constructed using the coordinates deduced by Blundell *et al.* (1983). The amino terminus of the peptide is near the top of the picture.

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SUMMARY

A highly potent analogue of insulin-like growth factor I (IGF-I) has been previously isolated from bovine colostrum (Francis *et al.*, 1986: *Biochem. J.* **233** 207-213). This peptide (des-(1-3)-IGF-I) was apparently identical to IGF-I except that it lacked the three amino-terminal amino acid residues (Gly-Pro-Glu). The highly purified variant IGF-I had been found to be five- to ten-fold more potent than IGF-I in a bioassay which measured the stimulation of protein synthesis in rat L6 myoblasts.

Direct chemical synthesis of IGF-I and analogues sequentially lacking between one and six residues from the amino terminus, analogues exhibiting small deletions near the amino terminus and analogues bearing substitutions at positions three and four of the IGF-I sequence was performed. Protocols for the assembly of the 70 residue IGF-I peptide by solid phase peptide synthesis and the subsequent formation of the native disulphide bonds of IGF-I were developed. The abilities of the synthetic peptides to stimulate protein synthesis in L6 myoblasts and to compete with radiolabelled IGF-I for binding to a polyclonal anti-IGF-I antiserum, cell-surface receptors and specific IGF-binding proteins were measured.

Chemically synthesized des-(1-3)-IGF-I was found to be as potent as natural des-(1-3)-IGF-I in the L6 bioassay indicating that the enhanced biological activity of des-(1-3)-IGF-I did not result from the presence of contaminants or differences other than the lack of the amino-terminal tripeptide.

Studies using a set of IGF-I analogues with sequential deletions from the amino terminus indicated that the removal of the third residue was required in order to observe increased activity in the bioassay and that removal of further residues did not produce any additional increase in the biological activity. The enhanced potency of des-(1-3)-IGF-I could not be explained by increased affinity for the IGF-I receptor but probably resulted from its inability to bind to IGF-binding proteins present in the assay medium which inhibit the action of IGF-I in this bioassay. In support of this hypothesis, L6 myoblasts were found to produce an IGF-binding protein which bound des-(1-3)-IGF-I sixtyfold less well than IGF-I and could inhibit the binding of radiolabelled IGF-I to these cells. Studies using analogues of IGF-I in which residue Glu^3 had been replaced or deleted confirmed the importance of this residue in mediating the binding of IGF-I to the binding proteins isolated from the medium conditioned by L6 myoblasts or MDBK kidney cells. The reduction in binding of analogues lacking Glu^3

to these binding proteins could be further enhanced by the introduction of a positively charged residue at positions three or four.

Analogues of IGFs possessing disulphide bond rearrangements were produced. The peptides des-(1-3)-[6-47-, 48-52-cystine]IGF-I and [6-47-, 48-52-cystine]IGF-I were found to bind to the IGF-I receptor of L6 myoblasts approximately 100-fold less well than IGF-I. The [6-47-, 48-52-cystine]IGF-I peptide also exhibited very weak binding to the IGF-binding protein produced by the L6 myoblasts while the des-(1-3)-[6-47-, 48-52-cystine]IGF-I showed no detectable binding to this binding protein. These results suggest that the correct folding of the IGF peptide is required for binding to both the IGF-I receptor and IGF-binding proteins.

These studies demonstrate that the increased biological activity of des-(1-3)-IGF-I results from a lack of inhibition by IGF-binding proteins present in the assay medium and that residue Glu^3 is important for the binding of IGF-I to some binding proteins.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge, it contains no material that has been previously published or written by another person except where due reference is made in the text.

Christopher. J. Bagley

ACKNOWLEDGEMENTS

I wish to thank Professor W.H.Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

I would like to thank my supervisor, Dr. John C. Wallace for his advice, encouragement and enthusiasm during the course of this study. I am grateful to Dr. F. John Ballard of the C.S.I.R.O. Division of Human Nutrition for the provision of the facilities of his laboratory and for his advice.

I would like acknowledge the assistance of Mr. Bruce May who synthesized and purified IGF-I peptides. I am also grateful to Ms. Marina Ross for performing many tedious cell culture assays, to Mr. Laszlo Szabo for providing IGF-binding proteins and performing competitive binding assays, to Mr. Peter McNamara and Mr. Michael Conlon for performing radioimmunoassays and Ms. Denise Turner for performing protein sequence analysis. I also wish to thank Dr. Fil Lim and Mr. Grant Booker for assisting with various computing tasks.

I am grateful to Mr. Geoff Francis of the C.S.I.R.O. Division of Human Nutrition who provided me with natural and radioiodinated IGF peptides as well as friendly advice and encouragement. I thank Ms. Tina Southwell, Dr. Ken Otteson, Ms. Sarah McCurdy and Ms. Laura Pierce of Applied Biosystems Inc. for the provision of IGF-I peptides and for their assistance in the task of chemical synthesis.

I thank the past and present members of the Growth Faction and Biotin Biocentre, especially David Mottershead, Laszlo Szabo and Briony Forbes and Grant Booker for their help and the many inhabitants of labs 109, 110 and 112 and the C.S.I.R.O. for their camaraderie.

Finally, I am compelled to acknowledge that there is only one "Motorhead".

ABBREVIATIONS

In addition to those abbreviations accepted for use in *The Biochemical Journal*, the following will be assumed;

Acm:	acetamidomethyl
Boc:	tert-butoxycarbonyl
Bzl:	benzyl
Ches	2-[N-cyclohexylamino]-ethanesulphonic acid
DCC:	N,N′-dicyclohexylcarbodiimide
DCM:	dichloromethane
DIEA:	diisopropylethylamine
DMF:	dimethylformamide
DMSO:	dimethylsulfoxide
Fmoc:	Fluorene-9-methoxycarbonyl
HOBt:	1-hydroxybenzotriazole
HPLC:	high performance liquid chromatography
IGF:	Insulin-like growth factor
Mts:	mesitylene-2-sulphonyl
NMP:	N-methylpyrrolidinone
MeCN:	acetonitrile
Mob:	2-methoxybenzyl
MOPSO:	3-[N-morpholino]-2-hydroxypropanesulphonic acid]
OBzl:	benzyl ester
PAM:	phenylacetamidomethyl
POPSO:	Piperazine-N,N′-bis[2-hydroxypropanesulphonic acid]
Taps:	tris[hydroxymethyl]methylaminopropanesulphonic acid
TFA:	trifluoroacetic acid
TFMSA:	trifluoromethanesulfonic acid.
Tricine:	N-tris[hydroxymethyl]-methylglycine
Z:	benzyloxycarbonyl

CHAPTER 1

INTRODUCTION

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CHAPTER 1: INTRODUCTION

The insulin-like growth factors (IGFs) constitute a group of peptides which are structurally related to insulin. Their major biological role differs from that of insulin in that they cause predominantly a stimulation of growth processes rather than a regulation of fuel homeostasis. In this chapter, I shall review the isolation and structural characterization of the IGFs, the structures and expression of their genes, the interactions between IGFs and other biological molecules and the roles of IGFs in stimulating growth responses *in vitro* and *in vivo*. I shall then describe the background to this research project as well as the approach that I have taken to investigate the biochemical properties of analogues of IGF-I.

1.1 EARLY HISTORY

1.1a Original isolation of the IGFs

Three lines of investigation lead to the discovery of what are now known as insulin-like growth factors. These were the investigation of (i) mediators of growth hormone action, (ii) non-suppressible insulin-like activity and (iii) serum mitogens.

1.1ai Somatomedins

Salmon and Daughaday observed that growth hormone (somatotropin) stimulated the incorporation of $^{35}SO_4$ into the cartilage of rats when injected *in vivo* but not when added to the culture medium *in vitro* (Salmon and Daughaday, 1957). This lead to the formulation of the "Somatomedin hypothesis" in which the sulphation factor was proposed to be produced in the body under the control of growth hormone and to circulate in the blood to the target tissues such as cartilage, thus mediating the effects of growth hormone (Daughaday *et al.*, 1972). These sulphation factors became known as somatomedins (Sm).

1.1aii Non-Suppressible Insulin-Like Activity

While measuring the the insulin-like effect of serum on adipose tissue, Froesch *et al.* (1963) found that a significant amount of the insulin-like activity could not be neutralized by anti-insulin antibodies. The low molecular weight, acid-soluble fraction of this material was termed Non-Suppressible Insulin-Like Activity (soluble) $NSILA_S$.

1.1aiii Multiplication Stimulating Activity

In the course of investigating the serum factors responsible for supporting the growth and division of cells in tissue culture, Pierson and Temin (1972), partially purified a

peptide from calf serum which exhibited mitogenic activity and it was denoted Multiplication Stimulating Activity (MSA).

1.1b IGFs as members of the insulin family of proteins

1.1bi Relationships between the isolated growth factors

That there was a relationship between these various factors was established with the observations that serum MSA and Sm both exhibited NSILA (Dulak & Temin, 1973; Hall & Uthne, 1971), the MSA purified from the conditioned medium of rat liver cells exhibited sulphation activity and that $NSILA_S$ had growth promoting activity (Morell & Froesch, 1973; Zingg & Froesch, 1973).

1.1bii Relationship between IGFs and insulin

Purified forms of $NSILA_S$ (NSILA I and II) were found to have potencies of approximately 2% that of insulin in an insulin bioassay but were 50 to 100 times more potent than insulin in stimulating DNA synthesis in chick embryo fibroblasts (Rinderknecht & Humbel, 1976a). Amino-terminal sequence analysis of these peptides indicated a high degree of structural similarity to insulin (Rinderknecht & Humbel, 1976b) and they were renamed Insulin-like Growth Factors. In 1978, the complete amino-acid sequences of human IGF-I and IGF-II were reported (Rinderknecht & Humbel, 1978a,b). These were found to be single-chain molecules of 70 and 67 amino-acid residues respectively and contained three intramolecular disulphides. Subsequently, somatomedin C was found to be structurally identical to IGF-I (Klapper *et al.*, 1983) and rat MSA was found to be closely related to IGF-II (Marquardt *et al.*, 1981). The three factors which had been characterized on the basis of functional properties were therefore found to be members of the insulin family of proteins.

1.2 STRUCTURE OF THE IGFs

IGFs have been detected in a wide range of mammalian species and in domestic fowl and turtle but were not detectable in frog serum (Zangger *et al.*, 1987). However, Rothstein *et al.* (1980) had previously detected IGF-I-like substances in bull-frog serum. IGFs have been isolated from many species and their amino acid sequences determined.

1.2a Primary Structure

Amino acid sequence data have been obtained for IGFs from man (Rinderknecht & Humbel, 1978a,b), cow (Francis *et al.*, 1986; Honegger and Humbel, 1986; Francis *et al.*, 1988), rat (Marquardt *et al.*, 1981; Rubin *et al.*, 1982), sheep (Francis *et al.*, 1989) and domestic fowl (Dawe *et al.*, 1988). In addition, the primary structures of the IGF-I molecules from mouse and pig have been inferred from the cDNA sequences (Bell *et al.*, 1986; Tavvakol *et al.*, 1988). A summary of these data is presented in table 1.1. The IGFs fall into two categories, IGF-I and IGF-II (Rinderknecht & Humbel, 1976a) which show 60-70% sequence similarity. Both classes of IGF are highly conserved between species.

The IGFs have approximately 40-50% sequence similarity to insulin. This includes conservation of the six cysteine residues found in insulin and thus the intramolecular disulphide bonds. The IGFs differ from insulin in that they consist of a single peptide chain analogous to that of proinsulin. In addition, there is a carboxy-terminal extension of 6 (IGF-II) or 8 (IGF-I) amino-acid residues known as the D-peptide.

1.2b Tertiary structures of the IGFs

Based on the high resolution X-ray crystallographic analysis of insulin, Blundell *et al.* (1983) proposed a model for the three dimensional structures of IGF-I and IGF-II. In these models, the positions of the cysteine residues and highly conserved residues constituting the hydrophobic core of insulin were assumed to occupy the same relative positions in the IGFs. Other conserved residues and conservatively changed residues were placed in positions similar to those of the homologous residues in the insulin model. Non-homologous parts of the structure were assigned on the basis of predictions using the method of Chou and Fasman (1974). The models were further refined by iterative energy minimization calculations. Recent nuclear magnetic resonance spectroscopic studies of IGF-I have largely supported the predictions of the Blundell model (Cooke, 1988). From this model, Blundell *et al.* (1983) found that the IGFs preserved structural elements thought to be required for binding to the insulin receptor in accordance with the observed crossreactivity. The region of insulin thought to be involved in binding anti-insulin antibodies was found to differ considerably in the IGFs accounting for the lack of suppression of their activity in the presence of such antibodies. Furthermore, Blundell *et al.* (1983) predicted that a hydrophobic

surface patch centered on residues Ala^{13}, Phe^{16}, Val^{17} and Leu^{54} along with the surrounding residues residues Glu^{3}, Leu^{5}, Glu^{9}, Gln^{15}, Glu^{58}, Arg^{55} and Asp^{53} might be involved in binding to IGF-binding proteins.

1.2c Variant IGFs

Although the known IGFs fall into the two classes defined by Rinderknecht and Humbel (1976a), several variant types of IGF molecule have been isolated. Yang *et al.* (1985) have characterized a precursor form of IGF-II from the conditioned medium of a rat liver cell-line. Also, a large molecular weight form of IGF-II has been purified from human serum (Gowan *et al.*, 1987). Two variants of IGF-II, containing amino-acid insertions have been identified (Zumstein *et al.*, 1985; Jansen *et al.*, 1985). Of these, one of the insertions occurs at an intron/exon boundary and may be formed as a result of alternative splicing (Jansen *et al.*, 1985). Variants of IGF-I lacking the three amino-terminal amino-acid residues have been purified from bovine colostrum (Francis *et al.*, 1986; Francis *et al.*, 1988), human brain (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986) and porcine uterus (Ogasawara *et al.*, 1989).

1.3 IGF GENE STRUCTURE

1.3a Isolation of IGF cDNA

The sequence of a cDNA encoding IGF-I was first reported by Jansen *et al.* (1983). This showed that IGF-I was synthesized as a precursor of at least 130 amino-acid residues which required proteolytic processing at both ends in order to release the IGF-I. Similarly, the cDNA sequence of human IGF-II indicated that the peptide was derived from a 180 amino acid prepropeptide (Bell *et al.*, 1987). In both cases, the precursor have amino-terminal leader peptides and carboxy-terminal extensions (E-peptides) which must be cleaved in order to release the mature IGF peptide.

1.3b IGF genes

1.3bi Chromosomal localization

The gene encoding IGF-I was localized to human chromosome 12 and that encoding IGF-II was found to be on the short arm of chromosome 11 which also contains the

insulin structural gene (Brissenden *et al.*, 1984; Tricoli *et al.*, 1984). In fact, a promoter for the human IGF-II gene has been identified only 1.4kb from the 3′ end of the insulin gene (de Pagter-Holthuizen *et al.*, 1987).

1.3bii IGF-I gene structure

In both rats and humans, the gene encoding IGF-I has been found to extend over at least 40kb and contain at least five exons (Rotwein *et al.*, 1986; Shimatsu and Rotwein, 1987). *In vitro*, translation has been shown to initiate in the first exon to produce a protein with a 48 residue signal peptide which is co-translationally removed (Rotwein *et al.*, 1987) although initiation of translation of the human mRNA *in vivo* may occur at Met^{-25} or Met^{-22}. In both the human and rat genes, exon 2 encodes part of the leader sequence as well as most of the B-domain while exon 3 encodes the remainder of the mature peptide and the amino-terminal region of the E-peptide. Exons 4 and 5, which encode carboxy-terminal regions of the E-peptide as well containing 3′ untranslated sequences, are introduced into the mRNA by alternative splicing mechanisms (Rotwein, 1986; Bell *et al.*, 1986; Shimatsu & Rotwein, 1987). In the rat, an alternative splicing mechanism apparently exists which introduces one of three exons into the first exon position of the mRNA in a tissue-specific manner (Roberts *et al.*, 1987; Lowe *et al.*, 1987).

1.3biii IGF-II gene structure

The rat IGF-II gene extends over at least 12kbp and contains at least five exons (Frunzio *et al.*, 1986). The human gene has been shown to consist of at least seven exons (De Pagter-Holthuizen *et al.*, 1987). In both species, several types of mRNA have been identified which result from alternative 5′ exons being spliced onto a common 3′ (coding) structure. These mRNAs were apparently transcribed from different promoters and although they were not found to exhibit tissue-specific or age-related regulation in the rat (Frunzio *et al.*, 1986), they were shown to be developmentally regulated in human liver (De Pagter-Holthuizen *et al.*, 1987).

1.4 IGF RECEPTORS

1.4a Biochemical characterization

Kasuga *et al.* (1981) and Massague & Czech (1982) investigated the receptors for the IGFs by chemically cross-linking radioiodinated IGF-I and IGF-II to the cell-surface.

This lead to the identification of two structural subtypes of IGF receptor. Type 1 IGF receptors have an $\alpha2\beta2$ subunit composition maintained by disulphide bonds where the extracellular α subunits (M_r=130k) bind the ligand and the β subunits (M_r=95k) span the cell membrane. This subunit structure is similar to that of the insulin receptor and suggests that the two receptors are homologous. A further similarity is that both the insulin and type 1 IGF receptors possess an intrinsic ligand-stimulated tyrosine-kinase activity (Jacobs *et al.*, 1983). Type 1 IGF receptors typically exhibit a higher affinity for IGF-I than IGF-II and bind insulin weakly (Massague & Czech, 1982). The type 2 receptor consists of a single polypeptide (M_r=250k), which binds IGF-II with considerably greater affinity than IGF-I and does not appreciably bind insulin (Massague & Czech, 1982; Rosenfeld *et al.*, 1987). Human brain exhibits a type 1 receptor with an atypical carbohydrate moiety (Gammeltoft *et al.*, 1985) which has been shown to bind the brain-derived IGF-I analogue (probably des-(1-3)-IGF-I) fivefold better than IGF-I (Sara *et al.*, 1986).

1.4b Amino acid sequence

1.4bi The type 1 receptor

Ullrich *et al.*, (1986) deduced the amino acid sequence of the type 1 receptor from the sequence of its cDNA clone. The type 1 receptor was found to be closely related to the insulin receptor. The presence of a tyrosine kinase domain and the overall primary structures allow the insulin and type IGF receptors to be considered as part of the tyrosine kinase family of receptor and receptor-related proteins (Yarden & Ullrich, 1988). The type 1 IGF receptor is synthesized as a precursor of approximately 1400 amino acid residues which becomes glycosylated and proteolytically processed to produce the mature α and β subunits (Ullrich *et al.*, 1986).

1.4bii The type 2 receptor

The amino acid sequence of the type 2 receptor has been deduced from that of its cDNA clone (Morgan *et al.*, 1987). The type 2 receptor is predicted to consist of approximately 2400 amino acid residues and shows no sequence similarity to the type 1 IGF receptor or to the insulin receptor (Morgan *et al.*, 1987). The majority of the extracellular domain consists of 15 repeat sequences containing a conserved pattern of cysteine and hydrophobic residues. A segment of 43 amino acid residues in the extracellular domain is

similar to the collagen-binding domain of fibronectin and most intriguingly, the whole receptor was found to have a sequence virtually identical to that of the bovine cation-independent mannose-6-phosphate receptor (Lobel *et al.*, 1987). Moreover, the purified human type 2 IGF receptor was found to bind antibodies to the mannose-6-phosphate receptor (Roth *et al.*, 1987). Mannose-6-phosphate was found to stimulate two-fold the binding of IGF-II to the receptor and this effect had the same specificity and affinity as the binding of mannose-6-phosphate to its receptor. The functional significance of this relationship is not presently known.

1.4c Mechanisms of receptor signalling

Attempts to understand the signalling mechanism of the type 1 IGF receptor have focussed on phenomena such as receptor aggregation and phosphorylation events.

Evidence that signal transduction by the type 1 IGF receptor may involve receptor aggregation has come from the observation that mouse antibodies (or F_{ab} fragments of them) which bind to the type 1 IGF receptor of human skin fibroblasts without stimulating amino acid transport are able to do so if anti-mouse IgG is subsequently added (Ikari *et al.*,1988). Also, Feltz *et al.* (1988) found that $\alpha\beta$ heterodimeric fragments of the type 1 IGF receptor, produced by reduction of the class 1 disulphide bonds, were able to bind IGF-I with a similar affinity to that of the intact receptor but were not stimulated to undergo autophosphorylation. Thus, binding of ligand *per se* is insufficient to activate an $\alpha\beta$ heterodimer.

In studies of the insulin receptor, substitution of a specific lysine residue in the kinase domain was found to abolish insulin-stimulated autophosphorylation (Chou *et al.*, 1987). When expressed in an appropriate cell-line, normal but not mutated receptors were able to elicit typical insulin responses on binding insulin. Like the insulin receptor, the type 1 IGF receptor possesses a ligand-stimulated protein kinase which phosphorylates tyrosine residues (Jacobs *et al.*, 1983; Catanese *et al.*, 1986). By analogy with the insulin receptor, it is thought that the tyrosine kinase activity of the type 1 IGF receptor is likely to be important for its biological activity. In this regard, both insulin and IGF-I have been shown to stimulate the phosphorylation of several, possibly the same, intracellular proteins (Madoff *et al.*, 1988). In the KB epidermoid carcinoma cell-line, both the insulin and type IGF 1 receptors were

shown to cause rapid membrane ruffling upon addition of low concentrations of insulin and IGF-I respectively (Kadowaki *et al.*, 1986). These effects could be blocked by addition of antibodies directed against the appropriate receptors. This effect may be the result of specific phosphorylation of components of the cytoskeleton. Recently, Farese *et al.* (1988) detected a phosphatidylinositol-glycan which is rapidly hydrolyzed in the presence of insulin, IGF-I or EGF suggesting that there may be a common mechanism, involving a phospholipase, by which several of the receptors of the tyrosine kinase family regulate cell metabolism or growth.

The type 2 IGF receptor does not possess a tyrosine kinase domain and, until recently, was not known to exhibit a discrete biological function. In a human hepatoma cell-line (HepG2), IGF-II has been shown to stimulate glycogen synthesis in the presence of antibodies that inhibit cellular responses mediated by the type 1 IGF of insulin receptors (Hari *et al.*, 1987; see section 1.7). The effect of IGF-II could be mimicked by anti-receptor antibodies or even F_{ab} fragments of them suggesting that receptor aggregation was not required. Similarly, Kojima *et al.* (1988) found that anti-(type 2 receptor) antibodies stimulated calcium influx and DNA synthesis in fibroblasts that had been primed with epidermal growth factor. The type 2 receptor may act by stimulating phospholipase C since low concentrations of IGF-II have been shown to stimulate the formation of inositol-trisphosphate and diacylglycerol in kidney proximal tubule basolateral membranes (Rogers & Hammermann, 1988). The liberated diacylglycerol could then activate protein kinase C and thus effect phosphorylation of cellular proteins and activation of the Na^+/H^+ antiport.

1.5 IGF-BINDING PROTEINS

The term IGF-binding protein refers to a class of proteins which specifically bind IGFs and does not usually include cell-surface receptors for the IGFs. Specific IGF-binding proteins have been observed in serum (Zapf *et al.*, 1975; Hintz & Liu, 1977), amniotic fluid (Chochinov *et al.*, 1977), lymph (Moses *et al.*, 1979), cerebrospinal fluid (Binoux *et al.*, 1982) and cell-conditioned medium (Moses *et al.*, 1979).

1.5a Structure of the IGF-binding proteins

Recently, the amino acid sequences of various binding proteins have been inferred from the sequences of their corresponding cDNA clones. Thus the binding proteins known as placental protein 12, human amniotic fluid binding protein, the small serum binding protein (BP-28) and that from HepG2 (hepatoma) cell-conditioned medium are apparently identical (Brewer *et al.*, 1988; Julkunen *et al.*, 1988; Brinkman *et al.*, 1988 & Lee, Y.-L. *et al.*, 1988). For reasons of clarity, I shall refer to this binding protein as IBP-1. The amino acid sequence of IBP-1 is shown in table 1.2. Furthermore, Brinkman *et al.* (1988) showed that the IBP-1 structural gene was present as a single copy in the genome located on chromosome 7. The cDNA for a second binding protein has been cloned, sequenced and expressed (Wood *et al.*, 1988). The protein encoded by this cDNA corresponds to the acid-stable subunit of the growth-hormone-dependent serum binding protein (BP-53) which I have denoted IBP-2 in table 1.2. Protein sequence data from the binding proteins produced by BRL-3A (rat liver) cells (Mottola *et al.*, 1986) and MDBK (bovine kidney) cells (Szabo *et al.*, 1988) suggest that these proteins constitute a third class of binding proteins, IBP-3. This interpretation is supported by studies on the specificity of ligand-binding of human IBP-1 and IBP-2 and the MDBK cell binding protein (Forbes *et al.*, 1988). Recently, the sequence of the BRL-3A protein has been inferred from the cDNA sequence (Brown *et al.*, 1989). As shown in table 1.2, the three classes of binding proteins exhibit significant sequence similarity with conservation of the 18 cysteine residues. No sequence similarity of these binding proteins to the type 1 or type 2 IGF receptors has been observed.

1.5b Binding proteins in serum

The existence of IGF-binding proteins in serum had been inferred from the heterogeneous distribution of $NSILA_S$ or Sm when subjected to gel-permeation chromatography under neutral conditions compared with the recovery of a single low molecular weight peak of activity when the chromatography was performed under acid conditions. Kaufmann *et al.* (1975) found that the major circulating form of IGF in serum has a M_r equal to 150k with a minor part of the IGF activity circulating with an apparent molecular weight of 30k-40k. The large circulating form of IGF was demonstrated to be a complex between small IGF molecules and large specific IGF-binding proteins (Zapf *et al.*,

1975; Hintz & Liu, 1977). This complex apparently consists of one molecule each of IGF, an acid-stable IGF-binding subunit and an acid-labile subunit which does not directly bind IGF (Furlanetto, 1980). All components of this complex are apparently under the control of growth hormone suggesting that there may be a coordinate role of both IGF and binding proteins in mediating the effects of growth hormone (Furlanetto, 1980).

Acid gel-filtration chromatography has identified IGF-binding proteins of 53kDa and 28kDa (Martin & Baxter, 1986). The 53kDa protein, which represents the acid-stable subunit of the 150kDa complex, has been called IBP-2. The small binding protein, which had been found to be immunologically related to the major binding protein from human amniotic fluid (Povoa *et al.*, 1984), has been subsequently described as IBP-1 (Drop *et al.*, 1988). In contrast to IBP-2, IBP-1 is not saturated with IGF *in vivo* and is not under growth hormone control (Furlanetto, 1980). In addition, serum concentrations of this small binding protein have been found to exhibit a marked diurnal rhythm which is unrelated to plasma growth hormone levels (Baxter & Cowell, 1987) . Further studies indicated that IBP-1 levels declined after food intake, an effect which could be mimicked by oral glucose administration whereas administration of insulin caused an increase in serum IBP-1 levels. Nevertheless IBP-1 accounts for only a small proportion of the IGF-binding capacity of serum (Cotterill *et al.*, 1988; Yeoh *et al.*, 1988).

Recent studies have detected the amino acid sequence of the small binding protein in a preparation of the 150kDa complex (Enberg, 1986) and a 200kDa complex has been shown to be immunologically related to the amniotic fluid binding protein (Lee, P.D.K. *et al.*, 1988). These data support the hypothesis of Wilkins & D'Ercole (1985) that the large complex may represent an oligomer of small binding proteins and IGFs. In addition, Kiess *et al.* (1987a) have detected the type 2 IGF receptor in rat serum. Perhaps, several IGF/binding protein complexes coexist in serum.

1.5c Binding proteins from non-serum tissues

Other *in vivo* sources of binding proteins are separated from the blood by barriers which may be impermeable to some components of serum. These tissues may therefore exhibit only a subset of the binding proteins found in serum but may also contain binding proteins synthesized at sites other than the liver.

Amniotic fluid has been shown to contain two binding proteins, the larger of which is related to IBP-2 from serum (Baxter *et al.*, 1987). The smaller and predominant binding protein (IBP-1) is identical to the small binding protein from serum (Drop *et al.*, 1988). The site of synthesis appears to be the decidualized endometrium (Bell *et al.*, 1988) where it may bind IGF released by the placental trophoblast cells and function to inhibit the invasion of the endometrium by these cells and thus restrict the growth of the placenta.

Lymph appears to contain a subset of the binding proteins present in serum lacking only the 150kDa complex presumably because it is too large to cross the capillary barrier (Binoux & Hossenlopp, 1988).

1.5d The role of binding proteins *in vivo*

Measurements of the level of IGF-I in human serum, after separation from binding proteins, give approximate values of 200ng/ml and 650ng/ml for IGF-I and IGF-II respectively (Zapf *et al.*, 1981; Bennett *et al.*, 1983), concentrations which would be expected to be maximally-stimulating for many target tissues. Thus, the identification of the 150kDa complex as the major circulating form of IGF-I suggested that serum binding proteins may act as a reservoir for IGFs (Kaufmann *et al.*, 1975). In this capacity, and as shown by Zapf *et al.* (1979), binding proteins were considered to be inhibitors of IGF action. Specific mechanisms by which IGFs may be released at sites of action are yet to be elucidated, although it is speculated that the small binding proteins may have a role in this process since they seem to be able to diffuse from the blood into the interstitial fluid whereas the 150kDa complex does not (Binoux & Hossenlopp, 1988). Thus the small binding proteins may be able to present IGFs to the target tissues. In this respect, the report of an IGF-binding protein potentiating the action of IGF-I has proved interesting (Elgin *et al.*, 1987). The mechanisms by which binding proteins may be able to inhibit or potentiate the action of IGFs are complex and will be discussed later.

1.6 STRUCTURE FUNCTION STUDIES OF IGFs

The IGFs are interesting molecules from the point of understanding the relationship between protein structure and function. They bind to the type 1 and type 2 IGF receptors and also to the insulin receptor. In addition, they bind to a variety of specific IGF-

binding proteins. However, insulin is unable to bind to any of the known IGF-binding proteins or to the type 2 IGF receptor. This suggests that the IGFs possess a number of distinct but possibly overlapping sites for interaction with these various receptors and other IGF-binding proteins.

Early attempts to identify the various sites within the IGF molecules responsible for interaction with receptors and binding proteins were hampered by (i) the lack of naturally occurring variants of IGF exhibiting altered biological properties, (ii) the difficulties of the direct chemical synthetic approach for a molecule the size of an IGF and (iii), the lack of an appropriate IGF gene expression system for the production of mutant IGF molecules.

The approach that was therefore taken was to use the insulin structure as a framework on which to attach various features of the IGF molecule and observe the insulin-like and IGF-like activities of these hybrids. In many cases, such hybrid molecules showed lower activity than insulin in both insulin and IGF assays, thus limiting the usefulness of such an approach. Some success was achieved in broadly defining the regions of IGF molecules which interact with their receptors and binding proteins. The structures of some of these insulin/IGF-I hybrid molecules are shown in figure 1.1.

King *et al.* (1982) found that a hybrid molecule in which the A-chain of insulin had been extended at the carboxy-terminus by the IGF-I D-region octapeptide was 2.8-fold more potent than insulin in the stimulation of thymidine incorporation into fibroblasts whereas the control A^{21}-asparaginimid insulin was only 10% as potent as insulin in this assay. The insulin receptor binding activity was reduced fourfold by the addition of this octapeptide. In a similar study, in which the carboxy-terminal hexapeptide of IGF-II was fused to insulin, the analogue did not exhibit increased growth promoting activity (Ogawa *et al.*, 1984). Further examination of this peptide indicated that it had no ability to bind to type 2 IGF receptors (De Vroede *et al.*, 1985). Hybrid molecules containing the IGF-I A- and D-domains linked to the insulin B-chain, were found to be fivefold more potent than insulin in the stimulation of thymidine incorporation into fibroblasts and exhibited a threefold enhanced reactivity at the type 1 receptor (Tseng *et al.*, 1987). This molecule was unable to bind to any of the IGF-binding proteins tested.

Hybrid molecules containing the 30 residue B-domain of IGF-I linked to the A-chain of insulin, were found to bind to crude preparations of IGF-binding proteins from a

number of sources but did not exhibit any greater mitogenic activity than insulin (De Vroede *et al.*, 1985).

The general conclusions from these studies is that the A and D domains of IGF-I are involved in binding to the type 1 receptor and that the B domain is involved in interactions with specific IGF-binding proteins. The residues involved in binding of IGFs to the type 2 receptor were not identified. The observation that a number of derivatives of insulin showed dramatic decreases in insulin-like activity without a corresponding increase in IGF activity (King *et al.*, 1982; De Vroede *et al.*, 1986) called into question the assumption that the 2 chain insulin structure was a suitable framework on which to build analogues of IGFs. It is likely that subtle differences occur in the receptor-binding regions due to the effects of residues not directly involved or due to the absence of the connecting peptide of the IGFs. In this regard, it is surprising that the experiment of making a two chain IGF lacking only the C-peptide has not been reported. If this molecule had strong IGF-like properties, the systematic investigation of the structural requirements for interaction with receptors and binding proteins using a chemical synthetic approach would have been feasible. In fact, as will be discussed later, production of variant IGFs via recombinant DNA techniques is likely to supersede chemical synthesis as a method for elucidating regions of the IGF molecules of biological significance.

1.7 BIOLOGICAL EFECTS OF IGFs

Many cells possess receptors for IGFs and for insulin. In considering the biological function of these growth factors, consideration must be given to the cross-reactivity of these peptides with heterologous receptors since, as will become apparent, insulin and IGF-II can both produce mitogenic responses via the type 1 IGF receptor and IGF-I and IGF-II can produce insulin-like responses via the insulin receptor.

The classical non-insulin-like effects of IGFs are the proliferative and anabolic responses such as stimulation of the synthesis of DNA and protein and the inhibition of protein degradation. These effects have been observed in many cell types including fibroblasts and chondrocytes (Zapf *et al.*, 1978), hepatocytes and osteoblasts (Ballard *et al.*, 1980), muscle cells (Janeczko & Etlinger, 1984; Ballard *et al.*, 1986), erythroid progenitor cells (Kurtz *et al.*, 1982) and glomerular mesangial cells (Conti *et al.*, 1988). Both IGF-I and

IGF-II cause a stimulation of thymidine incorporation and uptake of non-metabolizable amino acid analogues into cells, an effect which can be inhibited by antibodies directed against the type 1 IGF receptor (Shimizu *et al.*, 1986; Conover *et al.*, 1986, 1987; Nakanishi *et al.*, 1988). This suggests that the type 1 receptor plays a major role in mediating the biological responses to both IGFs.

In the human hepatoma cell-line HepG2, IGF-I, IGF-II and insulin are able to stimulate glycogen synthesis. The effect of low concentrations of insulin but not that of the IGFs is inhibited by the presence of anti-(insulin receptor) antibodies (Verspohl *et al.*, 1984). The inclusion of an anti-(type 1 receptor) antibody inhibited the stimulation of glycogen synthesis and amino acid uptake caused by IGF-I (Verspohl *et al.*, 1988). These results argue for a control of metabolism, to produce insulin-like effects, by IGF-I acting through the type 1 receptor in this cell-line.

IGF-I is able to promote differentiated function in a number of cell types. Low concentrations of IGF-I or high concentrations of insulin cause 3T3-L1 preadipocytes to differentiate into adipocytes suggesting that the activation of the type 1 receptor is important in this process (Smith *et al.*, 1988). In rat ovarian granulosa cells, low concentrations of IGF-I were found to cause an increase in progesterone synthesis (Adashi *et al.*,1984a). This effect was potentiated by coincubation with follicle stimulating hormone (Adashi *et al.*, 1984b). In cultured rat calvaria cells, prolonged incubation with IGF-I caused a significant increase in alkaline phosphatase activity which is a marker for the differentiated, osteoblast state (Schmid *et al.*, 1984). In contrast, platelet-derived growth factor (PDGF) caused an apparent dedifferentiation concomittant with an increase in cell number.

The biological role of circulating IGF-II has proved elusive since IGF-II has been shown to act via the type 1 receptor in many cell types including myoblasts (Kiess *et al.*, 1987b), myotubes (Shimizu *et al.*, 1986) and fibroblasts (Conover *et al.*, 1987,1988). In the H-35 (rat hepatoma) cell line, IGF-II apparently acts via the insulin receptor (Krett *et al.*, 1987). Using a cell-line deficient in type 1 IGF receptors (K562), Tally *et al.* (1987) demonstrated that low concentrations of IGF-II stimulated cell proliferation. Quantitatively, this effect was related to the concentration of type 2 IGF receptors in different clones of this cell-line. In a human hepatoma cell-line (HepG2), low concentrations of IGF-II have been

shown to stimulate glycogen synthesis (Hari *et al.*, 1987), an effect which appeared to be mediated by the type 2 IGF receptor.

In order to produce long-term effects, growth factors would be expected to alter gene expression in target cells. Zumstein & Stiles (1987) found that IGF-I regulated the levels of approximately 30 genes in Balb/c 3T3 cells. One group of such genes was apparently regulated at the level of transcription and responded more to IGF-I than to PDGF while the other group was found to be partly regulated at the level of mRNA stability and responded similarly to IGF-I and to PDGF and may be due to the tyrosine kinase function which is shared by the PDGF and type 1 IGF receptors (Yarden & Ullrich, 1988). In the rat L6 myoblast cell-line, IGF-I has been shown to cause an increase in the abundance of c-*fos* mRNA suggesting that this proto-oncogene may be involved in processes leading to proliferation or differentiation (Ong *et al.*, 1987). It is likely that the induction of c-*fos* expression occurs via the type 1 IGF receptor since high concentrations of insulin also caused a small effect.

The effects of IGFs are thus the stimulation of growth and maturation in bone, skeletal muscle and kidney, regulation of specific differentiation processes in adipocytes and granulosa cells and classical insulin-like effects on tissues such as the liver. These processes are primarily mediated by the type 1 IGF receptor. Both IGF-I and IGF-II appear to exert the majority of their biological effects via the type 1 IGF receptor.

1.8 THE SOMATOMEDIN HYPOTHESIS

1.8a The somatomedin hypothesis

The somatomedin hypothesis states that the physiological effects of pituitary growth hormone (somatotropin), particularly its growth promoting activities, are mediated by IGF-I (Daughaday *et al.*, 1972). Growth hormone is thought to act primarily by stimulating the liver to produce IGF-I which is released into the blood and carried to the classical target tissues such as bone, cartilage and muscle. IGF-I thus mediates the actions of growth hormone. Although possessing somatomedin-like properties, IGF-II synthesis does not appear to be directly regulated by growth hormone and thus it is not a somatomedin (Zapf *et al.*, 1981).

1.8b Autocrine or paracrine mechanisms of IGF action

Although the liver has been demonstrated to be the major site of IGF-I production (Schwander *et al.*, 1983) and to respond to growth hormone by increasing transcription of the IGF-I gene (Roberts *et al.*, 1986), other tissues have been shown to produce IGF-I mRNA which does not respond to growth hormone (Lund *et al.*, 1986; Mathews *et al.*, 1986). This mRNA may be translated to produce physiologically significant concentrations of IGF-I in the tissue. This suggests that some cells may be able to synthesize IGF *in vivo* and thus stimulate their own growth or that of neighbouring cells. These are known as the autocrine or paracrine mechanisms of IGF action. There is some evidence that the local production of IGF-I may be under the control of growth hormone in some tissues (cartilage, bone) but not in others (Schlechter *et al.*, 1986). This problem has proved difficult to resolve since it is not readily possible to distinguish between local production of IGF and sequestration from the blood *in vivo*. In many cases, it is possible that a tissue may respond to both locally produced and circulating IGF-I.

1.9 PRODUCTION OF IGFs *IN VIVO*

Originally detected in serum (Salmon & Daughaday, 1957), IGFs have also been found in amniotic fluid (Merimee *et al.*, 1984), brain (Haselbacher *et al.*, 1984), semen (Baxter *et al.*, 1984), colostrum (Francis *et al.*, 1986), bone (Stracke *et al.*, 1984; Frolik *et al.*, 1988), urine (Hizuka *et al.*, 1987), saliva (Costigan *et al.*, 1988) and in the medium conditioned by cells in tissue culture (Moses *et al.*, 1980).

Liver has been identified as the major site of IGF-I synthesis in the adult, and it is quantitatively able to account for the circulating IGF-I levels (Schwander *et al.*, 1983). IGF-I mRNA has been measured in several rat tissues and, with the exception of liver, has been found to decline to very low levels in the adult compared with the foetus (Lund *et al.*, 1986). IGF-I mRNA was present in much greater abundance in adult compared with foetal liver consistent with the observation that production by the liver was responsible for the increase in circulating IGF-I levels observed after parturition. Growth hormone has been shown to regulate production of IGF-I by the liver (Clemmons & Van Wyk, 1984) by causing increased abundance of IGF-I mRNA (Norstedt & Palmiter, 1984) apparently as a result of increased IGF-I gene transcription (Roberts *et al.*, 1986). Mathews *et al.* (1986) examined

IGF-I mRNA production by a number of adult rat tissues but found only liver to respond to growth hormone.

IGF-II mRNA has been measured in a large number of rat tissues with high levels apparent in the foetus decreasing to low or undetectable levels in the adult (Brown *et al.*, 1986). Accordingly, Moses *et al.* (1980) found a high level of IGF-II in foetal serum which declined soon after birth. This has suggested that IGF-II may be an important growth factor for foetal development. However, in man, the levels of IGF-II in the foetus are low relative to adult values and increase with gestational age. The site(s) of production of IGF-II in adult man have not been clearly defined although the brain is probably able to synthesize IGF-II since IGF-II has been detected in the brain (Haselbacher *et al.*, 1985) and IGF-II mRNA is abundant in the brain (Rotwein *et al.*, 1988). The source of serum IGF-II is not known since the adult liver shows only low expression of the IGF-II gene (Soares *et al.*, 1985; de Pagter-Holthuizen *et al.*, 1987). Circulating levels appear to be partly under the control of growth hormone since serum IGF-II levels are only threefold lower than control values in growth hormone deficient humans and are not elevated in acromegalics (Zapf *et al.*, 1981).

1.10 *IN VIVO* STUDIES OF IGF ACTION

1.10a Relationship between growth and circulating IGF

There are several genetically growth-deficient experimental animals (Beamer & Eicher, 1976; van Buul & van den Brande, 1978; Blair *et al.*, 1988) as well as growth-retarded states in man (Laron *et al.*, 1971; Merimee *et al.*, 1981). In many cases these result from a deficiency in growth hormone production or in the ability of the liver to produce IGF-I in response to growth hormone. In dogs, the size of the adult has been shown to correlate with circulating IGF-I levels (Eigenmann, 1984; Eigenmann *et al.*, 1988). Similar conclusions have been reached in studies of children of constitutionally variant stature which nevertheless do produce growth hormone (Binoux & Gourmelen, 1987). In the case of short children with low growth hormone levels, growth could be increased by growth hormone treatment and the increase in growth rate correlated with the measured increase in levels of IGF-I and IGF-II in the first ten days of treatment (Albertsson-Wikland & Hall, 1987).

1.10b *In vivo* administration of IGF

The short term effects of administration of IGF-I and IGF-II have been investigated by bolus injection. Zapf *et al.* (1986) found that infusion of either IGF-I or IGF-II caused a number of insulin-like effects such as the rapid induction of hypoglycaemia and stimulation of glycogen synthesis. Guler *et al.* (1987) found that IGF-I exhibited only 6% the potency of insulin for induction of hypoglycaemia suggesting that it may act via binding to the insulin receptor. It is likely that these acute effects result from the use of large amounts of IGF which exceed the binding capacity of binding proteins in the serum leading to supraphysiological concentrations of free IGF.

In order to measure a long term effect such as growth, it is necessary to provide chronic administration of the growth factor to a growth deficient animal. Suitable experimental animals are the genetically growth hormone-deficient mice (Snell and *lit/lit*) or hypophysectomized animals. Growth factors are usually administered by osmotic minipump or frequent sub-cutaneous injection. Early work using impure IGF preparations, containing both IGF-I and IGF-II, showed that injection into Snell mice caused an increase in body weight gain compared with saline-injected animals (van Buul-Offers *et al.*, 1979). In addition, these workers found that cartilage isolated from treated animals showed enhanced sulphate incorporation *in vitro*. Schoenle *et al.* (1982) showed that purified IGF-I stimulated the growth of hypophysectomized rats in a dose-dependent manner to an extent similar to that which could be caused by growth hormone. Similarly, biosynthetic IGF-I was found to stimulate both longitudinal growth and weight gain of Snell mice (van Buul-Offers *et al.*, 1986). Analysis of the increases in the weights of various organs showed that they were increased in a manner similar to that induced by growth hormone. The similarity between the effects of administration of either growth hormone or IGF-I lends further support to the somatomedin hypothesis.

Specific infusion of IGF-I or growth hormone into a single hindlimb of rats caused a stimulation of the growth of the epiphyseal plate in that limb but not in the contralateral limb (Schlechter *et al.*, 1986). The effect of growth hormone was abolished by coinfusion of anti-IGF-I antiserum suggesting that the stimulation of growth by growth hormone was mediated by local production of IGF-I. This is an elegant *in vivo* demonstration of the autocrine/paracrine mechanism of IGF action. A similar suggestion that IGFs are

involved in an autocrine or paracrine stimulation of muscle growth came from the studies of Turner *et al.* (1988) who implanted a growth hormone-secreting cell-line into adult rats and demonstrated a significant increase in the abundance of mRNAs for IGF-I and IGF-II in skeletal and cardiac muscle.

The potential use of IGFs for wound healing has been investigated by Lynch *et al.* (1987) who found that the combination of IGF-I and PDGF caused a significant increase in the amount of new connective tissue and epidermis at the site of the wound. IGF-I alone had no effect and PDGF alone caused only a small increase the width of the epidermis.

1.10c Transgenic animals

The first report of transgenic animals expressing an exogenous growth hormone gene was that of Palmiter *et al.* (1982). These mice grew to approximately twice the body weight of their control non-transgenic littermates. The increased growth rate of transgenic mice was not observed until three weeks postpartum, shortly after circulating IGF-I levels had started to increase above those in control mice (Mathews *et al.*, 1988a). This suggested that a major part of the growth response might be due to increased IGF-I production by these animals. However, mice bearing an IGF-I transgene did not exhibit as great an increase in body weight gain as those bearing growth hormone trangenes and showed a delayed onset of the increase in growth rate (Mathews *et al.*, 1988b).

The chronic elevation of the level of circulating growth hormone in mice expressing a growth hormone transgene results in pathogenic changes in various organs such as the liver and kidney, an effect that is not seen in similar mice expressing an IGF-I transgene (Doi *et al.*, 1988; Quaife *et al.*, 1989).

1.11 SYNTHETIC IGF PEPTIDES

1.11a Chemical synthesis of IGFs

Chemical synthesis of peptides is useful in order to verify that the measured activity is an intrinsic property of the protein that has been isolated and sequenced. In the case of IGF research, the heterogeneity of some preparations of IGF-I and IGF-II and the difficulty in effecting complete separation between the two IGFs has made synthesis especially useful.

In 1983, the complete synthesis of human IGF-I was reported (Li *et al.*, 1983). Biochemical comparison with natural IGF-I indicated that the two IGFs were equipotent in a radioimmunoassay and a radioreceptor assay and had similar potencies in a DNA synthesis bioassay (Van Wyk *et al.*, 1984). Further characterization showed that the synthetic peptide was able to compete for IGF-I binding to a preparation of binding proteins with approximately 60% the potency of natural IGF-I (Schalch *et al.*, 1984), a potency similar to that of an IGF-I analogue produced by recombinant DNA methods. The synthetic IGF-I, and recombinant [Thr^{59}]IGF-I, exhibited a much lower affinity than several preparations of natural IGF-I for binding to type 2 receptors (Rosenfeld *et al.*,1987). This suggested that even highly purified preparations of IGF-I contain material possessing IGF-II-like properties despite there being no IGF-II detectable by a C-peptide radioimmunoassay.

IGF-II has also been chemically synthesized (Yamashiro & Li, 1985; Li *et al.*, 1985) and this has allowed investigation of the biological role of IGF-II (Tally *et al.*, 1987).

1.11b Synthesis of IGFs by recombinant DNA methods

In 1985, Peters *et al.* reported the synthesis of an analogue of IGF-I by expression of a synthetic gene in *Escherichia coli.* In order to facilitate cleavage of the IGF from a leader peptide by the use of cyanogen bromide, Met^{59} was replaced by Thr. [Thr^{59}]IGF-I was found to be 80% as potent as natural IGF-I by both radioimmunoassay and radioreceptor assay and was mitogenic for Balb/c 3T3 cells. The production of IGF-I in *Escherichia coli* has also been reported by Nilsson *et al.* (1985) and Buell *et al.* (1985).

In order to improve the production of biosynthetic IGF-I, Moks *et al.* (1987) created a fusion protein containing the IgG-binding domain of staphylococcal protein A which allowed affinity purification of the fusion protein prior to cleavage at the $Asn^{-1}Gly^{1}$ peptide bond by hydroxylamine. Similarly, Saito *et al.* (1987) synthesized a fusion protein which made the IGF peptide less susceptible to host-derived proteases presumably by rendering it insoluble.

One of the difficulties of using *Escherichia coli* as a host for the biosynthesis of IGFs has been the need to chemically treat the the IGF to release it from its fusion partner and to promote formation of the correct intramolecular disulphide bonds. This problem may be avoided by the use of a secretion system. Bayne *et al.* (1988) created a synthetic gene

which, when expressed in yeast, directed the synthesis of a fusion protein which was secreted and processed to remove the leader peptide. The secreted IGF-I had the native disulphide-bonded structure. This research group has similarly produced Ala-IGF-I as a secreted protein in mouse L cells (Bayne *et al.*, 1987).

IGF-II has also been produced by recombinant DNA means (Furman *et al.*, 1987). The IGF-II peptide was synthesized as a fusion protein and cleaved from its fusion partner using cyanogen bromide.

In all cases, the biosynthetic IGFs were full agonists of natural IGFs and exhibited 80-100% the potency of the natural growth factors in biological assays.

1.12 INTRODUCTION TO THE PROJECT

Research within the group of Dr. F.J.Ballard (C.S.I.R.O. Division of Human Nutrition) initially focussed on the factors in milk which might be important for growth and maturation of the neonate and which might also serve to correct catabolic states in the clinical situation. In addition to epidermal growth factor and insulin, human milk was found to contain significant concentrations of other mitogenic components. In collaboration with the group of Dr. J.C.Wallace (this department), IGFs were identified as the major growth-promoting components of bovine colostrum. Routine use of a biological assay (the stimulation of protein synthesis in rat L6 myoblasts) allowed the detection and purification of IGF-I, IGF-II and a variant form of IGF-I (Francis *et al.*, 1986,1988). This variant lacked the amino-terminal three amino acid residues of IGF-I (Gly-Pro-Glu) and, most significantly, exhibited a 5- to 10-fold greater potency than IGF-I in the bioassay. The novel protein (des-(1-3)-IGF-I), its clinical and other uses were patented and the two groups have collaborated on the determination of the molecular basis for the enhanced potency of the variant molecule and on the identification of alternative, highly potent analogues of IGF-I. In collaboration with Dr. L.C.Read (Department of Animal Sciences, University of Adelaide), this work has been extended to include *in vivo* measurements of the efficacy of IGF-I and des-(1-3)-IGF-I as growth promotants.

The approach I took to investigate the molecular basis for the enhanced potency of des-(1-3)-IGF-I was to synthesize this and other analogues of IGF-I. Solid phase peptide synthesis was chosen as the method of production of IGF peptides because small amounts of

many N-terminally variant peptides could be conveniently made at the same time. Since synthesis proceeds from the carboxy terminus to the amino terminus of the peptide, samples of peptide with the same carboxy-terminal moiety could be removed and separately converted into the desired analogues. Additionally, at the time of commencement of this project, a suitable system for production of IGF-I, des-(1-3)-IGF-I and other analogues by recombinant DNA methods was not functional in this department. The chemically-synthesized analogues produced could then be compared with the natural IGF-I and des-(1-3)-IGF-I peptides in the L6 myoblast protein synthesis bioassay, IGF-I and IGF-II radioreceptor assays and in an IGF-I radioimmunoassay. The abilities of these peptides to bind to IGF-binding proteins which may be inhibitors of IGF action could also be measured.

In order to confirm that the enhanced potency of des-(1-3)-IGF-I was solely due to the lack of the amino-terminal tripeptide, I firstly produced synthetic IGF-I and des-(1-3)-IGF-I peptides and characterized them biochemically. I then systematically investigated the effects of truncation at the amino terminus by producing and characterizing a family of IGF-I peptides lacking between 1 and 6 residues from the amino terminus. Following this, I examined in greater detail the roles of residues three and four in the biological activity of IGF-I by the production and characterization of analogues exhibiting substitutions or deletions at these positions.

In the course of producing IGF-I peptides, I isolated disulphide-bond isomers which I also characterized with respect to their abilities to bind to IGF-I receptors, binding proteins and to stimulate protein synthesis in L6 myoblasts.

The potential commercial significance of these investigations as well as the knowledge that a protein similar to des-(1-3)-IGF-I had been isolated by other researchers (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986), necessitated a rapid, coordinated approach towards elucidating the *in vitro* and *in vivo* properties of des-(1-3)-IGF-I and other IGF analogues. Thus, the candidate received assistance in the performance of the work reported in this thesis. Mr. B.May performed the chemical synthesis of synthetic IGF-I peptides produced in this department. IGF-I was also synthesized by Dr. K.Otteson and Ms. S.McCurdy in the laboratories of Applied Biosytems Inc.. Ms. M.Ross maintained the cell-culture facility and performed many of the bioassays and radioreceptor assays for the estimation of potency of the IGF peptides reported in chapters 5-7. Mr. P.McNamara and Mr.

M.Conlon performed the radioimmunoassays reported in chapters 6 & 7. Some of the IGF-binding protein competitive binding assays reported in chapters 6 & 7 were performed by Mr. L.Szabo. The candidate performed the oxidation reactions and the purification of synthetic peptides. He performed all the biochemical assays reported in chapters 3, 4 & 8 as well as some of the bioassays and binding protein competitive binding assays reported in chapters 6 & 7.

Table 1.1: Sequences of the insulin-like growth factors

Those residues conserved in IGF-I or IGF-II are boxed and those residues that are also conserved in the insulin family are strippled

IGF-I	1 · 10 · 20 · 30 · 40 · 50 · 60 · 70
Man	G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Cow	G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Cow [des-(1-3)]	- - - T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Pig	G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Sheep	G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K A A K S A
Rat	G P E T L C G A E L V D A L Q F V C G P R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P T K S A
Mouse	G P E T L C G A E L V D A L Q F V C G P R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P T K A A
Domestic fowl	G P E T L C G A E L V D A L Q F V C G D R G F Y F S K P T G Y G S S S R R L X X K G I V D E C C F Q S C D L R R L E M Y C A P I K P X X X X

IGF-II	1 · 10 · 20 · 30 · 40 · 50 · 60 · 67
Man	A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P A - - S R V S R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Pig	A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P A - - S R V N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Cow	A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P S - - S R I N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Sheep	A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P S - - S R I N R R S R - - G I V E E C C F R S C D L A L L E T Y C A A - - P A K S E
Rat 1	A Y R P S E T L C G G E L V D T L Q F V C S D R G F Y F S R P S - - S R A N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Rat 2	A Y R P S E T L C G G E L V D T L Q F V C S D R G F Y F S R P G - - S R A N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Mouse	A Y G P G E T L C G G E L V D T L Q F V C S D R G F Y F S R P S - - S R A N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Domestic fowl	- Y G T A E T L C G G E L V D T L Q F V C G D R G F Y F S R P V - - G R N N R R I N - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Insulin	B1 · B10 · B20 · B30 / A1 · A10 · A20
Man	F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A / G I V E Q C C T S I C S L Y Q L E N Y C N

Table 1.2: Sequences of the IGF-binding proteins

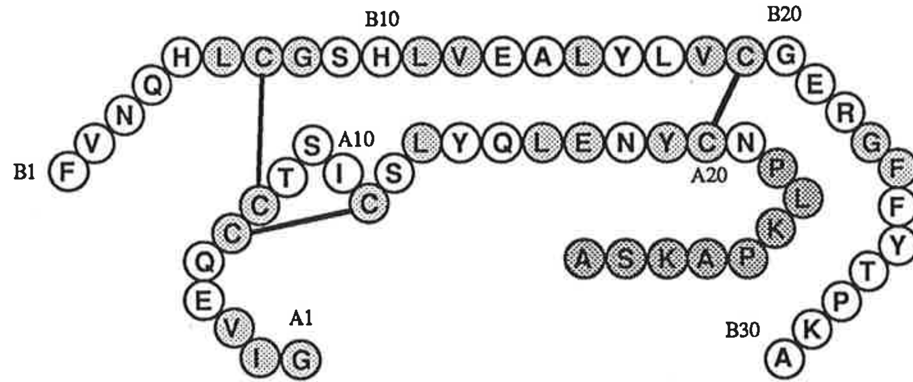
Protein	Sequence
IBP-1 (man)	A P W Q C A P C S A E K L A L C P P V S - -
IBP-2 (man)	G A S S G G L G P V V R C E P C D A R A L A Q C A P P P - -
IBP-3 (rat)	E V L F R C P P C T P E R L A A C G P P P D -
MDBK BP (cow)	E V L F R C P P C T P E S L A A C K P P P D G
IBP-1 (man)	- - - - - - - - - - A S C S E V T R S A G C G C C P M C A L P
IBP-2 (man)	- - - - - - - - - - A V C A E L V R E P G C G C C L T C A L S
IBP-3 (rat)	- - - - - - - - - - A P C A E L V R E P G C G C C S V C A R Q
MDBK BP (cow)	A A A G P A D A R V P C - E L V R E P G C G C C S V C A R L
IBP-1 (man)	L G A A C G V A T A R C A R G L S C R A L P G E Q Q P L H A
IBP-2 (man)	E G Q P C G I Y T E R C G S G L R C Q P S P D E A R P L Q A
IBP-3 (rat)	E G E A C G V Y I P R C A Q T L R C Y P N P G S E L P L K A
MDBK BP (cow)	E
IBP-1 (man)	L T R G Q G A C V Q E S D A S - - - - - - - - - A P H A - - -
IBP-2 (man)	L L D G R G L C V N A S A V S R L R A Y L L P A P P A P G N
IBP-3 (rat)	L V T G A G T C E K R R V G A T P Q Q V A D S E D D H S E G
IBP-1 (man)	A E - - - - - - - - A G S P E S P E S T E I T E E E L L D N F
IBP-2 (man)	A S E S E E D R S A G S V E S P - S V S S T H R V S D P K F
IBP-3 (rat)	G L V E N H V D G T M N M L G G S S A G R K P P K S G M K E
IBP-1 (man)	H L L M A P S - - - E E D H S I L W D A I S - T Y D G S K A
IBP-2 (man)	H P L H S K I I I I K K G H A K D S Q R Y K V D Y E S Q S T
IBP-3 (rat)	L A V F R E K V N E Q K R Q M G K G A K H L S L E E P - - -
IBP-1 (man)	L H V T N I K K W K - - - - - - E P C R I E L Y R V V E S L A
IBP-2 (man)	D T Q N F S S E S K R E T E Y G P C R R E M E D T L N H L K
IBP-3 (rat)	- - - - - - K K L R P P P A R T P C Q Q E L D Q V L E R I S
IBP-1 (man)	K A D E T S G E E I S K F Y L - - - - P N C N K N G F Y H S
IBP-2 (man)	F L N V L S P R G V - - - - - - - - H I P N C D K K G F Y K K
IBP-3 (rat)	T M R L P D D R G P L E H L Y S L H I P N C D K H G L Y N L
IBP-1 (man)	R Q C E T S M D G E A G L C W C V Y P W N G K R I P G S P E
IBP-2 (man)	K Q C R P S K G R K R G F C W C V D K Y G Q P L P G Y T T K
IBP-3 (rat)	K Q C N M S L N G Q R G E C W C V N P N T G K P I Q G A P T
MDBK BP (cow)	C W C V N P N T G K L I Q G A P T
IBP-1 (man)	I R G D P N C Q I Y F N V Q N
IBP-2 (man)	G K E D V H C Y S M Q S K
IBP-3 (rat)	I R G D P E C H L F Y N E Q Q E N D G V H A Q R V Q
MDBK BP (cow)	I R G D P E

Note that the illustrated sequence of the MDBK-cell binding protein differs from that reported by Szabo*et al.*(1988) in positions 13 (Ser) and 50 (Cys).

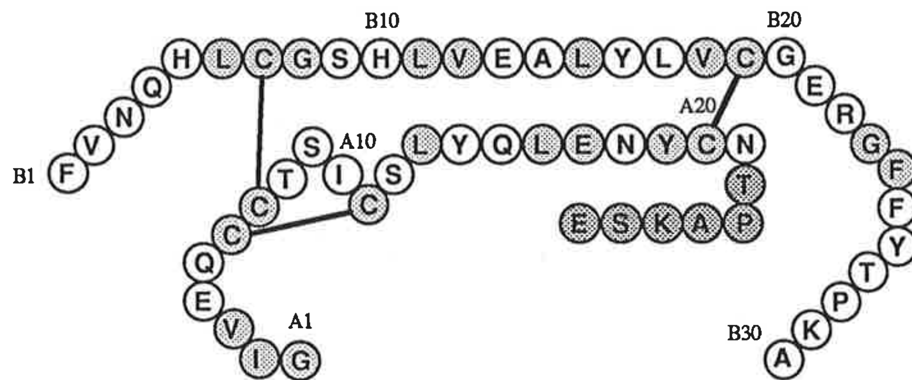
Figure 1.1: Insulin/IGF-I hybrids

Hybrid molecules containing parts of the IGFs (heavily strippled) linked to the insulin framework.Those residues in the insulin moiety which are also found in the IGFs are lightly strippled. Disulphide bonds are indicated by heavy lines. The insulin residues are numbered.

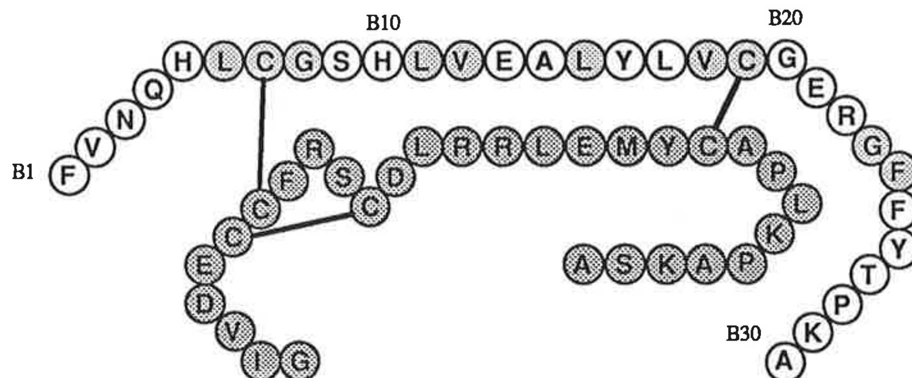
King *et al.*(1982): Insulin extended with the IGF-I D-domain



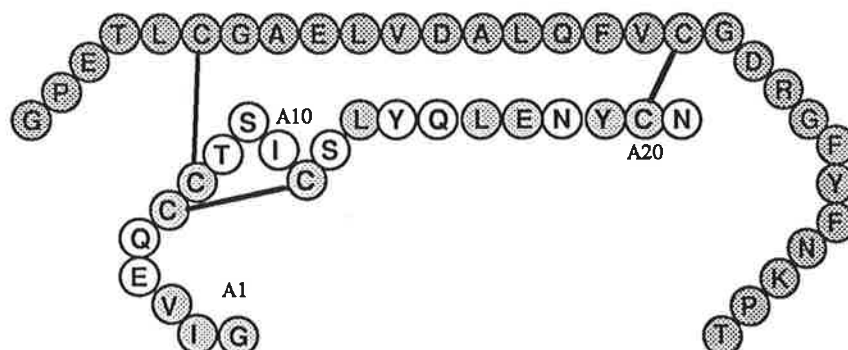
Ogawa *et al.* (1984): Insulin extended with the IGF-II D-domain



Tseng *et al.*(1982): Hybrid containing the IGF-I A- and D-domains



De Vroede *et al.* (1985): Hybrid containing the IGF-I B-domain



CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

The chemical synthesis of the IGFs was carried out on a Model 430A peptide synthesizer (Applied Biosystems Inc., Foster City, CA, U.S.A.). Reagents for chemical synthesis were obtained from Applied Biosystems Inc., Foster City, CA, U.S.A. except Boc-Cys(Acm) which was obtained from Bachem, Bubendorf, Switzerland. HPLC columns were obtained from Waters, Sydney, Australia or Brownlee, Santa Clara, CA, U.S.A.. FPLC apparatus and columns were obtained from Pharmacia Biotechnology Uppsala, Sweden. Acetonitrile, trifluoroacetic acid and heptafluorobutyric acid were of HPLC grade. Water was purified by Milli-Q apparatus (Millipore, Sydney, Australia). Recombinant human IGF-I was generously provided by Drs. H.H.Peters and K.Schiebli, CIBA-GEIGY, Basle, Switzerland. Synthetic IGF-I (ABI-1) and resin-bound IGF-I (ABI-2) were kindly provided by Dr. K.Otteson, Applied Biosystems Inc., Foster City, CA, U.S.A.. Natural IGF-I, des-(1-3)-IGF-I and IGF-II, purified from bovine colostrum by the method of Francis *et al.* (1988) as well as ^{125}I-recombinant human IGF-I and ^{125}I-IGF-II with specific activities of 100-150Ci/g, prepared by the chloramine T method (Van Obberghen-Schilling & Pouyssegur, 1983) were provided by Mr. G.Francis, C.S.I.R.O. Division of Human Nutrition. Recombinant human epidermal growth factor was provided by Dr. C.George-Nasciemento, Chiron Corp., Emeryville, CA, U.S.A.. Bovine serum albumin (RIA grade) and activated charcoal (250-350 mesh) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.. [4,5-^{3}H]leucine (specific activity 40-60Ci/mmol) was obtained from Amersham, Bucks., U.K.. Rat L6 myoblasts were kindly provided by Dr. J.M.Gunn, Texas A&M University, College Station, TX, U.S.A.. Foetal bovine serum was purchased from Flow Laboratories, Stanmore, N.S.W., Australia; amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; Hanks salts were from GIBCO, Grand Island, NY. U.S.A.; gentamycin was from Schering Corp., Kenilworth, NJ, U.S.A.. Other chemicals were of analytical reagent grade.

2.2 CHROMATOGRAPHIC METHODS

2.2a Solvents

Solvents were prepared in Milli-Q water. Solvents prepared from the dissolution

of solids were filtered through a 0.45μm membrane prior to use. Solutions prepared by the mixing of two or more liquids are described as volume/volume unless otherwise stated.

2.2b Reverse-phase HPLC

Analytical and small-scale preparative chromatography were performed on either a Waters high performance liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) or a Kortec high performance liquid chromatograph (*ETP*Kortec, Ermington, N.S.W., Australia). The Waters HPLC system consisted of two model 510 pumps, a dynamic mixer and either a lambda 481 variable wavelength detector or an M490 multiwavelength detector. A model 840 controller was used to form high pressure gradients and to collect data from the detector. Samples were loaded via a Rheodyne model 7125 injector (Rheodyne Inc., Cotati, CA, U.S.A.) but for some experiments a Kortec model K65B autoinjector was used. The Kortec HPLC system consisted of two model K25D pumps, a dynamic mixer, a model K45 controller, a Rheodyne model 7125 injector, a K95 variable wavelength detector and a chart recorder.

Preparative HPLC requiring the use of flow-rates of 5-10ml/min was performed on either a Varian model 5000 liquid chromatograph (Varian Associates Inc., Walnut Creek, CA, U.S.A.) or a Waters Deltaprep high performance liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) which both produce low-pressure gradients. Samples were loaded via the solvent A inlet line or by multiple injections using a 10ml sample loop attached to a Rheodyne model 7125 injector. Absorbance was monitored by a Kortec K95 UV detector (or similar) and recorded by chart recorder.

Elution from reverse-phase columns was usually achieved at linear flow-rates of approximately 6cm/min for all column sizes. The solvents were usually 0.11% TFA (solvent A) and 0.085% TFA in acetonitrile (solvent B) which produced a flat baseline absorbance when a gradient was created between 20% and 50% acetonitrile.

2.2c Estimation of protein amount by reverse-phase HPLC

The concentration of IGF peptide eluting during reverse-phase HPLC was estimated assuming $A^{1\%}_{215}=150$ or $A^{1\%}_{280}=6$ for the Waters M490 u.v. detector. When other detectors were used, they were calibrated by chromatography of a sample of a standard

solution of bovine serum albumin, a sample of which had also been chromatographed using the M490 u.v. detector.

2.3 CHEMICAL METHODS

2.3a Ninhydrin analysis of the resin-peptide

Ninhydrin analysis (Sarin *et al.*, 1981) was performed on the resin-bound peptide after the coupling of each amino acid and removal of the excess activated amino acid. A background absorbance value, estimated from ninhydrin analysis of proline couplings, was subtracted from the experimental data prior to calculation of the percentage of coupling.

2.3b Amino acid sequence analysis

Edman degradation sequence analysis was performed by Ms. D.Turner using a Model 470A protein sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line reverse-phase HPLC system for analysis of the liberated phenylthiohydantoin-amino acids. For quantitation of the amounts of each amino acid derivative liberated at each cycle, the areas of the chromatographic peaks were compared with those of standard phenylthiohydantoin-amino acids chromatographed under identical conditions.

In order to measure quantitatively the efficiency of the coupling of residues, preview sequence analysis was performed (Applied Biosystems, 1985). The principle of this method is that the amount of phenylthiohydantoin-amino acid n+1 present in cycle n of the sequence determination indicates the efficiency of coupling of residue n. For this analysis Edman degradation was performed on fully protected resin-bound peptide. Phenylthiohydantoin derivatives of the protected amino acids were identified by on-line reverse-phase HPLC. The areas of the peaks were estimated by comparison with standard amino acid derivatives and the amount of preview in each cycle was calculated.

2.3c Solid phase synthesis of peptides

Automated peptide synthesis was carried out on a Model 430A peptide synthesizer (Applied Biosystems Inc., Santa Clara, CA, U.S.A.) according to the manufacturer's instructions using the Boc protecting strategy. The side chain protecting groups used were Asp(OBzl), Glu(OBzl), Ser(Bzl), Thr(Bzl), Lys(Cl-Z), Tyr(Br-Z),

Arg(Mts), Met(O) and Cys(Acm) except in synthesis BIO-1 in which Cys(Mob) replaced Cys(Acm). Amino acids were activated as the symmetric anhydride except Asn and Gln which were activated as the HOBt ester. The actual protocols used for each cycle of coupling are given in chapter 4.

Manual synthesis was used to modify some resin-bound peptides in order to produce IGF-I analogues. Boc synthesis was performed using DCC to produce the symmetric anhydride with the coupling performed in DMF. Alternatively, Fmoc amino acids were coupled as the HOBt ester by the method of Bodansky *et al.* (1980).

2.3d Cleavage of the resin-peptide

TFMSA/TFA cleavage of the peptide from the resin was performed as follows. 100mg peptide-resin was dried under vacuum and reactions were carried out under nitrogen at 0°C. Ethanedithiol (50µl) and thioanisole (100µl) were added and stirred for 5min. TFA (1.0ml) was then added and after 10min, TFMSA (100µl) was added and the mixture stirred for a further 40min. The mixture was filtered into ether (30ml) and the resin washed with TFA (3 × 500µl). The precipitated peptide was collected by centrifugation, washed with ether and dried under vacuum. The crude peptide was immediately resuspended in 8M-urea or 6M-guanidine.HCl in the presence of 0.1M-TrisCl pH 8.5 and isolated by preparative reverse-phase HPLC. The purified Acm derivative of IGF-I was then dried using a Speed-vac concentrator (Savant Instruments Inc., Hicksville, NY, U.S.A.). Ninhydrin analysis of the spent resin typically showed that cleavage had proceeded with 80-90% yield.

2.3e Deprotection of Acm-derivative of synthetic IGF-I

Removal of the Acm protecting group from the cysteine residues was performed by the method of Kamber *et al.* (1980) using an extended reaction time. Typically, dried peptide (1mg) was redissolved in 80% acetic acid (74µl) and diluted with an equal volume of 30mM I_2 in 80% acetic acid. After incubation at 23°C for 2 hours, the solution was diluted with water (700µl) and extracted with tetrachloromethane (4 × 500µl). The sample was then dried under vacuum.

2.3f Reduction of peptides

Peptides for reduction were typically dissolved at a protein concentration of 0.1-

1mg/ml in a solution containing 6M-guanidine.HCl, 10mM-DTE and 0.3M-TrisCl at pH 8 and incubated at room temperature for at least 10min. The reduced peptide was then isolated by chromatography on a suitable reverse-phase HPLC column using a gradient of acetonitrile in the presence of approximately 0.1%TFA as described in section 2.2b. Although direct measurement of free thiol groups was not attempted, peptide prepared in this way could be derivatized with iodoacetic acid to give a peptide that eluted from reverse-phase HPLC with a retention time which was not altered by further exposure to DTE or to conditions that lead to disulphide bond formation in reduced IGF peptides. The peak fractions of reduced peptide could then be concentrated or dried under vacuum using a Speed-Vac (Savant Instruments Inc., Hicksville, NY, U.S.A.).

2.4 BIOCHEMICAL ASSAYS

2.4a Radioimmunoassay

Anti-IGF-I antibodies were produced in rabbits, immunized with a bovine IGF-I/ovalbumin conjugate, by Mr. P.McNamara. Radioimmunoassays were carried out at 4°C for 16h in 0.25ml of a solution containing 0.03M-potassium phosphate at a pH value of 7.5, 0.2% protamine sulphate, 0.2% NaN_3 and 0.25% bovine serum albumin, antiserum at a final dilution of 1:20 000, ^{125}I-IGF-I (50pg) and competing peptides. For the precipitation of antibodies, rabbit IgG (0.1µl) and goat anti-rabbit gamma-globulin (1.7µl) were added to each tube. After 30min at 4°C, cold 5% polyethylene glycol (1.5ml) was added and the tubes were immediately centrifuged at 4000g before removal of the supernatants by aspiration.

2.4b Radioreceptor assay

Confluent monolayers of L6 cells in 12 place multiwell dishes were washed twice with Hanks Minimal Essential Medium (1ml) containing 20mM-Hepes and 0.5% bovine serum albumin to remove serum and then incubated for 2h at 23°C in this medium (1ml). Subsequently, 400µl of a solution containing approximately 50pg of ^{125}I-labelled IGF was added to each well, together with unlabelled peptides in a solution containing 0.15M-NaCl, 0.05M-potassium phosphate and 0.5% bovine serum albumin at pH 7.4. The binding reaction was carried out for 18h at 4°C or 2h at 22°C, the cells were then washed four times with

Hanks salts at 0°C. The cell monolayers were dissolved by trituration in 0.5M-NaOH containing 0.1% Triton X-100 for the determination of cell-associated radioactivity.

2.4c Stimulation of protein synthesis in L6 myoblasts

Confluent monolayers of L6 myoblasts in 24-place multiwell dishes were incubated at 37°C for 2h under an atmosphere containing 5% CO_2 in humidified air in 1ml of a 1:1 mixture of Waymouth's MB721/1 and Dulbecco-modified Eagle's Minimal Essential Medium with the leucine concentration adjusted to 0.5mM. The medium in each well was replaced with 900µl of a similar solution but containing 1µCi/ml [4,5-^{3}H] leucine together with 100µl of sample dissolved in 10mM-potassium phosphate, 145mM-NaCl and 0.1% bovine serum albumin at pH 7.4. After incubation as described above for a further 15 to 18h, the monolayer were washed at 0°C, twice with Hanks salts, twice with 5% trichloroacetic acid over a 10min period and once with water before dissolution by trituration in 0.5M-NaOH containing 0.1% Triton X-100 for the determination of cell-associated radioactivity.

2.4d IGF-binding protein competitive binding assay

Binding proteins were purified from the medium conditioned by L6 myoblasts and MDBK cells by Mr. L.Szabo using the method of Szabo *et al.* (1988). Serum-free medium that had been incubated for 16h under the conditions used for the bioassay (section 2.4c) was also used as a source of IGF-binding proteins. For the compeptitive binding assay, ^{125}I-IGF-I (20pg) and unlabelled peptides were diluted into 0.25ml of a 50mM-sodium phosphate buffer containing 0.25% bovine serum albumin at pH 6.5. The assay was initiated by the addition of 50µl of a solution containing binding protein and incubated at room temperature for 1h. The amount of binding protein added was typically able to bind 30- to 40% of the tracer IGF-I and, in the case of the purified protein from MDBK cells, this was approximately 10ng. Unbound ^{125}I-IGF-I was precipitated by addition of 1ml of assay buffer containing 0.5% activated charcoal and 0.02% protamine sulphate at 0°C. After 30min the tubes were centrifuged at 10 000g for 3min and half the supernatant removed for the determination of radioactivity.

CHAPTER 3

OXIDATION AND FOLDING OF IGF PEPTIDES

CHAPTER 3: OXIDATION AND FOLDING OF IGF PEPTIDES

3.1 INTRODUCTION

It is generally accepted that the information required for the correct folding of a protein is contained within the amino acid sequence. Since Levinthal (1968) showed that a folding protein cannot sample all the possible conformations within a reasonable time in order to reach the folded state, it was concluded that folding occurs via specific pathways. The information known about the folding of many proteins falls into two broad classifications. The framework model of protein folding (Kim & Baldwin, 1982) proposes that early in the folding process, discrete units of secondary structure form which are then assembled into the native tertiary structure. The alternative jigsaw puzzle model (Harrison & Durbin, 1985) proposes that protein folding can occur via multiple pathways without formation of any unique structural intermediates. Until recently, experimental detection of intermediates in the folding pathway has been difficult since folding is generally too rapid for investigation by techniques such as n.m.r. and is a highly cooperative process. Some detailed information on folding pathways has been obtained using disulphide bonds as a marker of the folding process since they can be trapped by alkylation and analysed at leisure. The best example of this method is the elegant study on the folding pathway of bovine pancreatic trypsin inhibitor (Creighton, 1977). A more general approach is to observe the rate of exchange of amide protons with those from the medium during the folding process since proton exchange is inhibited by burial within the protein or by participation in hydrogen bonds. Recently, this approach has been adopted by Udgaonar & Baldwin (1988) and Roder *et al.* (1988) who used two dimensional n.m.r. to observe proton incorporation into specific amides of deuterated, refolding proteins.

In the case of the IGFs, little is known concerning the folding and oxidation of the reduced peptide. Obviously, in order to convert a crude synthetic IGF-I peptide into a biologically active molecule, it is necessary purify it and induce it to form the native disulphide bonds. Several techniques have been used to form disulphide bonds in reduced proteins. For example, exposure of the reduced ribonuclease A to atmospheric oxygen has been shown to restore enzymic activity (White, 1960). It is probable that this process was catalyzed by traces of metal ions present in the aqueous medium since Takagi & Isemura (1964) found that the inclusion of chelating agents inhibited the oxidation of Taka-amylase A

and the deliberate inclusion of divalent metal ions such as Cu^{II} caused the rate of recovery of enzymic activity to be increased. An alternative method of generating disulphide bonds is that of Ahmed *et al.* (1975) in which a simple organic disulphide is used to provide the oxidant. Free thiol is also present in the reaction, presumably to permit rearrangement of non-native disulphide bonds and thus encourage the formation of native disulphide bonds which are generally considered to be the most stable. Oxidation of the thiol groups of the protein can occur even in the presence of excess thiol because this process is an intramolecular reaction and consequently thermodynamically favoured by reason of the gain in entropy.

There are a number of considerations in ascertaining the most appropriate conditions for the correct oxidation of a protein such as IGF-I. The protein must be kept soluble and in a monomeric state which may require the use of denaturing conditions. However, the presence of excess denaturant will prevent the peptide from folding to form a structure in which the correct disulphide bonds are most likely to form. Additional variables to be considered are the concentration of peptide, the pH value and temperature of the oxidation reaction as well as the nature and concentrations of the oxidant and the free thiol reagent, if present.

For these studies, I used the limited amount of bovine IGF-I that was available for some initial studies which I report below. I then extended the investigation of the conditions required for optimum refolding of reduced IGF peptides using the best available synthetic IGF-I. This synthetic IGF-I (denoted ABI-1) was a gift from Applied Biosystems Inc. (Foster City, CA).

3.2 MATERIALS AND METHODS

The synthetic IGF-I (ABI-2) was synthesized in the laboratories of Applied Biosystems Inc. This peptide had been cleaved from the resin, fully deprotected, and the cysteine residues had been reduced. The peptide had then been separated from reagents by gel-permeation chromatography and dried. Natural IGF-I (bovine) and reagents were obtained as described in section 2.1. Reverse-phase HPLC was performed as described in section 2.2.

3.3 RESULTS

3.3a Reoxidation of natural IGF-I by Copper ions

I chose to investigate the efficacy of Cu^{II} as a reagent for the oxidation of reduced, natural IGF-I. For this series of experiments, I used concentrations of $CuSO_4$ and reduced peptide similar to those used successfully by Takagi & Isemura (1964) for the oxidation of Taka-amylase A. Bovine IGF-I (8μg) was reduced (section 2.3f), the peptide was then isolated by reverse-phase HPLC (section 2.2b) and dried under vacuum. The peptide was redissolved in 10mM-HCl and samples (2μl) were diluted with 50mM-TrisCl pH8.0 (4μl) and either 0.08mM- or 0.8mM-$CuSO_4$ (5μl). The amounts of Cu^{II} chosen represented 0.5 or 5 molar equivalents with respect to protein thiol groups. After time intervals of 5min or 1h, samples were acidified by addition of glacial acetic acid (2μl) and analysed by reverse-phase HPLC (fig. 3.1). All samples exhibited peptide species which eluted before reduced IGF-I and thus were probably oxidized forms of IGF although no discrete peak of absorbance corresponding to correctly oxidized IGF-I was observed. Fractions were subjected to L6 bioassay and samples from all treatments were found to exhibit some biological activity (fig. 3.1). The most significant amount of bioactivity was obtained after an incubation time of 1h in the presence of 0.8mM $CuSO_4$ in a fraction corresponding in elution time to native IGF-I.

In a further experiment, samples of reduced bovine IGF-I (1.2μg) were prepared as above, dried and redissolved in 0.1M-TrisCl pH 8.0 containing 2M-urea (12μl) and diluted with 50μM-$CuSO_4$ (12μl) in the same buffer. After either 30min or 2.5h, they were analysed by reverse-phase HPLC as shown in figure 3.2. Fractions of eluted peptide were subjected to L6 bioassay as shown. In both chromatograms, a peak corresponding to native IGF-I was observed and peptide corresponding to these peaks exhibited biological activity. The peak of correctly folded IGF was less prominent in the 2.5h sample and represented only a small fraction of the protein eluted from the column. These data suggested that the method of disulphide bond formation using Cu^{II} did not selectively produce correctly oxidized IGF-I and that the yield was inadequate for the purpose of producing analogues of IGF-I for biochemical evaluation.

3.3b Reoxidation of natural and synthetic IGFs by a glutathione redox buffer

The refolding of reduced, natural IGF-I was attempted using the glutathione redox buffer system of Ahmed *et al.* (1975). Reduced bovine IGF-I (2.5μg) was prepared as described in sections 2.3f and 2.2b, dried and redissolved in 8M-urea/0.1M-TrisCl at pH 8.0 (10μl). Two samples (4μl, 1μg) were diluted with 0.1M-TrisCl pH 8.0 (12μl) and then with 4μl of a solution containing 5mM-glutathione, 0.5mM-oxidized glutathione and 2M-urea in the TrisCl buffer. Samples were stood at 25°C for 30- or 140min prior to analysis by reverse-phase HPLC as shown in figure 3.3. In the chromatograms of both reoxidation experiments, two major peaks of peptide were observed, the second of which exhibited an elution time identical to that of native IGF-I and corresponded to the major peak of biological activity in the L6 bioassay. The sample which had been allowed to oxidize for 140min showed a greater degree of oxidation than the 30min sample with approximately 35% of the recovered protein corresponding to correctly oxidized IGF-I. It is therefore apparent that natural IGF-I is able to be reduced and reoxidized in acceptable yield.

A direct comparison of the refolding of natural and synthetic IGFs was then performed. Reduced bovine IGF-I (1.4μg) and des-(1-3)-IGFI (1μg) were prepared as above. Reduced synthetic IGF-I (0.6mg) was chromatographed on a 4.6 × 30mm reverse-phase HPLC column and the absorbance peak that eluted at 34-38%MeCN was collected. The reduced BIO-1 IGF-I (3.6mg) was similarly prepared. Although this material did not exhibit a discrete peak corresponding to reduced authentic IGF-I, the equivalent fractions were taken for oxidation. Samples of synthetic IGF-I (3μg) or natural IGF peptides (1.4 or 1μg) were dried, redissolved at a protein concentration of 100μg/ml in a solution containing 2mM-glutathione, 2M-urea, 1mM-EDTA in 0.1M-TrisCl pH 8.0, stood for 10-min at 25°C and subsequently diluted with an equal volume of a solution containing 0.2mM-oxidized glutathione, 2M-urea, 1mM-EDTA in 0.1M-TrisCl pH 8.0 and stood for a further 16h at 25°C. Samples were analysed by reverse-phase HPLC and fractions were collected and subjected to the L6 bioassay (fig. 3.4). The areas of the peaks corresponding to correctly oxidized IGF-I were estimated and expressed relative to the total area under the chromatogram trace of the peptide. The natural IGF-I peptides gave yields of correctly oxidized peptide approximately equal to 40% whereas the synthetic peptides gave yields of only 8-9% and these IGF-I peptides were not well resolved from other, presumably

incorrectly oxidized peptides. Furthermore, in the case of the natural peptides, the peak of material corresponding to correctly-oxidized IGF exhibited high biological potency (comparable with that of authentic IGF-I) in the L6 bioassay whereas, in the chromatograms of the synthetic peptides, material which corresponded to correctly-oxidized IGF-I exhibited relatively low biological potency. Thus, these preparations of reduced, synthetic IGF-I apparently contain a large fraction of peptide that is unable to oxidize correctly.

Natural IGF-I and des-(1-3)-IGF-I are able to oxidize in good yield although several discrete oxidation products are observed. For the purpose of comparison, I reoxidized reduced recombinant human epidermal growth factor under the same conditions as those used for the IGF-I oxidation (fig. 3.5). In this experiment, virtually complete reoxidation to the native form (containing 3 disulphide bonds) was observed suggesting that the reoxidation conditions *per se* do not necessarily promote the formation of non-native disulphide bonds and therefore heterogeneous oxidation products.

Although the synthetic IGF-I peptides oxidized poorly, some significant correct oxidation could be observed if purified reduced (ABI-1) IGF-I was used as the starting material. At this time, adequate amounts of natural IGF-I with which to optimize the conditions for correctly folding IGF-I were not available so I proceeded to investigate various parameters of the oxidation reaction using the ABI-1 IGF peptide.

3.3c The time course of oxidation of synthetic IGF-I

Reduced ABI-1 IGF-I was chromatographed on a preparative scale and a fraction corresponding to the peak of eluted IGF was taken to give 50μg in a volume of 400μl which was concentrated to 50μl. Samples (2μg) were diluted to a concentration of 0.2mg/ml in a solution of 2mM-glutathione, 2M-urea in 0.1M-TrisCl pH8.0 and oxidation commenced by addition of an equal volume of a solution of 0.2mM-oxidized glutathione, 2M-urea in 0.1M-TrisCl pH8.0. Samples were incubated for 1min, 12min, 1h or 16h prior to acidification and reverse-phase HPLC analysis (fig. 3.6). Partial oxidation of the reduced IGF-I was evident at the earliest time-point with some correctly folded IGF-I apparent at 12min. A significant peak of correctly oxidized IGF-I was evident at 1h which was only slightly increased in the 16h sample.

3.3d The effect of pH value and temperature on oxidation of synthetic IGF-I

Synthetic ABI-1 IGF-I (0.5mg) was reduced as described in section 2.3f and chromatographed on a 4.6 × 30mm reverse-phase HPLC column using a gradient of acetonitrile from 20% to 50% in 15min as described in section 2.2b. A broad profile was observed with a major peak of peptide eluting at 35-37% acetonitrile which was subsequently used for reoxidation studies. Samples equivalent to 2μg of peptide were dried under vacuum, redissolved in 10μl of solutions containing 2mM-glutathione in a buffer of 67mM-TrisCl, 67mM-glycine, 67mM-potassium phosphate, 2M-urea at pH values of 6, 7, 8, 9 or 10 and diluted with an equal volume of a solution containing 0.2mM-oxidized glutathione in the same buffer. One set of samples was incubated at 25°C for 2h and another set of peptides was similarly treated at pH8 and incubated at 37°C, 50°C or 65°C for 2h or 4°C for 4h. After the indicated incubation times, the samples were acidified by addition of glacial acetic acid (2μl) and analysed by reverse-phase HPLC (fig. 3.7). In each chromatogram, the peak corresponding to correctly oxidized IGF-I was identified by comparison with a natural IGF-I standard and its protein content was estimated from the absorbance trace and expressed relative to the total amount of the recovered protein. A sample of reduced synthetic IGF-I which had been redissolved in 10mM-HCl was found to comigrate with reduced natural IGF-I indicating that significant oxidation had not occurred during the drying process (data not shown). The yields of correctly oxidized IGF, assessed as the areas of the absorbance peaks corresponding to natural IGF-I relative to the areas corresponding to the total recovered protein, are given in table 3.1.

Table 3.1: Effect of pH value and temperature on oxidation

CONDITIONS		ANALYSIS	
pH value	temperature (°C)	time (h)	yield of IGF-I (%)
6.0	25	2	0
7.0	25	2	6.0
8.0	25	2	20
9.0	25	2	21
10.0	25	2	21
8.0	4	4	13
8.0	37	2	19
8.0	50	2	11
8.0	65	2	5

In this experiment, the highest yields of correctly oxidized IGF-I (approximately 20%) were obtained using pH8/25°C, pH8/37°C and pH9/25°C and pH10/25°C. The reoxidized IGF-I from the experiment performed at a pH value equal to 10 chromatographed as a doublet suggesting that some undesirable modification of the peptide had occurred under these conditions. The results of this experiment were confirmed in an additional, similar experiment (data not shown).

3.3e The requirement for oxidized and reduced glutathione.

Samples of reduced synthetic IGF-I (2μg) were prepared as above (section 3.3a) and oxidized in the presence of 2M-urea and 0.1M-TrisCl pH8.0 containing the indicated concentrations of glutathione and oxidized glutathione. They were incubated at 22°C for 2h, acidified and analysed by reverse-phase HPLC (fig. 3.8). The yields of correctly oxidized IGF, assessed as the areas of the absorbance peaks corresponding to natural IGF-I relative to the areas corresponding to the total recovered protein, are given in table 3.2.

Table 3.2: Effect of oxidized and reduced glutathione on oxidation

CONDITIONS		ANALYSIS
concn. of glutathione (mM)	concn. of oxidized glutathione (mM)	yield of IGF-I (%)
1	0.05	14
1	0.1	20
1	0.2	20
1	1.0	23
0.2	0.1	17
0.4	0.1	21
2	0.1	19
4	0.1	15

In this experiment, a broad optimum for the concentrations of the thiol and disulphide reagents was observed. When glutathione was present at 1mM, concentrations of oxidized glutathione between 0.1 and 1mM were found to cause a similar extent of correct oxidation (about 20%). When the concentration of oxidized glutathione was held at 0.1mM, concentrations of glutathione between 0.4 and 2mM were found to give approximately 20% correct oxidation. Somewhat poorer oxidation was observed at high ratios of reduced/oxidized glutathione such as 1/0.05 and 4/0.1. Similar results were obtained in a separate experiment using a different preparation of reduced ABI-1 IGF-I as starting material.

3.3f The effects of protein concentration and urea concentration on oxidation of synthetic IGF-I

The ability of reduced IGF-I to oxidize correctly is expected to depend inversely on the urea concentration of the oxidizing solution since the peptide must adopt a native-like structure in order to favour the formation of the native disulphide bonds. Non-native intramolecular and intermolecular bonds may form especially at high peptide concentrations. Preliminary experiments indicated that the reduced IGF peptides showed poor solubility at concentrations of urea below 1 molar. I therefore performed a series of oxidation experiments in the presence of different concentrations of urea and peptide. Samples of reduced synthetic IGF-I (2μg) were oxidized in the presence of 1.0mM-glutathione and 0.1mM-oxidized glutathione and 0.1M-TrisCl pH8.0 at the indicated concentrations of peptide and urea for a period of 2h at 22°C prior to acidification and reverse-phase HPLC analysis (fig. 3.9). The yields of correctly oxidized IGF, assessed as the areas of the absorbance peaks corresponding to natural IGF-I relative to the areas corresponding to the total recovered protein, are given in table 3.3.

Table 3.3: Effect of urea concentration and protein concentration on oxidation

CONDITIONS		ANALYSIS
protein concn. (μg/ml)	urea concn. (M)	yield of IGF-I (%)
25	2	16
50	2	16
100	2	15
50	1	17
50	3	18

Similar percentage yields of correctly oxidized IGF-I were observed under each set of conditions tested. In a further experiment using higher concentrations of urea, an optimum of between 1 and 3 molar was again observed with low yields of correctly oxidized IGF-I at high concentrations of urea (data not shown).

3.4 DISCUSSION

Using reduced natural IGF-I as a starting material, Cu^{II} was not found to be an effective oxidizing agent for forming the native disulphide bonds of IGF-I. This may result from the irreversible formation of incorrect disulphide bonds since no free thiol is added to

the reaction to facilitate disulphide bond rearrangement. By contrast, the glutathione redox buffer system of Ahmed *et al.* (1975) was found to correctly oxidize reduced IGF-I and des-(1-3)-IGF-I with a yield of approximately 40% (fig. 3.4). These peptides were found to exhibit high biological potency in the L6 protein synthesis bioassay as would be expected if they possessed the correct disulphide bonds. A significant peak of oxidized IGF-I was found to elute under reverse-phase HPLC at a slightly lower concentration of acetonitrile than authentic IGF-I (fig. 3.4). This peptide is presumably the same as that observed by Tamura *et al.* (1988) and Raschdorf *et al.* (1988) who identified it as [6-47-, 48-52-cystine]IGF-I. and is denoted isomer 1 in figure 3.4a. The peak of oxidized IGF-I that eluted after the native IGF-I peak in figure 3.4a, denoted isomer 3, is probably [6-52-, 47-48-cystine]IGF-I as observed by Tamura *et al.*. The structures of [6-47-, 48-52-cystine]IGF-I, [6-52-, 47-48-cystine]IGF-I and native IGF-I are shown in figure 3.10. A peptide that is probably [6-47-, 48-52-cystine]IGF-I has been previously observed by Saito *et al.* (1987) in their chromatograms of reoxidized recombinant IGF-I. The ratio of occurrence of these two disulphide-bonded isomers did not appear to be altered significantly by changes in the pH value, urea concentration or temperature of the oxidizing reaction (see figs. 3.7 & 3.9). Recently, Tamura *et al.* (1988) reported that the inclusion of organic solvents in their air oxidation of reduced recombinant IGF-I improved the yield of native IGF-I.

In contrast to the reoxidation of natural IGFs, both the ABI-1 and the BIO-1 preparations of synthetic IGF-I gave low yields of correctly oxidized IGF-I as assessed by reverse-phase HPLC or the L6 myoblast bioassay (fig. 3.4). It is apparent that the low yields of correctly oxidized peptide in preparations of synthetic IGF-I do not result from inappropriate conditions for the oxidation reaction since reduced natural IGF-I reoxidizes well under these conditions. It is unlikely that the reduced natural IGF-I retains any secondary structure since it is prepared using strongly denaturing conditions and is apparently fully reduced (see section 2.3f). Rather, it is probable that some inherent property of the synthetic peptide preparation such as undesired chemical modification or the presence of failure sequence peptides rendered it unable to fold correctly in high yield. One possibility was that cysteine residues were becoming modified either during the chain assembly or the cleavage reactions. This could occur if the 4-methoxybenzyl protecting group were labile under the conditions used for deprotection of Nα*t*-butoxycarbonyl group leading to alkylation

during the synthesis or if alkylation could occur during the trifluoroacetic acid/ trifluoromethanesulphonic acid cleavage reaction in spite of the presence of scavengers. It was not feasible to test directly for the presence of modified cysteine residues since Edman degradation of the peptide or amino acid analysis may not liberate identifiable derivatives of cysteine. In order to prevent undesirable modification of cysteine residues, the acetamidomethyl protecting group was used for cysteine protection in subsequent syntheses. This protecting group is stable under even the strong acid conditions of the cleavage reaction but may be removed oxidatively using molecular iodine (Kamber *et al.*, 1980). As I will describe in the next chapter, IGF-I peptide produced by a second synthesis, using acetamidomethyl protection of cysteine, was able to be oxidized to form disulphide bonds in better yield than those from syntheses ABI-1 or BIO-1.

Experiments using purified reduced (ABI-1) IGF-I indicated that correctly folded IGF-I can be produced under a variety of buffer conditions using the glutathione redox system. A pH optimum for the oxidation reaction was found between values of 8 and 9 (fig. 3.7). The yields of correctly folded IGF-I were reduced at pH values of 6 and 7 and the peptide corresponding to correctly oxidized IGF-I in the reaction at a pH value of 10 chromatographed as a doublet. Urea concentrations between 1- and 3M were found to be suitable for the oxidation reaction and an optimum range for the temperature of the reaction from 25- to 37°C was found (fig. 3.9). For IGF peptides at concentrations up to 0.1mg/ml, concentrations of 0.4- to 2mM-glutathione with 0.1- to 1.0mM-oxidized glutathione were found to be suitable for the oxidation reaction. From these data, I concluded that a suitable protocol for the preparative oxidation of IGF-I and the various analogues that I intended to produce would be as described in table 3.4.

TABLE 3.4

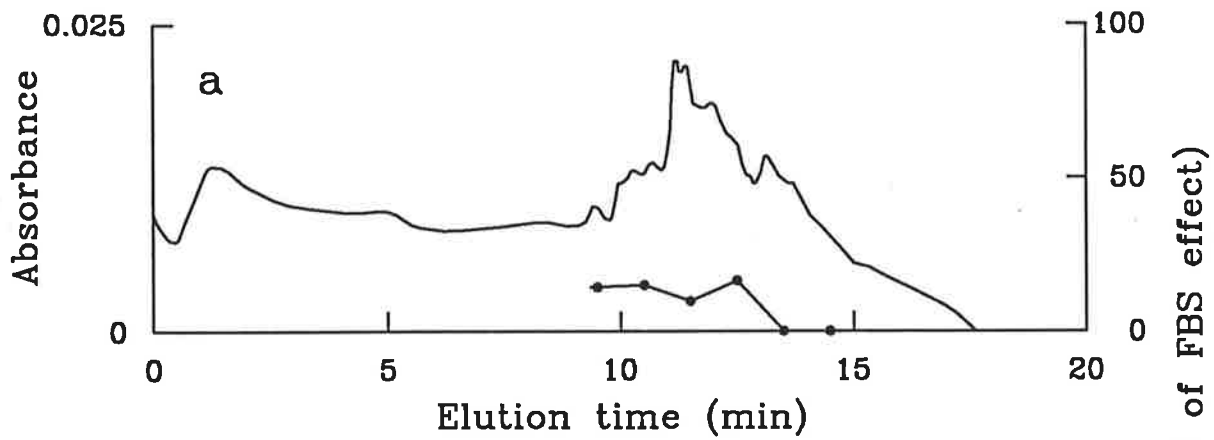
1) Reduced peptide is prepared, isolated by reverse-phase HPLC and dried.
2) Peptide is redissolved at a concentration of 0.4mg/ml in a solution of 4mM-glutathione/8M-urea/0.1M-TrisCl at a pH value of 8.0 or 8.5.
3) The solution is diluted with 3vol. of 0.133mM-oxidized glutathione in 0.1M-TrisCl pH 8.0 (or 8.5) and stood at 25°C overnight.

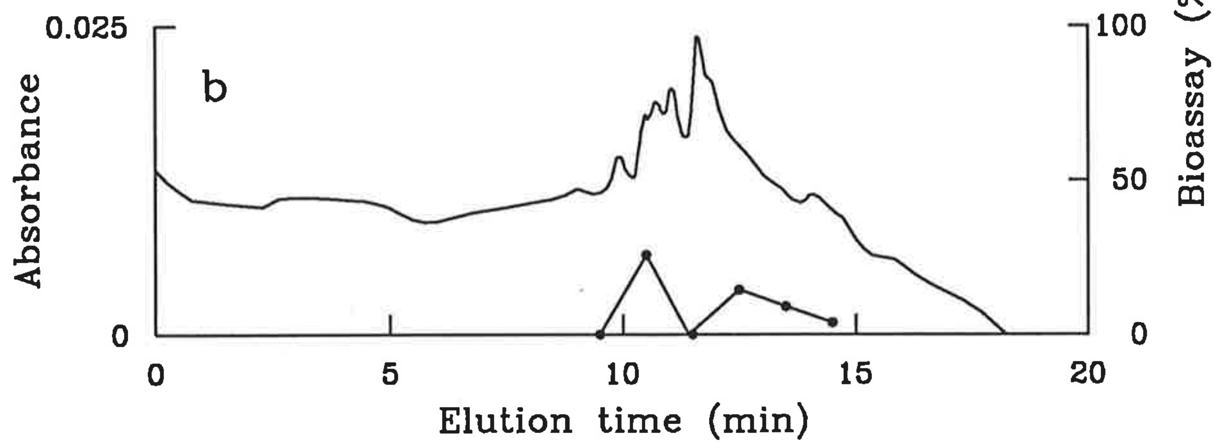
For convenience, and to ensure that equilibrium had been reached, the oxidation reaction was performed overnight for the routine production of peptides.

The formation of two major reoxidation products from a single reduced peptide is unusual. Other growth factors such as transforming growth factor α (Tam *et al.*, 1985) and epidermal growth factor (fig. 3.5) apparently give the native disulphide-bonded structure when reoxidized. It appears that, under the conditions which I have used for the folding of IGF-I, the reduced peptide can exist in two different folded states of similar energy which can be oxidized to produce the two isomers. Using CD, Tamura *et al.* (1988) found that [6-47-, 48-52-cystine]IGF-I exhibited less helix and ß-turn and greater sheet and random structure than IGF-I possessing the native disulphide bonds suggesting that a significant structural difference exists between these isomers. In studies using chemically-synthesized disulphide bond isomers of insulin, Sieber *et al.* (1978) found that, in the presence low concentrations of 2-mercaptoethanol, the isomer equivalent to [6-47-, 48-52-cystine]IGF-I rearranged to form some native insulin but not *vice versa* suggesting that it was less stable than native insulin. It is of interest that the most productive folding/oxidation pathway for reduced bovine pancreatic trypsin inhibitor includes the formation of non-native disulphide bonds which are subsequently broken via intramolecular rearrangement (Creighton, 1977). Perhaps, in the case of IGF-I, a non-native disulphide bond that forms transiently can become fixed by the rapid formation of another non-native bond. This may be promoted in the case of IGF-I if the transition between two different folded states of the molecule is slow (perhaps due to proline isomerization) and if the formation of the second non-native disulphide bond is essentially irreversible. Similarly, a stable isomer of bovine pancreatic trypsin inhibitor has been isolated in which two cysteine residues have been trapped as free thiols by being stably buried in the molecule by the formation of the three other native disulphide bonds (States *et al.*, 1984). There is no evidence of the formation of mis-folded IGFs *in vivo* suggesting that the mode of oxidation in the endoplasmic reticulum encourages the formation of the correct disulphide bonds or that the carboxy-terminal E-peptide may play a role in folding of the IGF-I molecule. Alternatively, mis-folded IGF peptides may be formed *in vivo* but rapidly degraded and thus not readily observed.

Figure 3.1: Reoxidation of natural IGF-I by $CuSO_4$

IGF-I peptides were chromatographed on a 2.1 × 30mm C4 cartridge at a flow rate of 0.5ml/min using a resolving gradient from 20-48%MeCN over 14min. A_{215} was monitored (—) and fractions were collected at time intervals of 1min. 90% of each fraction was subjected to the L6 bioassay (●). Bioassay data are expressed as a percentage of the stimulation observed in the presence of 5% foetal bovine serum (2.3-fold). *Sample a:* reduced IGF incubated with 0.08mM-$CuSO_4$ for 5min. *Sample b:* reduced IGF incubated with 0.8mM-$CuSO_4$ for 5min. *Sample c:* reduced IGF incubated with 0.08mM-$CuSO_4$ for 1h. *Sample d:* reduced IGF incubated with 0.8mM-$CuSO_4$ for 1h.


a
0.025
Absorbance
0
0
5
10
15
20
Elution time (min)
100
50
0
Bioassay (% of FBS effect)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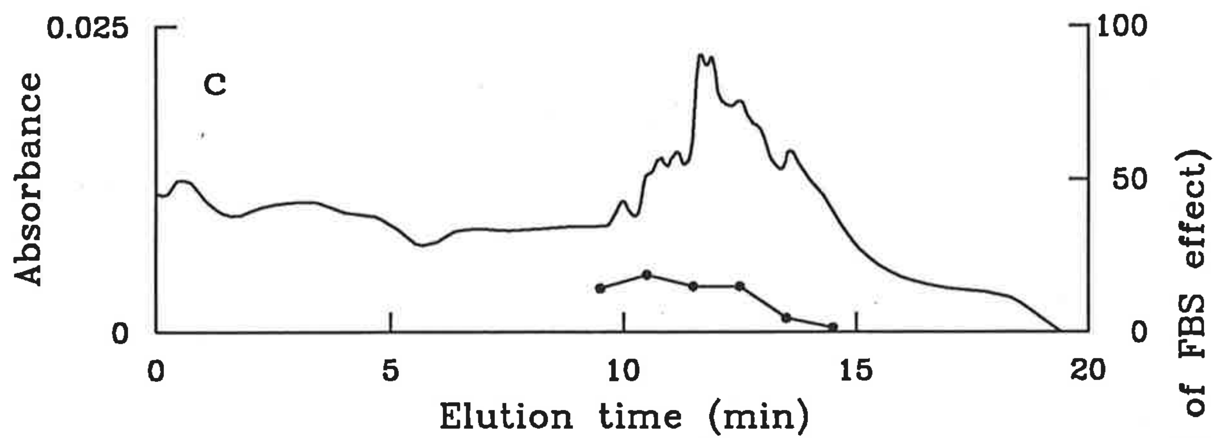
Absorbance
0
0
5
10
15
20
Elution time (min)
100
50
0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10
15
20
Elution time (min)
100
50
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Bioassay (% of FBS effect)

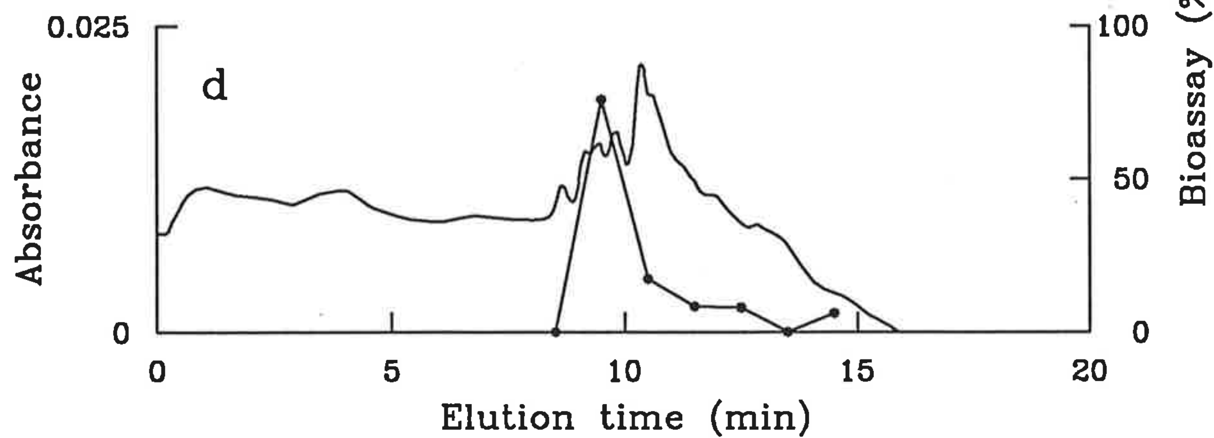
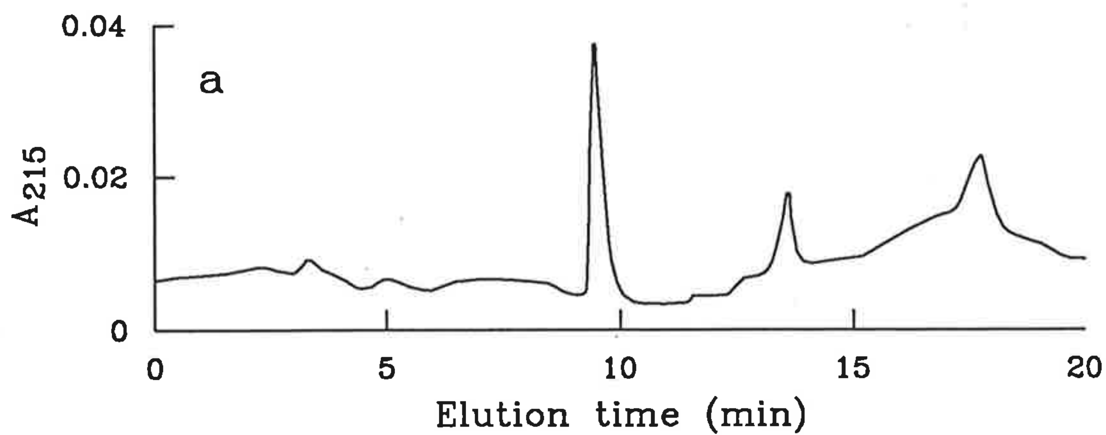
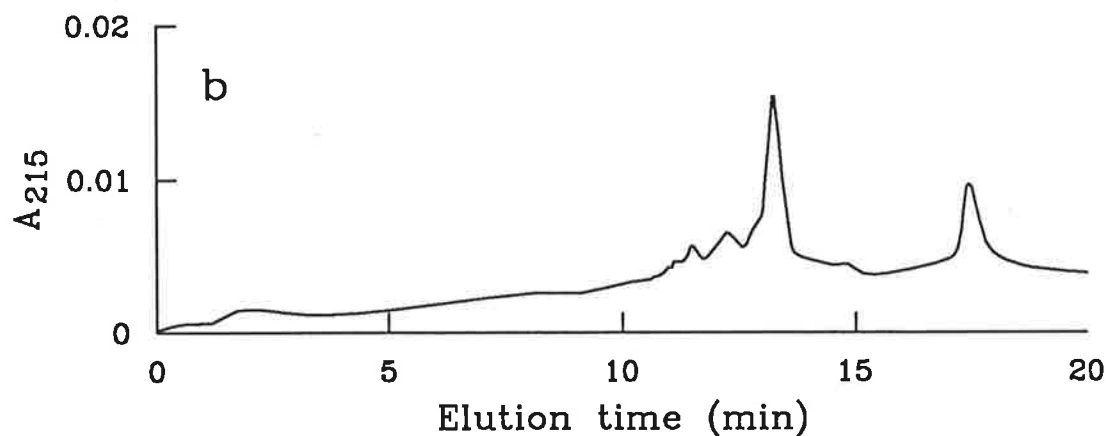

d
0.025
Absorbance
0
0
5
10
15
20
Elution time (min)
100
50
0

Figure 3.2: Time course of reoxidation of natural IGF-I by $CuSO_4$

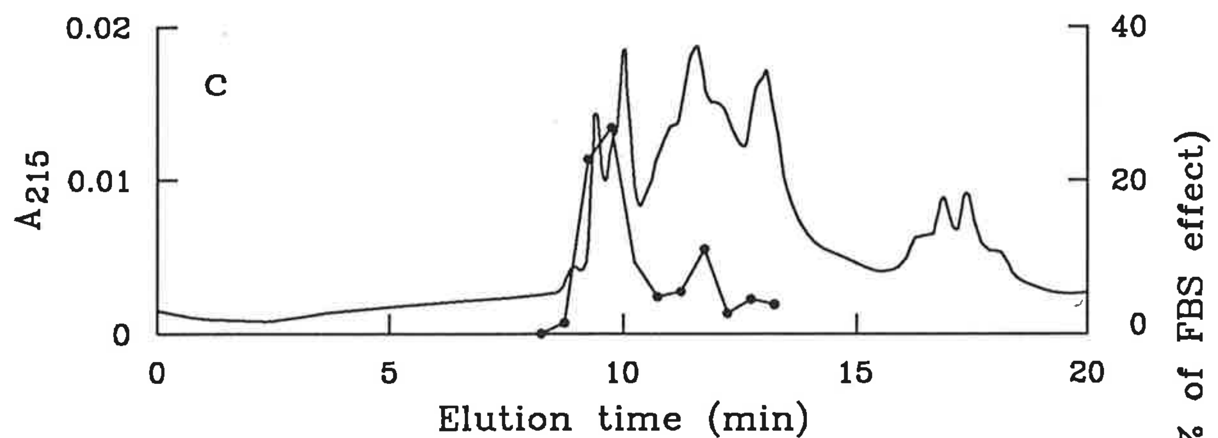
IGF-I peptides were chromatographed on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-50%MeCN over 15min. A_{215} was monitored (—) and fractions were collected at time intervals of 0.5min. Duplicate samples equivalent to 40% of each fraction were subjected to the L6 bioassay (●). Bioassay data are expressed as a percentage of the stimulation observed in the presence of 5% foetal bovine serum. Incorporation of radioactivity was equal to 1.4% in the absence of added growth factor and 5.2% in the presence of 5% foetal bovine serum. *Sample a:* natural IGF-I standard. *Sample b:* reduced IGF-I. *Sample c:* reduced IGF-I incubated with 0.01mM-$CuSO_4$ for 30min. *Sample d:* reduced IGF incubated with 0.01mM-$CuSO_4$ for 2.5h.



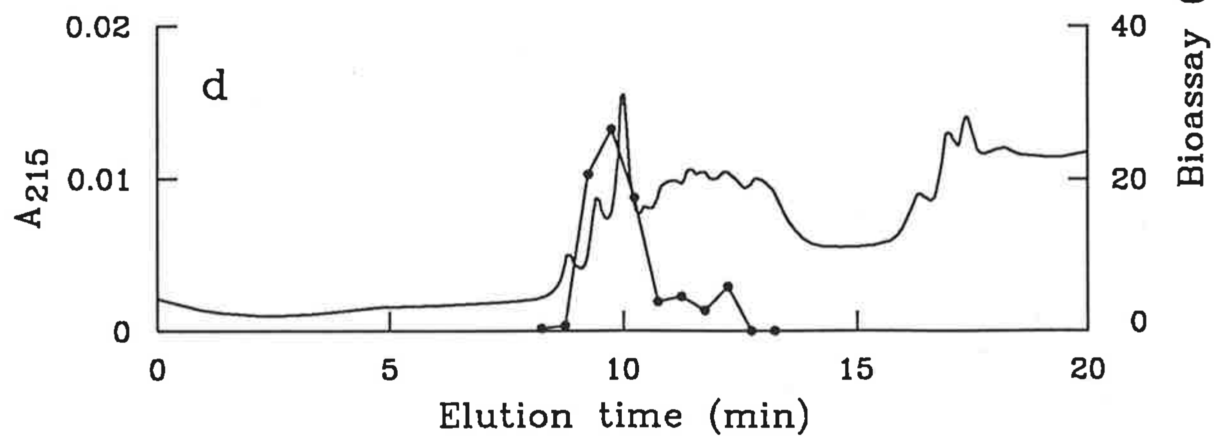
0.04
0.02
0
A215
a
0
5
10
15
20
Elution time (min)



0.02
0.01
0
A215
b
0
5
10
15
20
Elution time (min)



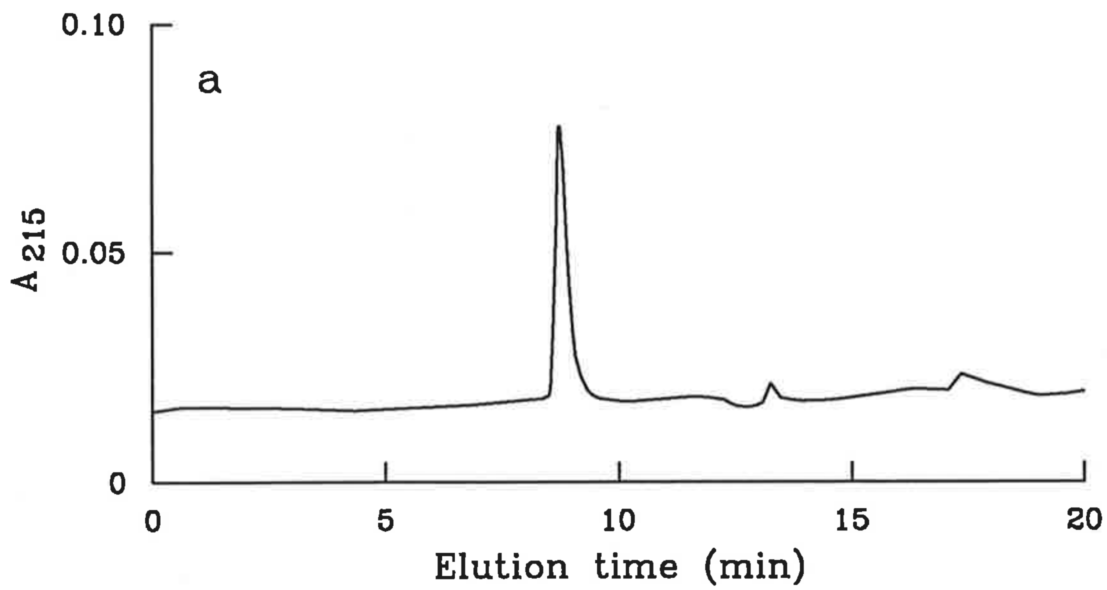
0.02
0.01
0
A215
c
40
20
0
0
5
10
15
20
Elution time (min)

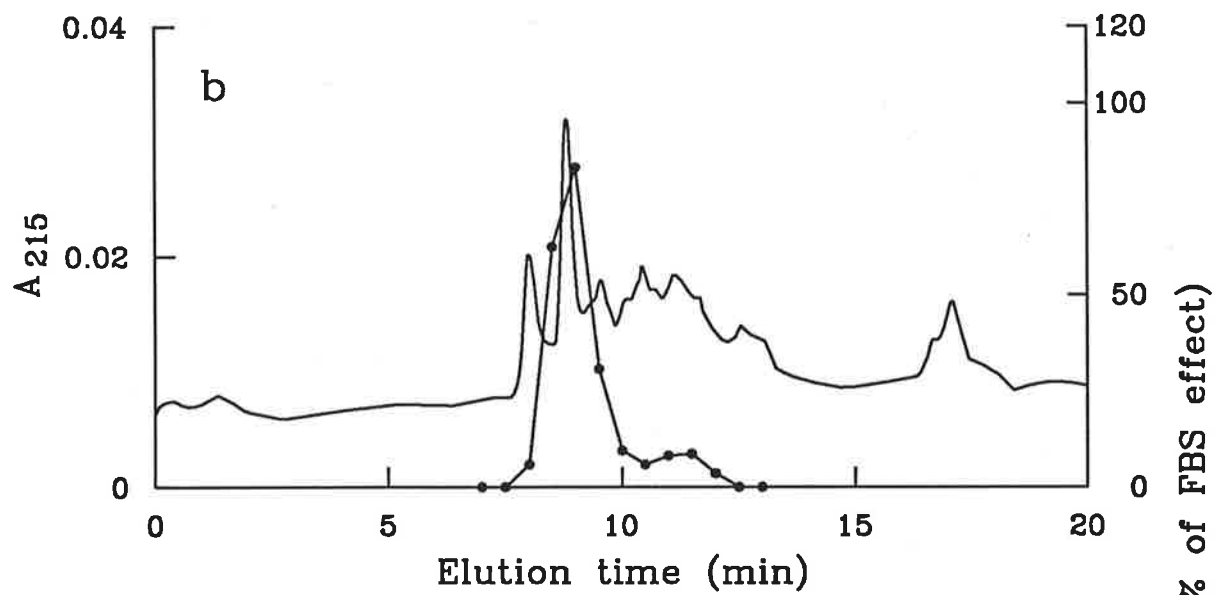


0.02
0.01
0
A215
d
40
20
0
Bioassay (% of FBS effect)
0
5
10
15
20
Elution time (min)

Figure 3.3: Reoxidation of natural IGF-I using a glutathione redox buffer

IGF-I peptides were chromatographed on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-50%MeCN over 15min. A_{215} was monitored (—) and fractions were collected at time intervals of 0.5min. Duplicate samples equivalent to 30% of each fraction was subjected to the L6 bioassay (●). Bioassay data are expressed as a percentage of the stimulation observed in the presence of 5% foetal bovine serum. Incorporation of radioactivity was equal to 1.3% in the absence of added growth factor and 4.6% in the presence of 5% foetal bovine serum. *Sample a:* natural IGF-I. *Sample b:* reduced IGF-I incubated in glutathione redox buffer for 30min. *Sample c:* reduced IGF-I incubated in glutathione redox buffer for 140min.


0.10
a
A 215
0.05
0
0
5
10
15
20
Elution time (min)


0.04
b
A 215
0.02
0
0
5
10
15
20
Elution time (min)
120
100
50
0
Bioassay (% of FBS effect)

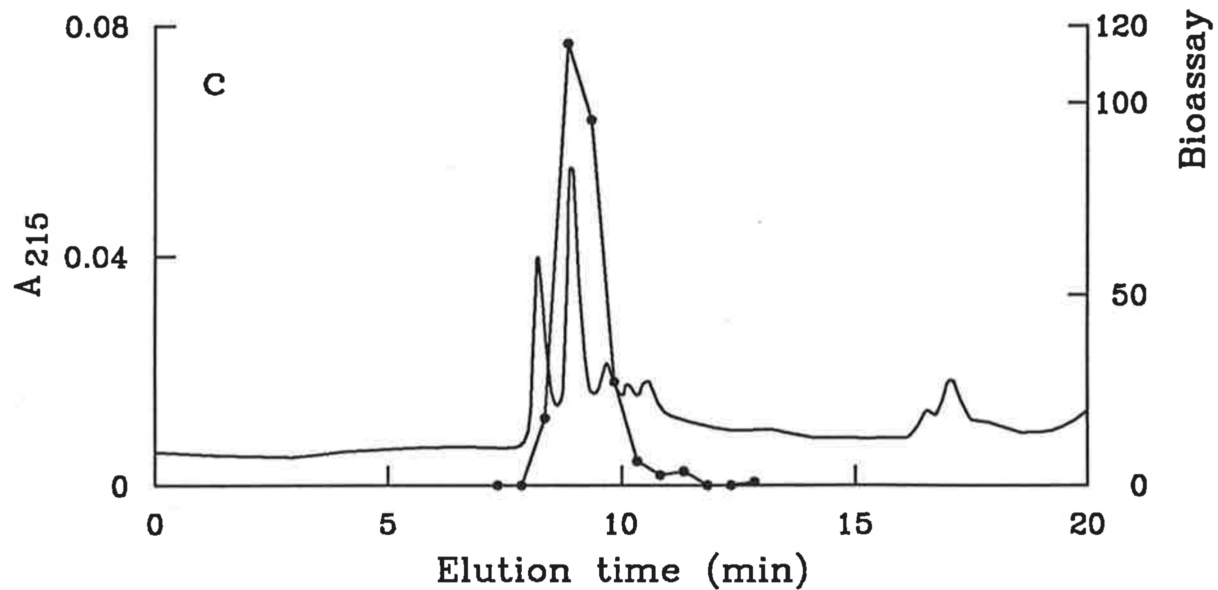
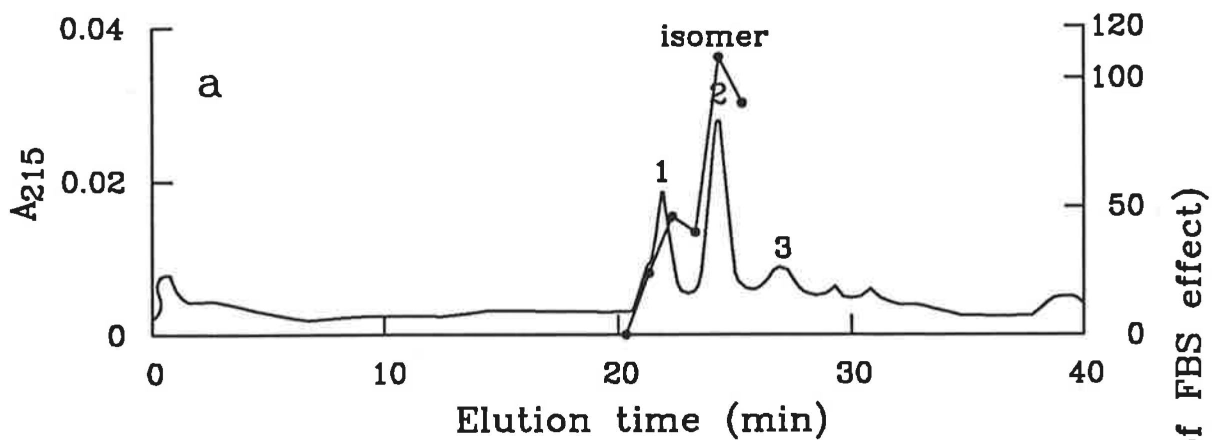
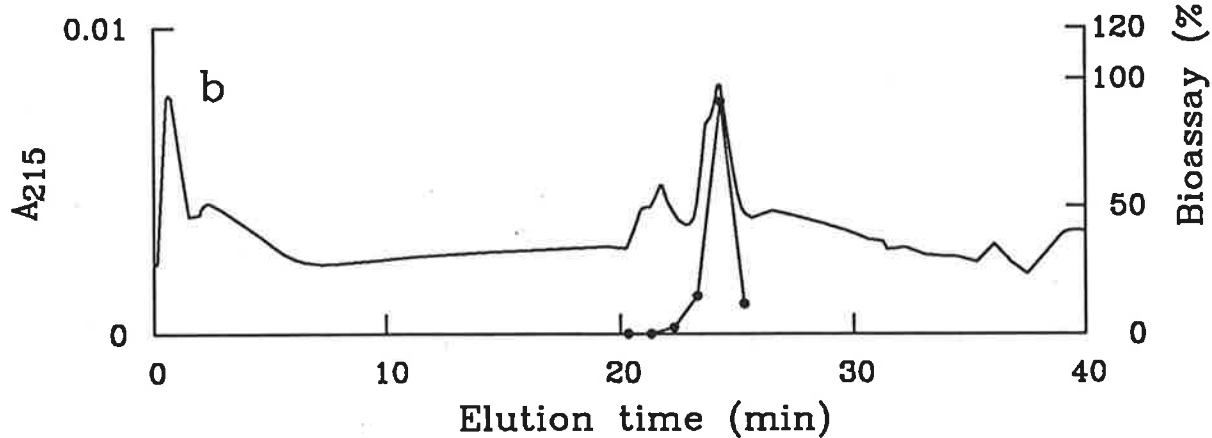
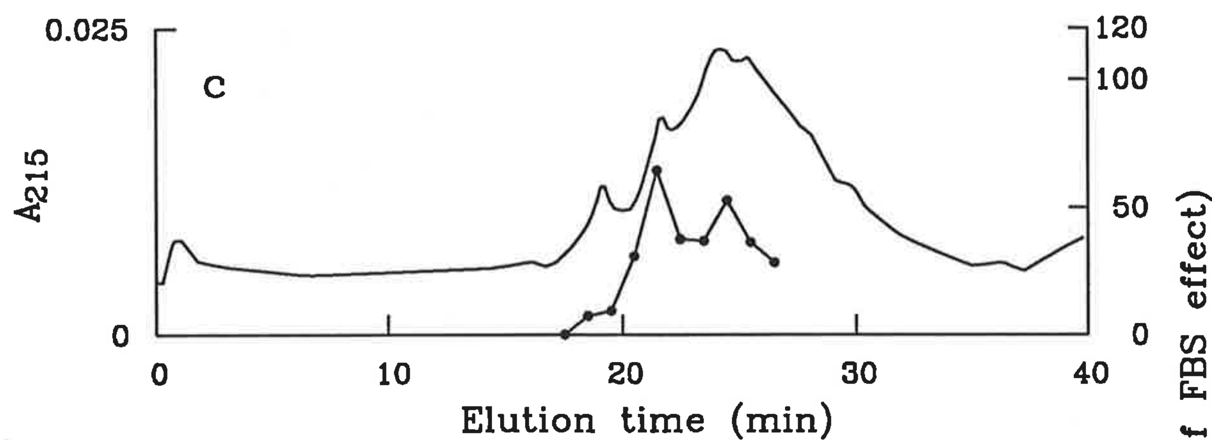

0.08
c
A 215
0.04
0
0
5
10
15
20
Elution time (min)
120
100
50
0

Figure 3.4: Oxidation of natural and synthetic IGF-I peptides

IGF-I peptides were chromatographed on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-25%MeCN over 5min followed by a gradient from 25-40%MeCN over 30min. A_{215} was monitored (—) and fractions were collected at time intervals of 1min. 90% of each fraction was subjected to the L6 bioassay (●). Bioassay data are expressed as a percentage of the stimulation observed in the presence of 5% foetal bovine serum. Incorporation of radioactivity was equal to 2.5% in the absence of added growth factor and 6.6% in the presence of 5% foetal bovine serum. *Sample a:* reoxidation of reduced bovine IGF-I. *Sample b:* reoxidation of reduced natural des-(1-3)-IGF-I. *Sample c:* oxidation of reduced ABI-1 IGF-I. *Sample d:* oxidation of reduced BIO-1 IGF-I. Note that the slightly earlier elution times for the two synthetic IGF-I peptides probably result from correction of a minor solvent leak after sample b.


0.04
0.02
0
A215
a
isomer
1
2
3
120
100
50
0
0
10
20
30
40
Elution time (min)


0.01
0
A215
b
120
100
50
0
0
10
20
30
40
Elution time (min)
Bioassay (% of FBS effect)


0.025
0
A215
c
120
100
50
0
0
10
20
30
40
Elution time (min)

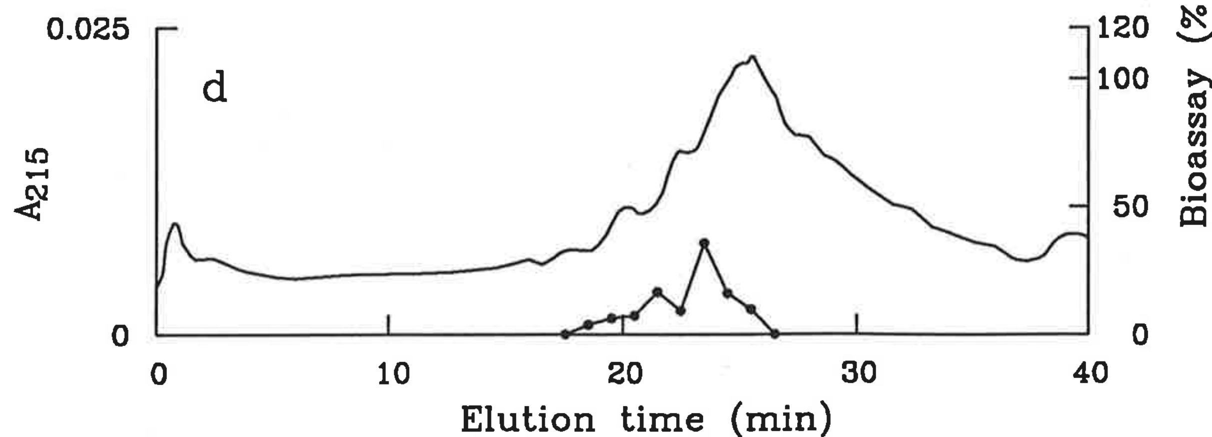
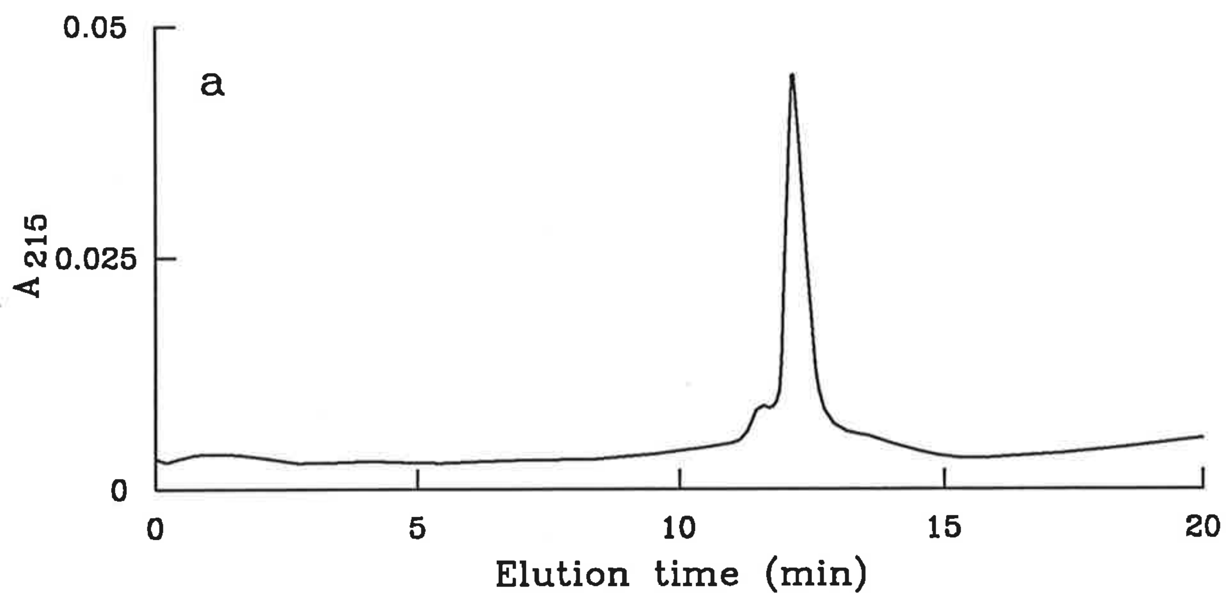
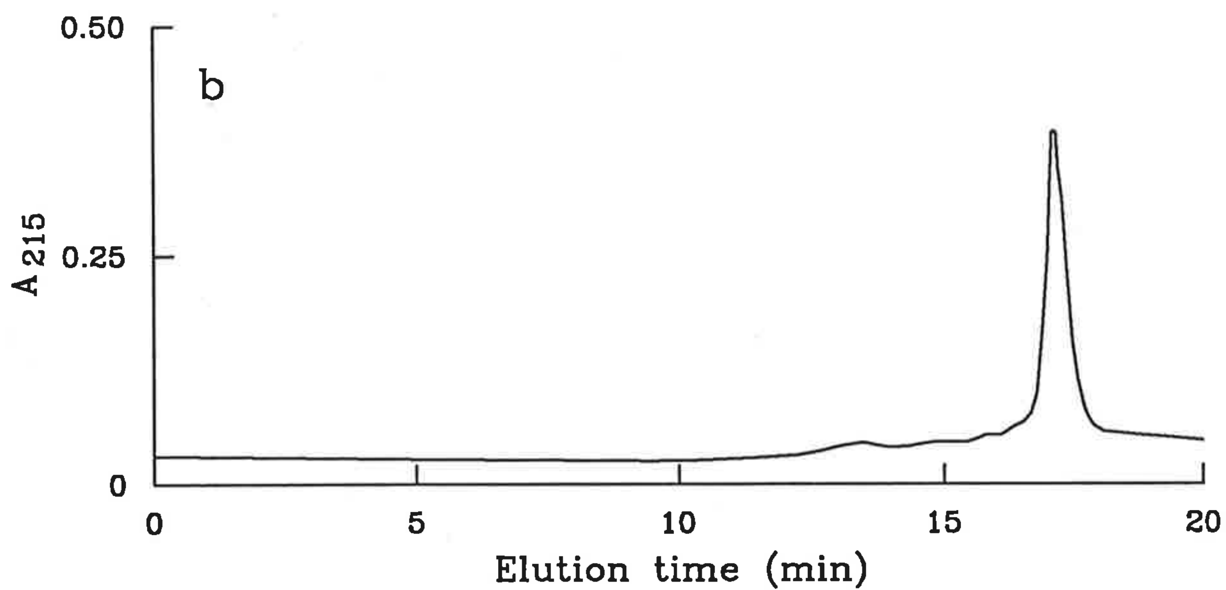

0.025
0
A215
d
120
100
50
0
0
10
20
30
40
Elution time (min)
Bioassay (% of FBS effect)

Figure 3.5: Reoxidation of epidermal growth factor

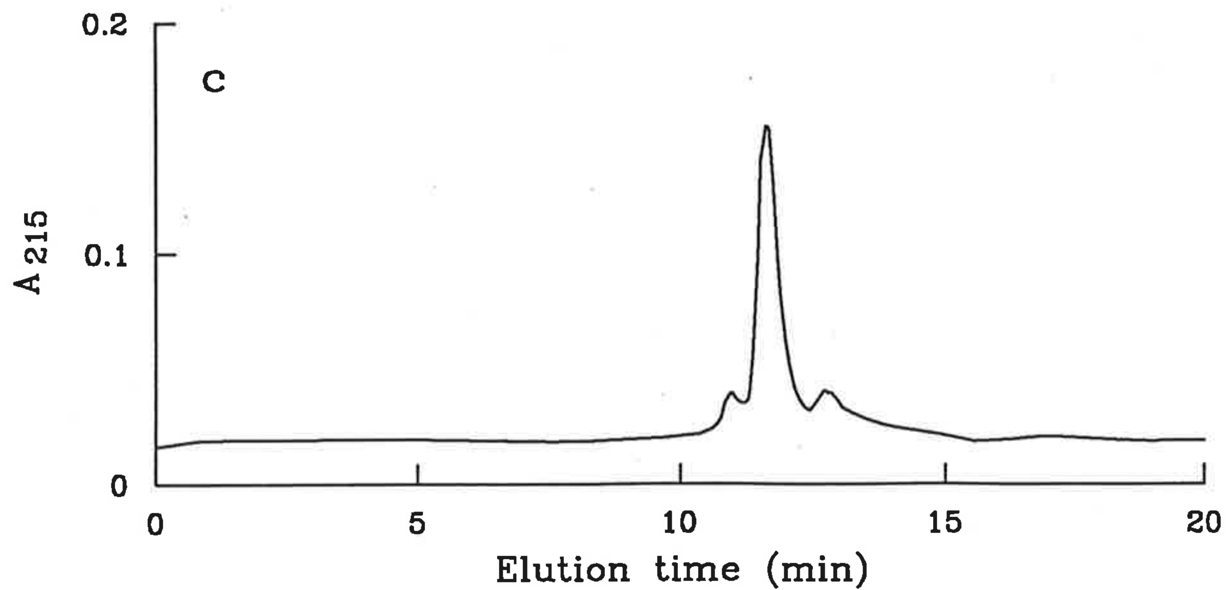
EGF peptides were chromatographed on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-50%MeCN over 15min. A_{215} was monitored (—). *Sample a:* epidermal growth factor. *Sample b:* reduced epidermal growth factor. *Sample c:* oxidation of reduced epidermal growth factor.



a
A_{215}
0.05
0.025
0
0
5
10
15
20
Elution time (min)



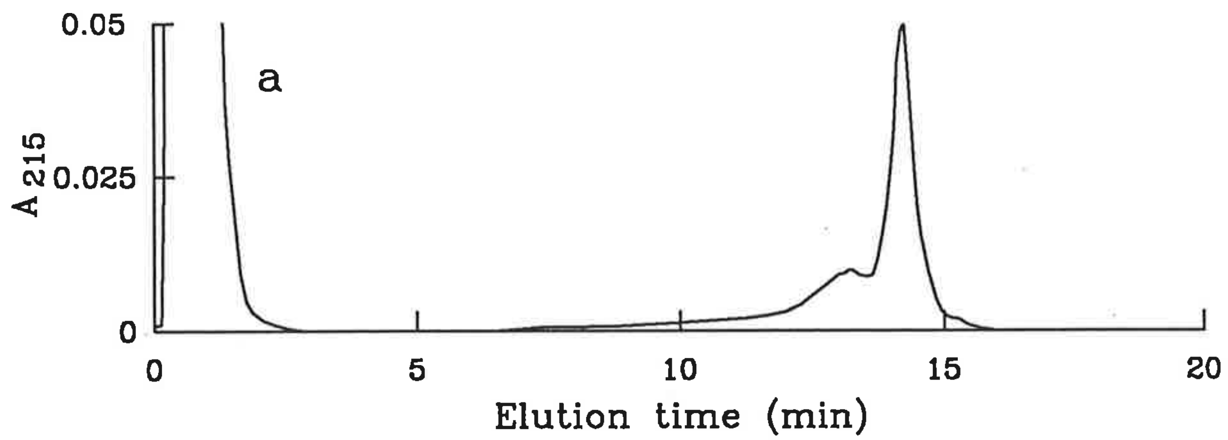
b
A_{215}
0.50
0.25
0
0
5
10
15
20
Elution time (min)



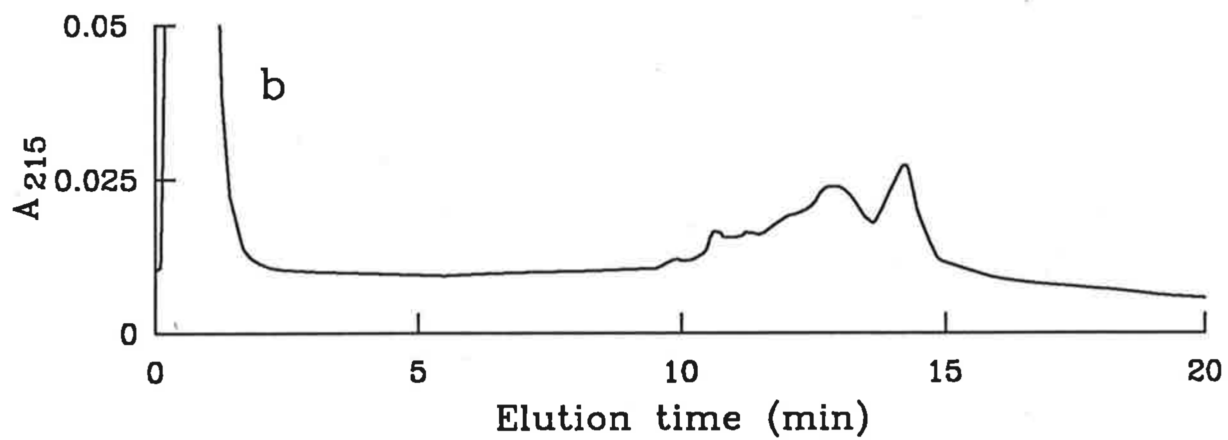
c
A_{215}
0.2
0.1
0
0
5
10
15
20
Elution time (min)

Figure 3.6: Time course of oxidation

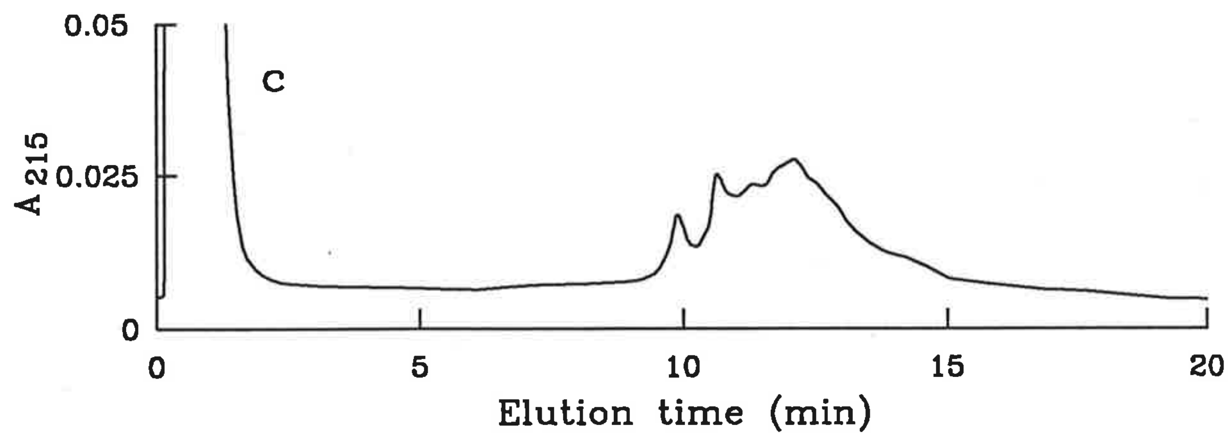
Reduced IGF-I peptides were allowed to oxidize for the indicated times prior to chromatography on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-50%MeCN over 15min. A_{215} was monitored (—). *Sample a:* IGF after an oxidation time of 1min. *Sample b:* IGF after an oxidation time of 12min. *Sample c:* IGF after an oxidation time of 1h. *Sample d:* IGF after an oxidation time of 18h.



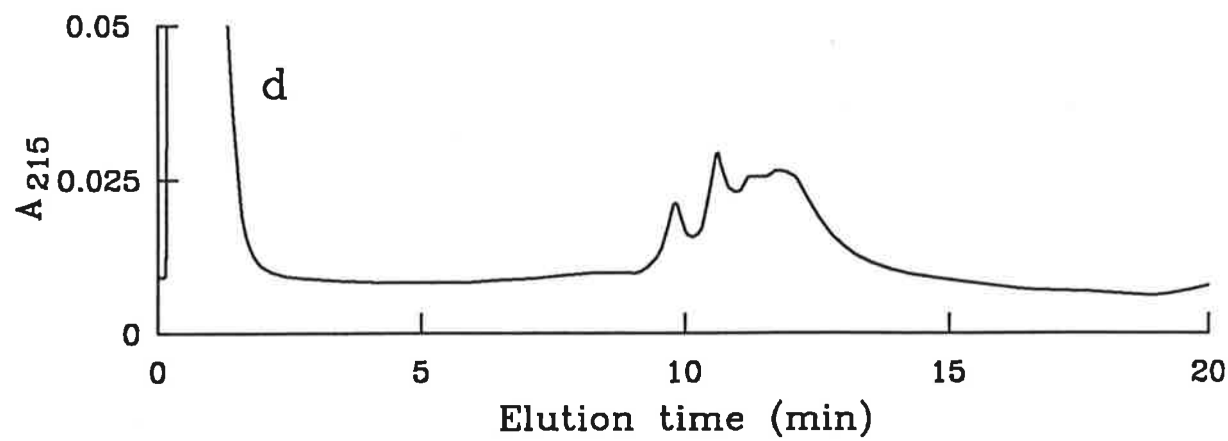
0.05
a
A 215
0.025
0
0
5
10
15
20
Elution time (min)



0.05
b
A 215
0.025
0
0
5
10
15
20
Elution time (min)



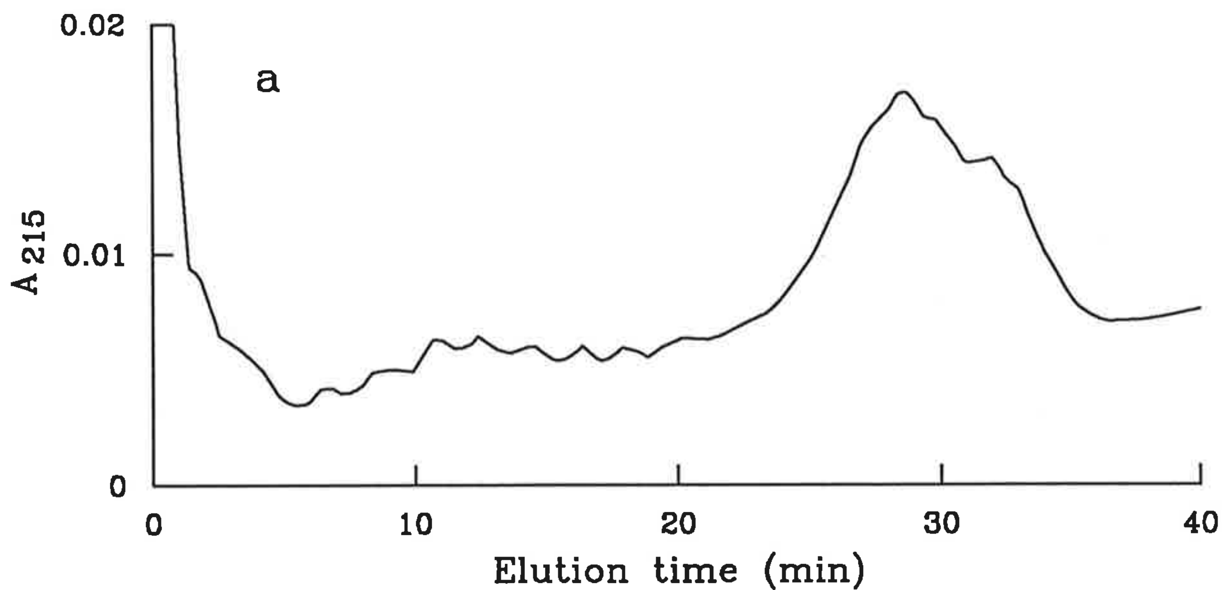
0.05
c
A 215
0.025
0
0
5
10
15
20
Elution time (min)



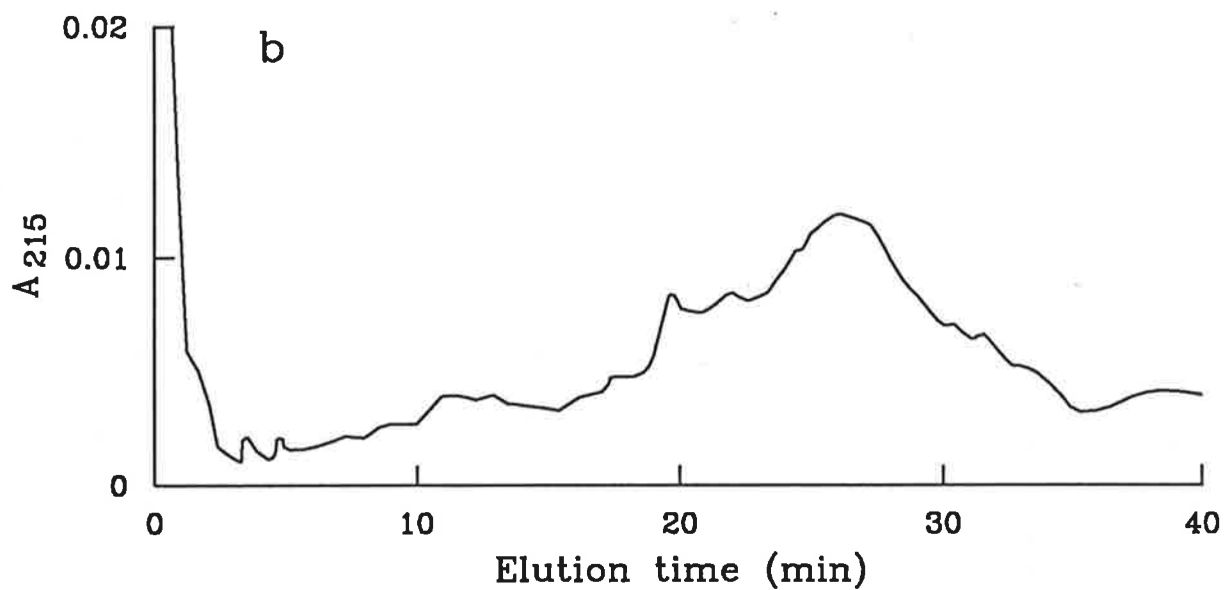
0.05
d
A 215
0.025
0
0
5
10
15
20
Elution time (min)

Figure 3.7: The effect of pH value and temperature

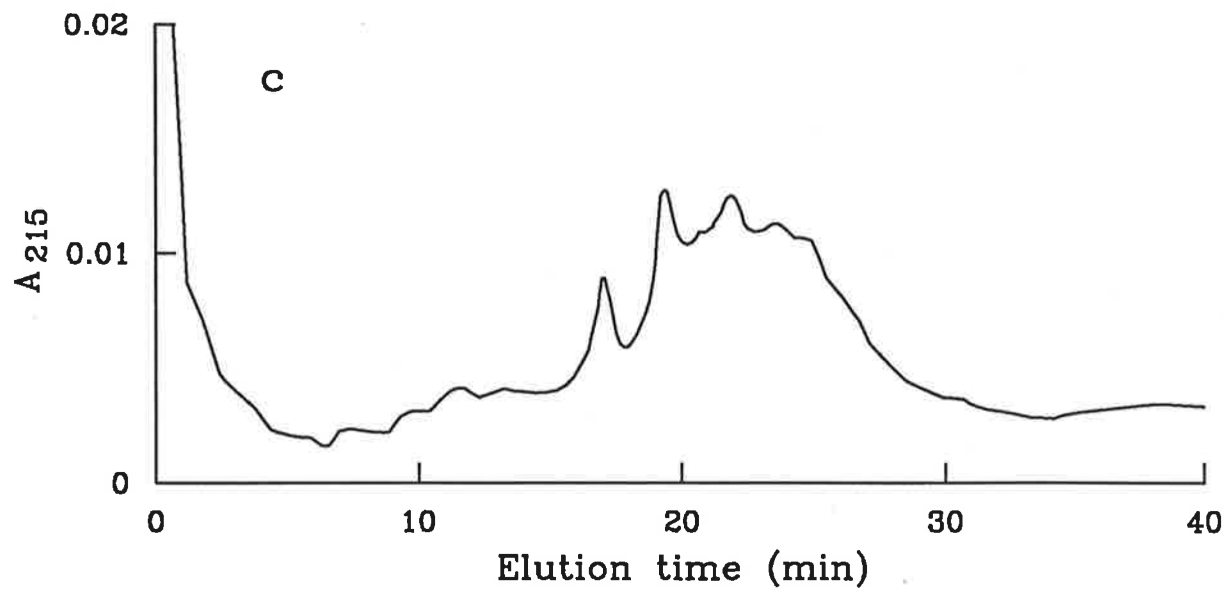
Reduced IGF-I peptides were allowed to oxidize for the indicated times prior to chromatography on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-25%MeCN over 5min followed by a gradient from 25-40%MeCN over 30min. A_{215} was monitored (—). *Sample a:* oxidation at pH 6.0 and 25°C. *Sample b:* oxidation at pH 7.0 and 25°C. *Sample c:* oxidation at pH 8.0 and 25°C. *Sample d:* oxidation at pH 9.0 and 25°C. *Sample e:* oxidation at pH 10.0 and 25°C. *Sample f:* oxidation at pH 8.0 and 4°C. *Sample g:* oxidation at pH 8.0 and 37°C. *Sample h:* oxidation at pH 8.0 and 50°C. *Sample i:* oxidation at pH 8.0 and 65°C.



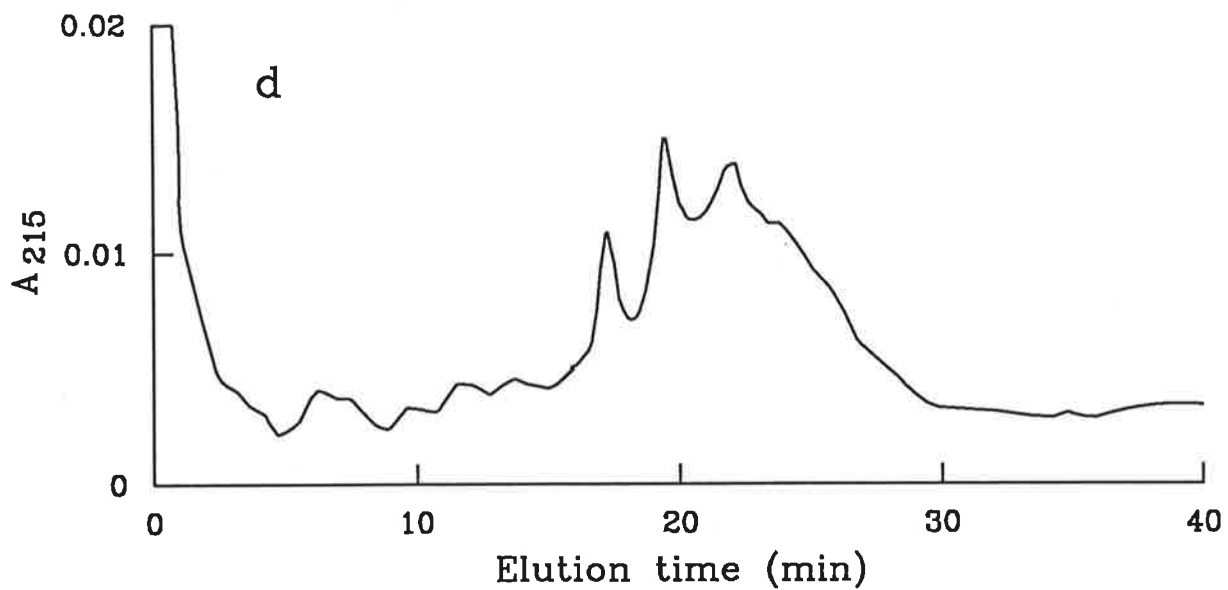
0.02
a
A 215
0.01
0
0
10
20
30
40
Elution time (min)



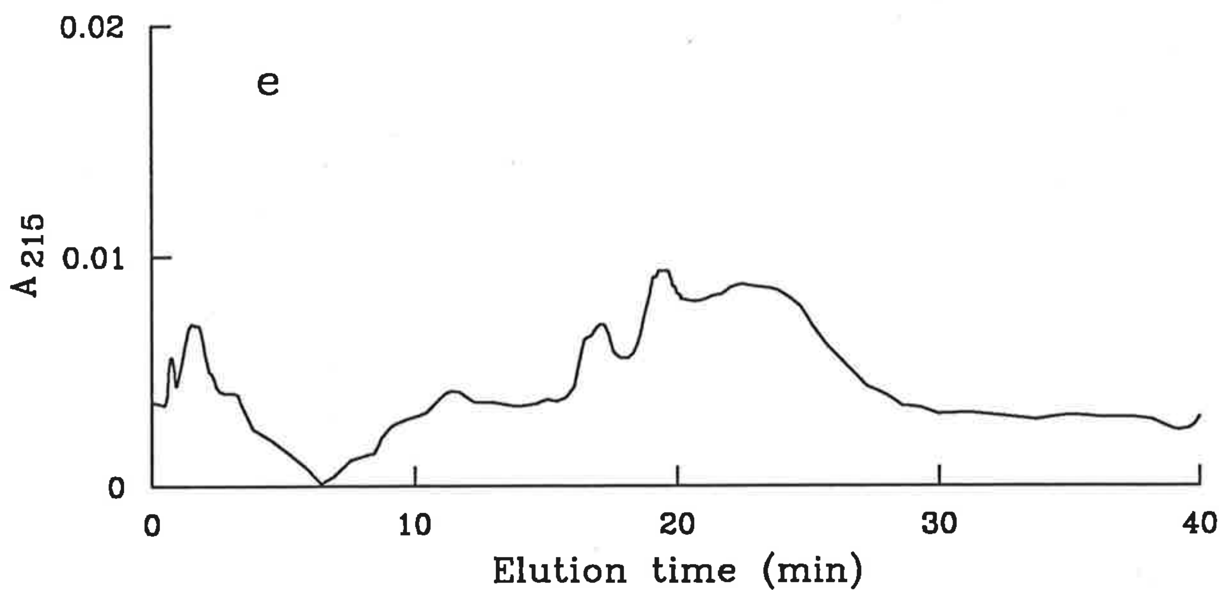
0.02
b
A 215
0.01
0
0
10
20
30
40
Elution time (min)



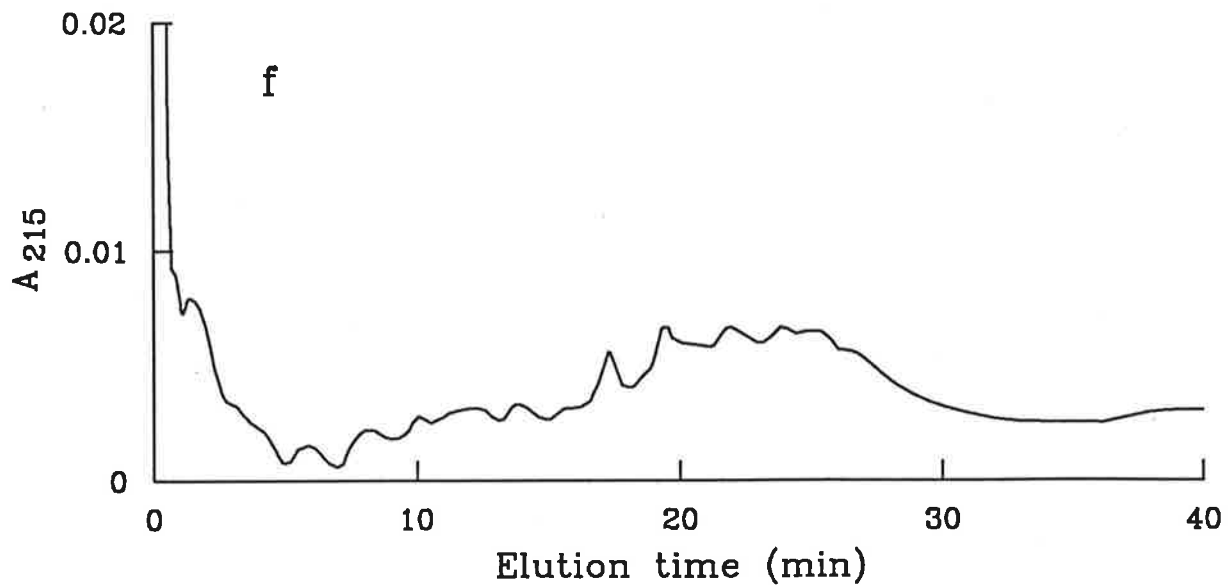
0.02
c
A 215
0.01
0
0
10
20
30
40
Elution time (min)



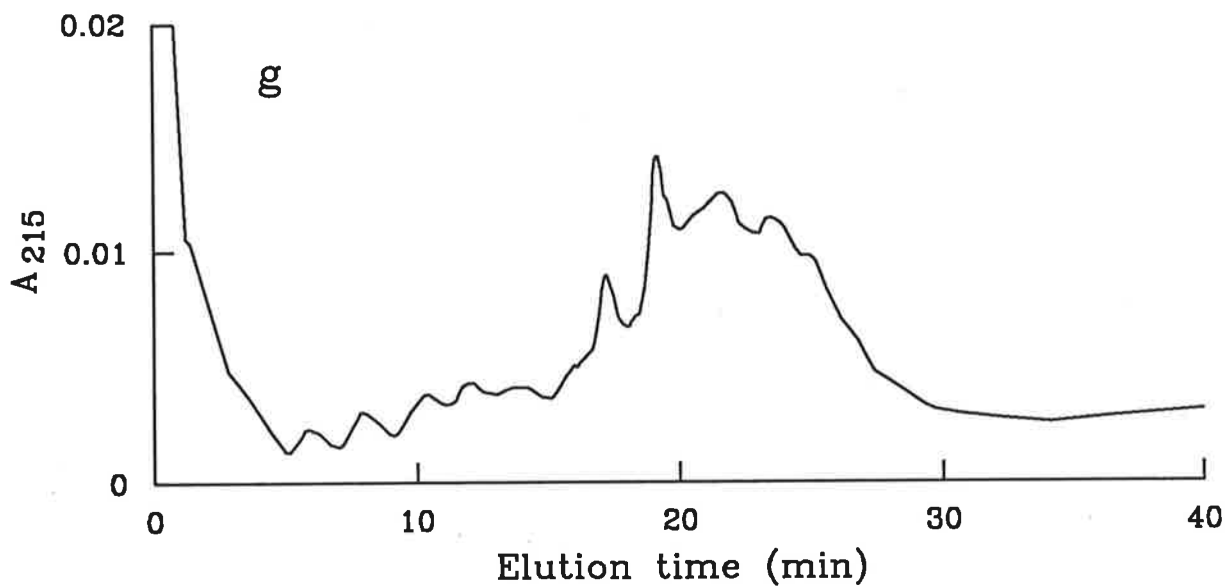
0.02
d
A 215
0.01
0
0
10
20
30
40
Elution time (min)



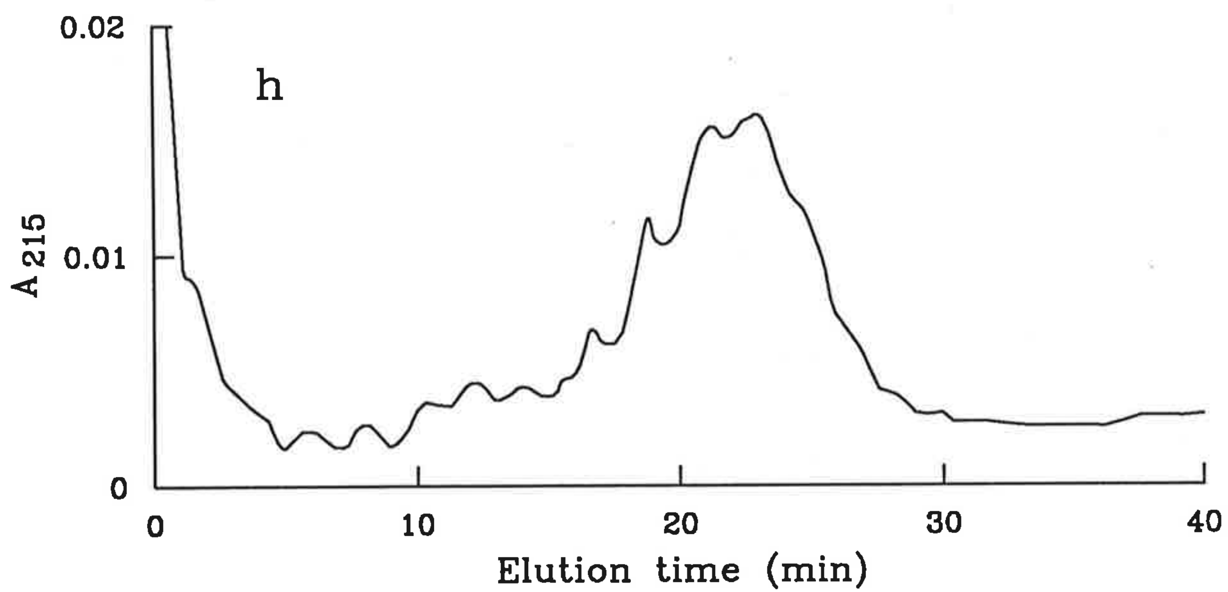
0.02
e
A 215
0.01
0
0
10
20
30
40
Elution time (min)



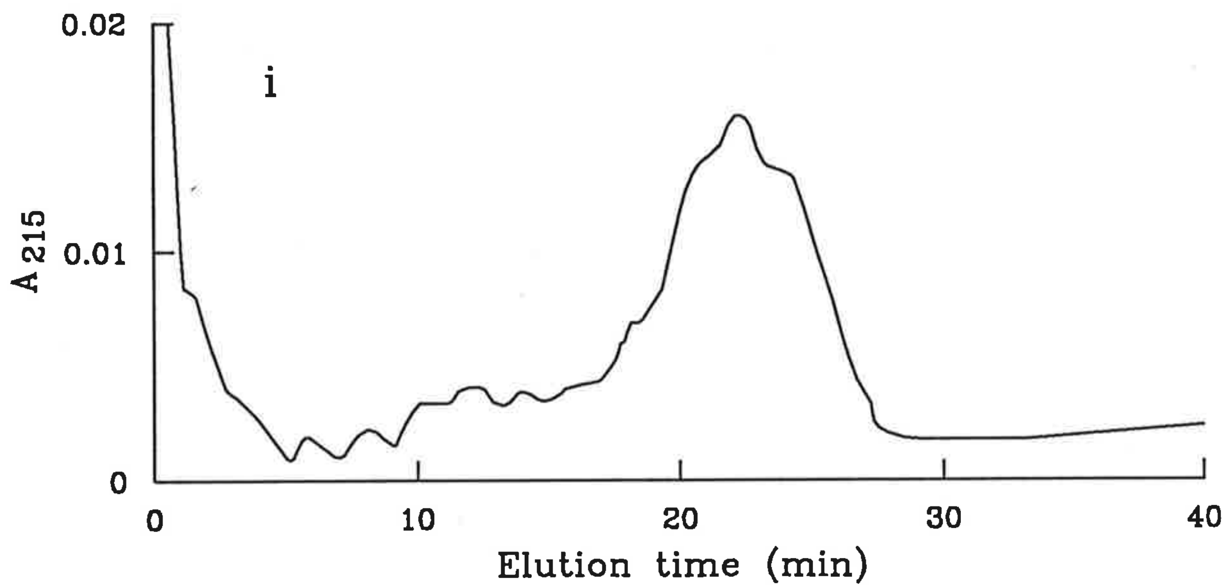
0.02
f
A 215
0.01
0
0
10
20
30
40
Elution time (min)



g
A 215
0.02
0.01
0
0
10
20
30
40
Elution time (min)

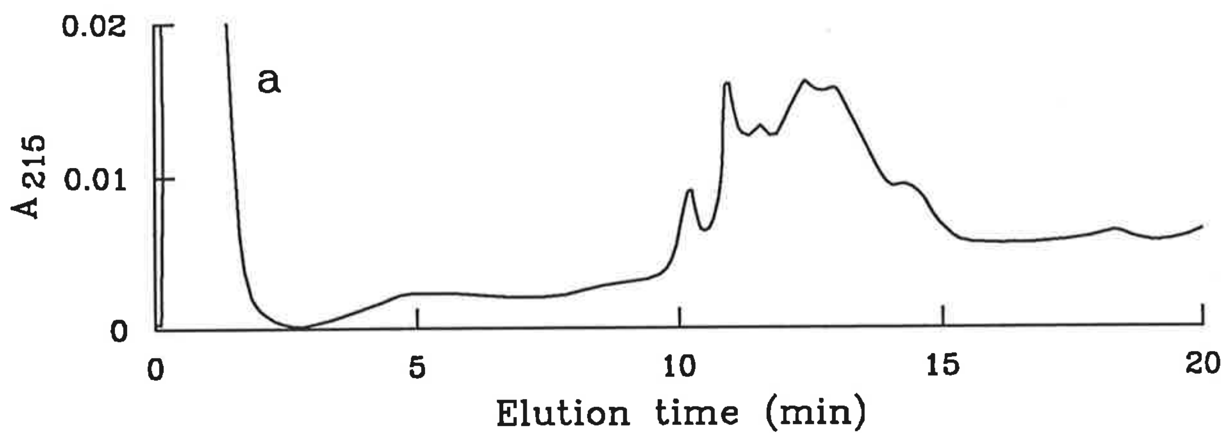


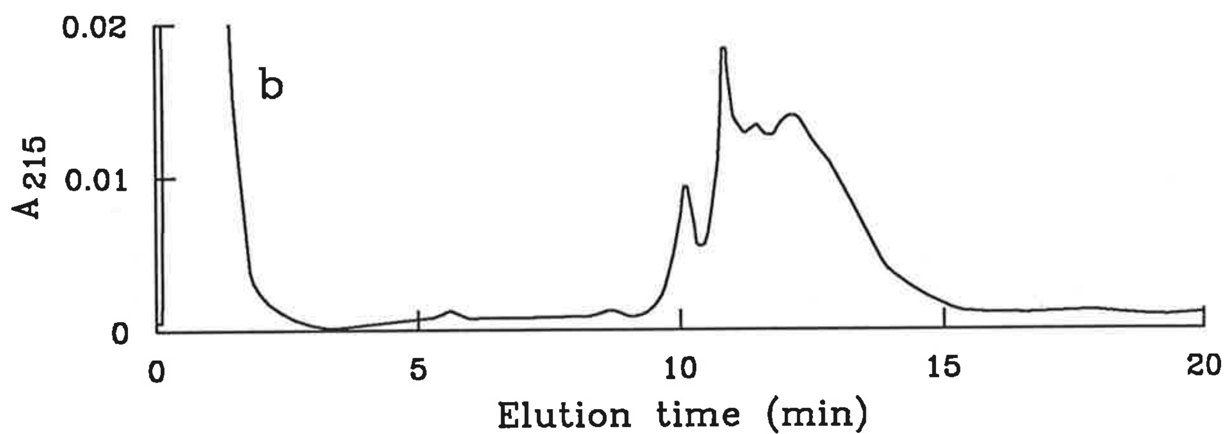
h
A 215
0.02
0.01
0
0
10
20
30
40
Elution time (min)

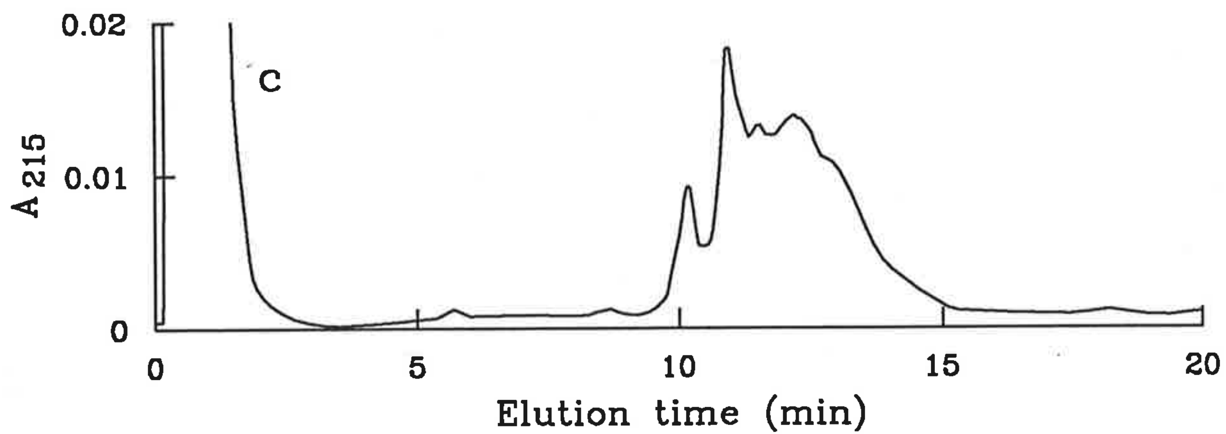


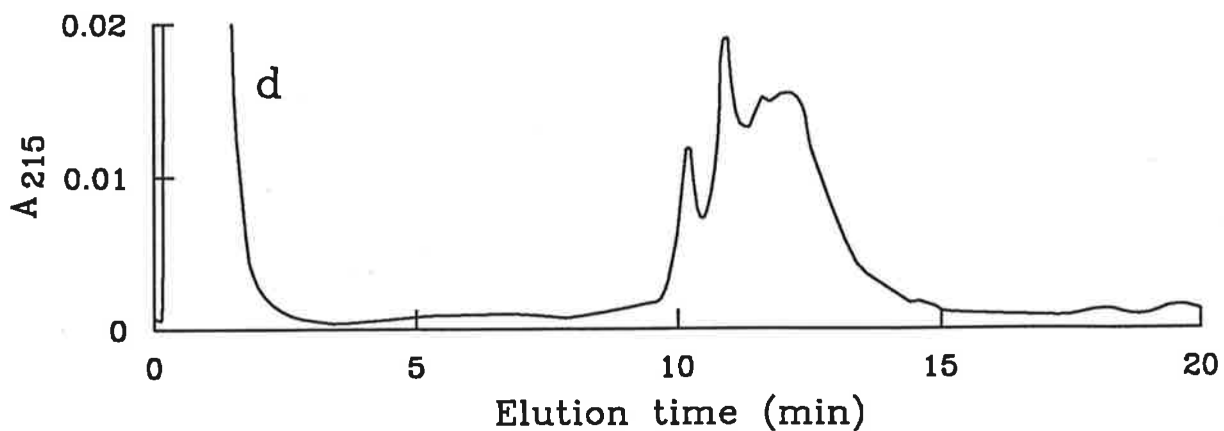
i
A 215
0.02
0.01
0
0
10
20
30
40
Elution time (min)

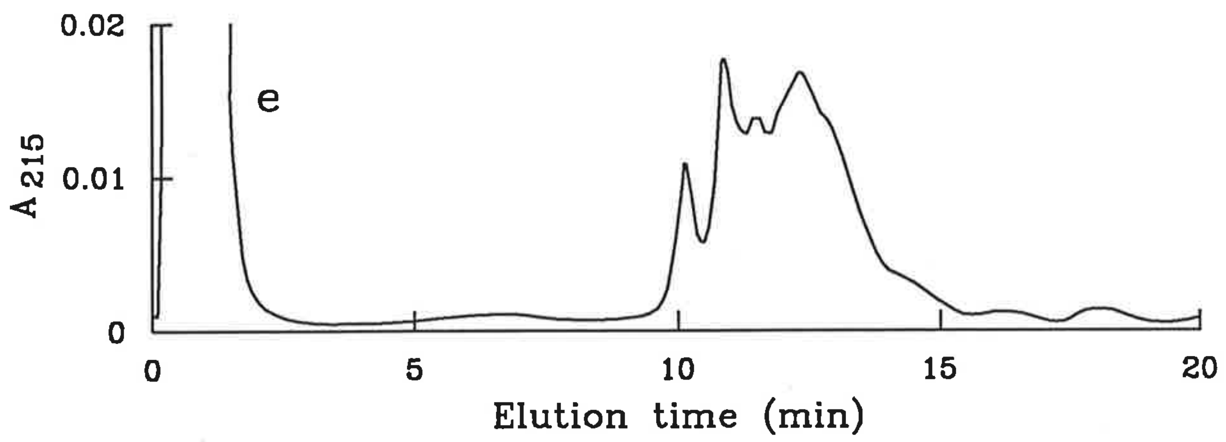
Figure 3.8: The effect of various concentrations of oxidized and reduced glutathione
Reduced IGF-I peptides were allowed to oxidize for the indicated times prior to chromatography on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-50%MeCN over 15min. A_{215} was monitored (—). *Sample a:* oxidation in the presence of 1mM-reduced glutathione and 0.05mM-oxidized glutathione. *Sample b:* oxidation in the presence of 1mM-reduced glutathione and 0.1mM-oxidized glutathione. *Sample c:* oxidation in the presence of 1mM-reduced glutathione and 0.2mM-oxidized glutathione. *Sample d:* oxidation in the presence of 1mM-reduced glutathione and 1.0mM-oxidized glutathione. *Sample e:* oxidation in the presence of 0.2mM-reduced glutathione and 0.1mM-oxidized glutathione. *Sample f:* oxidation in the presence of 0.4mM-reduced glutathione and 0.1mM-oxidized glutathione. *Sample g:* oxidation in the presence of 2mM-reduced glutathione and 0.1mM-oxidized glutathione. *Sample h:* oxidation in the presence of 4mM-reduced glutathione and 0.1mM-oxidized glutathione.

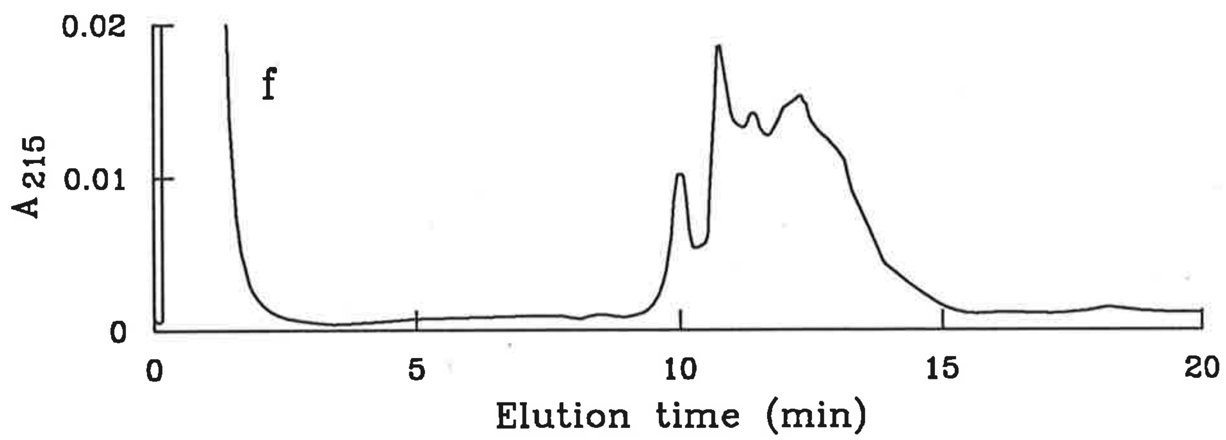

0.02
a
A 215
0.01
0
0
5
10
15
20
Elution time (min)

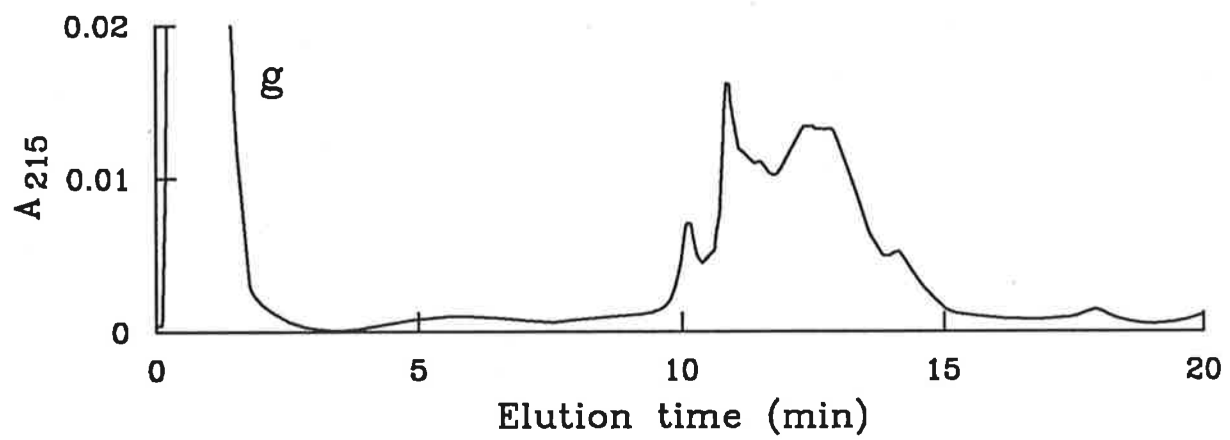

0.02
b
A 215
0.01
0
0
5
10
15
20
Elution time (min)


0.02
c
A 215
0.01
0
0
5
10
15
20
Elution time (min)


0.02
d
A 215
0.01
0
0
5
10
15
20
Elution time (min)


0.02
0.01
0
A 215
e
0
5
10
15
20
Elution time (min)


0.02
0.01
0
A 215
f
0
5
10
15
20
Elution time (min)


0.02
0.01
0
A 215
g
0
5
10
15
20
Elution time (min)

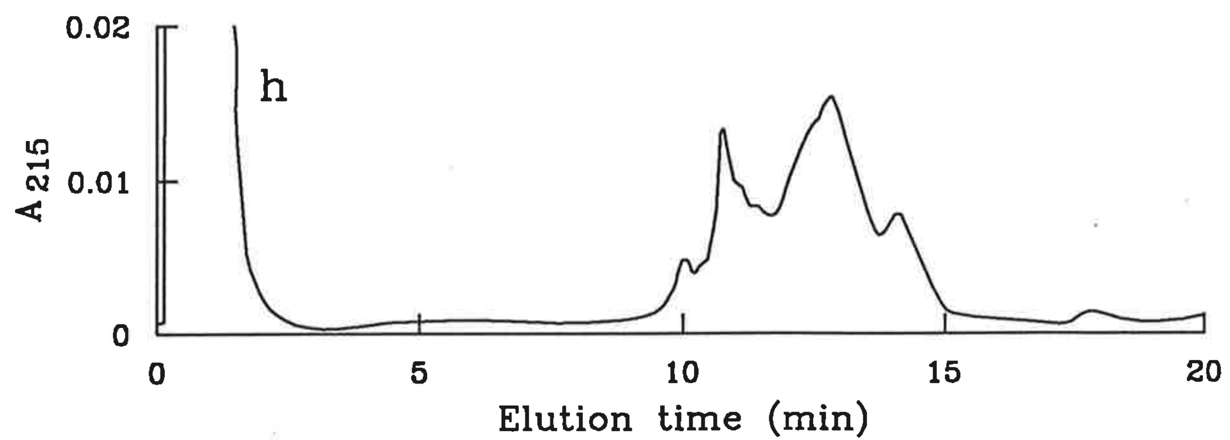
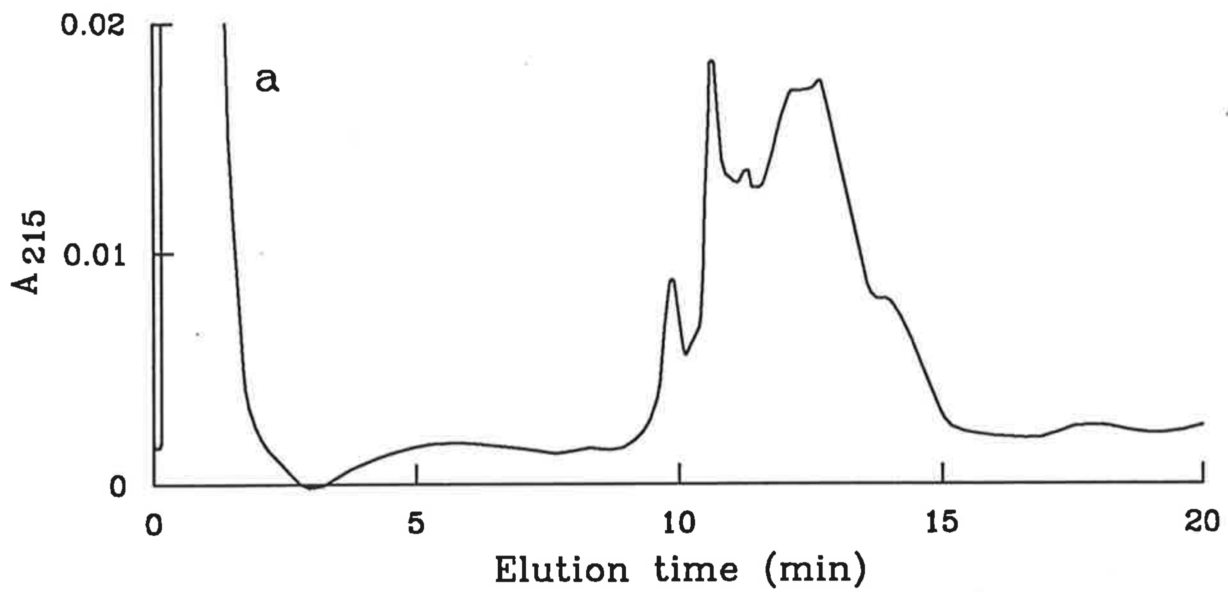
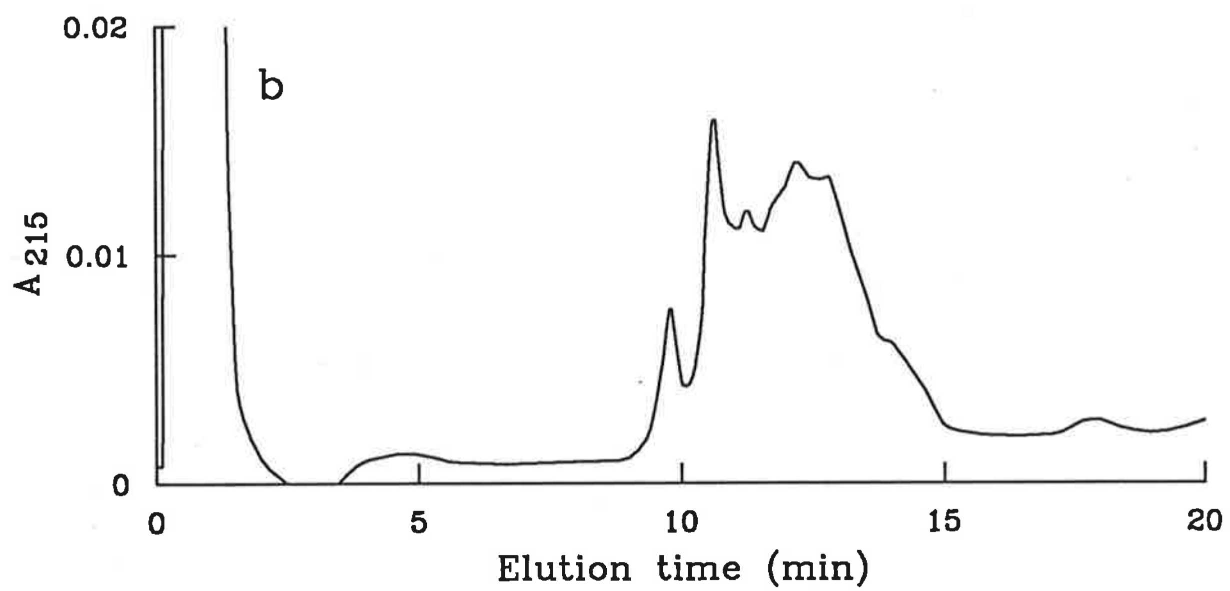

0.02
0.01
0
A 215
h
0
5
10
15
20
Elution time (min)

Figure 3.9: The effect of the concentration of urea and protein

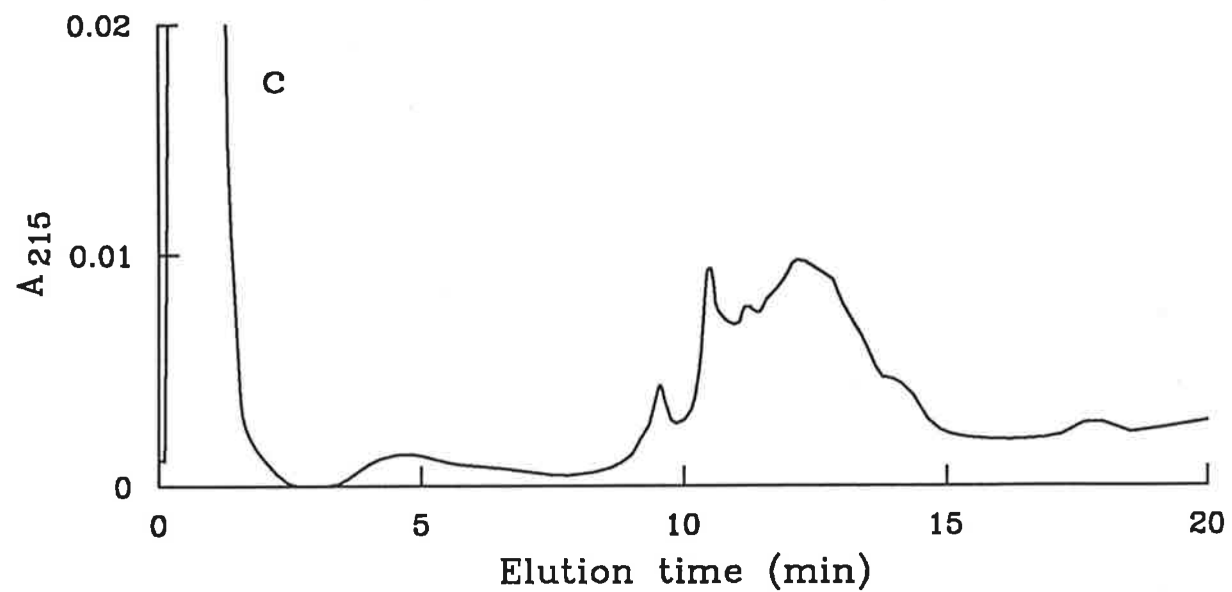
Reduced IGF-I peptides were allowed to oxidize for the indicated times prior to chromatography on a 2.1 × 30mm C4 cartridge at a flow rate of 0.2ml/min using a resolving gradient from 20-50%MeCN over 15min. A_{215} was monitored (—). *Sample a:* oxidation at a peptide concentration of 25µg/ml in the presence of 2M-urea. *Sample b:* oxidation at a peptide concentration of 50µg/ml in the presence of 2M-urea. *Sample c:* oxidation at a peptide concentration of 100 µg/ml in the presence of 2M-urea. *Sample d:* oxidation at a peptide concentration of 50µg/ml in the presence of 1M-urea. *Sample e:* oxidation at a peptide concentration of 50µg/ml in the presence of 3M-urea.



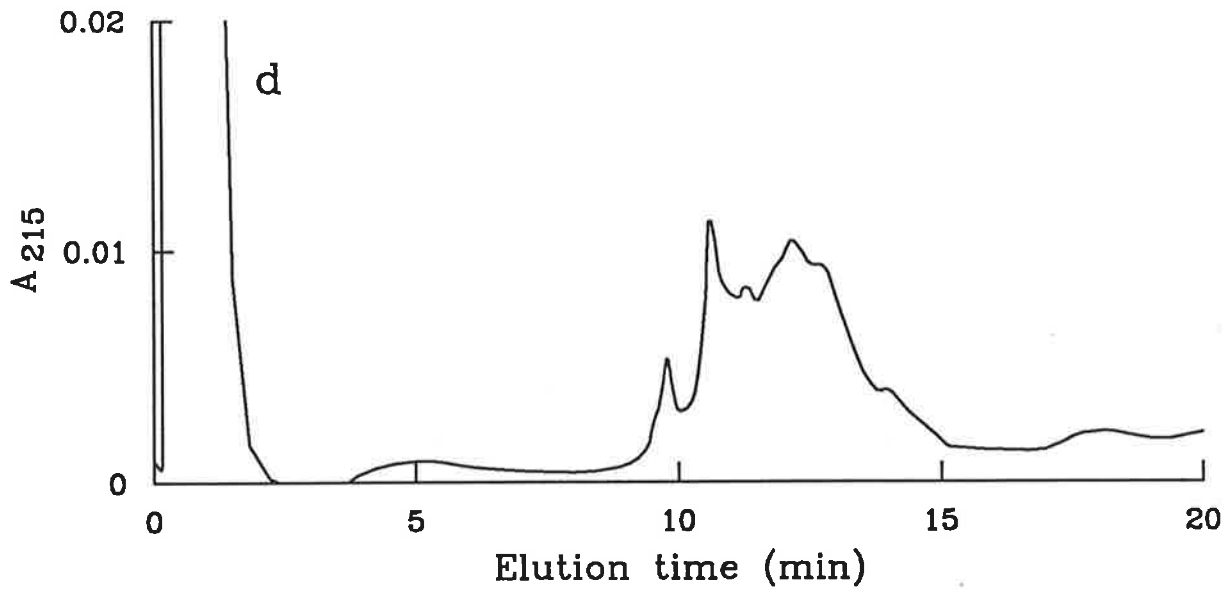
a
A 215
0.02
0.01
0
0
5
10
15
20
Elution time (min)



b
A 215
0.02
0.01
0
0
5
10
15
20
Elution time (min)



c
A 215
0.02
0.01
0
0
5
10
15
20
Elution time (min)


0.02
d
A_{215}
0.01
0
0
5
10
15
20
Elution time (min)

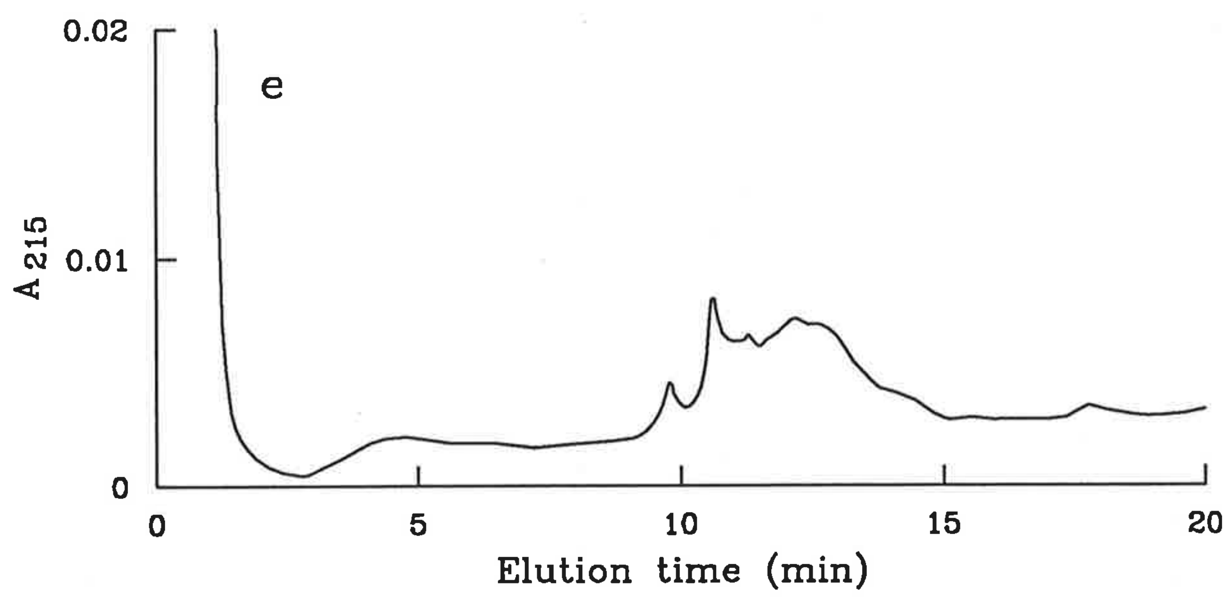
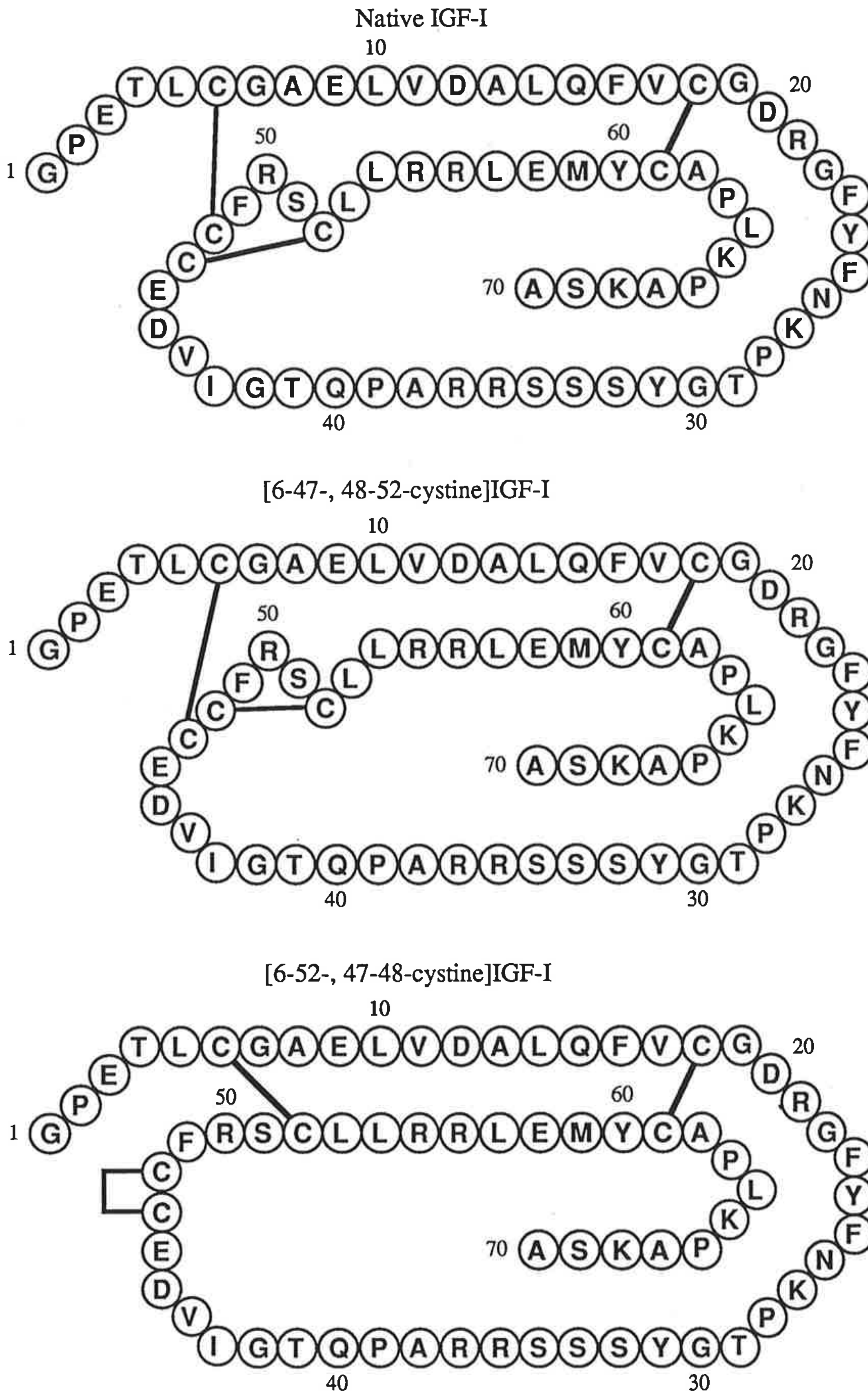

0.02
e
A_{215}
0.01
0
0
5
10
15
20
Elution time (min)

Figure 3.10: Disulphide-bond isomers of IGF-I

The structures of the two major isomers of IGF-I which are formed when the reduced peptide is oxidized. Disulphide bonds are indicated by heavy lines. The structures are from Tamura *et al.* (1988).



CHAPTER 4

CHEMICAL SYNTHESIS OF IGF-I PEPTIDES

CHAPTER 4: CHEMICAL SYNTHESIS OF IGF-I PEPTIDES

4.1 INTRODUCTION

In order to investigate the role of the amino terminus of IGF-I in the mediation of its biological activities, it was necessary to produce a number of analogues with substitutions or truncations in the amino-terminal region. Since there was not a system available in this laboratory for the expression of genes encoding IGF-I, solid phase peptide synthesis was considered the most suitable method for producing variants of IGF-I for biological studies. A particular advantage of solid phase peptide synthesis is that chain assembly proceeds from the carboxy terminus to the amino terminus and thus amino-terminally truncated analogues of IGF-I could be readily produced by removal of peptide at appropriate stages of the chain assembly.

In 1963, Merrifield introduced the technique of solid phase peptide synthesis (Merrifield, 1963). This method has the distinct advantage, compared with solution phase chemistry, of allowing the nascent polypeptide to be readily separated from reagents by physical means independent of specific knowledge of the physical properties of the peptide. Solid phase peptide synthesis is a step-wise process commencing with the coupling of the C-terminal amino acid to an insoluble support (resin) followed by sequential coupling of the desired amino acid residues. Each amino acid is chemically blocked at the $N\alpha$ position by a labile protecting group and on the sidechain (if necessary) by a protecting group which is inert under the conditions of chain assembly but can be removed later. In a cycle of synthesis, the $N\alpha$ protecting group is firstly removed and a derivative of the desired amino acid, activated at the α-carboxyl group, is then added. Reaction to form a peptide bond is allowed to occur for a time determined by either previous experience or by direct measurement of unreacted amino function. The excess amino acid is then removed and a second cycle of synthesis can then commence. After assembly of the desired sequence of amino acid residues, the resin-peptide is then subjected to a more harsh chemical environment which removes (usually) all the side-chain protecting groups, the $N\alpha$ protection and cleaves the peptide from the resin.

There are two common methods for the direct chemical synthesis of peptides. One of these, known as the Fmoc strategy involves the use of base-labile $N\alpha$ protection and side-chain protection allowing the use of relatively mild conditions for the synthesis. As yet,

this method has not been successfully applied to long peptides (such as IGF) and at the time of commencement of this project, suitable automated apparatus was not available for this chemistry. The research groups involved in this project therefore purchased a machine capable of carrying out automated chemical synthesis by the Boc strategy as originally described by Merrifield (1963). This method of solid phase peptide synthesis involves the use of the moderately acid-labile *t*-butoxycarbonyl (Boc) group for N^{α} protection. Side-chain protection is achieved by groups which are only labile under strong acid conditions such as liquid HF or trifluoromethanesulphonic acid/ trifluoroacetic acid (TFMSA/TFA). Likewise, the cleavage of the peptide from the resin only occurs under these strong acid conditions.

One of the difficulties encountered with the chemical synthetic approach has been the efficient synthesis of peptides of greater than 40 amino acid residues. The main problems that occur during such a synthesis are (i) the accumulation of failure sequence peptides resulting from incomplete coupling of activated amino acids to the nascent peptide chain, and (ii) the subsequent purification and folding of the peptide to its native conformation. Other potential problems include loss of the peptide from the resin during chain assembly and the undesirable chemical modification of the peptide during the synthesis or during cleavage from the resin.

Strategies for improving the chain assembly include optimization of the reaction conditions via automation of the amino acid activation and coupling processes as well as the use of stable support resins which allow good solvation of the protected peptide. Recently, N-methylpyrrolidinone (NMP) has been introduced to solid phase peptide synthesis as a solvent for the coupling reaction (Tam, 1987). NMP has been shown to promote greater solvation of peptide-resin complexes than does dimethylformamide (DMF) or dichloromethane (DCM), which have been widely used as coupling solvents, and therefore presumably facilitates more complete reaction of the peptide with the activated amino acid (Geiser *et al.*, 1988). As an alternative to the use of liquid HF, TFMSA/TFA has been introduced as a reagent with which to both remove the side-chain protecting groups and cleave the peptide from the resin (Tam *et al.*, 1986; Bergot *et al.*, 1988).

The total synthesis of IGF-I by the solid phase method has been previously reported by Li *et al.* (1983). In their synthesis, all the amino acids were double-coupled. Likewise, the syntheses carried out by the candidate and Mr. May used a double-coupling

protocol and could be compared with the synthesis of IGF-I performed by Dr. K.Otteson, Ms. S.McCurdy and Ms. L.Pierce in the laboratories of Applied Biosystems Inc. which used only single couplings in NMP for all the amino acids except residue 4 (Thr) which was double-coupled. Due to the inconvenience and cost of installing apparatus for the handling of liquid HF, TFMSA/TFA was chosen as the reagent for the final deprotection of the peptides and cleavage from the resin.

In this chapter, I will report the chemical synthesis and purification of IGF-I and analogues. I was also supplied with a gift of resin-bound, protected IGF-I by Applied Biosystems Inc. which allowed a direct comparison of the quality of syntheses described in this chapter with that obtained by an expert laboratory and facilitated a collaborative investigation of two protocols for producing relatively long peptides. In this chapter, I will describe the synthesis of IGF-I and the biochemical comparison of synthetic, natural and recombinant-DNA-derived peptides in order to validate the usefulness of the chemical synthetic approach. I shall also describe the synthesis and folding of analogues of IGF-I truncated at the amino terminus and the production of further peptides exhibiting deletions or substitutions in the N-terminal region of IGF-I.

I wish to acknowledge the assistance of Mr. B.May who operated the automated peptide synthesizer, performed cleavage and deprotection reactions and the assistance of Ms. D.Turner who performed amino acid sequence analysis. I wish to acknowledge Dr. K.Otteson, Ms. S McCurdy and Ms. L.Pierce who provided me with IGF-I synthesized in the laboratories of Applied Biosystems Inc., Foster City, CA, U.S.A..

4.2 MATERIALS AND METHODS

Reagents for chemical synthesis were obtained as described in section 2.1. TFMSA/TFA cleavage of the peptide from the resin was performed as described in section 2.3d. Ninhydrin analysis and Edman degradation were performed as described in sections 2.3a and 2.3b respectively. Acetamidomethyl protecting groups were removed from peptides using the method described in section 2.3e. Oxidation of peptides to form the correct disulphide bonds was performed as described in table 3.4. Purification and analysis of peptides by reverse-phase HPLC was performed as described in section 2.2b. The protein contents of peaks observed on reverse-phase HPLC were estimated by absorbance as

described in section 2.2c. Radioimmunoassays, radioreceptor assays, protein synthesis bioassays and binding protein competitive binding assays were performed as described in sections 2.4 (a-d).

4.3 RESULTS

The peptides described below were produced by three independent syntheses performed in this laboratory, denoted BIO-1, BIO-2 and BIO-3. In addition, the synthesis of IGF-I in the laboratories of Applied Biosystems Inc. is described (denoted ABI-2). This peptide is derived from a different synthesis from that of the ABI-1 peptide used for some experiments reported in chapter 3. A listing of the synthetic peptides is given in table 4.1.

4.3a Synthesis BIO-1

The first synthesis of IGF-I performed by the candidate and Mr. May used the standard double-coupling protocol recommended by the instrument manufacturer including the use of 4-methoxybenzyl (Mob) protection of the cysteine residues. The crude cleavage product from this synthesis was highly heterogeneous by reverse-phase HPLC analysis. A discrete peak corresponding to the target peptide could not be identified and peptide corresponding major area of peptide on reverse-phase HPLC was poorly soluble at the neutral pH required for disulphide bond formation. The average repetitive coupling yield was 99.2% by ninhydrin analysis of the first 51 couplings and preview sequence analysis of the resin-bound peptide indicated an average repetitive yield of 99.0% for cycles 52 to 69 which indicated an expected yield of 50% for the target peptide. When the isolated reduced peptide was subjected to conditions favouring the formation of native disulphide bonds, a very low yield of bioactive IGF-I was obtained (see fig. 3.4d). It was possible that some modification to the peptide had occurred either during the synthesis or during cleavage and deprotection. One possibility was that the Mob protection was not adequately stable during the chain assembly and that some cysteine residues had become alkylated which prevented disulphide bond formation. Peptide from this synthesis was not used further.

4.3b Syntheses BIO-2 and ABI-2

A second synthesis (BIO-2) was performed using acetamidomethyl (Acm) protection of cysteine rather than Mob protection. This protecting group is stable to even strong acids such as TFMSA but can be removed oxidatively by the use of I_2. This synthesis was performed using the double-coupling protocol shown in table 4.2. Boc-Ala-PAM resin (658mg, 0.500mmol) was subjected to 69 couplings of the IGF-I sequence. After 29 cycles of coupling, half the resin was removed from the reaction vessel in order to facilitate better mixing of the resin-peptide with the reagents. A plan of the synthesis is shown in figure 4.1. The resin-bound peptide remaining in the reaction vessel was coupled with another 37 cycles of the IGF-I sequence at which point, half of the peptide-resin was removed for the production of des-(1-3)-IGF-I. The remainder was coupled with the remaining three cycles of the IGF-I sequence. Resin samples were taken at the completion of each cycle of the IGF-I synthesis and subjected to ninhydrin analysis. This indicated that the average repetitive yield was 99.4% after correction for the background determined as the apparent coupling to proline residues. The total weight gain was 50% of theoretical. Significant coupling problems were observed for residue 54 (Leu, 95.8%), residue 41 (Thr, 97.8%) and residue 4 (Thr, 96.9%).

Synthesis ABI-2 was performed by Applied Biosystems in its laboratory using coupling cycles in the presence of NMP protocol as shown in table 4.2. Boc-Ala-PAM resin (549mg, 0.417mmol) was subjected to 69 couplings of the IGF-I sequence and the resin samples (total resin removed was 0.131mmol) subjected to quantitative ninhydrin analysis. This indicated that the average repetitive yield was 99.6% after correction for the background in the ninhydrin analysis. The only significant coupling problem occurred with residue 4 (Thr) which coupled in 98.1% yield despite double coupling. The weight of peptide-resin obtained was 2.80g (75% of theoretical).

Both resin-peptides were deprotected and cleaved from the resin by Mr. Bruce May using the TFMSA/TFA protocol. The crude products were analysed by reverse-phase HPLC as shown in figure 4.2. A major protein peak corresponding to IGF-Acm was observed in both cases. Comparison of the crude cleavage products indicated that the ABI-2 synthesis had given a better yield of the desired product than the BIO-2 synthesis and that there was also better separation from the failure sequence peptides. In both cases, the desired peptide

was purified by reverse-phase HPLC and dried under vacuum using a Speed-Vac concentrator.

Amino-terminal sequence analysis of the purified Acm-derivatives of the (ABI-2) and (BIO-2) IGF-I peptides was performed for 10 cycles and indicated a purity of 95%. Recombinant IGF-I was found to be approximately 80% pure by this method. The natural IGF-I was not sequenced on this occasion but this material had been purified to apparent homogeneity by a method which routinely gives IGF-I of greater than 95% purity by sequence analysis.

For the purpose of comparing the quality of the ABI-2 and BIO-2 synthetic products, samples of the synthetic Acm-protected IGF peptides (1mg each) were treated to remove the Acm protecting groups from the cysteine residues. Although this method generates an oxidized product, the majority of the material lacked the correct cystine bridges and did not comigrate with authentic IGF-I on analytical reverse-phase HPLC. It therefore required reduction and reoxidation.

IGF-I from preparations ABI-2 and BIO-2 was reduced and subjected to oxidation as described in table 3.4. After 18h incubation under conditions favouring the formation of the correct disulphide bonds, the IGFs were chromatographed as shown in figure 4.3 and samples of fractions corresponding to the major area of protein were tested for biological activity using the L6 protein synthesis bioassay. The protein which comigrated with authentic IGF-I was found to be the most biologically active species. The material which eluted as a discrete peak before the IGF-I probably corresponds to a peptide which is fully oxidized but has apparently has non-native disulphide bonds as observed by Saito *et al.* (1987). These fractions could be reduced and oxidized to produce some IGF peptide that would then comigrate with native IGF-I. Later-eluting material gave a poor yield of correctly-folded IGF when subjected to a second cycle of oxidation. This may be due to incomplete deprotection of the cysteine residues or this material may be enriched in failure sequence peptides or modified peptides. The most biologically active material was rechromatographed on reverse-phase HPLC (fig. 4.4) and the protein contents of the peaks estimated. A summary of the step-wise yields for the production of the IGF-I peptides is given in table 4.3. The yield of active IGF-I from the resin was 0.5% in the case of the ABI-2 peptide and 0.6% in the case of the BIO-2 peptide.

4.3c IGF-I analogues from synthesis BIO-2

The major purposes of this synthesis were to produce des-(1-3)-IGF-I in order to confirm that the enhanced biological potency of this peptide represents a true molecular property of the peptide (ch. 5) and to examine other truncated analogues of IGF-I for enhanced biological activity (ch. 6).

As shown in figure 4.1, half the resin-bound peptide was removed after 29 cycles of coupling and the remainder was used to produce IGF-I and des-(1-3)-IGF-I. Typical results from the cleavage and oxidation reactions for IGF-I are shown in figures 4.2b and 4.3b. Both peptides gave major peaks of target peptide after cleavage from the resin which could be converted into correctly-oxidized IGF by the procedures described in table 3.4. The isolated IGF-I peptides were rechromatographed on reverse-phase HPLC and used for the studies reported in chapter 5.

The resin-bound peptide which had been removed after cycle 29 and stored under DMF at -20°C was used to produce further analogues of IGF-I (see fig. 4.1). The resin-bound peptide was subjected to 40 further couplings with resin samples of approximately 300mg being removed after cycles 63-69 of the IGF-I sequence. These were used for the production of truncated IGF-I peptides which were studied biochemically as reported in chapter 6. The chromatograms of the crude oxidized peptides are shown in figure 4.5 and the most biologically active material was rechromatographed as shown in figure 4.6. Some of the remaining resin was subjected to further, manual couplings using the Fmoc procedure to produce des-(2-5)-IGF-I, des-(2-4)-IGF-I, des-(2,3)-IGF-I and [Gly^3]IGF-I which were used for some of the biochemical studies reported in chapter 7. Reverse-phase chromatograms of these purified peptides are shown in figure 4.7(a-d). Des-(1-3)-IGF-I resin-bound peptide from the first half of the synthesis was subjected to additional manual couplings using the Boc method to produce [Asp^3]IGF-I, [Gln^3]IGF-I, [Leu^3]IGF-I, [Lys^3]IGF-I and [Phe^3]IGF-I which were used for further biochemical studies reported in chapter 7. Reverse-phase chromatograms of these purified peptides are shown in figure 4.7(e-i).

4.3d Synthesis BIO-3

This synthesis was performed in order to make a family of IGF-I analogues bearing substitutions at positions three and/or four. Boc-Ala-PAM resin was subjected to 65

cycles of coupling of the IGF-I sequence before portions were removed for the production of IGF-I analogues as shown in figure 4.8. The conditions of synthesis were essentially the same as for the BIO-2 synthesis (table 4.2). Peptides were cleaved from the resin using TFA/TFMSA, deprotected on cysteine using iodine and oxidized to form the correct disulphide bonds. Analytical chromatograms of the oxidized IGF peptides indicated that the peptides had refolded to a similar extent to that seen with the IGF-I preparations from the BIO-2 synthesis (figs. 4.3b, 4,5a) except for the des-(2-4)-IGF-I for which only a small peak of bioactive peptide was recovered, poorly resolved from incorrectly oxidized peptides, and for the des-(2-5)-IGF-I which exhibited a chromatographic profile similar to that of des-(1-5)-IGF-I and had correspondingly low biological activity. After preparative chromatography of the oxidation products, peptide corresponding to correctly-oxidized IGF was rechromatographed for estimation of protein amount as shown in figure 4.9. In some cases where separation of the correctly-folded IGF species from contaminants proved difficult, a sample of oxidized peptide was reduced and subjected to a second round of oxidation. A higher percentage yield of correctly-oxidized IGF was normally observed and these preparations were probably of increased purity. An example of this for the IGF-I peptide is shown in figure 4.10.

4.3e Purification of the synthetic IGF-I peptides

For the routine production of IGF-I peptides, purification was carried out in three steps. First, the crude cleavage product was chromatographed on reverse-phase HPLC to remove substantial quantities on non-peptide material and to achieve separation from some failure-sequence peptides. Analytical chromatograms of IGF-I cleavage products are shown in figure 4.2. Second, further purification was achieved by reverse-phase HPLC following removal of the Acm protecting groups and reduction of the peptide. Third, the refolded and oxidized peptide was chromatographed on reverse-phase HPLC to separate correctly-oxidized IGF from incorrectly-oxidized forms. An example of this is shown in figure 4.3.

4.3f Biochemical comparison of synthetic, natural and recombinant IGF-I peptides

In order to estimate the protein contents of samples of natural IGF-I, recombinant IGF-I and the synthetic IGF-I peptides, they were chromatographed by reverse-phase HPLC (fig. 4.4). The protein contents of the peaks corresponding to IGF-I were determined by u.v. absorbance measurement.

The natural, recombinant and synthetic IGF-I peptides were subjected to radioimmunoassay as shown in figure 4.11. All the IGFs were able to displace at least 90% of the specifically bound radioligand. The potencies (measured as the concentration required to cause 50% displacement of radioligand) were natural IGF-I (180pg/ml), recombinant IGF-I (190pg/ml), ABI-2 IGF-I (280pg/ml) and BIO-2 IGF-I (320pg/ml).

In order to assess the utility of the synthetic forms of IGF-I as analogues of natural IGF-I, I firstly tested the abilities of the IGFs to bind to specific IGF-I cell-surface receptors using a radioreceptor assay as shown in figure 4.12. All the IGFs were able to displace at least 80% of the radioligand bound. The potencies of the various IGFs, determined as the concentration required to cause 50% competition for radioligand binding, were ABI-2 IGF-I (20ng/ml), recombinant human IGF-I (21ng/ml), natural IGF-I (22ng/ml) and BIO-2 IGF-I (24ng/ml). This demonstrates that the two synthetic forms of IGF-I have folded and oxidized to give a product with an essentially native conformation which is therefore able to bind to the type 1 IGF receptor with high affinity.

On binding to its receptor, IGF-I elicits a pleiotropic response leading to cell growth. I therefore tested the abilities of the IGF-I peptides to stimulate protein synthesis in rat L6 myoblasts as shown in figure 4.13. Each of the IGFs was able to stimulate protein synthesis to a level similar to that achieved by a maximally-stimulating (5%) concentration of foetal bovine serum. The potencies, determined as the concentration of peptide required to produce an effect equal to 50% of that caused by 5% foetal bovine serum were recombinant human IGF-I (10ng/ml), ABI-2 IGF-I & natural IGF-I (12ng/ml) and BIO-2 IGF-I (14ng/ml).

I also tested the abilities of the IGF-I peptides to compete with tracer IGF-I for binding to a purified form of the IGF-binding protein produced by MDBK cells in culture as shown in figure 4.14, since this protein is well-characterized (Szabo *et al.*, 1988; Forbes *et al.*, 1988). The potencies of the various IGF-I peptides, determined as the concentration

required to cause 50% competition for radioligand binding, were ABI-2 IGF-I (5.7ng/ml), BIO-2 IGF-I (8.3ng/ml), natural and recombinant human IGF-I (9.2ng/ml).

4.4 DISCUSSION

4.4a Production of IGF-I by chemical synthesis

A major challenge in peptide synthesis is to make long peptides quickly, cheaply and of high purity. In collaboration with Applied Biosystems Inc., we wished to study the efficacy of NMP as a solvent for the automated synthesis of a relatively large peptide (70 amino acid residues). IGF-I is a protein of considerable interest and serves as a model for proteins whose structures rely on the presence of intramolecular disulphide bridges. We chose to use TFA/TFMSA for cleavage of the synthetic peptide from the resin in order to avoid difficulties in handling HF and because it has been reported to be useful in avoiding some of the possible side reactions which may result from the use of HF (Tam *et al.*, 1986).

NMP is a very useful solvent in solid phase peptide synthesis because it is able to effectively solvate the growing peptide chain and thus allow for more complete coupling. The NMP protocol in table 4.2 also includes the addition of dimethylsulphoxide (DMSO) and diisopropylethylamine (DIEA) to the NMP coupling solution near the end of the coupling step. The DMSO and DIEA additions also help to increase the coupling efficiencies, especially on syntheses with difficult sequences again by helping to break up the self-aggregation of peptides (Toniolo *et al.*, 1985). Usually this NMP/DMSO/DIEA approach gives couplings above 99%. Occasionally, 94-98% couplings do occur and these failed couplings are capped with acetic anhydride. On a short peptide (less than 30 residues), this approach of single couplings and capping almost always leads to a successful synthesis. On syntheses of peptides with more than 30 residues, it is advantageous to double-couple these poor couplings, which is why residue 4 (Thr) was double-coupled.

The weight gain for the synthesis in NMP was 75% of theoretical as compared with the 87% weight gain obtained by Li *et al.* (1983). This might indicate that more amino acids should have been double coupled on this long synthesis in order to get a higher weight gain.

Both syntheses, described in Table 4.2, were performed in an automated instrument with ninhydrin analysis being used to examine the progress of the syntheses. The

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NMP protocol (ABI-2) was found to give a higher average repetitive yield (99.6%) than the protocol using double-coupling in DMF and DMF/DCM (BIO-2) which gave an average repetitive yield of 99.4%.

Comparison of the crude cleavage products indicated that the ABI-2 synthesis had given a better yield of the desired product than the BIO-2 synthesis and that there was a better separation from the failure sequence peptides.

The removal of the Acm protecting groups from the cysteine residues required the use of extended reaction times in order to obtain a nearly homogeneous product after reduction. This may be due to either (i) the high number of cysteine residues (6 per molecule) present which require quantitative removal, or to (ii) problems relating to the solubility of the peptide and thus its accessability to the reagent.

The two synthetic peptides exhibited similar abilities to oxidize correctly to the native structure, as assessed by retention time on reverse-phase HPLC and by their bioactivities. The extent of correct folding (10-15%) is less than that normally seen using reduced natural IGF-I (30-40%) (see figs. 3.4a & 4.4) suggesting that failure sequence- or modified peptides may be present.

In the biochemical assays, I compared the synthetic IGF-I peptides with recombinant human IGF-I and with bovine IGF-I which has an identical amino acid sequence to human IGF-I (Honegger & Humbel, 1986). Both synthetic IGFs showed qualitatively similar effects in the radioreceptor assay, the bioassay and the MDBK binding protein competitive binding assay, although preparation ABI-2 was approximately 20% more potent than preparation BIO-2. This may have resulted in part from an error in the estimation of the amount of IGF in preparation BIO-2 or from contaminating less active peptides since this material was less well separated from contaminants than the IGF from preparation ABI-2. From these results, both synthetic IGF-I peptides were found to (i) be full agonists of natural IGF-I, (ii) exhibit at least 80% of its potency in the bioassay, (iii) closely mimic its interactions with the type 1 IGF receptor and with the IGF-binding protein purified from the medium conditioned by MDBK cells. Other workers have found synthetic IGF-I to be identical to human IGF-I by radioimmunoassay and radioreceptor assay (Li *et al.*, 1983) but only 60% as potent in an IGF-binding protein competitive binding assay (Schalch *et al.*, 1984).

4.4b Production of analogues of IGF-I

In the course of this project, IGF-I, des-(1-3)-IGF-I and 20 novel IGF-I analogues were produced by solid phase peptide synthesis. No special problems were encountered with these peptides in the chain assembly or during the cleavage from the resin. However, not all the peptides folded correctly to form the correct product during the oxidation reaction. The peptides, des-(1-6)-IGF-I and des-(1-5)-IGF-I, did not produce a discrete peak of peptide with the chromatographic properties expected of correctly oxidized IGF-I peptides and thus were probably not correctly-folded. The des-(2-5)-IGF-I peptide refolded in low yield to produce a peptide that was difficult to purify. Des-(1-6)-IGF-I lacks a cysteine residue and therefore cannot oxidize correctly whereas des-(1-5)-IGF-I and des-(2-5)-IGF-I both lack residue Leu^5 which is highly conserved in insulin and the IGFs and may be involved in the hydrophobic core of the IGF molecule (Blundell *et al.*, 1983; Cooke, 1988). For each analogue that was able to form a correctly-oxidized IGF species as determined by analytical reverse-phase HPLC, a corresponding peak of biologically active material was observed. After further purification and quantitation, these peptides were able to be characterized biochemically as will be described in later chapters.

The purification of the synthetic peptides presented some difficulties since only reverse-phase HPLC was found to be suitable for the purification of peptides at all stages of preparation. Attempts to use ion exchange methods for the purification of reduced peptides prior to the oxidation step were hampered by low recoveries of peptide. The reduced IGF peptide also exhibited somewhat poor chromatographic behaviour on reverse-phase HPLC since the application a second gradient of organic solvent routinely eluted a peak of reduced IGF equivalent to 3% of that previously observed. Some resolution of disulphide-bond isomers produced during the oxidation reaction could be achieved by chromatofocussing (fig. 4.15) but this method was not superior to reverse-phase HPLC. For the routine preparation of IGF peptides only reverse-phase HPLC was used.

Table 4.1: List of syntheses

Biochemistry Department

BIO-1	IGF-I
BIO-2	IGF-I
	des-(1)-IGF-I
	des-(1,2)-IGF-I
	des-(1-3)-IGF-I
	des-(1-4)-IGF-I
	des-(1-5)-IGF-I
	des-(1-6)-IGF-I
	des-(2,3)-IGF-I
	des-(2-4)-IGF-I
	des-(2-5)-IGF-I
	[Gly^3]IGF-I
	[Asp^3]IGF-I
	[Gln^3]IGF-I
	[Leu^3]IGF-I
	[Lys^3]IGF-I
	[Phe^3]IGF-I
BIO-3	IGF-I
	des-(1-3)-IGF-I
	des-(2-4)-IGF-I
	des-(1-3)-[Val^4]IGF-I
	des-(1-3)-[Arg^4]IGF-I
	[Gly^3]IGF-I
	[Arg^3]IGF-I
	[Gly^3, Gly^4]IGF-I
	[Gly^3, Arg^4]IGF-I

Applied Biosystems Inc.

ABI-1	IGF-I
ABI-2	IGF-I

Table 4.2: Synthetic protocols

<table>
<tr><th colspan="2">Synthesis ABI-2</th><th colspan="2">Synthesis BIO-2</th></tr>
<tr><td colspan="2">Deprotection</td><td colspan="2">Deprotection</td></tr>
<tr><td>30% TFA in DCM</td><td>3min</td><td>30% TFA in DCM</td><td>3min</td></tr>
<tr><td>50% TFA in DCM</td><td>16min</td><td>50% TFA in DCM</td><td>17min</td></tr>
<tr><td colspan="2">Neutralization</td><td colspan="2">Neutralization</td></tr>
<tr><td>DCM washes</td><td>(5 times)</td><td>DCM washes</td><td>(3 times)</td></tr>
<tr><td>5% DIEA in NMP</td><td>(2 times)</td><td>5% DIEA in DMF</td><td>(2 times)</td></tr>
<tr><td>NMP washes</td><td>(5 times)</td><td>DMF washes</td><td>(6 times)</td></tr>
<tr><td colspan="2">Coupling</td><td colspan="2">Coupling</td></tr>
<tr><td>Single couple with preformed HOBt ester in NMP</td><td>30min</td><td>First couple with preformed symmetric anhydride in DMF</td><td>40min</td></tr>
<tr><td>with 20% DMSO</td><td>16min</td><td>DMF washes</td><td>(3 times)</td></tr>
<tr><td>with 4.7 equivalents DIEA</td><td>7min</td><td>5% DIEA/DMF</td><td>(1 time)</td></tr>
<tr><td colspan="2">Resin sampling</td><td>Second couple with preformed symmetric anhydride in DCM</td><td>30min</td></tr>
<tr><td colspan="2">Capping</td><td>DCM washes</td><td>(7 times)</td></tr>
<tr><td>10% acetic anhydride and 5% DIEA in DCM</td><td>6min</td><td></td><td></td></tr>
<tr><td>DCM washes</td><td>(5 times)</td><td colspan="2">Resin sampling</td></tr>
</table>

Table 4.3: Summary of yields

ABI-2 Peptide			BIO-2 Peptide		
Step	peptide amount	cumulative yield (%)	Step	peptide amount	cumulative yield (%)
resin-peptide	84.2mg	100	resin-peptide	56mg	100
crude cleavage product	90.4mg		crude cleavage product	63.7mg	
purified Acm-peptide	14.4mg	30	purified Acm-peptide	8.7mg	27

1mg of each Acm-peptide was deprotected and reduced

purified reduced peptide	126μg	3.7	purified reduced peptide	174μg	4.7
reoxidized IGF-I after purification	16.7μg	0.5	reoxidized IGF-I after purification	16.7μg	0.6

Figure 4.1: Plan of the BIO-2 synthesis

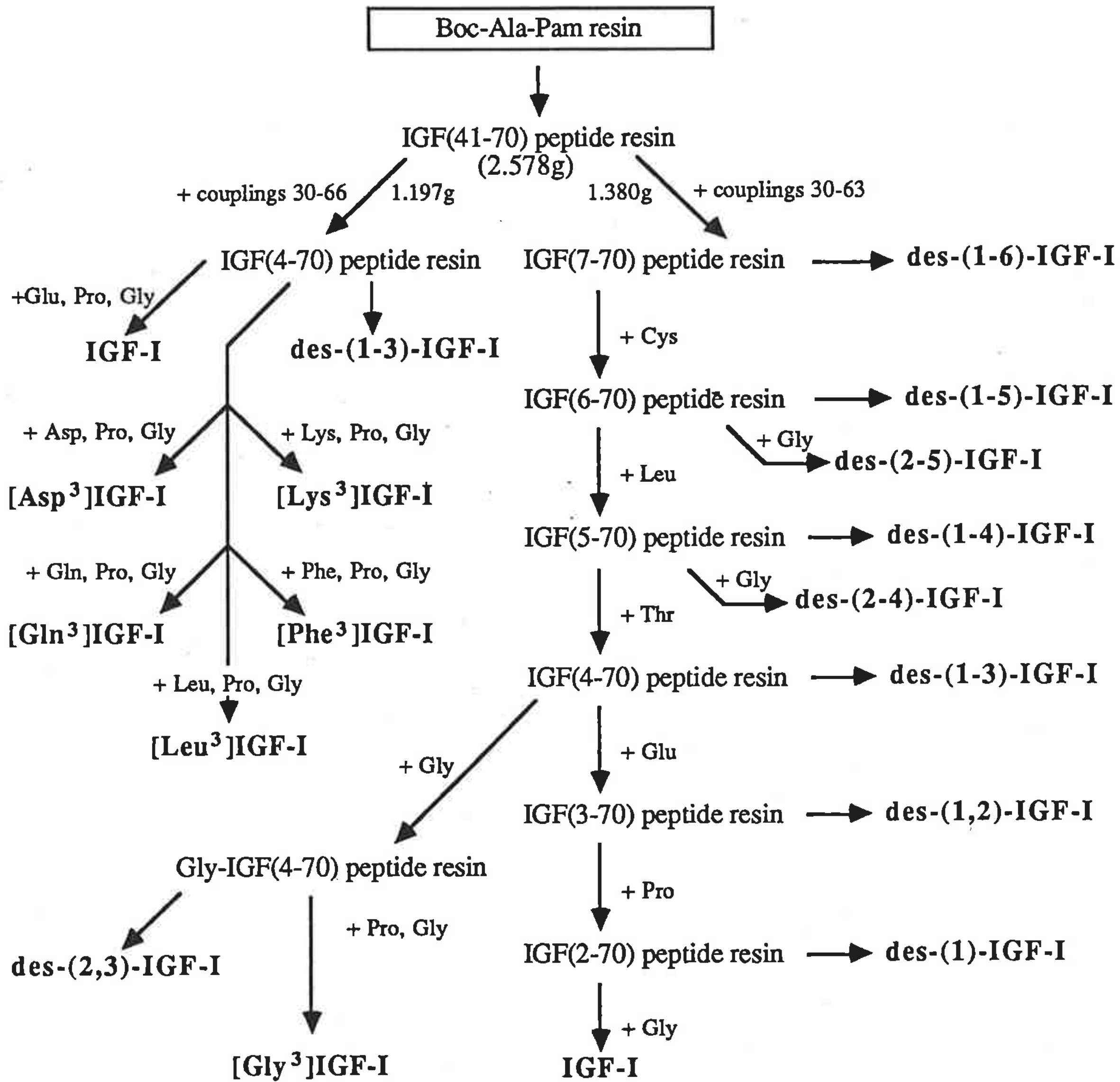
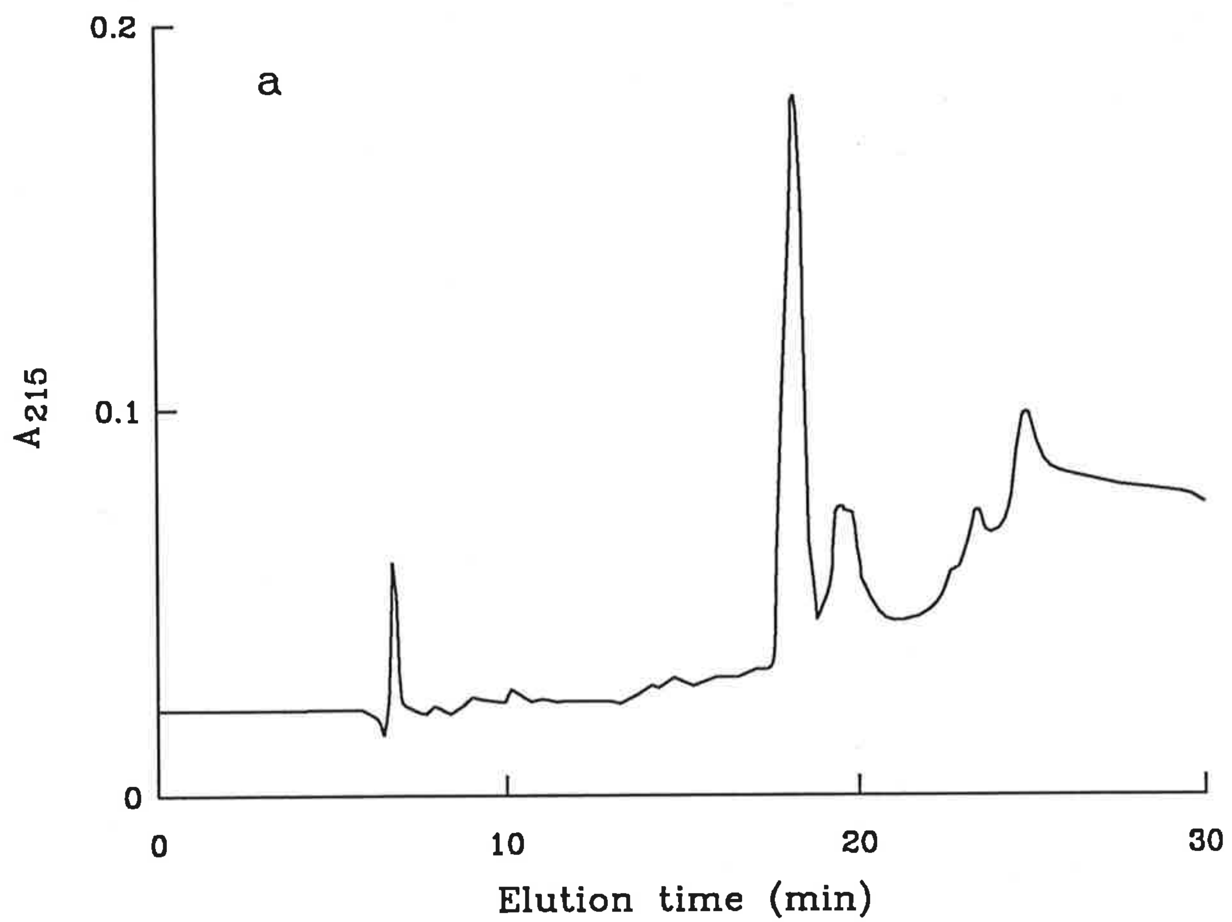


Figure 4.2: Reverse-phase HPLC of the crude cleavage products from the ABI-2 and BIO-2 syntheses of IGF-I

Samples of the crude products from the TFA/TFMSA cleavage reaction were analysed by reverse-phase HPLC and the A_{215} was monitored (—). *Panel a:* Crude cleavage products from synthesis ABI-2 were analysed on a 7.8 × 300mm μbondapak column at a flow rate of 1ml/min. Elution was effected by a linear gradient of acetonitrile from 0% to 60% over 30min in the presence of 0.1%TFA. *Panel b:* Crude cleavage products from synthesis BIO-2 were analysed on a 4.6 × 30mm butyl-silica column at a flow rate of 1ml/min. Elution was effected by a linear gradient of acetonitrile from 20% to 50% over 15min in the presence of 0.1%TFA.


0.2
a
0.1
A_{215}
0
0
10
20
30
Elution time (min)

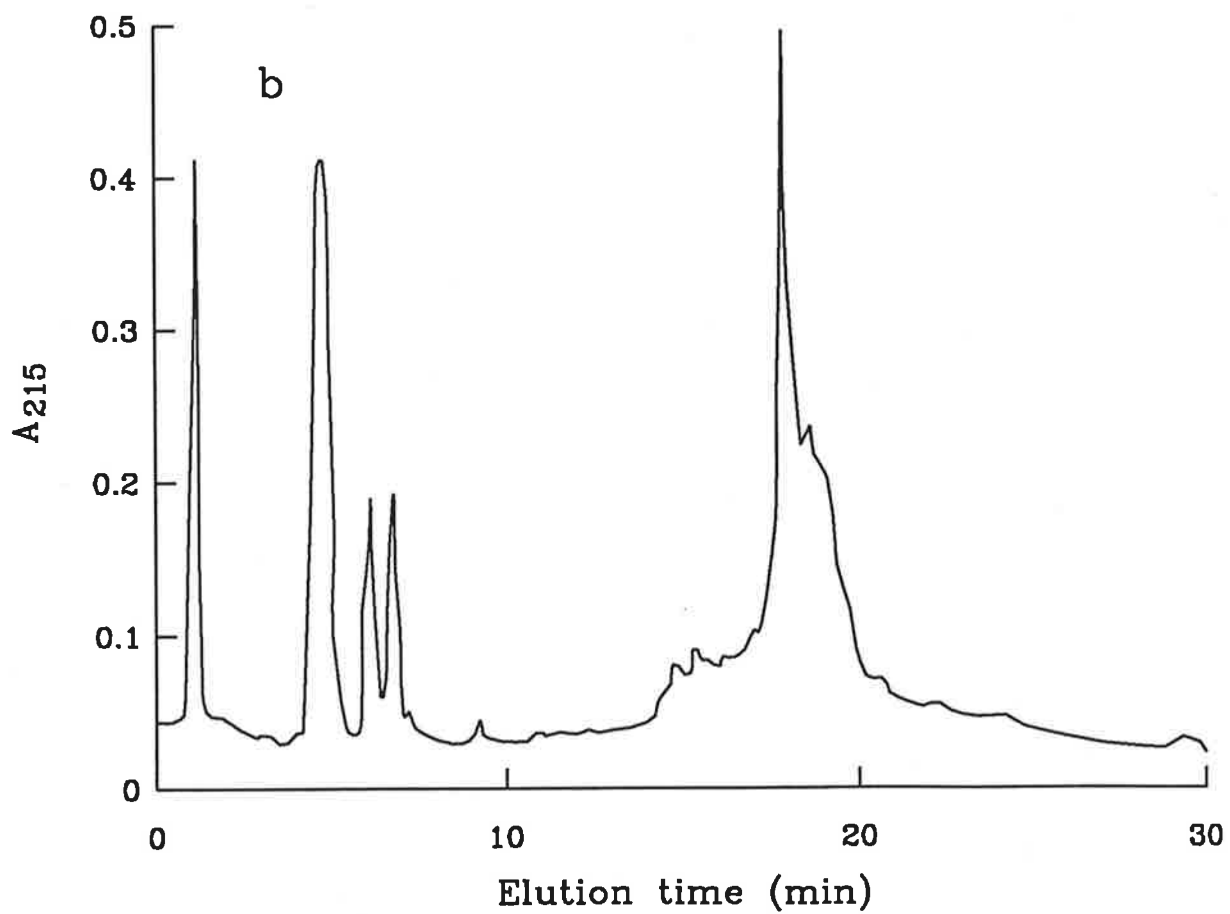
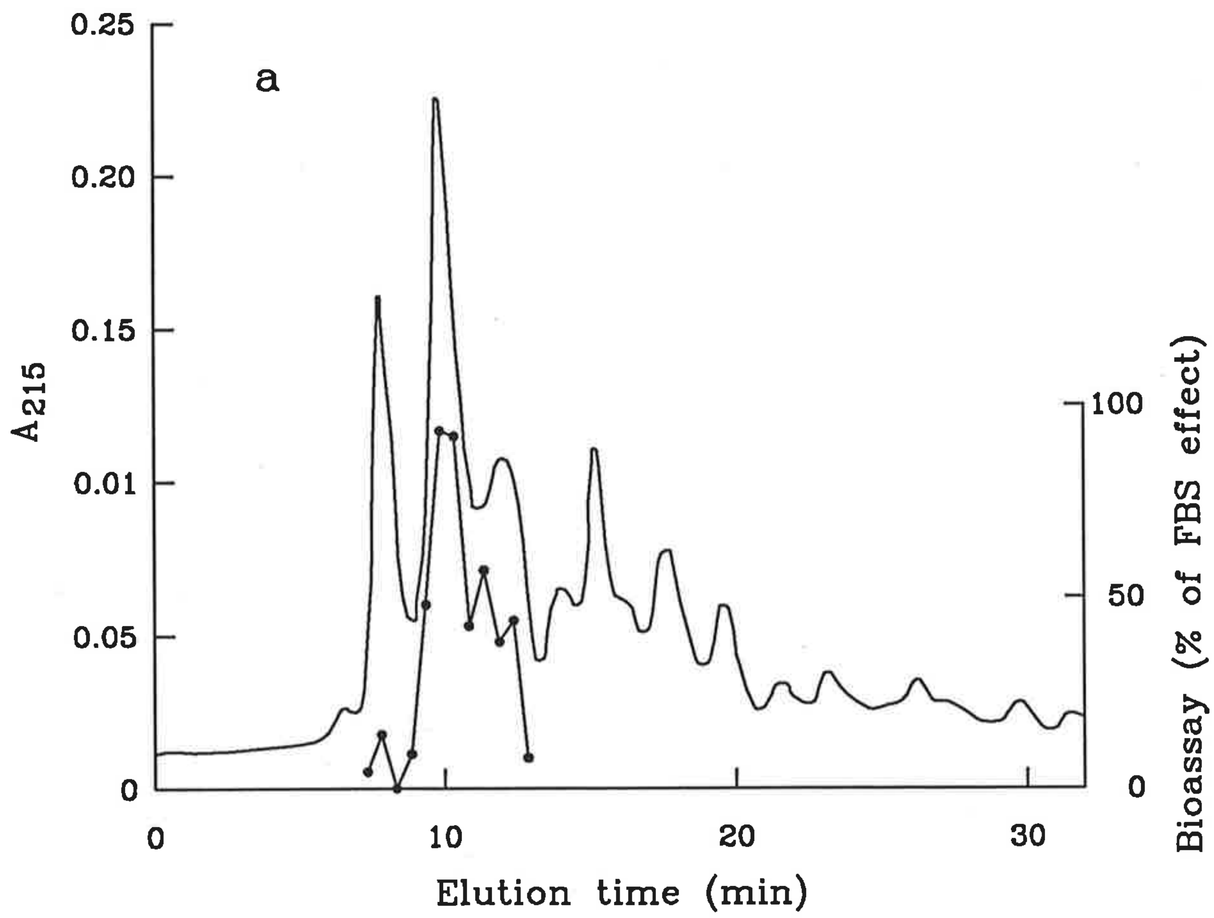

0.5
b
0.4
0.3
A_{215}
0.2
0.1
0
0
10
20
30
Elution time (min)

Figure 4.3: Reverse-phase HPLC of the crude oxidized IGF-I from syntheses ABI-2 and BIO-2

This was performed on a 4.6 × 30mm butyl-silica column at a flow rate of 2ml/min. Elution was achieved by a gradient of acetonitrile from 20 to 35% over 30min in the presence of 0.1% TFA (- -) and the A_{215} monitored (—). Fractions were collected and 4μl subsamples tested in the protein synthesis bioassay (●). Data are expressed as a percentage of the stimulation of protein synthesis caused by 5% foetal bovine serum, which was 2.5-fold over the incorporation of radiolabelled leucine into protein in the absence of added growth factor. *Panel a:* oxidized ABI-2 IGF-I.*Panel b:* oxidized BIO-2 IGF-I. The indicated fractions were pooled for rechromatography as shown in figure 4.4.


a
A215
0.25
0.20
0.15
0.01
0.05
0
0
10
20
30
Elution time (min)
100
50
0
Bioassay (% of FBS effect)

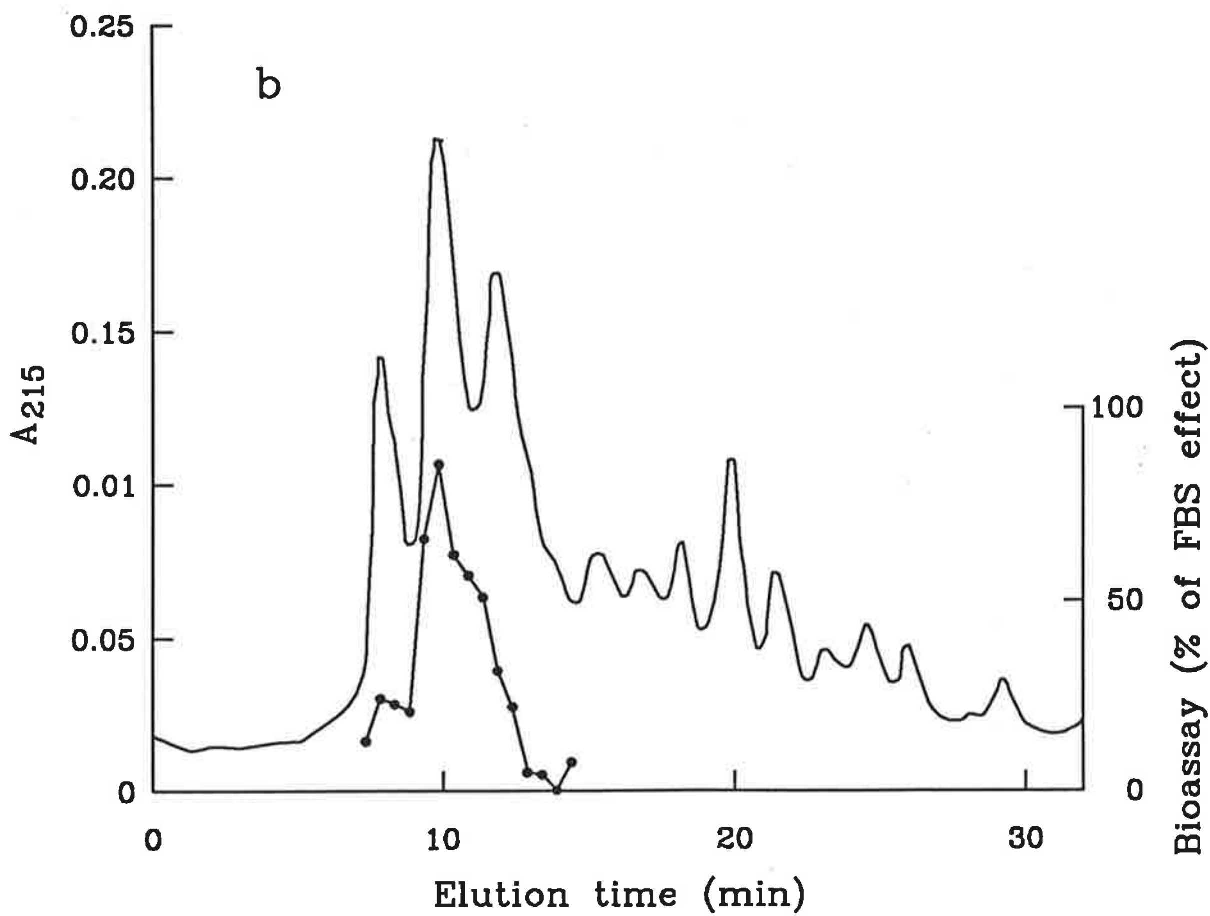

b
A215
0.25
0.20
0.15
0.01
0.05
0
0
10
20
30
Elution time (min)
100
50
0
Bioassay (% of FBS effect)

Figure 4.4: Reverse-phase HPLC of IGF-I peptides

Natural (a), recombinant (b), ABI-2 (c) and BIO-2 (d) IGF-I peptides from figure 4.3 were rechromatographed on a 4.6 × 30mm RP-300 column at a flow rate of 1ml/min. Elution was achieved using a gradient of acetonitrile from 20% to 50% in 15min and the A_{215} monitored (—). The indicated fractions were pooled for further use.

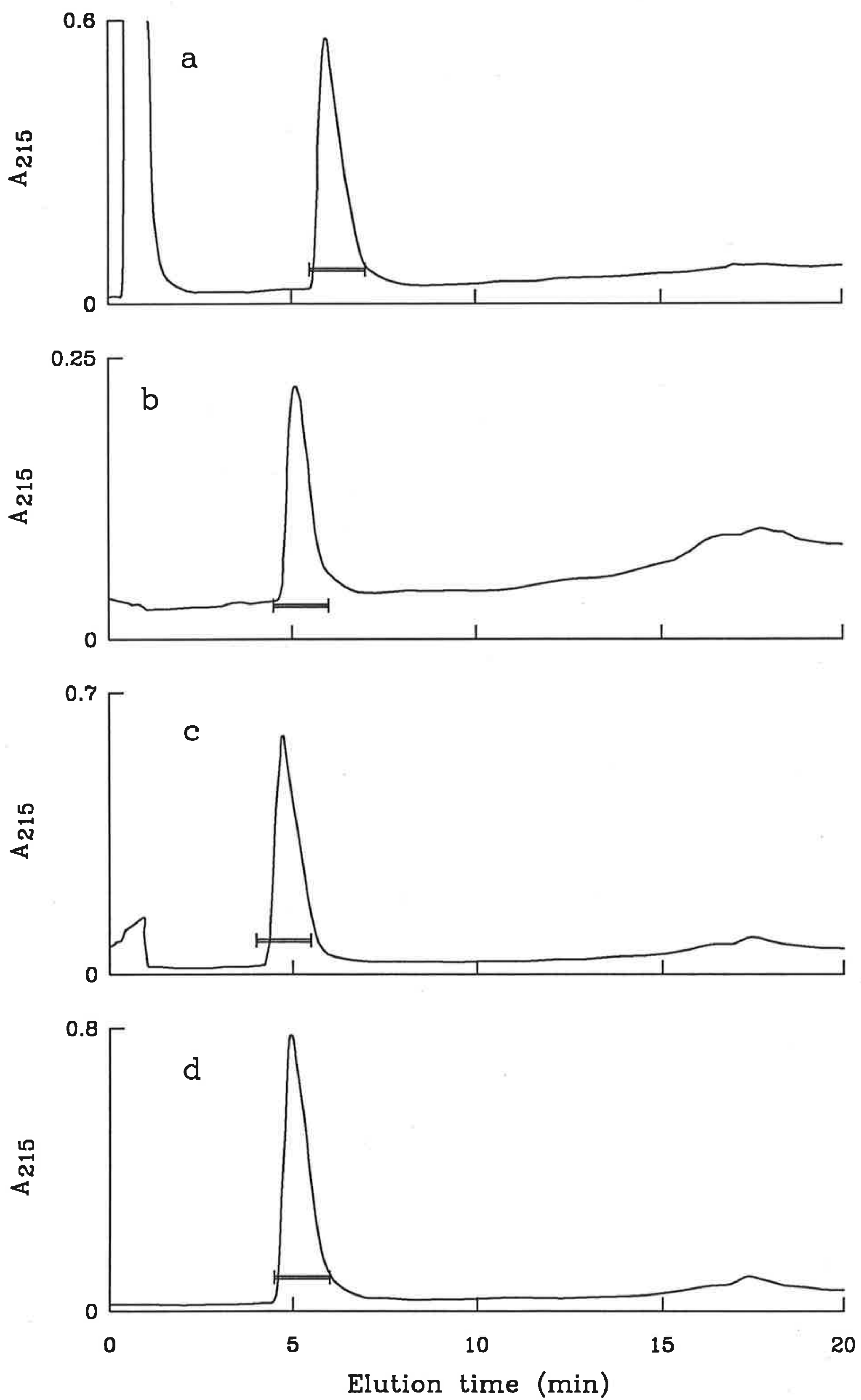
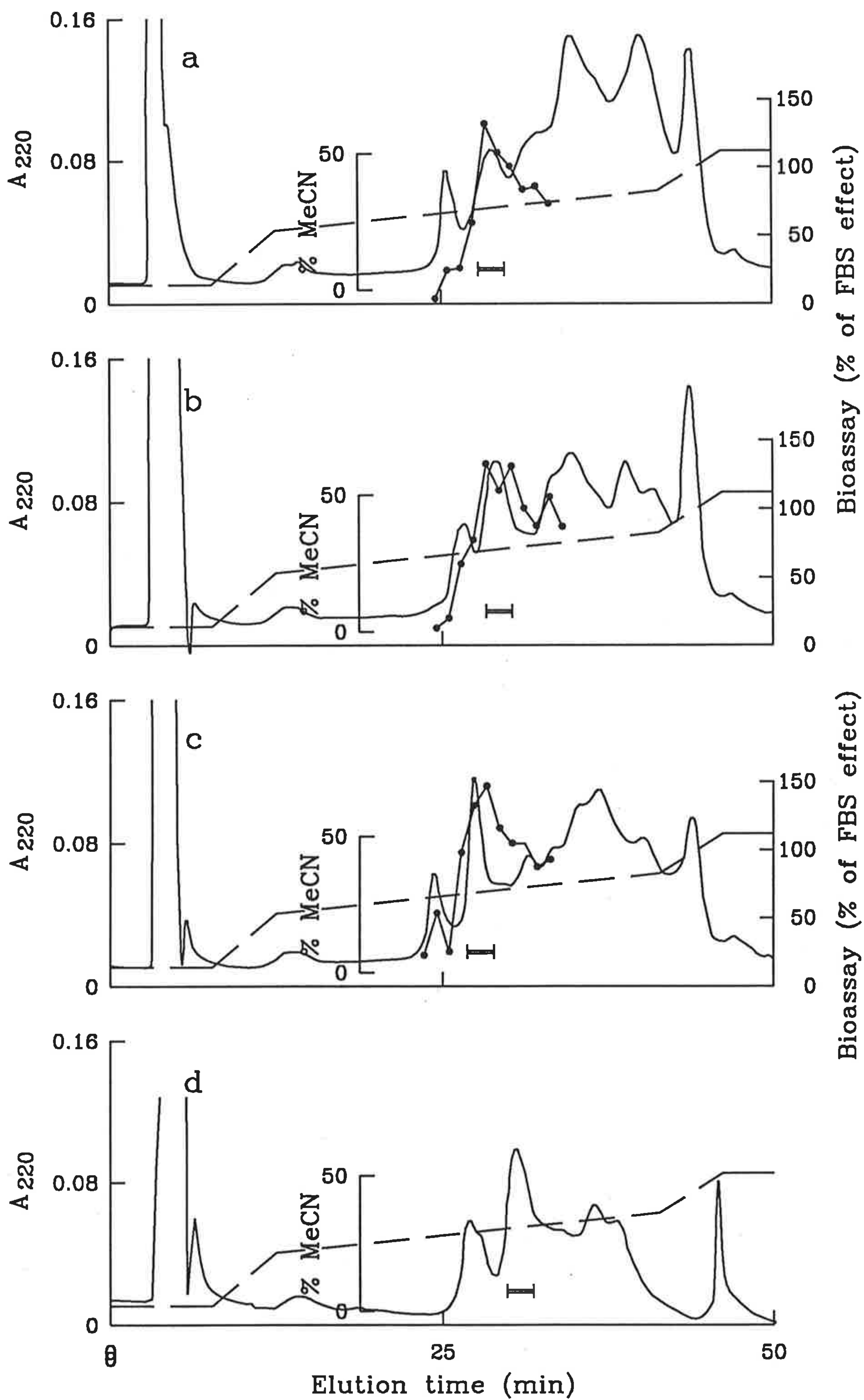
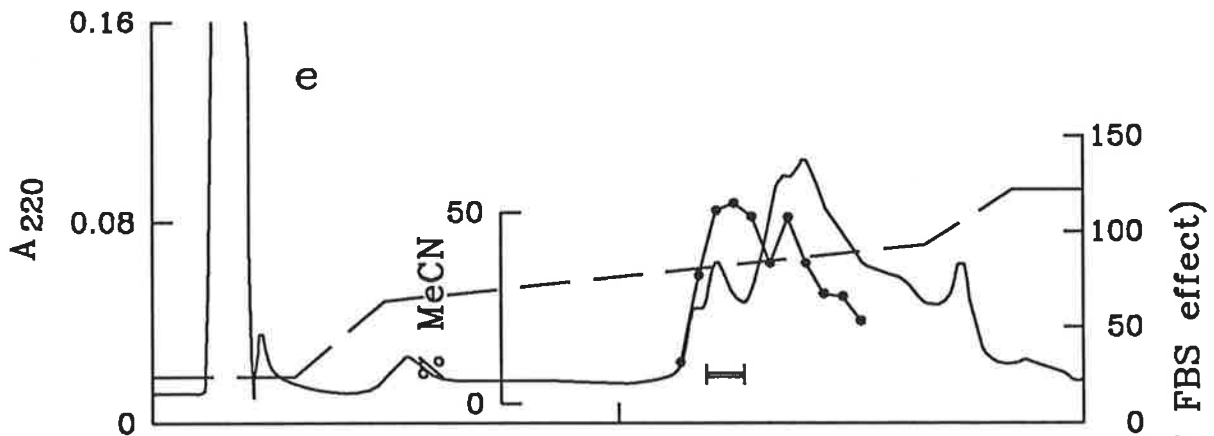

0.6
a
A215
0
0.25
b
A215
0
0.7
c
A215
0
0.8
d
A215
0
0
5
10
15
20
Elution time (min)

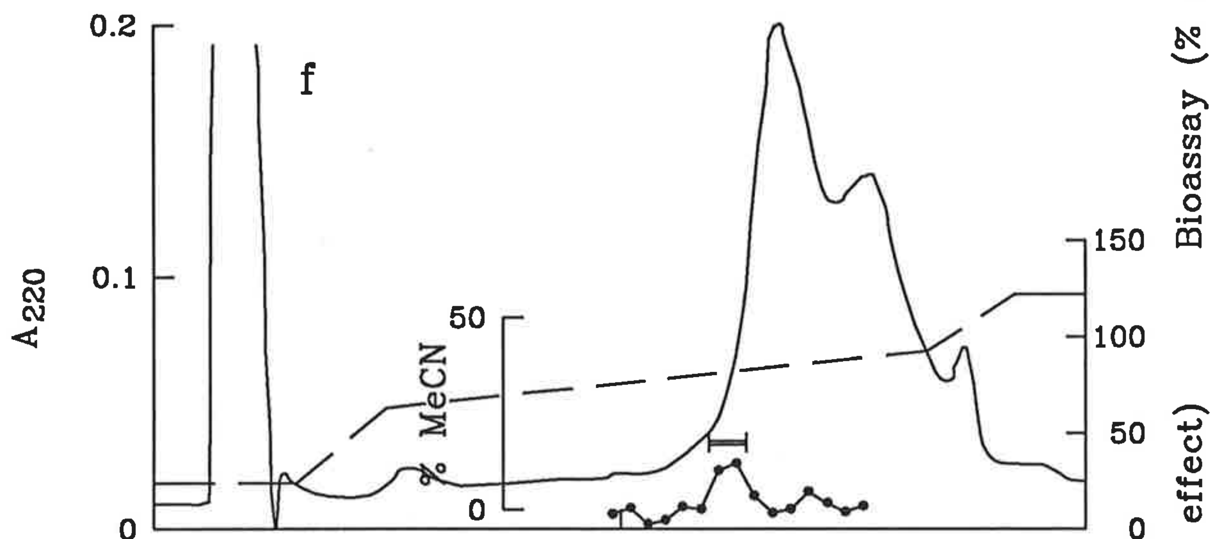
Figure 4.5: **Reverse-phase HPLC of the truncated oxidized IGF-I peptides from synthesis BIO-2**

The crude oxidized peptides were chromatographed on a 1 × 10cm butyl-silica column at a flow rate of 2ml/min. Elution was achieved by the indicated gradient of acetonitrile in the presence of 0.1% TFA (- -) and the A_{215} monitored (—). The peptides chromatographed were a) IGF-I, b) des-(1)-IGF-I, c) des-(1,2)-IGF-I, d) des-(1-3)-IGF-I, e) des-(1-4)-IGF-I, f) des-(1-5)-IGF-I and g) des-(1-6)-IGF-I. Fractions were collected and 10µl subsamples tested in the protein synthesis bioassay (●). Data are expressed as a percentage of the stimulation of protein synthesis caused by 5% foetal bovine serum. Incorporation of radiolabelled leucine into protein was 1.8% of added radioactivity in the absence of added growth factors and 4.6% in the presence of 5% FBS. The indicated fractions were pooled for rechromatography as shown in figure 4.6.

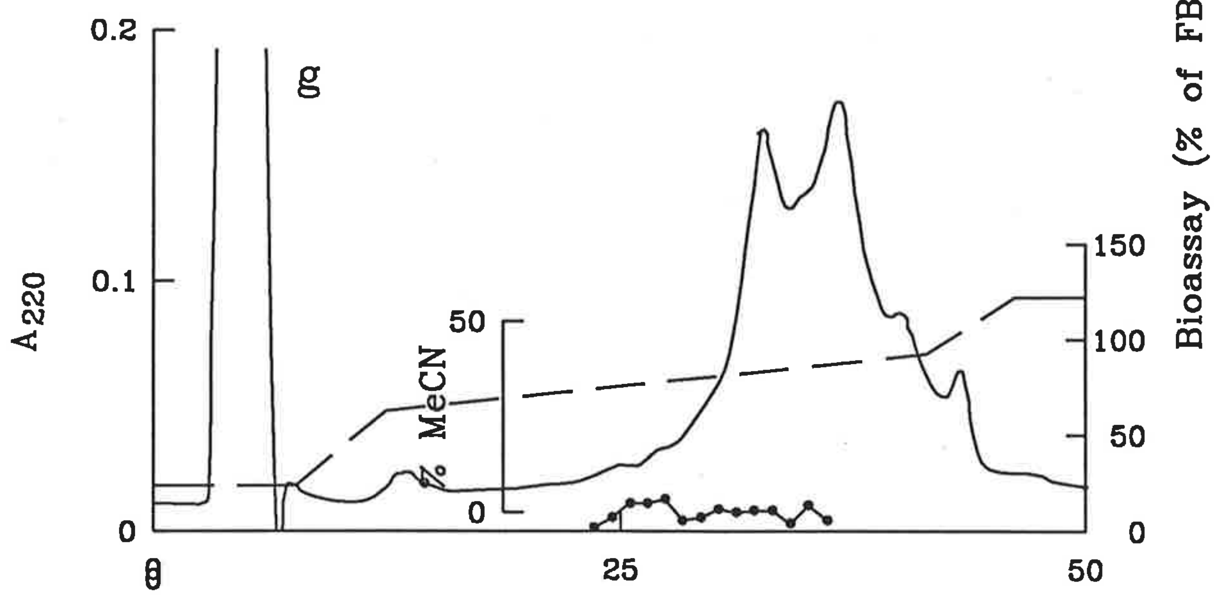

a
b
c
d
A 220
0.16
0.08
0
% MeCN
50
0
Bioassay (% of FBS effect)
150
100
50
0
25
50
Elution time (min)



e
A220
0.16
0.08
0
% MeCN
50
0
150
100
50
0



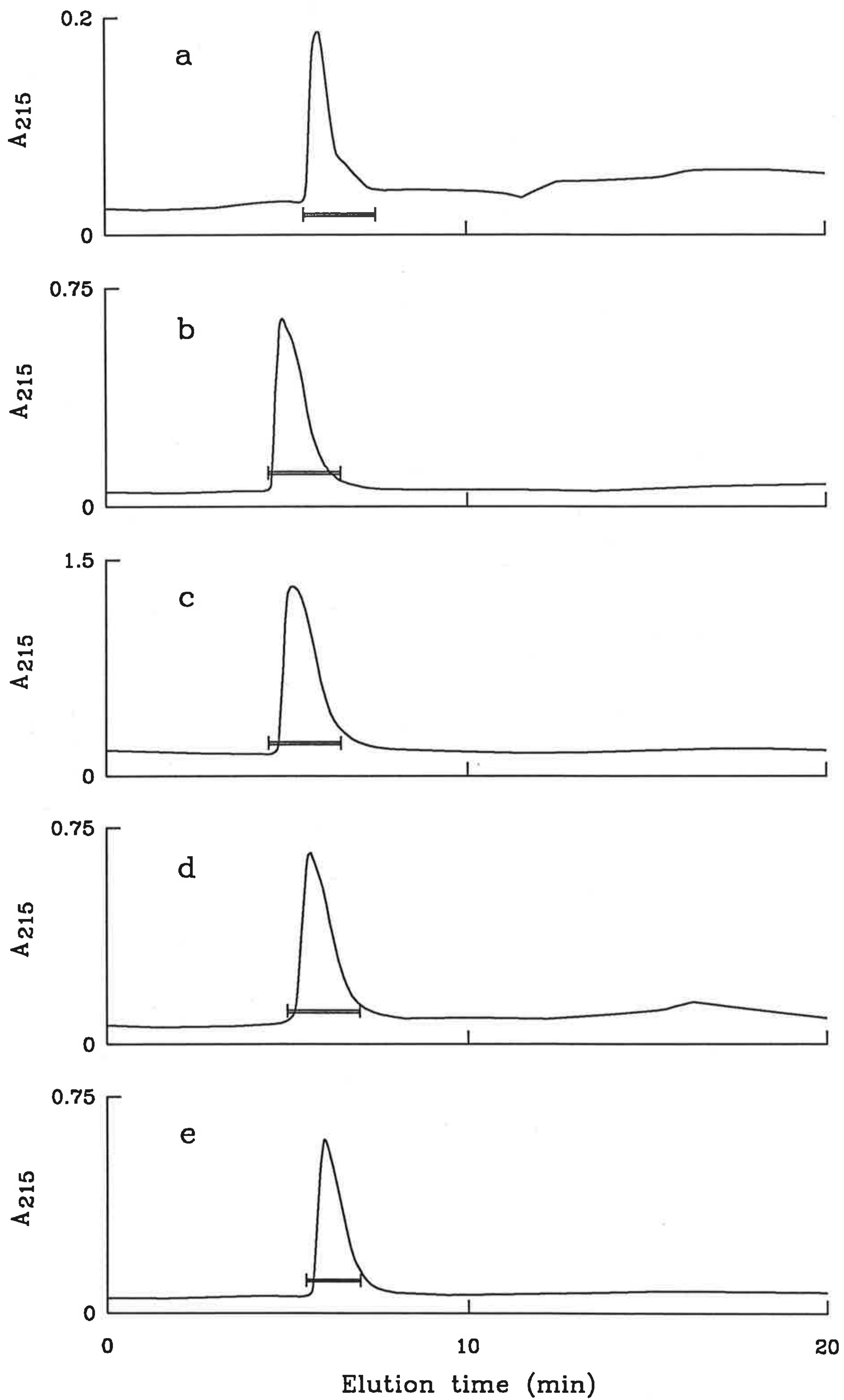
f
A220
0.2
0.1
0
% MeCN
50
0
150
100
50
0
Bioassay (% of FBS effect)



g
A220
0.2
0.1
0
% MeCN
50
0
150
100
50
0
Bioassay (% of FBS effect)
0
25
50
Elution time (min)

Figure 4.6: Reverse-phase HPLC of IGF-I peptides from figure 4.5

The IGF-I peptides from figure 4.5 (a-e) (denoted preparation A) as well as from a similar set of oxidation reactions (preparation B) were rechromatographed on a 4.6 × 30mm RP-300 column at a flow rate of 1ml/min. Elution was achieved using a gradient of acetonitrile from 20% to 50% in 15min and the A_{215} monitored (—). The samples from preparation A were a) IGF-I, b) des-(1)-IGF-I, c) des-(1,2)-IGF-I, d) des-(1-3)-IGF-I, and e) des-(1-4)-IGF-I, and from preparation B were f) IGF-I, g) des-(1)-IGF-I, h) des-(1,2)-IGF-I, i) des-(1-3)-IGF-I, and j) des-(1-4)-IGF-I. The indicated fractions were pooled and used for the biochemical studies reported in chapter 6.


0.2
a
A_{215}
0
0.75
b
A_{215}
0
1.5
c
A_{215}
0
0.75
d
A_{215}
0
0.75
e
A_{215}
0
0
10
20
Elution time (min)

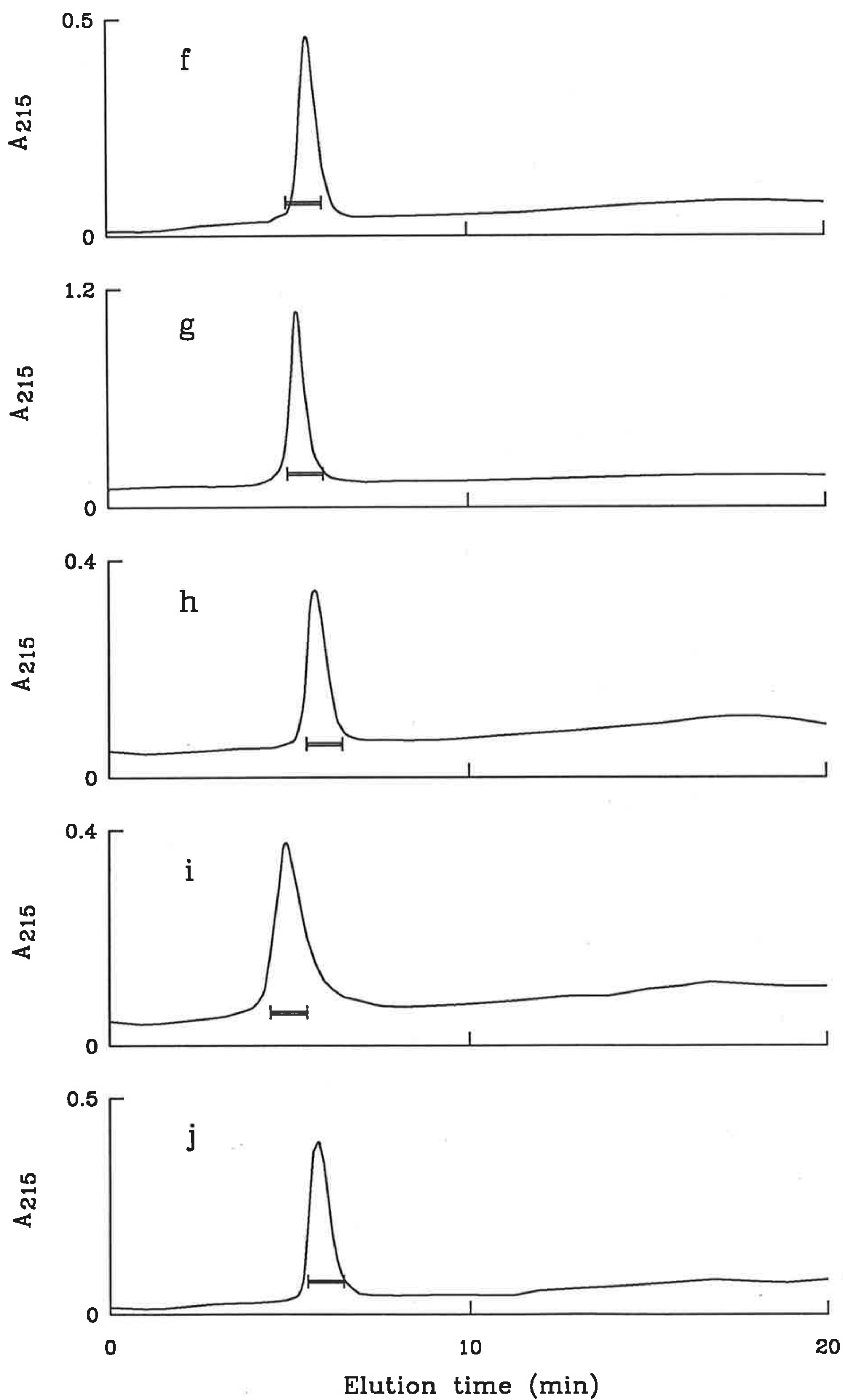
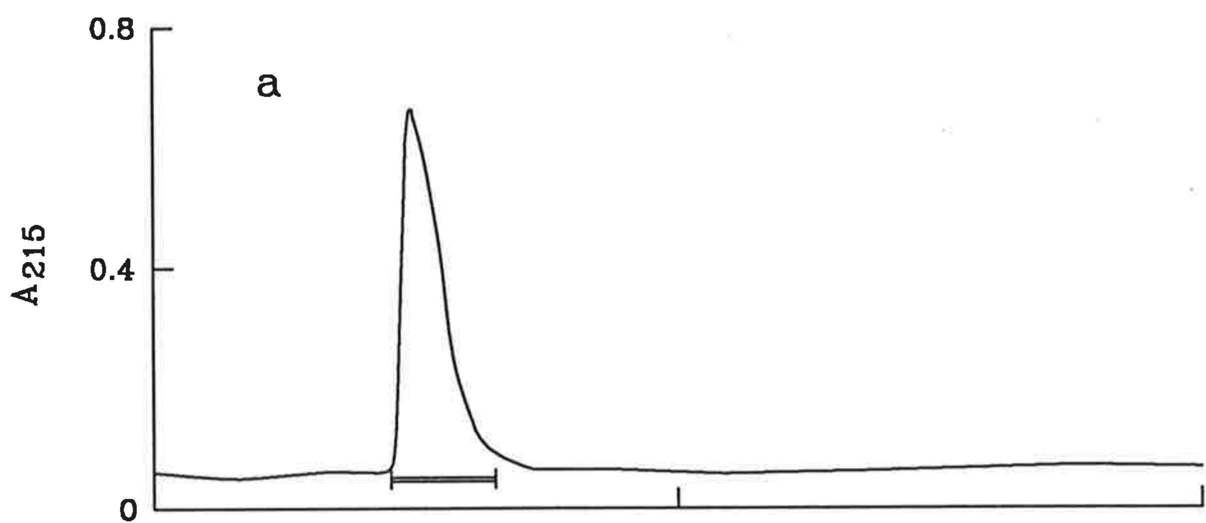
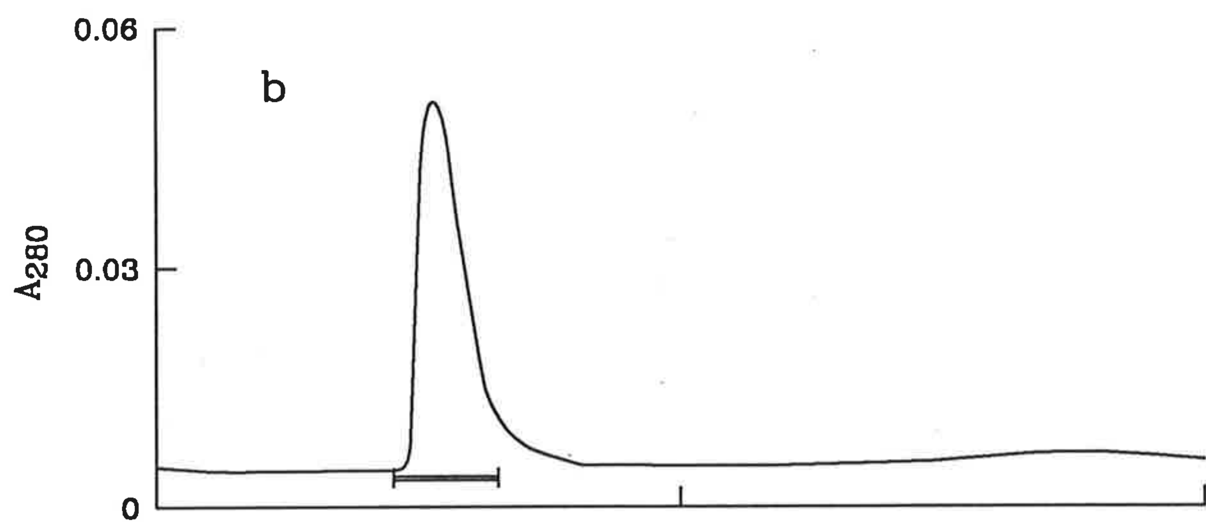
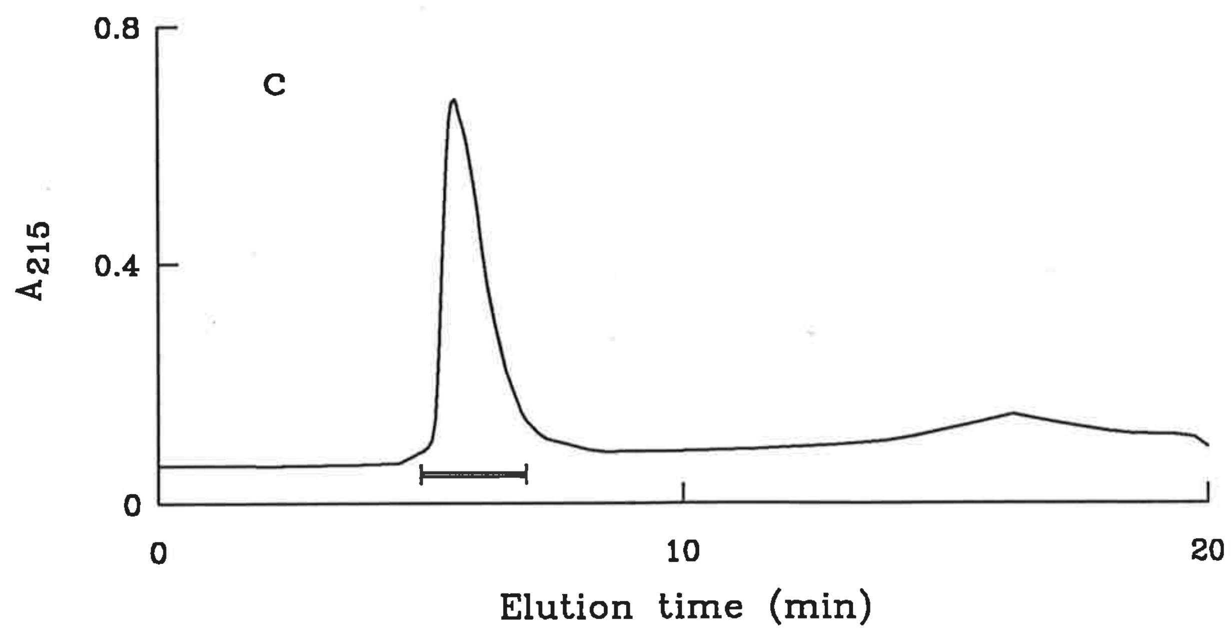

0.5
f
A215
0
1.2
g
A215
0
0.4
h
A215
0
0.4
i
A215
0
0.5
j
A215
0
0
10
20
Elution time (min)

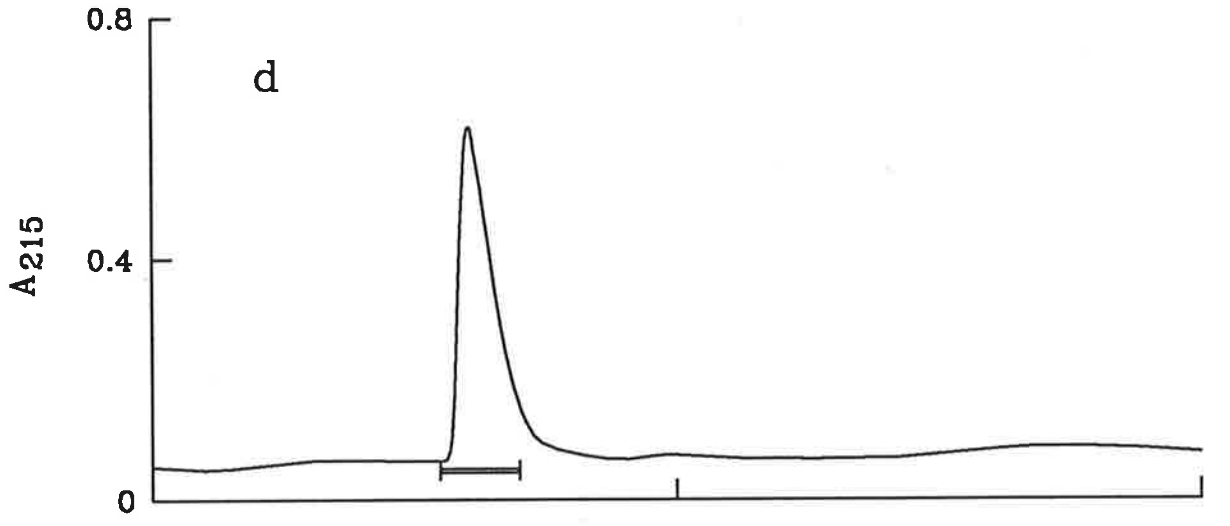
Figure 4.7: Reverse-phase HPLC of substituted and truncated IGF-I peptides from synthesis BIO-2

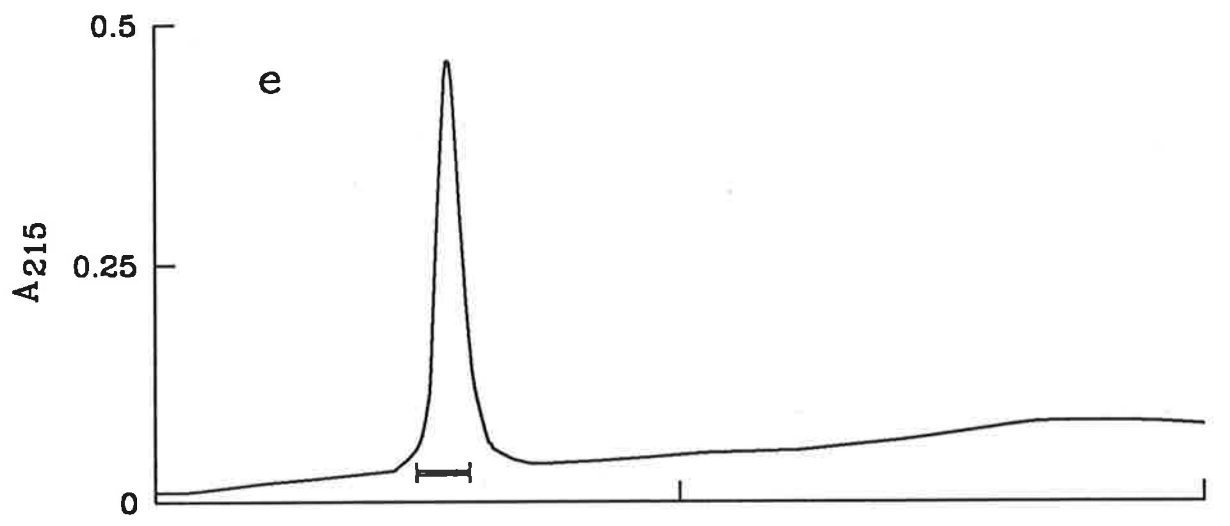
Peptides corresponding to correctly oxidized IGF were rechromatographed on a 4.6 × 30mm RP-300 column at a flow rate of 1ml/min. Elution was achieved using a gradient of acetonitrile from 20% to 50% in 15min and the A_{215} monitored (—). The samples were a) des-(2,3)-IGF-I, b) des-(2-4)-IGF-I, c) des-(2-5)-IGF-I, d) [Gly^3]IGF-I, e) [Asp^3]IGF-I, f) [Gln^3]IGF-I, g) [Leu^3]IGF-I, h) [Lys^3]IGF-I and i) [Phe^3]IGF-I. The indicated fractions were pooled and used for the biochemical studies reported in chapter 7.

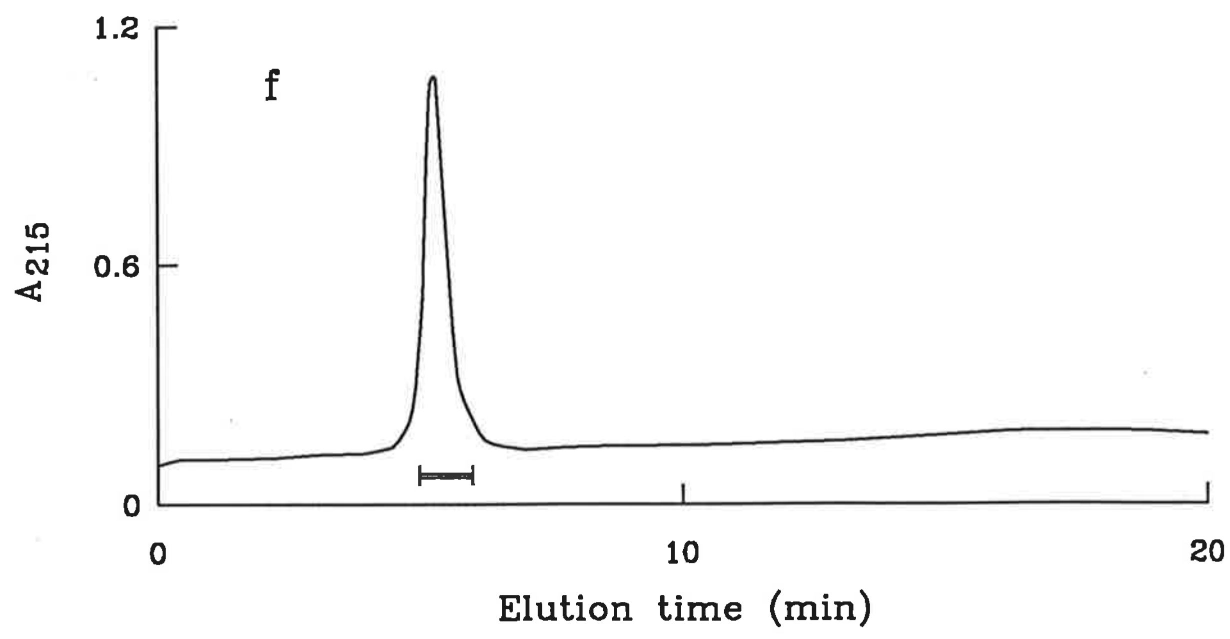

0.8
a
A215
0.4
0

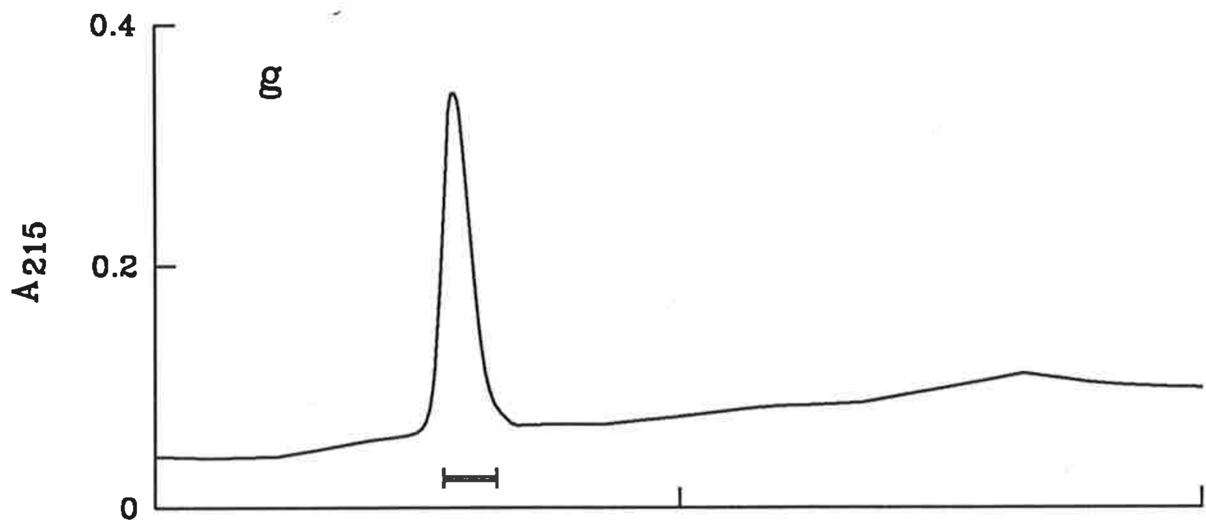

0.06
b
A280
0.03
0

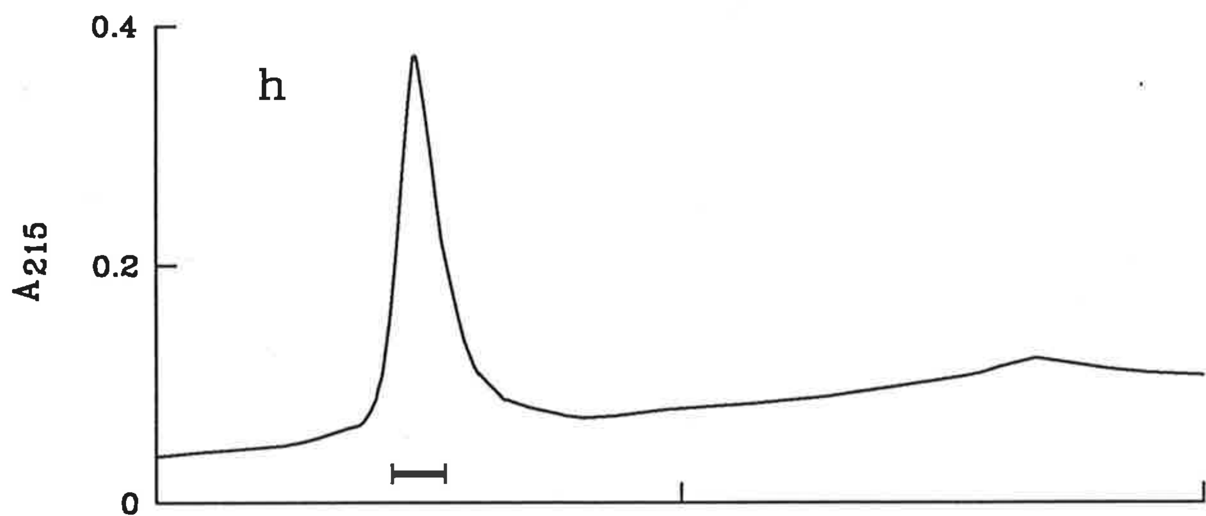

0.8
c
A215
0.4
0
0
10
20
Elution time (min)


0.8
d
A_{215}
0.4
0


0.5
e
A_{215}
0.25
0


1.2
f
A_{215}
0.6
0
0
10
20
Elution time (min)


0.4
g
A215
0.2
0


0.4
h
A215
0.2
0

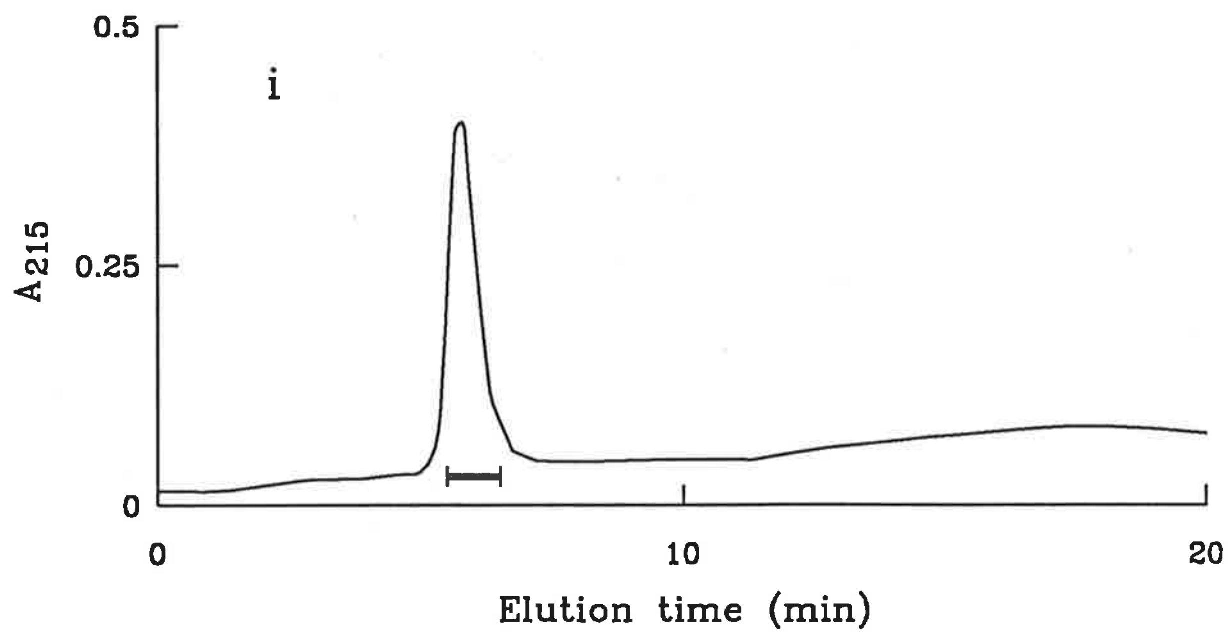

0.5
i
A215
0.25
0
0
10
20
Elution time (min)

Figure 4.8: Plan of the BIO-3 synthesis

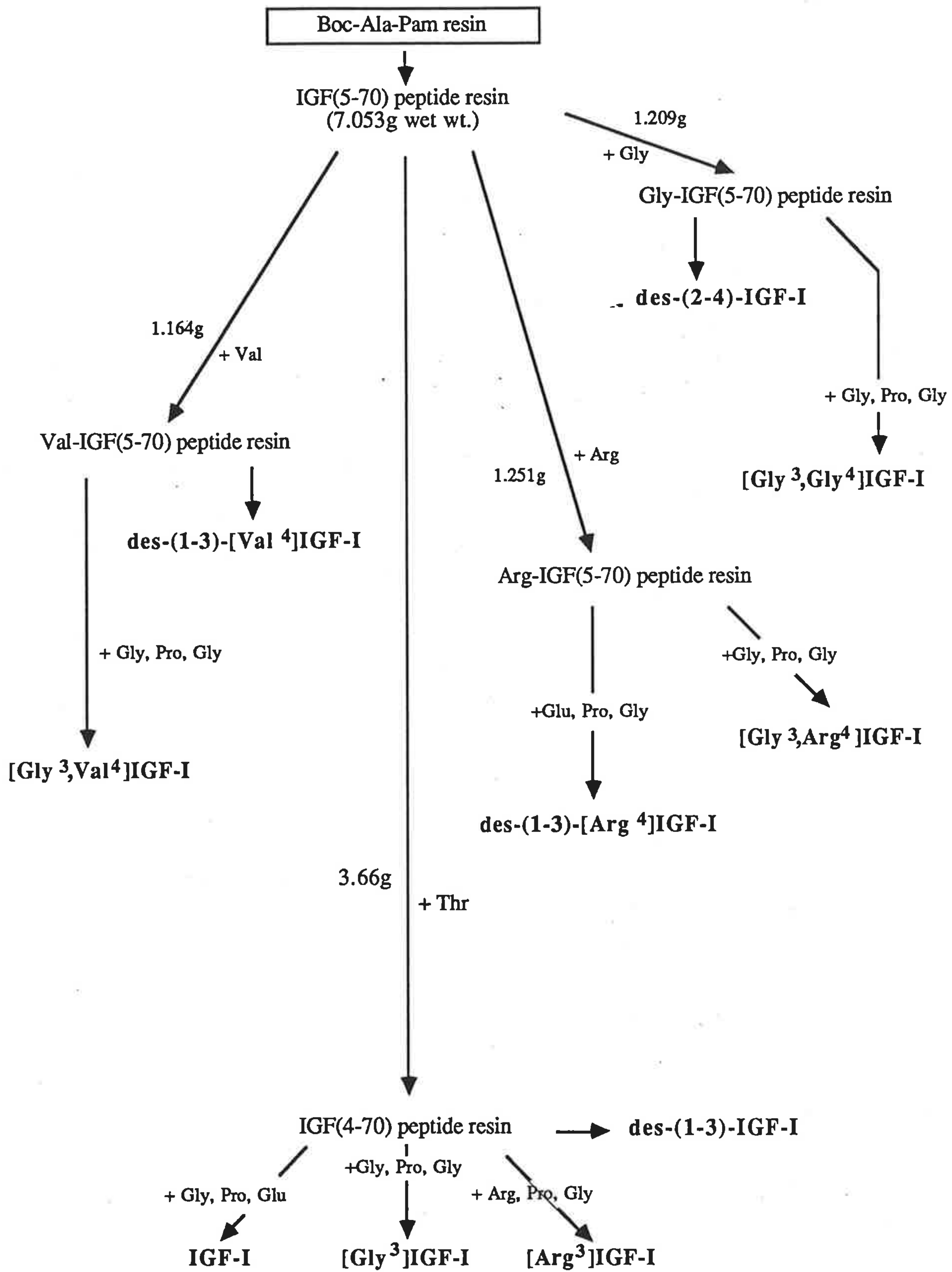
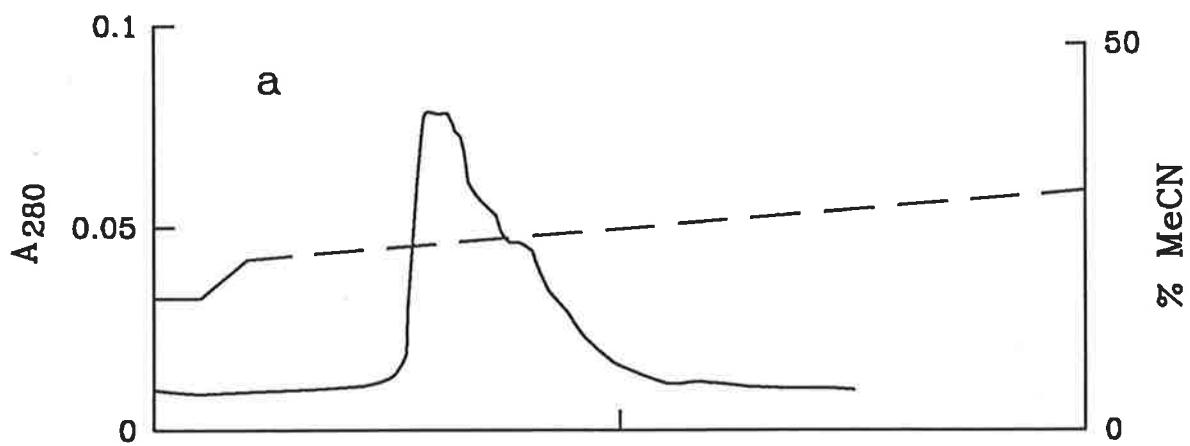
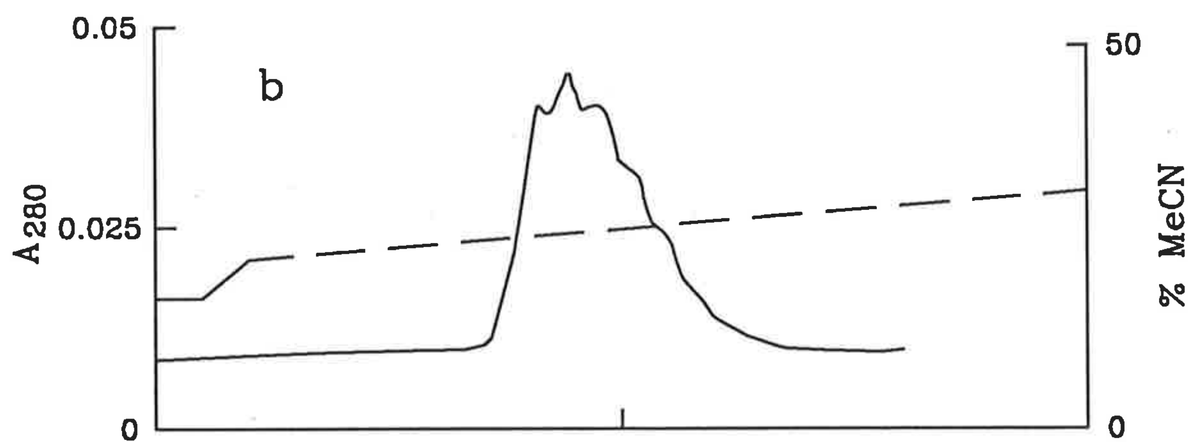
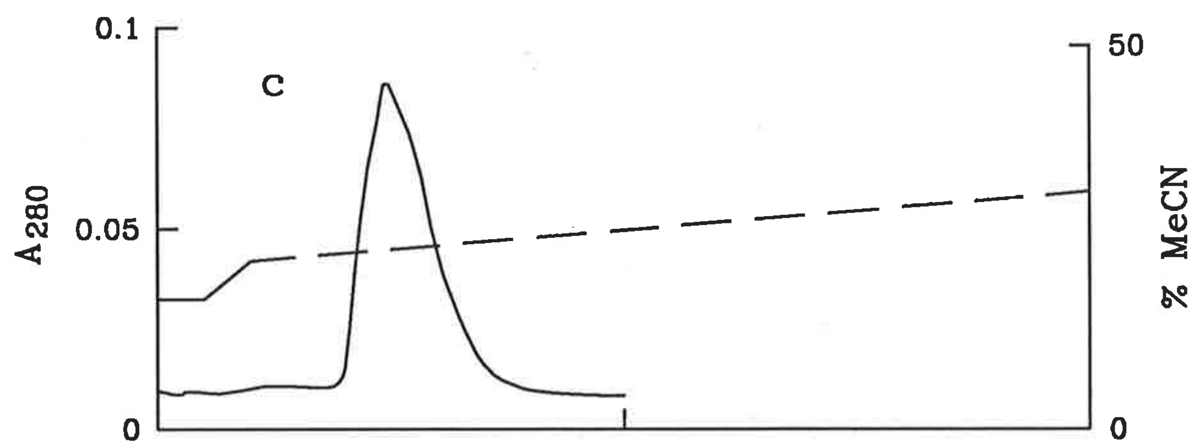


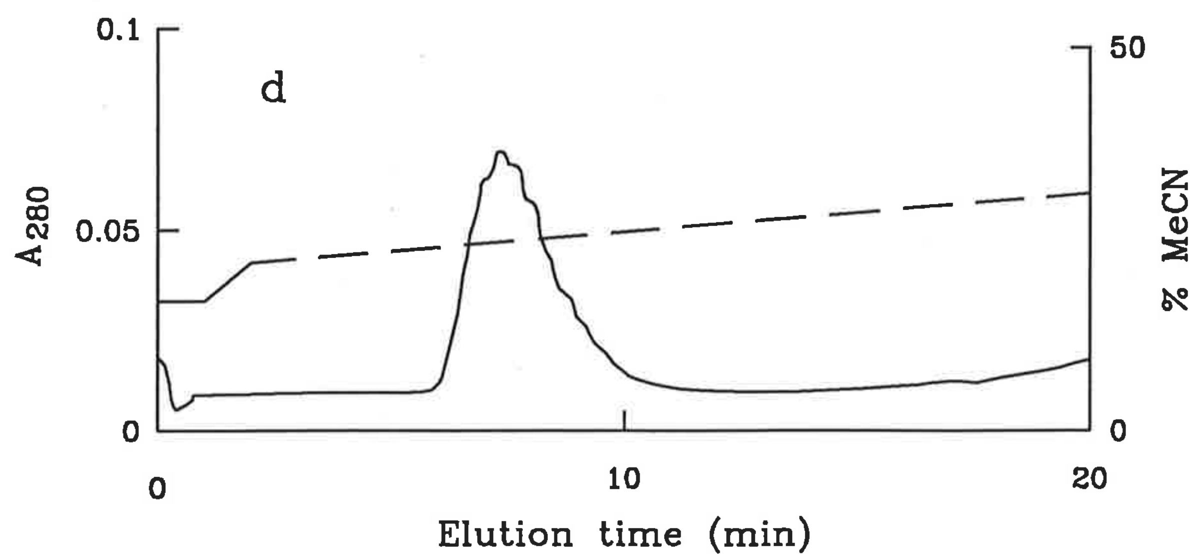
Figure 4.9: Reverse-phase HPLC of IGF-I peptides from synthesis BIO-3

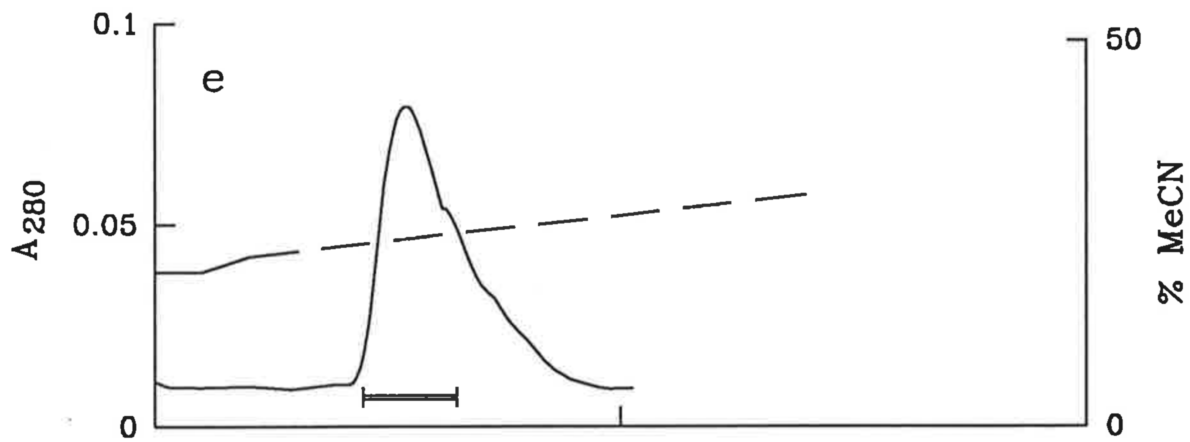
Peptides corresponding to correctly oxidized IGF were rechromatographed by reverse-phase HPLC on a 4.6 × 30mm RP-300 column at a flow rate of 1ml/min. Elution was achieved using the indicated gradient of acetonitrile (- -) and the A_{215} monitored (—). The samples were a) des-(1-3)-IGF-I, b) des-(2-4)-IGF-I, c) des-(1-3)-[Arg^4]IGF-I, d) des-(1-3)-[Val^4]IGF-I, e) [Gly^3]IGF-I, f) [Arg^3]IGF-I, g) [Gly^3, Gly^4]IGF-I, h) [Gly^3, Arg^4]IGF-I, i) IGF-I, j) reprocessed des-(1-3)-IGF-I, k) reprocessed des-(2-4)-IGF-I, l) reprocessed des-(1-3)-[Arg^4]IGF-I, m) reprocessed des-(1-3)-[Val^4]IGF-I, n) reprocessed [Gly^3, Gly^4]IGF-I, o) reprocessed [Arg^3]IGF-I, p) reprocessed [Gly^3]IGF-I. The indicated fractions were pooled and used for the biochemical studies reported in chapter 7.


0.1
0.05
0
A280
a
50
0
% MeCN

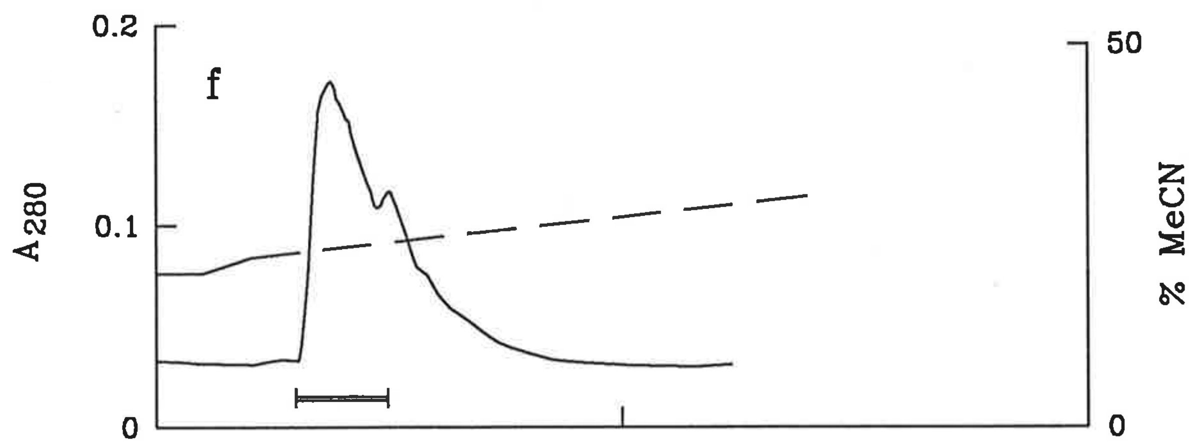

0.05
0.025
0
A280
b
50
0
% MeCN


0.1
0.05
0
A280
c
50
0
% MeCN

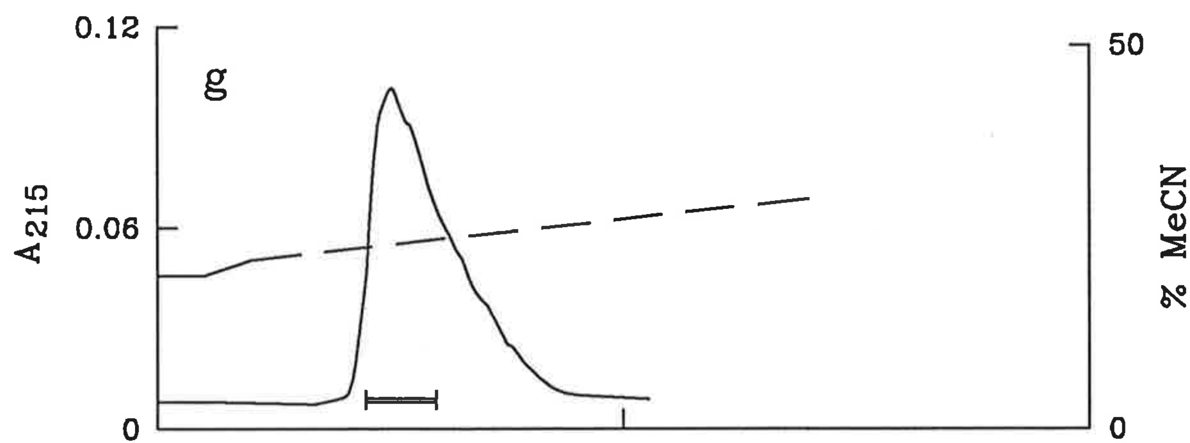

0.1
0.05
0
A280
d
50
0
% MeCN
0
10
20
Elution time (min)



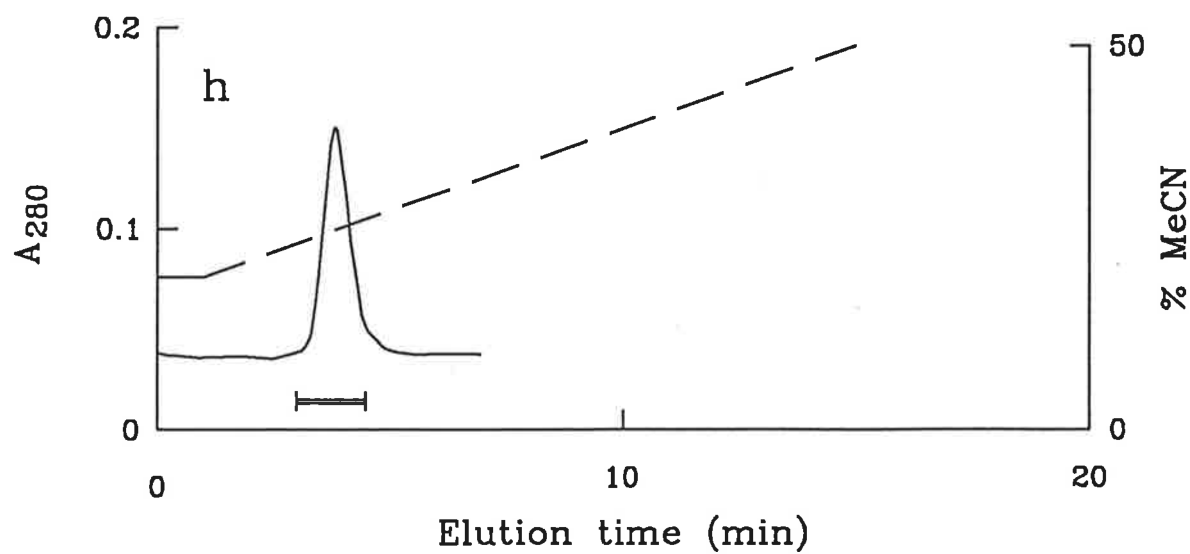
0.1
e
A280
0.05
0
50
% MeCN
0



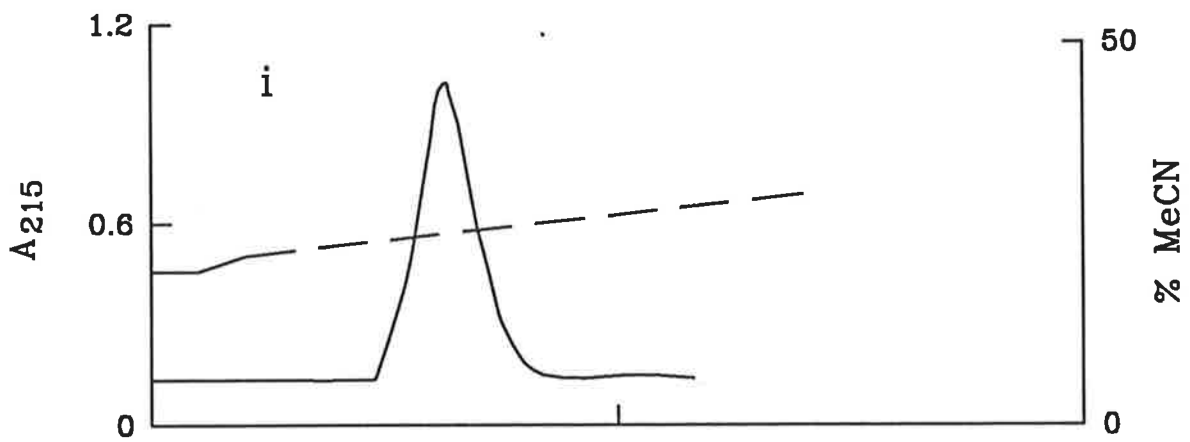
0.2
f
A280
0.1
0
50
% MeCN
0

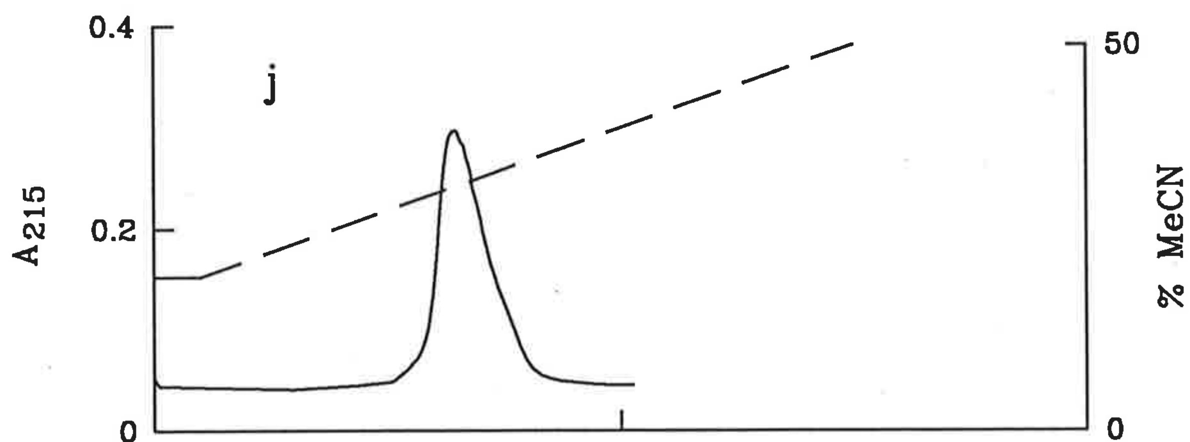


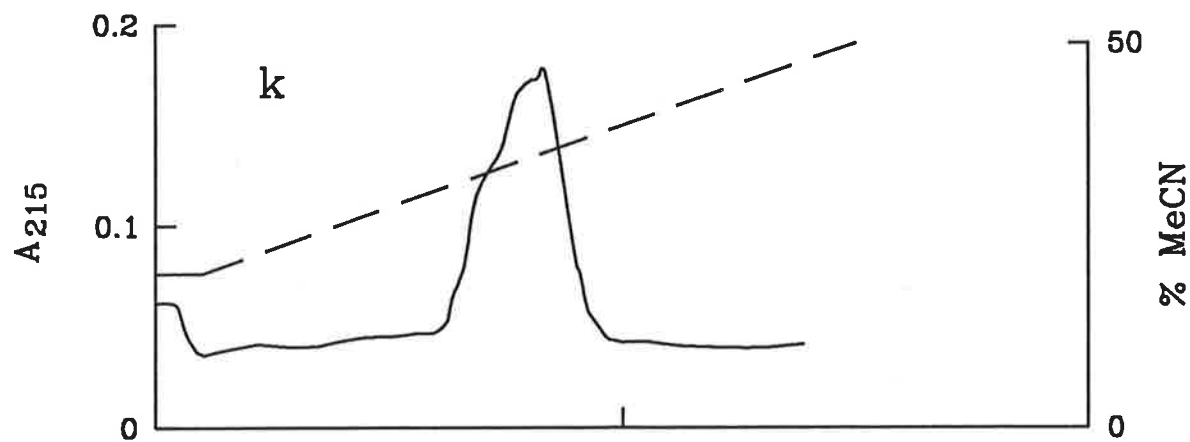
0.12
g
A215
0.06
0
50
% MeCN
0

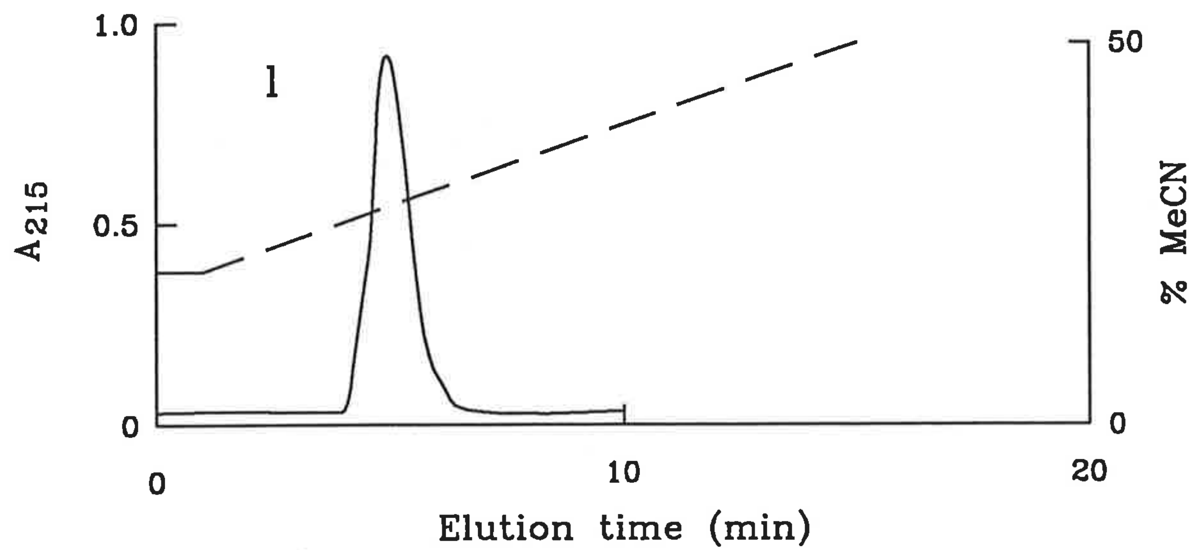


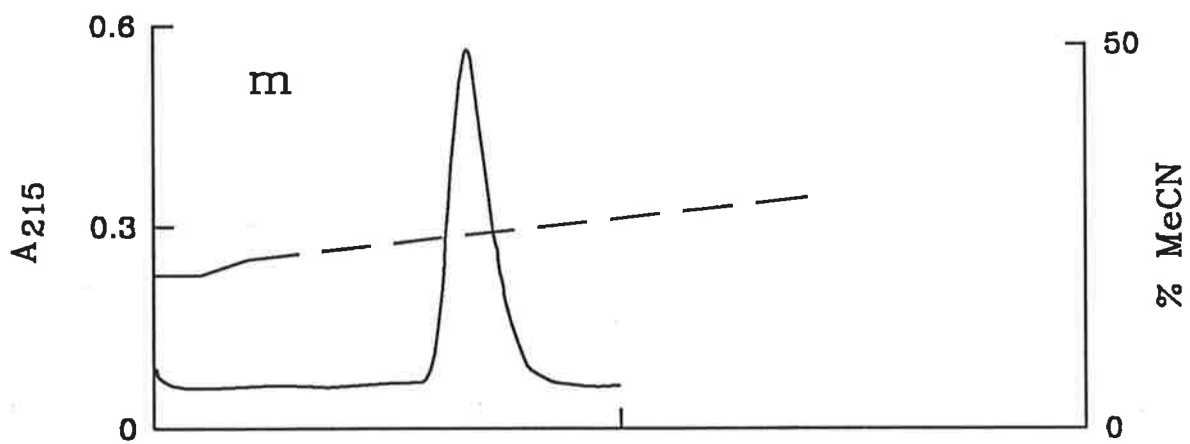
0.2
h
A280
0.1
0
50
% MeCN
0
0
10
20
Elution time (min)

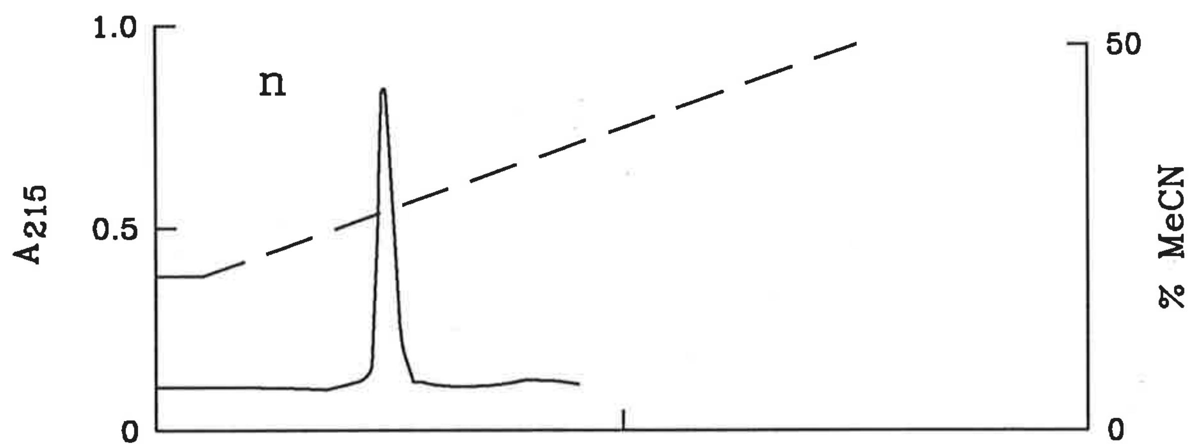

1.2
i
A_{215}
0.6
0
50
% MeCN
0

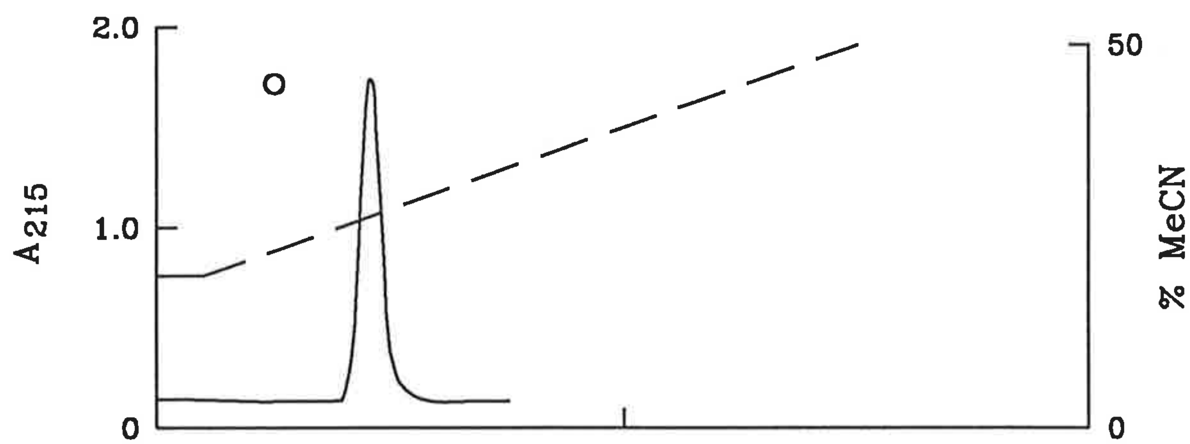

0.4
j
A_{215}
0.2
0
50
% MeCN
0


0.2
k
A_{215}
0.1
0
50
% MeCN
0


1.0
l
A_{215}
0.5
0
50
% MeCN
0
0
10
20
Elution time (min)


m
0.6
0.3
0
A_{215}
50
0
% MeCN


n
1.0
0.5
0
A_{215}
50
0
% MeCN


o
2.0
1.0
0
A_{215}
50
0
% MeCN

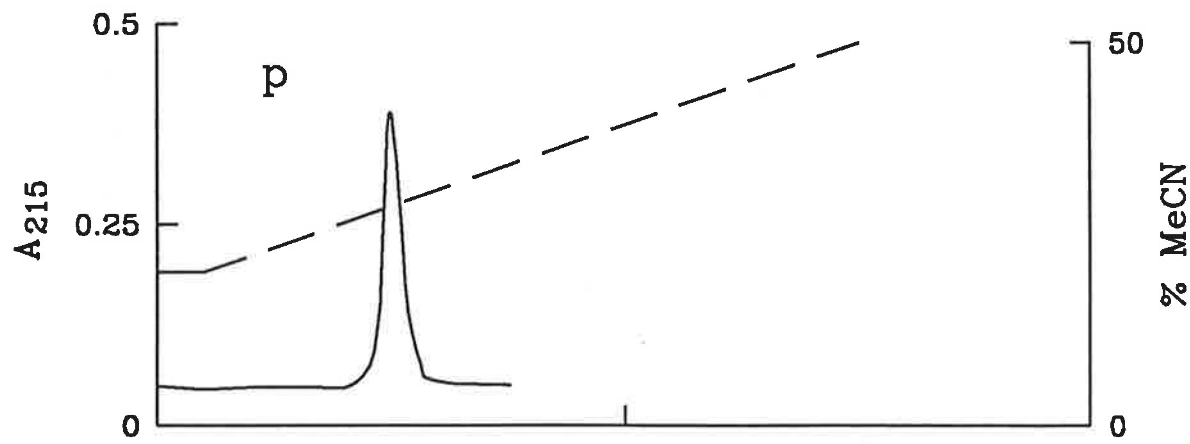
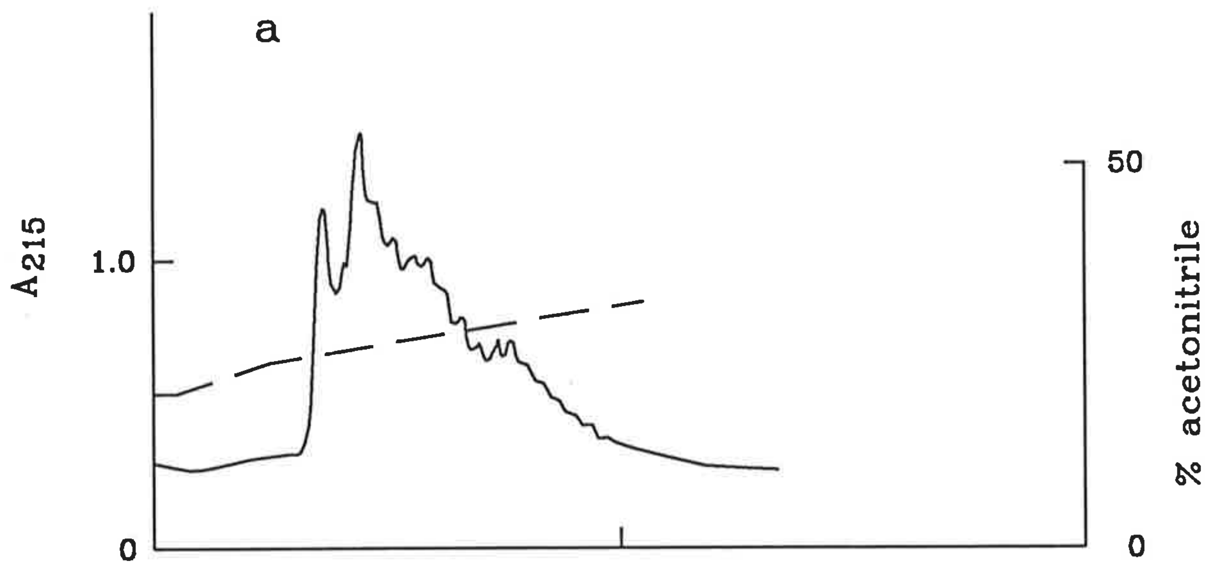

p
0.5
0.25
0
A_{215}
50
0
% MeCN
0
10
20
Elution time (min)

Figure 4.10: Reprocessing of IGF-I from synthesis BIO-3

Reduced IGF-I peptide from synthesis BIO-3 was oxidized under the standard conditions and chromatographed on a 4.6 × 30mm RP-300 column at a flow rate of 1ml/min (panel a). Elution was achieved with the indicated gradient of acetonitrile and the A_{215} monitored (—). Fractions containing correctly-oxidized IGF-I were pooled and half of this material was reduced and then subjected to a second oxidation step. The reprocessed peptide was chromatographed as above (panel b).


a
A215
1.0
0
50
% acetonitrile
0

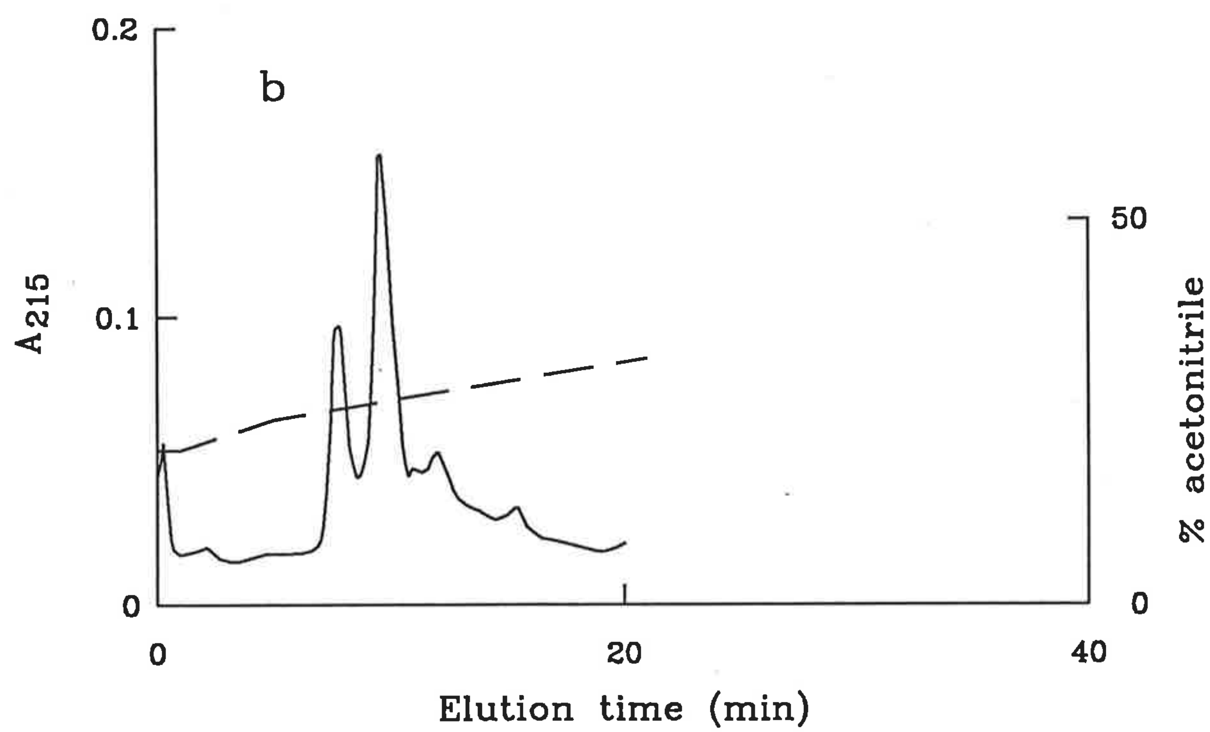
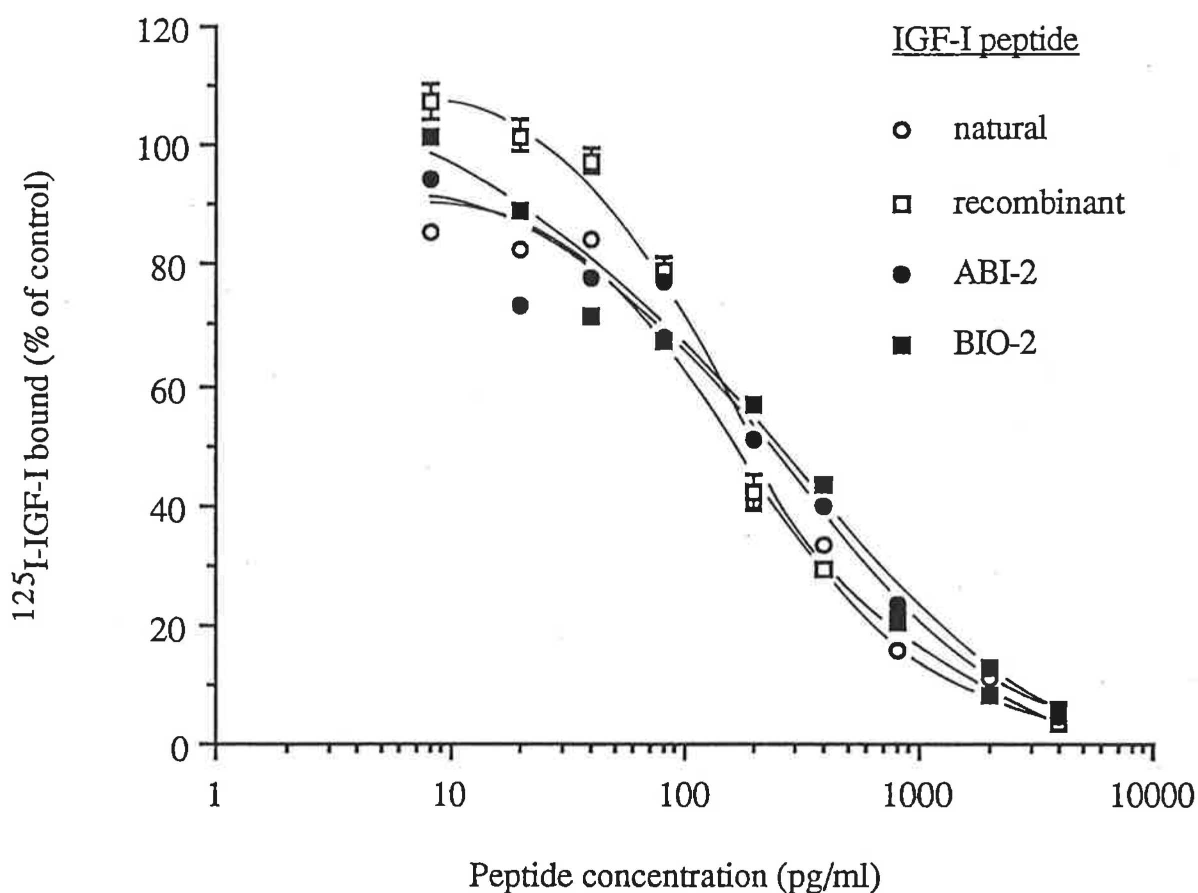
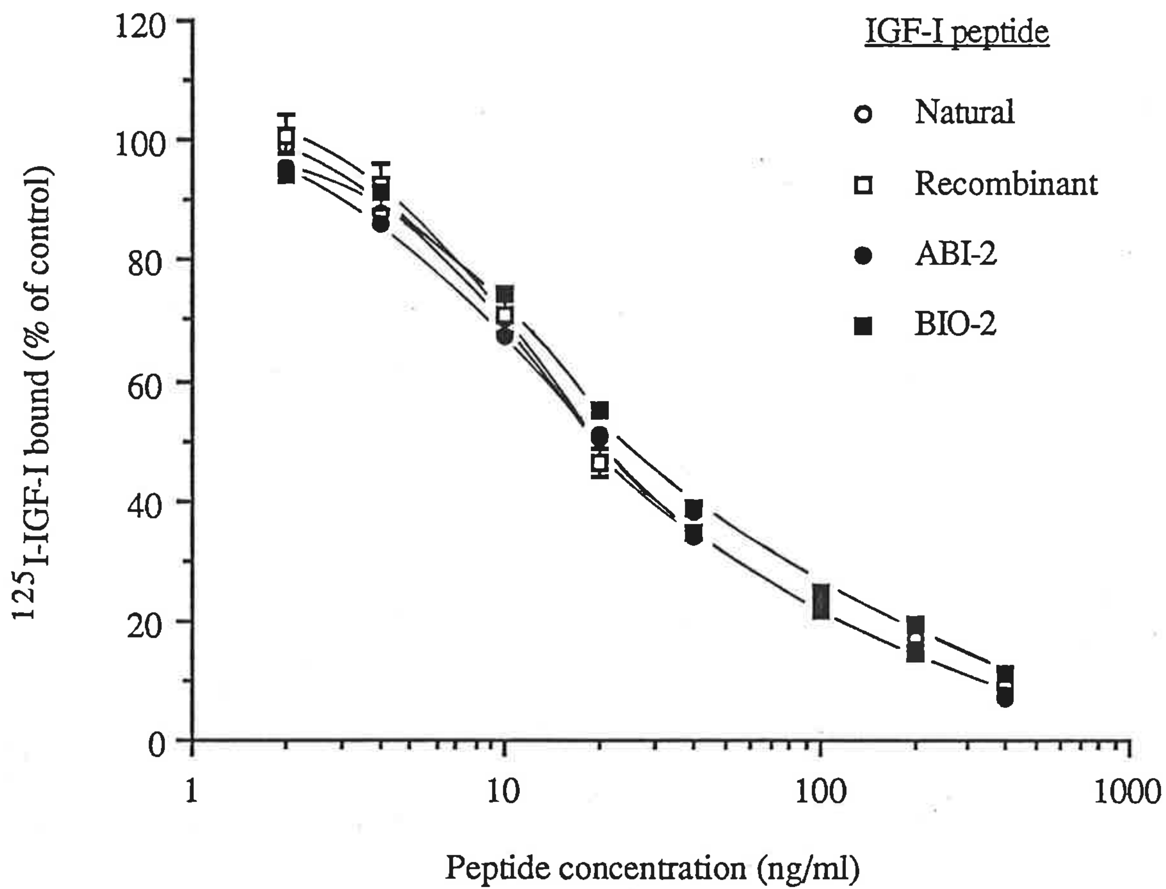

0.2
b
A215
0.1
0
50
% acetonitrile
0
0
20
40
Elution time (min)

Figure 4.11: Radioimmunoassay of natural, recombinant and synthetic IGF-I peptides



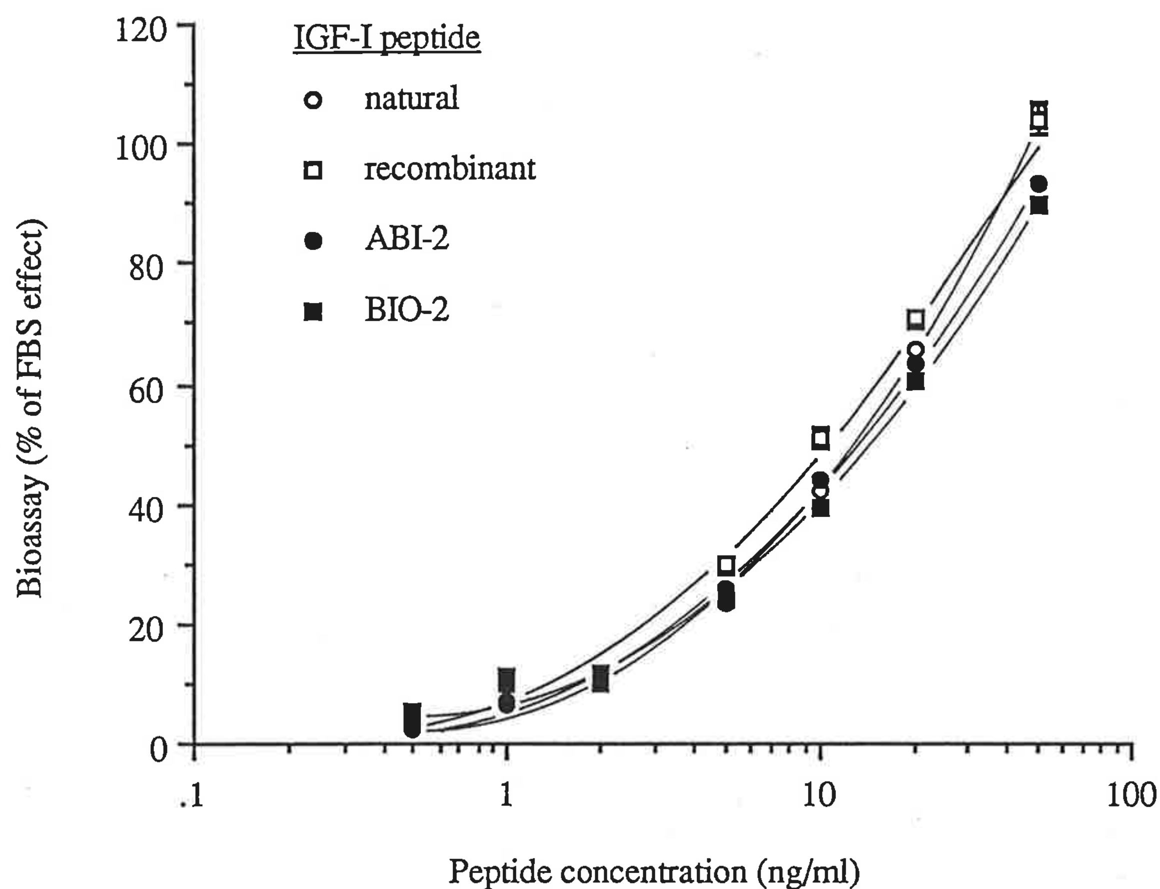
The indicated peptides were tested for their abilities to compete with ^{125}I-IGF-I for binding to anti-IGF-I antiserum. Binding of tracer IGF-I was equal to 28% of counts added and a blank (determined in the absence of antibody) equal to 0.3% has been subtracted. Data shown represent the mean of triplicate determinations and are expressed as percentages of the binding evident in the absence of competing ligand. Standard errors of the mean are indicated for the recombinant IGF-I data.

Figure 4.12: Radioreceptor assay of natural, recombinant and synthetic IGF-I peptides



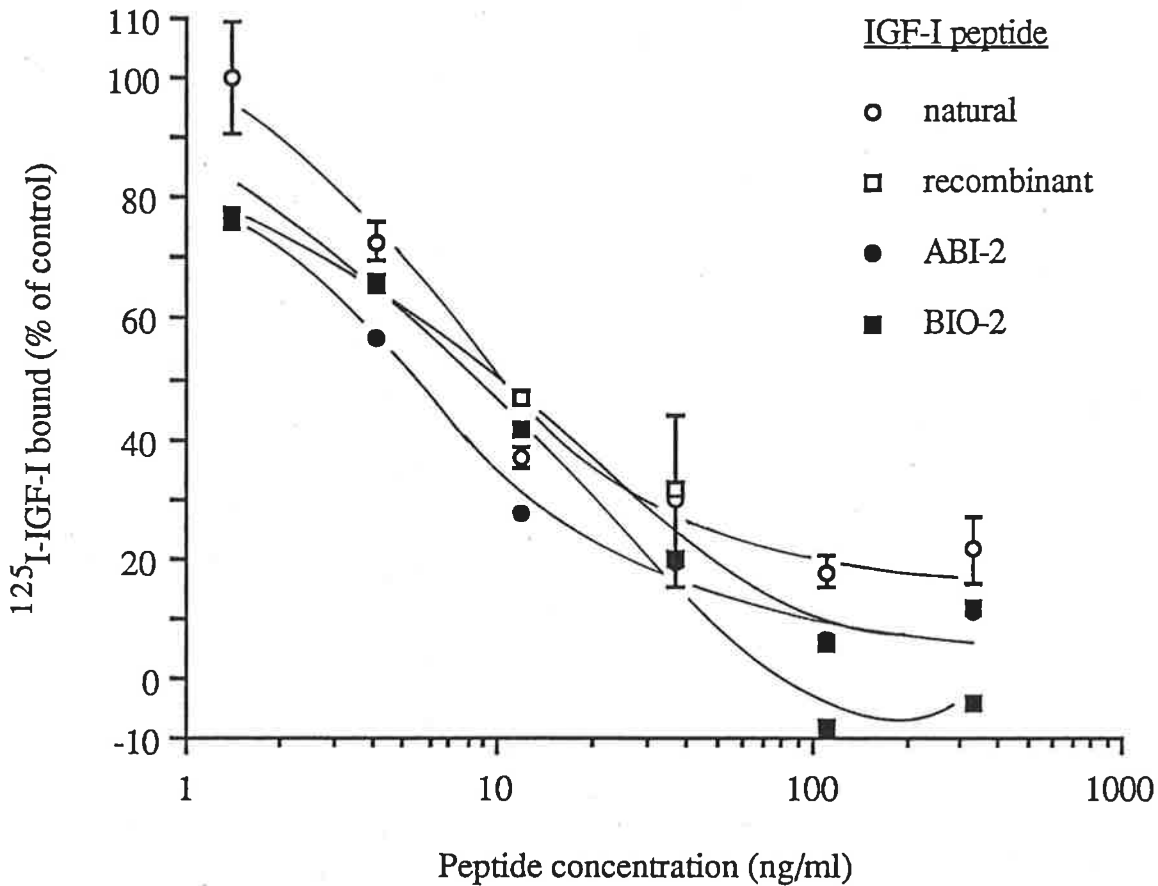
The indicated peptides were tested for their abilities to compete with ^{125}I-IGF-I binding to L6 myoblasts. Binding of tracer IGF-I was equal to 12.0% of counts added and nonspecific binding was not measured. Data shown represent the means of triplicate determinations and are expressed as percentages of the binding evident in the absence of competing ligand. Standard errors of the mean are indicated for the recombinant IGF-I data.

Figure 4.13: Bioassay of natural, recombinant and synthetic IGF-I peptides



The indicated peptides were tested for their abilities to stimulate protein synthesis in L6 myoblasts. Incorporation of radioactivity into protein in the absence of added growth factor was 1.6% and in the presence of 5% foetal bovine serum was 5.5%. Data shown represent the means of triplicate determinations and are expressed as a percentage of the effect caused by 5% foetal bovine serum. Standard errors of the mean are indicated for the recombinant IGF-I data.

Figure 4.14: Binding protein competitive binding assay of natural, recombinant and synthetic IGF-I peptides using MDBK cell binding protein



The indicated peptides were tested for their abilities to compete with ^{125}I-IGF-I for binding to the binding protein purified from MDBK cell-conditioned medium. Total binding of tracer IGF-I was 18.5% and a blank (determined in the absence of binding protein) equal to 11.1% has been subtracted. Data plotted represent the means of triplicate determinations and are expressed as percentages of the binding evident in the absence of competing ligand. Standard errors of the mean are indicated for the natural IGF-I data.

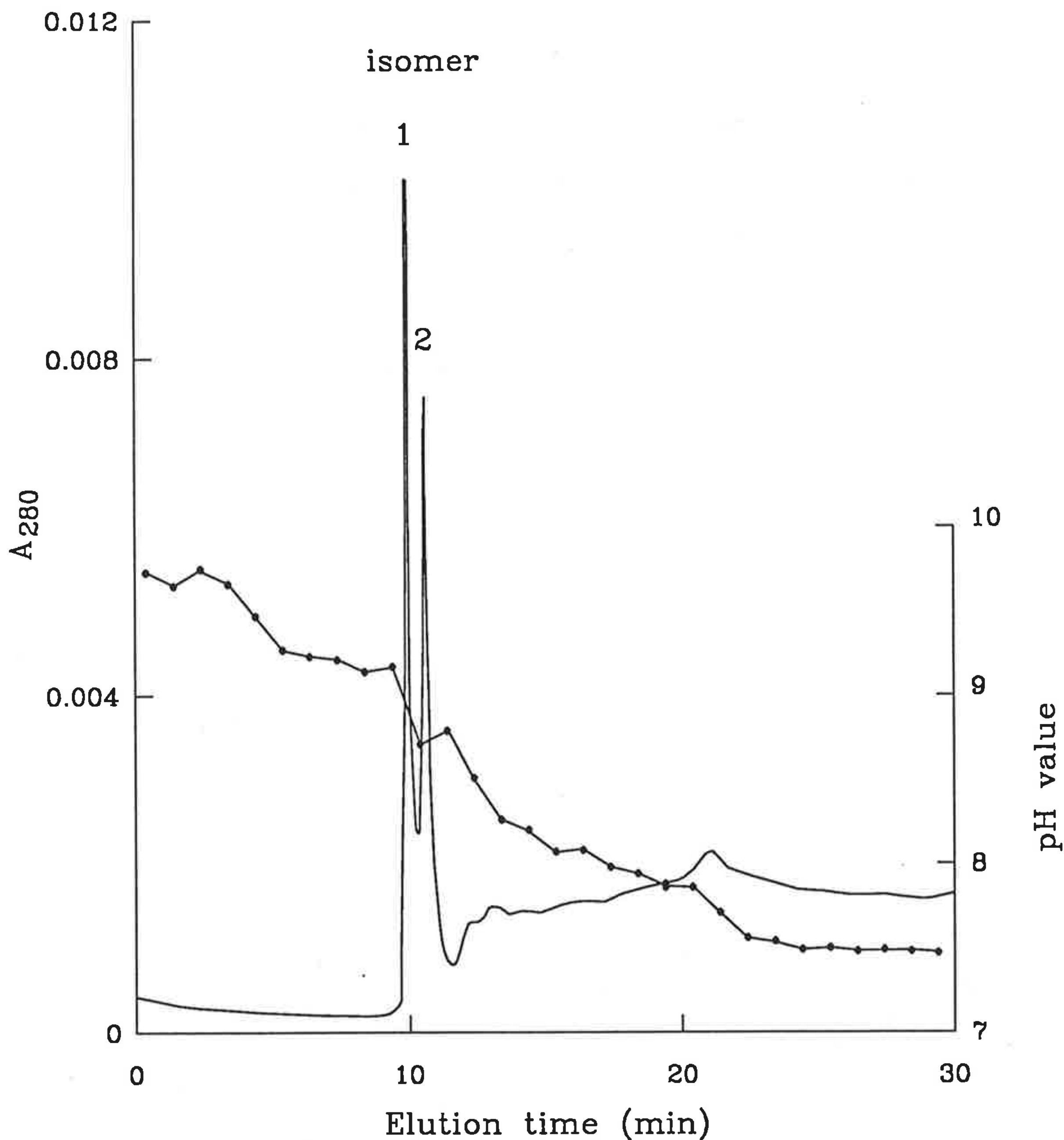


Figure 4.15: Separation of refolded IGF-I species by chromatofocussing

The Mono-P HR5/20 column was equilibrated with 25mM ethanolamine and eluted at a flow rate of 1ml/min with 40ml of a mixture of Ches, Lysine, Taps, Tris, Tricine, Hepps, Popso, Hepes, Mopso, Mops (1.8mM each) in 10%(v/v) acetonitrile pH 7.36. Samples of [6-47-, 48-52-cystine]IGF-I (28µg) and native IGF-I (34µg) peptides were mixed, dried, redissolved in 25mM ethanolamine, loaded onto the column and eluted as above. Fractions (1ml) were collected, their pH values determined and the content of the two isomers assessed by analytical reverse-phase HPLC.

CHAPTER 5

COMPARISON OF NATURAL AND SYNTHETIC DES-(1-3)-IGF-I PEPTIDES

CHAPTER 5: COMPARISON OF NATURAL AND SYNTHETIC DES-(1-3)-IGF-I PEPTIDES

5.1 INTRODUCTION

Using an incompletely purified preparation, Ballard *et al.* (1986) have previously observed bovine des-(1-3)-IGF-I to be twice as potent as human IGF-I in causing the stimulation of protein synthesis and DNA synthesis and the inhibition of protein degradation in rat L6 myoblasts. Sara *et al.* (1986) have purified a similar IGF-I variant and shown it to bind to a preparation of type 1 receptors from brain fivefold better than IGF-I, a binding potency which could account for its enhanced biological activity. However, further experiments in the laboratory of Dr. Ballard, using highly purified preparations of bovine des-(1-3)-IGF-I and IGF-I (identical amino acid sequence to human IGF-I), have shown des-(1-3)-IGF-I to be two-fold more potent than IGF-I for competing with radiolabelled IGF-I binding to these cells (Ballard *et al.*, 1988) whereas it was eight-fold more active in stimulating protein synthesis (Francis *et al.*, 1988). These data suggested that increased receptor binding does not entirely account for the enhanced biological potency of des-(1-3)-IGF-I. Likewise, studies using insulin/IGF-I hybrid molecules have suggested that the carboxy-terminal (A and D) domains of IGF-I might be involved in receptor binding rather than the amino terminus of IGF-I (see section 1.6). In order to confirm that the higher potency of des-(1-3)-IGF-I was solely due to the amino-terminal truncation and not due to the presence of contaminants or other modifications to the peptide and to establish whether the enhanced biological potency resulted from increased receptor binding, it was decided to chemically-synthesize IGF-I and des-(1-3)-IGF-I and evaluate these peptides biochemically. For the assays reported in this chapter, the L6 myoblast cell-line was used since this has been characterized previously with respect to its binding of IGFs (Ballard *et al.*, 1986). The abilities of the various growth factors to compete for binding to type 1 and type 2 IGF receptors and to stimulate protein synthesis was investigated using the L6 myoblast cell-line. I am grateful to Ms. M.Ross for performing the assays reported in this chapter.

The results presented in this chapter have been published: Ballard, F.J., Francis, G.L., Ross, M., Bagley, C.J., May, B.L. & Wallace, J.C. (1987) "Natural and synthetic forms of Insulin-like growth factor-1 (IGF-1) and the potent derivative, destripeptide IGF-1: Biological activities and receptor binding" *Biochem. Biophys. Res. Commun.* **149** 398-404.

5.2 MATERIALS AND METHODS

IGF-I and des-(1-3)-IGF-I were synthesized as described in section 4.3c and purified by reverse-phase HPLC as described in section 4.3e. Natural IGF-I, des-(1-3)-IGF-I and IGF-II and recombinant IGF-I were obtained as described in section 2.1. ^{125}I-labelled peptides were obtained as described in section 2.1. Radioreceptor assays were performed as described in section 2.4b. The stimulation of protein synthesis in L6 myoblasts was measured as described in section 2.4c.

5.3 RESULTS

5.3a IGF-I Radioreceptor assay

The natural and synthetic peptides were first examined for their abilities to inhibit the binding of ^{125}I-IGF-I to confluent monolayers of L6 myoblasts during a 2h incubation at 22°C. As shown in figure 5.1, approximately 15-20 ng/ml of natural IGF-I was required to cause 50% competition for binding of labelled IGF-I. The natural and synthetic peptides competed with similar potencies although the natural IGFs were slightly more potent. Recombinant IGF-I competed slightly more effectively than natural IGF-I. In this assay, there was no significant difference between IGF-I and des-(1-3)-IGF-I whereas IGF-II was about 2-fold less potent than IGF-I.

5.3b IGF-II Radioreceptor assay

When ^{125}I-IGF-II was used as the radioligand, IGF-II was found to be the most potent competing ligand requiring 6ng/ml for 50% competition (see fig. 5.2). Although the IGF-I species did not cause 50% competition at the levels tested, it could be seen that they exhibited similar potencies which were approximately 1000-fold less than that of IGF-II. Synthetic des-(1-3)-IGF-I was slightly more potent than IGF-I whereas bovine des-(1-3)-IGF-I was 2- to 3-fold more active.

5.3c Protein synthesis bioassay

The natural, synthetic and recombinant peptides were compared in the L6 myoblast protein synthesis bioassay (see fig. 5.3). All the peptides were able to elicit the same maximum level of response but exhibited different potencies. The three forms of IGF-I

exhibited a half-maximal effect at 5ng/ml while the des-(1-3)-IGF-I peptides were approximately 7-fold more potent and the IGF-II was fourfold less active. The dose-response curve of the des-(1-3)-IGF-I peptides exhibited a lower slope than that of the IGF-I peptides, a feature which has been regularly observed in the laboratory. Thus, at low levels of stimulation, des-(1-3)-IGF-I was found to be nearly 10-fold more potent than IGF-I.

5.4 DISCUSSION

These experiments indicated that natural, synthetic and recombinant IGF-I were equipotent in L6 myoblast radioreceptor assays using either IGF-I or IGF-II as the radioligand and exhibited equal potencies in the protein synthesis bioassay. In particular, it is noteworthy that the IGF-I shows a very low binding to the type 2 receptor since others have observed significantly greater cross-reactivities (Ewton *et al.*, 1987; Rosenfeld *et al.*, 1987). It is likely that some preparations of natural IGF-I are contaminated with IGF-II since the cross-reactivity is variable (Rosenfeld *et al.*, 1987) and labelled natural IGF-I but not recombinant IGF-I was able to bind to type 2 receptors (Ewton *et al.*, 1987). The source of this IGF-II-like material is unknown since a radioimmunoassay for the IGF-II C-peptide failed to detect any IGF-II (Rosenfeld *et al.*, 1987).

The natural des-(1-3)-IGF-I cross-reacted more strongly than either the synthetic des-(1-3)-IGF-I or the natural IGF-I in the IGF-II radioreceptor assay (fig. 5.2). It is probable that this results from a contamination of the natural preparation by approximately 1% IGF-II since this is difficult to separate during the purification (Francis *et al.*, 1988). It is unlikely that the observed differences in binding to the type 2 receptor result from true molecular differences between the natural and synthetic des-(1-3)-IGF-I peptides.

The increased biological potency of the des-(1-3)-IGF-I isolated from bovine colostrum does not result from the binding of carbohydrate or other moieties to the growth factor since the chemically-synthesized des-(1-3)-IGF-I is equipotent (see fig. 5.3). At the time when these studies were performed, the complete amino acid sequence of bovine des-(1-3)-IGF-I had not been determined. The equipotency of synthetic des-(1-3)-IGF-I and natural des-(1-3)-IGF-I in the bioassay demonstrated that the removal of the amino-terminal tripeptide of IGF-I was sufficient to cause an increase in the biological potency. In these experiments, the higher potency of des-(1-3)-IGF-I compared with IGF-I cannot be explained

by increased binding to the type 1 IGF receptor on L6 myoblasts (see fig 5.1). This contrasts with the report of Sara *et al.* (1986) in which a variant of IGF-I lacking the amino-terminal tripeptide bound to the type 1 receptor from brain fivefold better than did IGF-I and had a correspondingly higher biological potency. Perhaps the differences in binding potencies in the two series of investigations can be explained by true molecular differences between receptors, because the type 1 receptor in brain has an altered carbohydrate composition (Gammeltoft *et al.*, 1985). One possible explanation of the potency of des-(1-3)-IGF-I is that its biological activity is less affected by the presence of IGF-binding proteins in the L6 myoblast culture medium than is that of IGF-I. This interpretation is supported by the difference in slope of the dose-response curves in the bioassay (fig 5.3). The influence of IGF-binding proteins on the bioassay was investigated further with the series of IGF-I analogues described in the next chapter.

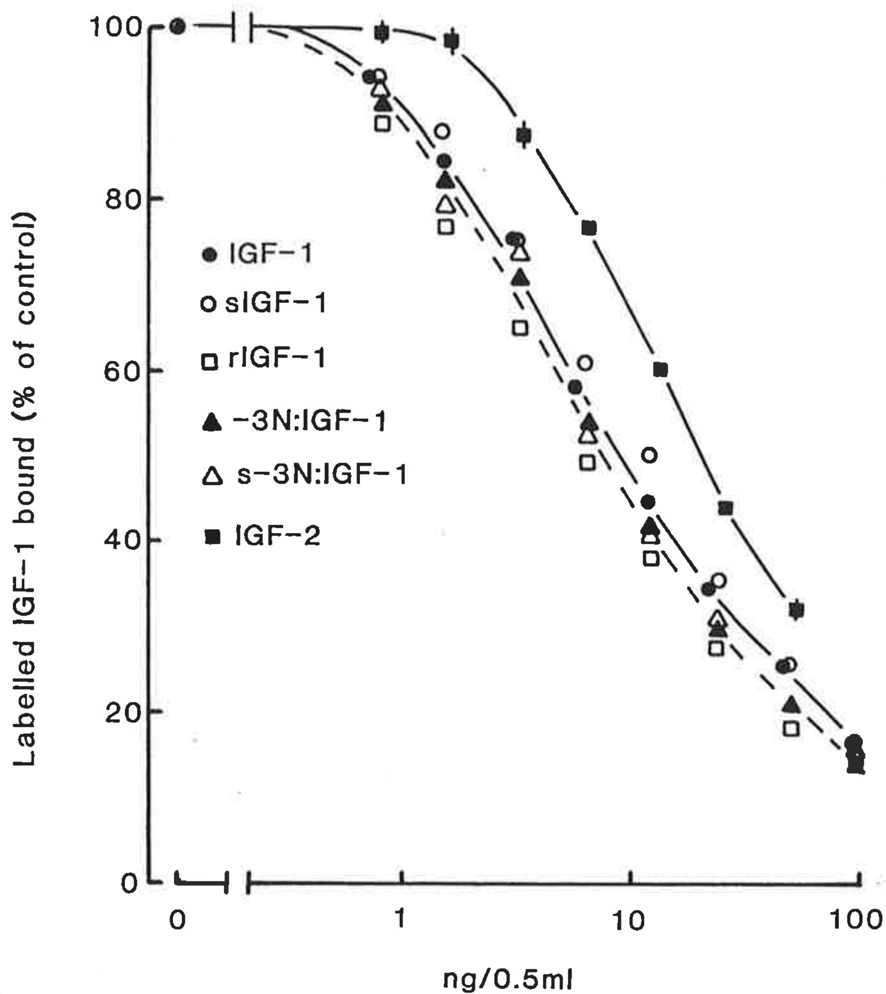


Figure 5.1: IGF-I radioreceptor assay

The radioreceptor assay was performed using ^{125}I-IGF-I as the radioligand. Binding is expressed as the percentage of that occurring in the absence of competing peptide which represented 11% of the added radioactivity. Each point is the mean of triplicate determinations with standard errors indicated for the IGF-II data, where they are adequately large in relation to the symbols, as an indication of assay variability.

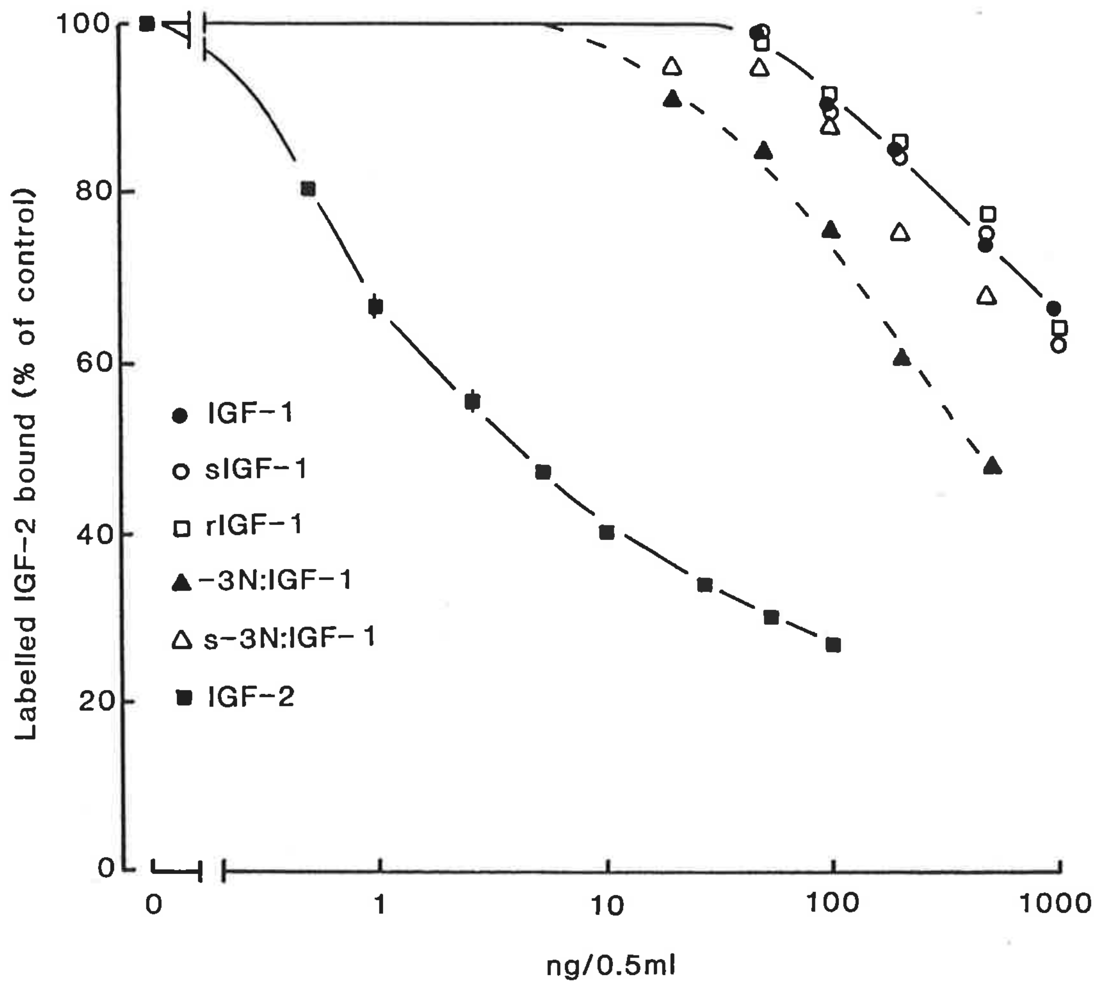


Figure 5.2: IGF-II radioreceptor assay

The radioreceptor assay was performed using ^{125}I-IGF-II as the radioligand. Binding is expressed as the percentage of that occurring in the absence of competing peptide which represented 21% of the added radioactivity. Each point is the mean of triplicate determinations with standard errors indicated for the IGF-II data, where they are adequately large in relation to the symbols, as an indication of assay variability.

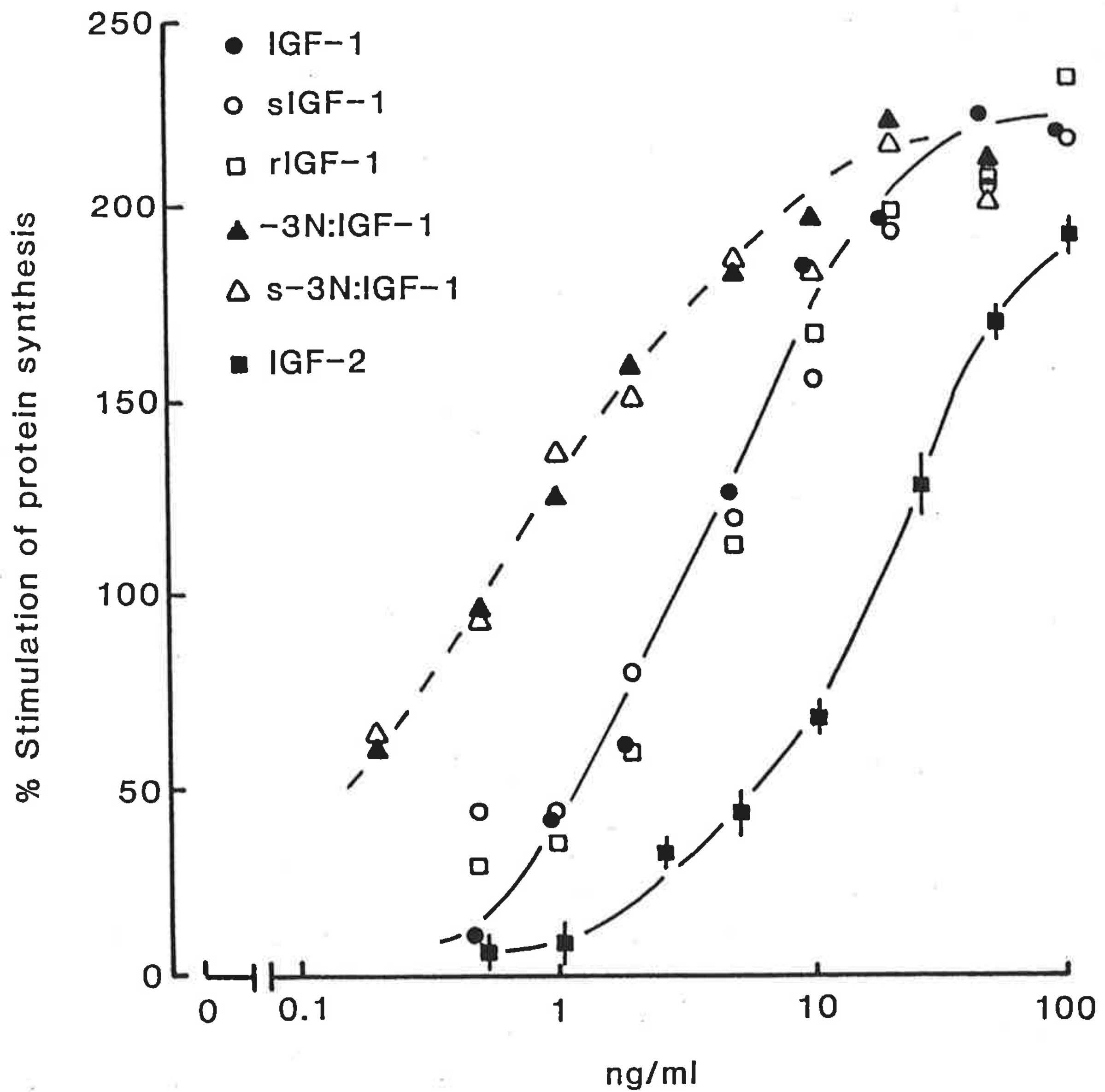


Figure 5.3: L6 myoblast protein synthesis bioassay

Values represent the percentage stimulation caused by added growth factors over the basal state in medium without growth factors. Each point is the mean of triplicate determinations with standard errors indicated for the IGF-II data, where they are adequately large in relation to the symbols, as an indication of assay variability.

CHAPTER 6

ANALOGUES OF IGF-I TRUNCATED AT THE N-TERMINUS

CHAPTER 6: ANALOGUES OF IGF-I TRUNCATED AT THE N-TERMINUS

6.1 INTRODUCTION

In the previous chapter, I demonstrated that both natural and synthetic forms of des-(1-3)-IGF-I have a sevenfold greater potency than IGF-I in the protein synthesis bioassay using rat L6 myoblasts. The increased potency could not be accounted for by an enhanced affinity for the IGF-I receptor of these cells. Rather the enhanced potency of this peptide may result from its lack of binding to a protein secreted by cells in tissue culture, a binding that would otherwise reduce the amount of peptide available for interaction with cell receptors. Indeed, experiments within this laboratory have shown that natural des-(1-3)-IGF-I binds poorly to an IGF-binding protein purified from the medium conditioned by MDBK cells (Szabo *et al.*, 1988).

The loss of capacity to bind to an IGF-binding protein observed with des-(1-3)-IGF-I coupled with the enhanced biological potency prompted me to investigate amino-terminal truncations in more detail. The amino-terminal sequence of IGF-I is Gly-Pro-Glu-Thr-Leu, with a half-cystinyl residue at position 6 being presumably essential for maintenance of the correctly folded structure. In particular, I wished to determine whether the absence of a specific residue was sufficient to confer the enhanced potency of des-(1-3)-IGF-I and whether further deletion could yield analogues of IGF-I with even greater biological potency. Hence, I have synthesized IGF-I as well as analogues lacking 1 to 5 amino acid residues and characterized them by radioimmunoassay, radioreceptor assay, bioassay and binding protein competitive binding assay in order to define the roles of particular amino acid residues. I wish to acknowledge the assistance of Mr. P.McNamara for performing radioimmunoassays, Ms. M.Ross for performance of some of the cell-culture assays and Mr. L.Szabo for performing IGF-binding protein competitive binding assays.

The research presented in this chapter has been published: Bagley, C.J., May, B.L., Szabo, L., McNamara, P.J., Ross, M., Francis, G.L., Ballard, F.J. & Wallace, J.C. (1989) "A key functional role for the insulin-like growth factor-1 amino-terminal pentapeptide" *Biochem. J.* **259** 665-671.

6.2 MATERIALS AND METHODS

Synthetic IGF-I and amino-terminally truncated analogues were prepared as

described in section 4.2c. For each synthetic IGF peptide, at least two separate oxidations and purifications were carried out (denoted A and B). The chromatograms of oxidized peptide (from preparation A) lacking up to four amino acid residues from the amino terminus exhibited a discrete peak corresponding the the biological activity (see fig. 4.5). The des-(1-5)-IGF-I did not oxidize to give a discrete peak of protein corresponding to the bioactivity, a result suggesting that this preparation may contain material lacking the correct disulphide bonds. The des-(1-6)-IGF-I showed no biological activity at a concentration of 250ng/ml and accordingly was not tested further. Typically, synthetic preparations of full-length IGF-I could elicit the same maximal level of response as recombinant IGF-I in the L6 myoblast protein synthesis bioassay but exhibited only 80% the potency.

Radioimmunoassays were performed as described in section 2.4a. The radioreceptor assays and bioassays were performed as described in sections 2.4b and 2.4c respectively. IGF-binding proteins were obtained as described in section 2.1 and used for competitive binding experiments by the method described in section 2.4d.

6.3 RESULTS

6.3a Radioimmunoassay of IGF-I peptides

The cross-reactivities of the synthetic IGF-I and analogues were compared using a polyclonal anti-IGF-I antiserum (figure 6.1). This experiment showed that all the IGF peptides competed for binding of the ^{125}I-IGF-I tracer. The des-(1-3)-IGF-I and des-(1-4)-IGF-I peptides competed slightly less effectively than IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I, especially at high concentrations of competing ligand, perhaps suggesting that there may be a small subpopulation of antibodies to which these IGFs do not bind. The des-(1-5)-IGF-I was approximately fourfold less potent than IGF-I in this assay.

6.3b Protein synthesis bioassay of IGF-I peptides

In order to determine which analogue was the most biologically active, I tested the synthetic IGF peptides in the L6 myoblast protein synthesis bioassay (figure 6.2). Although there were differences in the potencies of the two batches of growth factor preparations evaluated, as assessed by the concentrations required to produce half-maximal effects, within each assay the relative potencies of the various IGF peptides were similar. The

potencies, expressed as the geometric mean of the two experiments in which the concentrations required for half-maximal effects were determined, were des-(1-3)-IGF-I(1.5ng/ml); des-(1-4)-IGF-I(5.1ng/ml); des-(1)-IGF-I(10ng/ml); IGF-I, des-(1,2)-IGF-I(13ng/ml). Des-(1-5)-IGF-I was approximately one hundred-fold less potent than IGF-I.

6.3c Radioreceptor assay of IGF-I peptides

Previous reports (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986), using a foetal brain radioreceptor assay, have suggested that the enhanced potency of the des-(1-3)-IGF-I compared with IGF-I was due to its fivefold greater affinity for binding to the type 1 IGF receptor. However, I have previously noted that des-(1-3)-IGF-I was equipotent to IGF-I in the L6 myoblast ^{125}I-IGF-I radioreceptor assay (see section 5.3a). The truncated IGF peptides were therefore tested for their abilities to compete with ^{125}I-IGF-I for binding to L6 cells (see fig. 6.3). At 3°C, all the peptides competed effectively, with the concentration required for half maximal displacement being approximately 20ng/ml for IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I. Des-(1-3)-IGF-I was 1.5-fold more potent and des-(1-4)-IGF-I twofold less potent than IGF-I. Des-(1-5)-IGF-I was approximately one hundred-fold less potent than IGF-I.

When the radioreceptor assay was performed at 25°C, IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I produced anomalous displacement curves in which low concentrations of IGF (4-20ng/ml) stimulated radioligand binding up to 150% of the control. Des-(1-3)-IGF-I and des-(1-4)-IGF-I did not elicit this effect but exhibited displacement curves more similar to those obtained at 3°C. Des-(1-5)-IGF-I caused no displacement of labelled IGF-I at concentrations of up to 1μg/ml. The binding measurements carried out at 25°C suggested that there may have been an IGF-binding protein present which modified the binding of IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I but not des-(1-3)-IGF-I nor des-(1-4)-IGF-I to the cell receptor.

6.3d IGF-binding protein competitive binding assay of IGF-I peptides

Specific IGF-binding proteins, secreted by cells in tissue culture, have been shown to alter the binding of IGF-I to the cell-surface (De Vroede *et al.*,1986; Clemmons *et al.*, 1986). Since conditioned medium from MDBK cells has been found to be a convenient

source of an IGF-binding protein which can be readily purified to homogeneity (Szabo *et al.*, 1988), I first tested the abilities of IGF-I and its analogues to compete with ^{125}I-IGF-I for binding to a purified preparation of the MDBK-cell binding protein. As shown in figure 6.4a, IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I exhibited similar potencies in this assay, whereas des-(1-3)-IGF-I, des-(1-4)-IGF-I and des-(1-5)-IGF-I. were at least 100-fold less potent for binding to the MDBK protein. When the assay was performed using medium conditioned by L6 cells as a source of binding proteins, IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I again exhibited similar potencies (see figure 6.4b). On the other hand, des-(1-3)-IGF-I was 60-fold less potent and des-(1-4)-IGF-I was 280-fold less potent than IGF-I.

6.4 DISCUSSION

The truncated IGF-I peptides compared in this chapter fit into three groups. The first group, comprising IGF-I together with des-(1)-IGF-I and des-(1,2)-IGF-I, bind equally to the L6 myoblast receptor, have equal potencies in the myoblast protein synthesis assay and bind equally to an IGF-binding protein purified from MDBK-cell-conditioned medium as well as to a crude preparation of binding protein secreted by L6 myoblasts. Moreover, all three peptides exhibit an anomalous binding pattern when the myoblast receptor assay is performed at 25°C (figure 6.3a). The second group of truncated peptides consists of des-(1-3)-IGF-I and des-(1-4)-IGF-I. These are more potent than the intact molecule in the protein synthesis bioassay, do not significantly compete for ^{125}I-IGF-I binding to either preparation of binding protein and do not exhibit the anomalous receptor binding at 25°C. Of these peptides, des-(1-3)-IGF-I is more potent biologically because it exhibits a higher affinity for the cell receptor (figure 6.4a). The third group of peptides tested comprises molecules with five or six amino acid residues omitted from the amino-terminus. Neither peptide shows significant biological activity, no doubt because they bind so poorly to the cell receptors. However, it is unlikely that this preparation of des-(1-5)-IGF-I possesses the correct disulphide bonds since a discrete peak of folded peptide was not found upon chromatography of the material after reoxidation. Generation of des-(1-5)-IGF-I by an alternative means such as proteolysis of des-(1-4)-IGF-I may lead to a preparation exhibiting a greater receptor-binding activity. Des-(1-6)-IGF-I lacks the cysteine residue at position 6 and therefore was also unable to form the correct disulphide bonds.

Only a small part of the increased potency of des-(1-3)-IGF-I can be explained by enhanced binding of the growth factor to receptors on L6 myoblasts, in accord with results using both natural and synthetic preparations of this peptide (ch. 5). On the other hand, Sara and coworkers, who are probably investigating the same peptide based on their published amino-terminal sequences (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986), find that it competes for ^{125}I-IGF-I binding to the human brain receptor some fivefold more effectively than IGF-I. This increased receptor potency matches the higher biological potency (Sara *et al.*, 1986). Perhaps the differences in binding potencies in the two series of investigations can be explained by true molecular differences between receptors, because the type 1 receptor in brain has an altered carbohydrate composition (Gammeltoft *et al.*, 1985).

The modest reduction in receptor binding with des-(1-4)-IGF-I suggests that there is an involvement of the amino-terminal region in IGF-I binding to the cell receptor. Studies with insulin, which binds to a homologous receptor (Ullrich *et al.*, 1986), have suggested that the major receptor binding region of this molecule consists of the carboxy-terminal pentapeptide of the B chain together with various residues of the A-chain (Pullen *et al.*, 1976; Nakagawa & Tager, 1987). The removal of up to four residues from the amino-terminus of the insulin B-chain caused only a slight reduction in biological activity whereas the further removal of His^{B5} to give des-(B^{1-5})-insulin gave a protein which exhibited only 5% the activity of insulin in an isolated fat-cell assay (Schwartz & Katsoyannis, 1978). This histidine residue, which is highly conserved between species (Blundell & Wood, 1975), is homologous to Thr^4 of IGF-I and may represent a portion of the insulin molecule which is important for distinguishing it functionally from IGF-I. The threonine residue at position 4 of IGF-I appears to play only a minor role in receptor binding since des-(1-4)-IGF-I binds to the receptor only threefold less well than des-(1-3)-IGF-I (figure 6.4a). Moreover, this conclusion is in agreement with studies in which [Gln^3, Ala^4]IGF-I was found to be equipotent to IGF-I for binding to the type 1 receptor (Bayne *et al.*, 1988) while on the other hand, the replacement of residue Tyr^{24} by a non-aromatic residue caused a large decrease in affinity for the type 1 receptor (Cascieri *et al.*, 1988). Other workers have implicated the carboxy-terminal (A) domain (Tseng *et al.*, 1987) and the tyrosine residues at positions 24, 31 and 60 in the binding of IGF-I to cell-receptors (Maly & Luthi, 1988).

Although affinity is the major determinant of receptor-binding activity, these results suggested that L6 myoblasts produced a substance which caused cell-associated ^{125}I-IGF-I to be increased by addition of low concentrations of unlabelled IGF-I, des-(1)-IGF-I or des-(1,2)-IGF-I in a radioreceptor assay performed at 25°C. Similarly, De Vroede *et al* (1986) showed that low concentrations of IGF-I or an insulin-IGF hybrid caused an increase in the binding of ^{125}I-IGF-I to the cell surface of fibroblasts. They proposed that the extra binding resulted from displacement of tracer from a secreted binding protein, thus allowing more to bind at the cell surface. They simultaneously measured the amount of radioligand bound to the cell surface and to medium proteins in the presence of increasing concentrations of unlabelled ligand and found that maximal binding of the radioligand to the cell surface occurred at concentrations of unlabelled ligand which caused 30-80% displacement of radioligand from medium proteins. De Vroede *et al* (1986) further demonstrated that the medium had an adequate binding capacity for this process to occur. This interpretation is supported by results reported in this chapter because only those peptides which were able to bind significantly to proteins present in medium conditioned by L6 cells could cause an increased association of radioligand with the cell-surface.

The binding differences between the various truncated IGF-I peptides with either the MDBK-cell binding protein or the myoblast-conditioned medium are dramatic. Thus, virtually a total loss of binding occurs when Glu^3 is absent. The possibility that the effect of removal of Glu^3 is due to the perturbation of the IGF tertiary structure seems unlikely since firstly, the reduced protein was able to be oxidized correctly to give a protein of the expected chromatographic properties, and secondly this protein bound well in the radioreceptor assay performed at 3°C. It is more likely that residue Glu^3 is directly involved in binding to the IGF-binding protein, perhaps as part of an ion pair with a residue on the binding protein, although the altered position of the positively charged amino-terminal amino group may also act to inhibit interaction between des-(1-3)-IGF-I or des-(1-4)-IGF-I and the binding proteins. My results are consistent with the proposal by Blundell *et al* (1983) that the surface homologous to the hexamer formation region of insulin might be involved in binding to binding proteins. This region centered around residues Ala^{13}, Phe^{16}, Val^{17} and Leu^{54} also includes residues Glu^3, Leu^5, Glu^9, Gln^{15}, Asp^{53}, Arg^{55} and Glu^{58} of IGF-I. Moreover, studies with hybrid insulin-IGF-I molecules have identified the amino-terminal (B) domain

of IGF-I as being important for peptide association with binding proteins (De Vroede *et al.*, 1985). In support of the proposal that the N-terminal region of IGF-I is important for association with binding proteins, Bayne *et al.* (1988) found that [Gln^3, Ala^4, Tyr^{15}, Leu^{16}]IGF-I was 600-fold less potent than IGF-I for binding to a crude preparation of human serum binding proteins. Replacement of various residues of the A-domain of IGF-I with residues found in insulin, which does not bind IGF-binding proteins, did not cause any dramatic changes in affinity for serum binding proteins (Cascieri *et al.*, 1989).

The findings reported in this chapter point to the lack of association between IGF-I peptides and binding proteins as leading to enhanced biological potency. Thus, both des-(1-3)-IGF-I and des-(1-4)-IGF-I, which are more potent than IGF-I in the bioassay, do not bind significantly to the binding protein present in medium conditioned by L6 myoblasts. Moreover, the enhanced biological response is particularly evident at low concentrations of these truncated peptides (figure 6.2), conditions where binding protein secreted into the medium would be expected to reduce more effectively the concentration of free IGF-I, des-(1)-IGF-I and des-(1,2)-IGF-I. Ritvos *et al.* (1988) have demonstrated that IBP-1 from human decidua inhibits both the binding of IGF-I to a choriocarcinoma cell line and also IGF-I-stimulated α-[^{3}H]aminoisobutyrate uptake. Similarly, Rutanen *et al.* (1988) found that human secretory phase endometrium cells produced IBP-1 which inhibited the binding of IGF-I to the type 1 IGF receptor of these cells. This binding protein was secreted in a cyclic manner during the menstrual cycle and may be physiologically important in regulating the biological actions of IGF-I by an autocrine or paracrine mechanism. This postulate that it is the concentration of free IGF-I peptide that determines the biological potency, seems at variance with a report by Elgin *et al* (1987) in which the same IBP-1 from human amniotic fluid potentiated IGF-I stimulation of DNA synthesis in several fibroblast cell-lines and in aortic smooth muscle cells. However, this IBP-1 has been found to inhibit IGF-I effects on protein degradation, protein synthesis and DNA synthesis on chick embryo fibroblasts (Ross *et al.*, 1989). It is noteworthy that the cells used by Elgin *et al* (1987) were particularly insensitive to IGF-I in the absence of binding protein. Also, the IGF-I binding to the cell monolayer was predominantly to a surface protein other than the type 1 receptor, since it had a lower molecular weight than expected and neither anti-(type 1 receptor) antibodies nor insulin inhibited IGF-I binding (Clemmons *et al.*, 1986). A comparison of the amino-acid sequence

of human IBP-1 with the partial sequence of the MDBK-cell binding protein used in this study, has shown significant similarity (see fig. 1.2). The MDBK-cell protein is probably the bovine homologue of the rat IBP-3 which is derived from the conditioned medium of BRL-3A cells (Brown *et al.*, 1989; Upton, 1989). The amino-terminal region of the MDBK binding protein possesses in insertion of ten residues relative to IBP-1 but the internal peptide of the MDBK binding protein exhibits sequence identity to a carboxy-terminal region of IBP-1 in 15 out 23 positions and includes the Arg-Gly-Asp motif which Brewer *et al.* (1988) suggest is involved in attachment to cells and potentiation of IGF-I action. However, De Mellow & Baxter (1988) have recently found that the growth hormone-dependent IBP-2 inhibits IGF-I-stimulated thymidine incorporation when coincubated with the IGF-I but potentiates the action of IGF-I if added 8-48h prior to the addition of IGF-I. This effect apparently resulted from an increase in the maximal rate of [^{3}H]thymidine incorporation rather from a decrease in the basal rate of incorporation as might be expected if added binding protein sequestered IGFs produced by the cells. It should be noted that the IBP-2 used in this study exhibits only limited similarity to the IBP-1 used by Ritvos *et al.* (1988) and Rutanen *et al.* (1988). These data suggest IGF-binding proteins may be either inhibitory or stimulatory depending the mode of their presentation to cells and the cell-culture conditions employed.

I have identified des-(1-3)-IGF-I as being the most potent amino-terminally truncated form of IGF-I in L6 myoblasts and propose that the increased potency is due to its inability to bind to IGF-binding proteins produced by the cells. As yet, no physiological role for des-(1-3)-IGF-I has been established. This peptide has only been detected in human brain (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986), human foetal circulation (Carlsson-Skwirut *et al.*, 1987), bovine colostrum (Francis *et al.*, 1986,1988) and porcine uterus (Ogasawara *et al.*, 1989). Des-(1-3)-IGF-I was not able to be purified from foetal bovine serum, suggesting that it does not occur artefactually during the purification procedures (Francis *et al.*, 1988). It is not clear whether des-(1-3)-IGF-I is produced by action of a specific protease in the liver, brain, mammary gland or uterus.

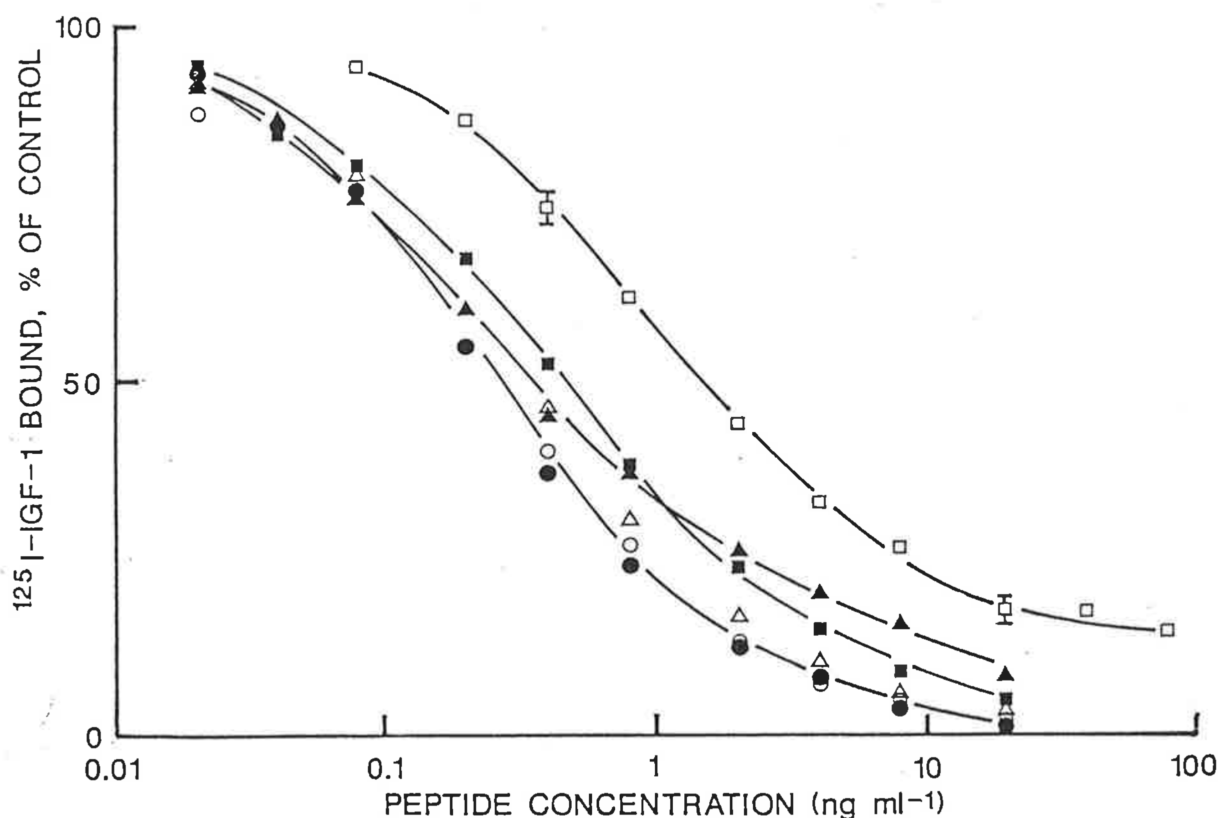


Fig. 6.1: IGF-I Radioimmunoassay of IGF-I and analogues

The ability of IGF-I and analogues from reference preparation B to compete with ^{125}I-IGF-I for binding to the anti-IGF-I antiserum was measured. Displacement by IGF-I (●); des-(1)-IGF-I (○); des-(1,2)-IGF-I (△); des-(1-3)-IGF-I (■); des-(1-4)-IGF-I (▲); des-(1-5)-IGF-I (□). Total binding was 35% a blank, determined in the absence of antibody, equal to 1.9% of total added radioactivity had been subtracted. Data plotted represent the means of triplicate determinations and are expressed as percentages of the binding evident in the absence of competing ligand. As an indication of assay variability, standard errors of the mean are indicated for the des-(1-5)-IGF-I data when they are sufficiently large in relation to the symbols.

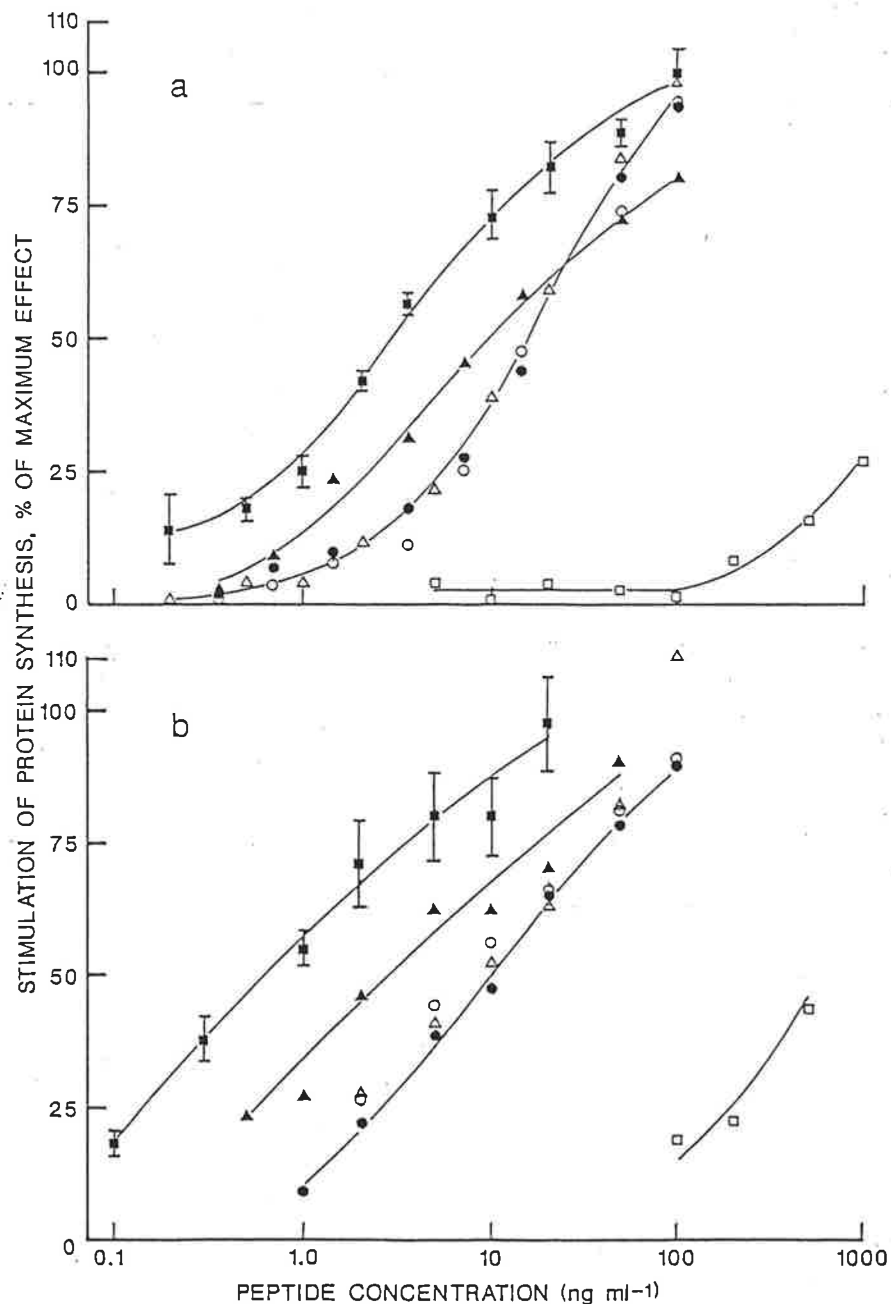


Fig. 6.2: Protein synthesis bioassay of IGF-I and analogues

The abilities of IGF-I and analogues to stimulate protein synthesis in L6 myoblasts was measured. *Panel a*: bioassay of peptides of reference preparation A. *Panel b*: bioassay of peptides of reference preparation B. Stimulation by IGF-I (●); des-(1)-IGF-I (○); des-(1,2)-IGF-I (△); des-(1-3)-IGF-I (■); des-(1-4)-IGF-I (▲); des-(1-5)-IGF-I (□). Data represent the means of triplicate determinations. Incorporation of radioactivity in the absence of added growth factor was 1.0% of total added radioactivity in experiment A and 2.3% in experiment B. Incorporation caused by 5% foetal bovine serum was 2.8% in experiment A and 4.9% in experiment B. As an indication of assay variability, standard errors of the mean are indicated for the des-(1-3)-IGF-I data.

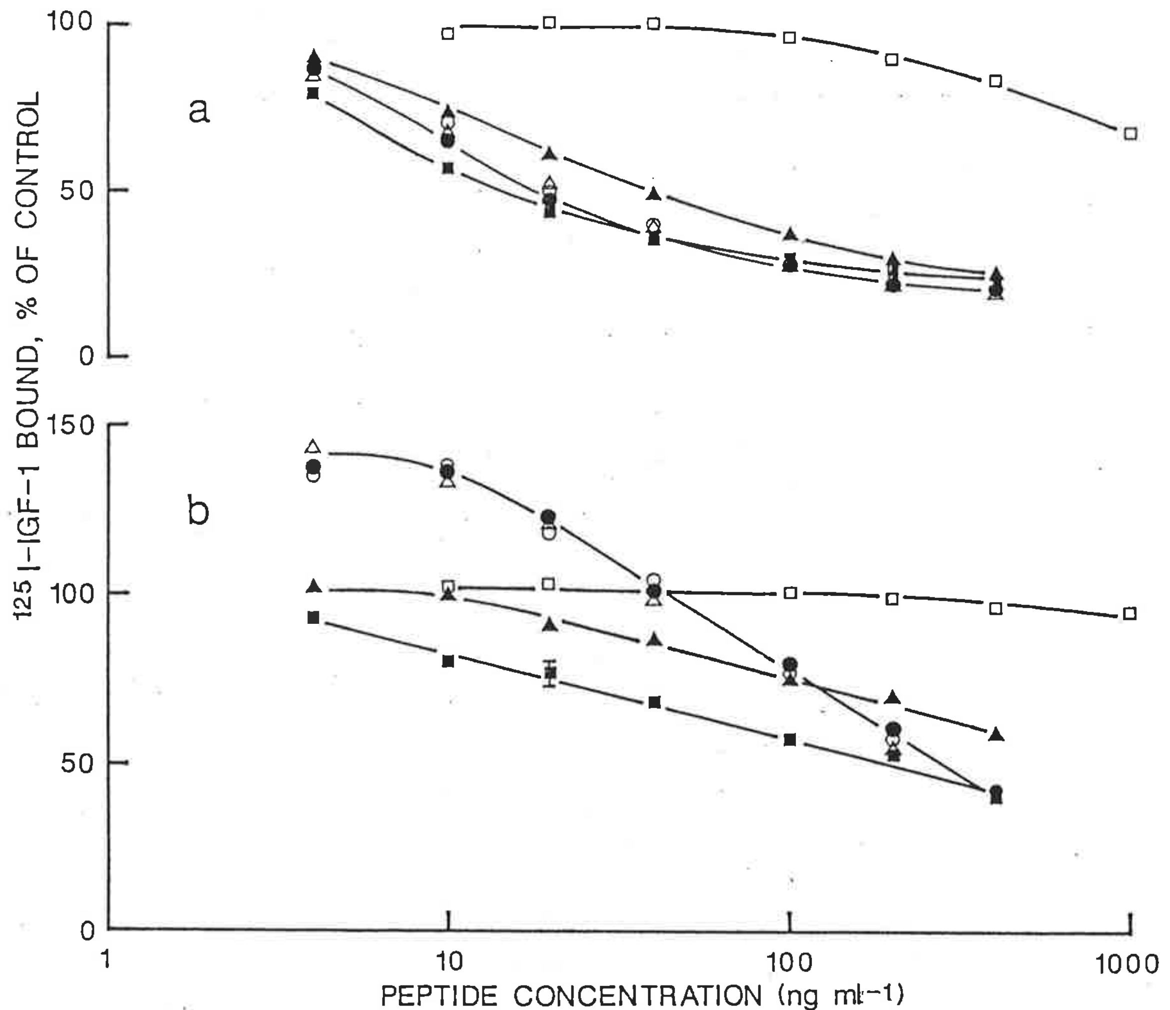


Fig. 6.3: IGF-I Radioreceptor assay using L6 myoblasts

The abilities of IGF-I and analogues from reference preparation B to inhibit binding of ^{125}I-IGF-I to L6-cell monolayers were determined. Inhibition by IGF-I (●); des-(1)-IGF-I (○); des-(1,2)-IGF-I (△); des-(1-3)-IGF-I (■); des-(1-4)-IGF-I (▲); des-(1-5)-IGF-I (□). *Panel a*: receptor assay incubation performed at 3°C for 6h. 13.6% of added radioactivity was bound in the control wells. *Panel b*: receptor assay incubation performed at 25°C for 2h. 6.9% of added radioactivity was bound in the control wells. Non-specific binding equal to has not been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. As an indication of assay variability, standard errors of the mean are indicated for the des-(1-3)-IGF-I data when they are sufficiently large in relation to the symbols.

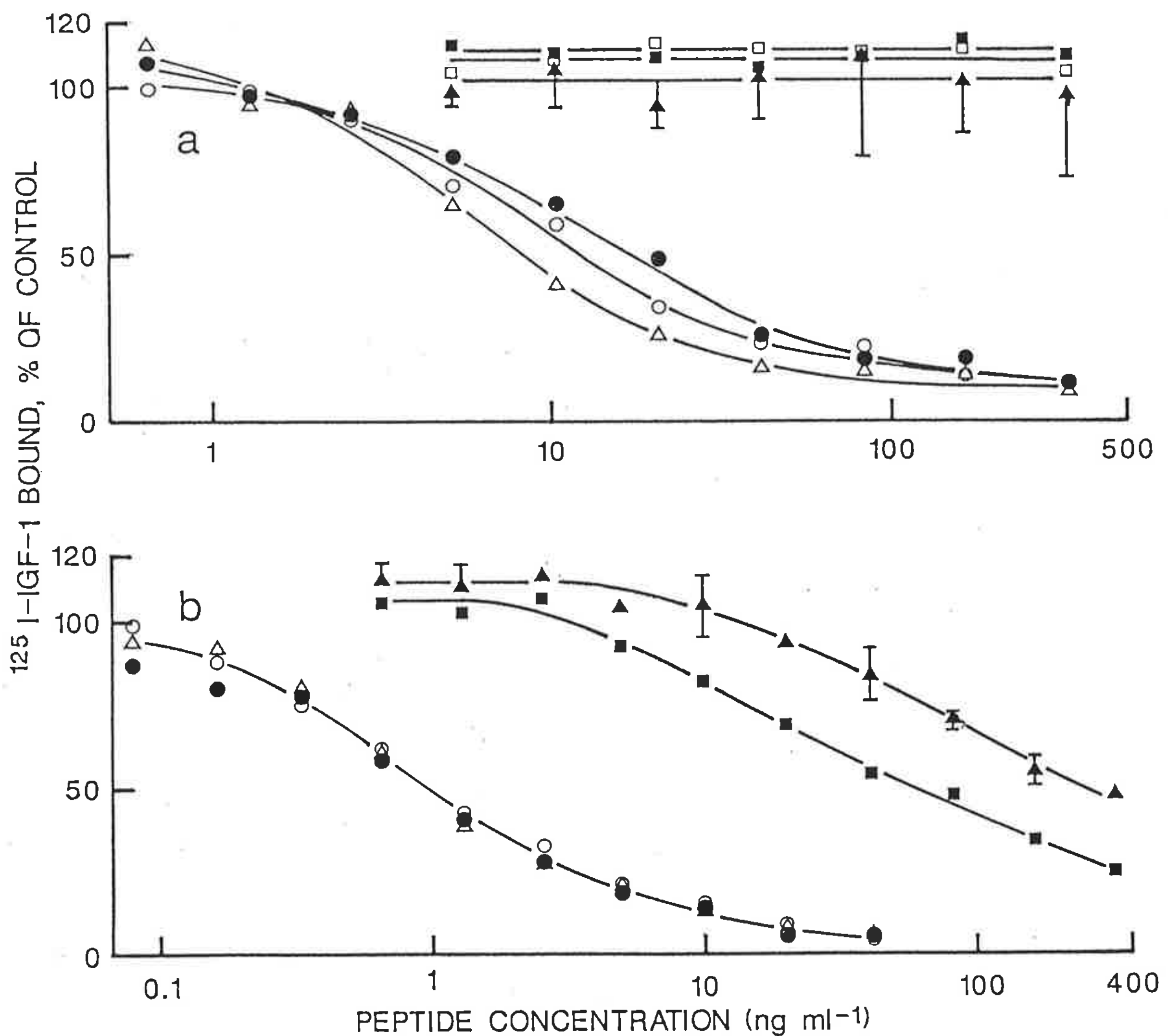


Fig. 6.4: **Binding assay using MDBK-cell binding protein and L6-myoblast-conditioned medium**

Competition for binding of ^{125}I-IGF-I to the purified MDBK binding protein or to L6-conditioned medium by IGF-I and analogues from reference preparation B (except where noted) was measured. The competing peptides were IGF-I (●); des-(1)-IGF-I (○); des-(1,2)-IGF-I (△); des-(1-3)-IGF-I (■); des-(1-4)-IGF-I (▲); des-(1-5)-IGF-I (□). *Panel a:* binding assay using MDBK binding protein. Total binding was 23% after a blank, determined in the absence of binding protein, equal to 19% of added radioactivity, had been subtracted. *Panel b:* binding assay using L6-conditioned medium. In this assay, recombinant IGF-I was substituted for synthetic IGF-I and a different preparation of des-(1-3)-IGF-I was used. Total binding was 26% after a blank equal to 7.8% had been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. As an indication of assay variability, ranges are indicated for the des-(1-4)-IGF-I data when they are sufficiently large in relation to the symbols.

CHAPTER 7

THE ROLE OF GLUTAMATE-3

CHAPTER 7: THE ROLE OF GLUTAMATE-3

7.1 INTRODUCTION

As discussed in chapter 6, it is possible residue Glu^3 is directly involved in binding to the IGF-binding protein perhaps via an ionic bond. Alternatively, the altered position of the positively charged N-terminal amino group may act to inhibit interaction between des-(1-3)-IGF-I or des-(1-4)-IGF-I and the binding proteins. In order to distinguish between these possibilities, analogues of IGF-I were synthesized which possessed substitutions or deletions in the amino-terminal region. A further aim was to create analogues of IGF-I which were more potent biologically than des-(1-3)-IGF-I. In view of the hypothesis that it is the IGF-binding proteins which inhibit the action of IGF-I and that Glu^3 is important for this interaction (ch. 6), it was proposed that the incorporation of a positively charged amino acid in place of Glu^3 might further enhance the biological activity of the IGF-I molecule.

In order to investigate the importance of the position of the N-terminal amino group, I first prepared the analogues des-(2)-IGF-I des-(2,3)-IGF-I and des-(2-4)-IGF-I which differ from the truncated peptides analysed in chapter 6 by the addition of an N-terminal Gly residue. These peptides might also have the additional advantage that they can be produced by recombinant DNA methods using hydroxylamine to cleave the IGF analogue from a fusion partner. In order to investigate the role of residue Glu^3, analogues of IGF-I containing substitutions at position 3 were made. The substitutions chosen to replace Glu^3 were Gly (no side-chain), Asp (negatively charged with a shorter side-chain), Gln (hydrophilic neutral and almost isosteric), Leu (hydrophobic), Lys (positively charged) and Phe (aromatic). In the light of the results obtained with the earlier series of peptides, the role of Thr^4 was also investigated using Gly, Val (hydrophobic and almost isosteric) and Arg (positively charged) to substitute for this residue. Analogues possessing double substitutions, [Gly^3, Gly^4]IGF-I & [Gly^3, Arg^4]IGF-I, and both truncation and substitution, (des-(1-3)-[Val^4]IGF-I & des-(1-3)-[Arg^4]IGF-I, were also synthesized . Each of these peptides was evaluated for its ability to stimulate protein synthesis in rat L6 myoblasts, to compete for binding of radiolabelled IGF-I to these cells and to compete for binding of radiolabelled IGF-I to the IGF-binding proteins from the media conditioned by MDBK cells or L6 myoblasts. A radioimmunoassay was used to confirm the estimations of protein amount. This last method

is useful since the natural, recombinant and synthetic peptides are quantified differently during their preparation. The radioimmunoassay may also serve to correct for the presence of impurities in the preparation of peptide. This is probably a conservative correction since the polyclonal antiserum probably recognizes incorrectly folded peptide better than does the IGF-I receptor (compare the des-(1-5)-IGF-I peptide in figs. 6.1 & 6.3a). However, it is possible that some peptides exhibit a binding to the antiserum that is intrinsically weaker than that of IGF-I, leading to an underestimation of protein amount.

An additional advantage of producing potent analogues of IGF-I by substitution, rather than truncation, is that the approach may facilitate the production of IGFs by recombinant DNA methods where the IGF-I analogue can be liberated from a fusion partner by a chemical method or by the action of a specific protease. The inclusion of specific residues at the amino terminus of a truncated IGF-I molecule may interfere with the ability of the peptide to fold to form a biologically active molecule whereas residues one and two of IGF-I have little effect on the biological properties of the molecule (ch. 6).

I wish to acknowledge the assistance of Ms. M.Ross in the performance of some of the bioassays and radioreceptor assays, Mr. M.Conlon for the radioimmunoassays and Mr. L.Szabo who performed some of the binding protein competitive binding assays.

7.2 MATERIALS AND METHODS

IGF peptides were synthesized as described in sections 4.3c and 4.3d. After oxidation of the peptides to form the correct disulphide bonds and purification by reverse-phase HPLC as described in section 4.3e, the peptides were rechromatographed on reverse-phase HPLC (fig. 4.9) and the amounts of protein were estimated by the method described in section 2.2c. Radioimmunoassays, radioreceptor assays, bioassays and binding protein competitive binding assays were performed as described in section 2.4.

7.3 RESULTS

7.3a Truncated peptides with an N-terminal Glycine residue

Resin-bound IGF(4-70), IGF(5-70) and IGF(6-70) peptides from synthesis BIO-2 were subjected to the addition of a glycine residue, deprotected and reoxidized by the

techniques as described in section 4.3c. The N-terminal sequences of these peptides and those of IGF-I and des-(1-3)-IGF-I were;

des-(2,3)-IGF-I	Gly-Thr-Leu-Cys
des-(2-4)-IGF-I	Gly-Leu-Cys
des-(2-5)-IGF-I	Gly-Cys
IGF-I	Gly-Pro-Glu-Thr-Leu-Cys
(1-3)-IGF-I	Thr-Leu-Cys

A reverse-phase HPLC chromatogram of the reoxidized des-(2-5)-IGF-I peptide did not indicate a discrete peak of peptide corresponding to correctly-oxidized peptide and hence the preparation of that particular peptide is probably impure.

The peptides were tested for their abilities to stimulate protein synthesis in the L6 bioassay, as shown in figure 7.1. The potencies of the peptides were assessed as the concentrations required to elicit an effect equal to 40% of that caused by 10% foetal bovine serum. The concentrations (ng/ml) of peptide required to cause this effect were;

des-(2-4)-IGF-I,	2.2;
des-(1-3)-IGF-I,	4.5;
des-(2,3)-IGF-I,	6.2;
recombinant IGF-I,	15;
des-(2-5)-IGF-I,	approx. 530.

The peptides were then tested for their abilities to compete with radiolabelled IGF-I for binding to L6 myoblasts, as shown in figure 7.2. The potencies of the peptides were assessed as the concentrations required to cause a 50% competition for tracer binding to the cells. The recombinant, des-(1-3)- and des-(2,3)-IGF-I were equipotent, requiring a concentration of 84ng/ml for 50% competition, whereas des-(2-4)-IGF-I was 1.3-fold less potent and des-(2-5)-IGF-I was approximately 13-fold less potent than recombinant IGF-I.

The peptides were also tested for their abilities to compete with radiolabelled IGF-I for binding to the IGF-binding protein purified from the medium conditioned by MDBK cells. As shown in figure 7.3, IGF-I caused 50% competition for radioligand binding at a concentration of 8ng/ml whereas des-(2,3)-IGF-I was 200-fold less potent and des(2-4)-IGF-I and des(2-5)-IGF-I showed no competitiion at concentrations of up to 2.4μg/ml.

A separate set of bioassays was performed to directly compare des-(1-3)-IGF-I and des-(1-4)-IGF-I with their homologues derivatized at the N-terminus by a Gly residue. Thus, des-(1-3)-, des-(2,3)-, des-(1-4)-, des-(2-3)- as well as recombinant IGF-I were tested in the L6 bioassay (fig. 7.4). The potencies, assessed as the concentrations (ng/ml) of peptide required to elicit an effect equal to 50% of that caused by 10% foetal bovine serum, were;

des-(2-4)-IGF-I,	1.0;
des-(1-3)-IGF-I,	1.3;
des-(2,3)-IGF-I,	1.6;
recombinant IGF-I,	6.7;
des-(1-4)-IGF-I,	10.

7.3b Substitutions at position 3 of IGF-I

Analogues of IGF-I in which residue Glu^3 was replaced by Gly, Asp, Gln, Leu, Lys or Phe were produced from BIO-2 IGF(4-70)-resin as described in section 4.3c. The N-terminal sequences of these peptides were;

[Gly^3]IGF-I	Gly-Pro-**Gly**-Thr-Leu-Cys
[Asp^3]IGF-I	Gly-Pro-**Asp**-Thr-Leu-Cys
[Gln^3]IGF-I	Gly-Pro-**Gln**-Thr-Leu-Cys
[Leu^3]IGF-I	Gly-Pro-**Leu**-Thr-Leu-Cys
[Lys^3]IGF-I	Gly-Pro-**Lys**-Thr-Leu-Cys
[Phe^3]IGF-I	Gly-Pro-**Phe**-Thr-Leu-Cys.

No special difficulties were encountered in the deprotection and reoxidation of these peptides, although the peaks of correctly-oxidized peptide eluted from reverse-phase HPLC exhibited greater peak-width than usual (fig. 4.8). This may have resulted from deterioration of the peptide during storage of the IGF(4-70) peptide-resin for approximately six months prior to the performance of the final three coupling cycles.

The six peptides substituted at position three were compared with IGF-I and des-(1-3)-IGF-I in the L6 myoblast bioassay as shown in figure 7.5. The potencies of the peptides, assessed as the concentrations (ng/ml) required to cause a stimulation of protein synthesis equal to 100% above control, were;

[Gly^3]IGF-I,	1.1;
des-(1-3)-IGF-I,	1.1;
[Lys^3]IGF-I,	2.3;
[Leu^3]IGF-I,	2.3;
[Gln^3]IGF-I,	2.4;
IGF-I,	5.1;
[Phe^3]IGF-I,	6.5;
[Asp^3]IGF-I,	10.5.

These peptides were also examined in the L6 myoblast radio-receptor assay as shown in figure 7.6. The potencies of the peptides, assessed as the concentrations (ng/ml) required to cause a 50% competition for tracer binding to the cells, were;

des-(1-3)-IGF-I,	7.2;
[Gly^3]IGF-I,	8.1;
IGF-I,	10;
[Phe^3]IGF-I,	18;
[Leu^3]IGF-I,	19;
[Gln^3]IGF-I,	20;
[Lys^3]IGF-I,	30;
[Asp^3]IGF-I,	48.

These peptides were further examined for their abilities to compete with ^{125}I-IGF-I for binding to the IGF-binding protein purified from MDBK-cell-conditioned medium and to the binding proteins present in L6-myoblast-conditioned medium as shown in figure 7.7. IGF-I was able cause 50% competition for tracer IGF-I binding to the MDBK protein at a concentration of 13ng/ml whereas [Phe^3]IGF-I was sixfold less potent. Des-(1-3)-IGF-I and the other peptides bearing substitutions at position 3 competed at least 50-fold less well than IGF-I. Using the L6 myoblast conditioned medium as the source of binding protein, the concentrations of peptide (ng/ml) required for 50% competition for tracer binding were;

recombinant IGF-I,	3.2;
[Phe^3]IGF-I,	5.1;
[Gly^3]IGF-I,	13;
[Asp^3]IGF-I,	17;
[Gln^3]IGF-I,	18;
[Leu^3]IGF-I,	50;
des-(1-3)IGF-I,	120;
[Lys^3]IGF-I,	220.

The data from the above experiments could be adjusted for the protein amount estimated by radioimmunoassay, which serves to correct for the presence of impurities, as shown in table 7.1.

7.3c Further investigation of the functional role of the N-terminus of IGF-I

A further chemical synthesis (BIO-3) was performed in order to test the effect of replacement of residues 3 or 4 with positively charged amino acid residues and to investigate the role of Thr^4 which is apparently involved in the interactions of IGF-I with both the cell surface receptor and the IGF-binding protein present in the medium conditioned by L6 myoblasts (see chapter 6). The peptides produced by Mr. May and me were;

des-(1-3)-[Val^4]-IGF-I	**Val**-Leu-Cys
des-(1-3)-[Arg^4]-IGF-I	**Arg**-Leu-Cys
des-(2-4)-IGF-I	**Gly**-Leu-Cys
[Gly^3]-IGF-I	Gly-Pro-**Gly**-Thr-Leu-Cys
[Arg^3]-IGF-I	Gly-Pro-**Arg**-Thr-Leu-Cys
[Gly^3, Gly^4]-IGF-I	Gly-Pro-**Gly**-**Gly**-Leu-Cys
[Gly^3, Arg^4]-IGF-I	Gly-Pro-**Gly**-**Arg**-Leu-Cys
IGF-I	Gly-Pro-Glu-Thr-Leu-Cys
des-(1-3)-IGF-I	Thr-Leu-Cys

The IGF-I, des-(1-3)-, des-(2-4)- and [Gly^3]-IGF-I peptides were resynthesized to serve as control peptides to allow evaluation of the quality of the synthesis. The production of these peptides is described in section 4.3d.

These peptides were tested for their abilities to stimulate protein synthesis in L6 myoblasts in the series of assays shown in figure 7.8. The peptides were also tested in the IGF-I radioreceptor assay as shown in figure 7.9. Radioimmunoassays were also performed on these peptides in order to confirm the estimates of protein amount. The data are summarized in table 7.2. In order to compare the potencies of the peptides between the three sets of bioassays and radioreceptor assays, the data were normalized against the recombinant IGF-I reference peptide as shown in table 7.3. The most biologically active peptides were des-(1-3)-[Arg^4]IGF-I and [Gly^3, Arg^4]IGF-I which were threefold more active than natural des-(1-3)-IGF-I and 50-fold more active than recombinant IGF-I.

I also tested the abilities of these peptides to compete for binding of tracer IGF-I to the IGF-binding proteins present in medium conditioned by L6 myoblasts and to the purified L6 myoblast IGF-binding protein (fig. 7.10). The IGF peptides competed for tracer binding with similar potencies in both assays with the exception of the [Gly^3]IGF-I peptide which competed strongly in the assay using purified binding protein and the [Gly^3, Gly^4]IGF-I peptide which competed weakly for binding to L6 cell-conditioned medium. In both cases, the competition curves were not parallel to those of other IGF-I analogues. The potencies of these peptides, assessed as the concentration required for 50% competition are shown in table 7.4.

7.4 DISCUSSION

The experiments described in this chapter were designed to investigate the role of residue Glu^3 in the modulation of the biological activity of IGF-I.

The biochemical assays themselves exhibit significant interassay variability due to the degree of confluency of the L6 myoblasts (bioassay and receptor assay) or variation in the specific radioactivity of the tracer IGF-I (radioreceptor assay and binding protein assays). In order to compare potencies of the different peptides which were assayed at different times, the potency values can be normalized against the value obtained for the recombinant IGF-I standard. A further method of normalizing the data is to express the potency in the bioassay relative to the potency in the radioreceptor assay. This ratio, which is independent of the estimate of protein amount, may provide a measure of the extent of inhibition of biological activity by the presence of binding proteins. Thus, a low value for this ratio suggests that the

biological activity of the peptide is only weakly inhibited by the L6-myoblast binding protein.

The first series of experiments involved the addition of a Gly residue to the truncated, des-(1-3)-, des-(1-4)- and des-(1-5)-IGF-I peptides. Des-(1-3)-IGF-I but not des-(1,2)-IGF-I has been previously shown to exhibit enhanced biological potency (fig. 6.2). When the N-terminal Glu residue of des-(1,2)-IGF-I was replaced by a Gly residue, the modified peptide, des-(2,3)-IGF-I, showed the enhanced biological potency observed with des-(1-3)-IGF-I. These data indicate that it is the lack of the side-chain of residue Glu^3, rather than the position of the N-terminal amino group which confers the enhanced biological potency to des-(1-3)-IGF-I and presumably to the other potent analogues of IGF-I. The des-(2-4)-IGF-I peptide was 1.5- to 2-fold more potent than the des-(1-3)-IGF-I and des-(2,3)-IGF-I peptides in the bioassay (figs. 7.1 & 7.3), suggesting that the replacement of residue Thr^4 may further enhance the biological potency of analogues of IGF-I. Unlike des-(1-4)-IGF-I, which showed threefold reduced competition for binding to the IGF-I receptor, des-(2-4)-IGF-I was only 1.3-fold less potent than IGF-I in the radioreceptor assay. As discussed in chapter 4, the des-(2-5)-IGF-I did not apparently oxidize correctly and this peptide exhibited low activity in both the bioassay and the radioreceptor assay.

The role of residue Glu^3 was further investigated by the production of peptides exhibiting single amino acid replacements at this position. After correction for the estimate of protein amount based on a radioimmunoassay, the peptides [Gly^3]-, [Lys^3]-, [Gln^3]- and [Leu^3]-IGF-I were approximately 6- to 7-fold more active than IGF-I in the bioassay, a potency similar to that of des-(1-3)-IGF-I (table 7.1) whereas [Asp^3]- and [Phe^3]IGF-I were of similar potency to IGF-I. With the exception of [Phe^3]IGF-I, these peptides competed poorly for binding to the IGF-binding protein from MDBK cells (fig. 7.7a). Surprisingly, even the [Asp^3]-analogue, in which the side-chain negative charge is retained, was unable to effectively bind to the MDBK protein whereas the [Phe^3]IGF-I peptide was able to do so only sixfold less well than IGF-I. A different pattern of binding was observed when medium conditioned by L6 myoblasts was used as a source of IGF-binding proteins (fig. 7.7b). [Phe^3]IGF-I was equipotent to IGF-I and the [Gly^3]-, [Asp^3]- and [Gln^3]-IGF-I peptides were only threefold less potent. The [Leu^3]-IGF-I was 10-fold less potent than IGF-I while the [Lys^3]- and des-(1-3)-IGF-I peptides were a further threefold less potent. The result with the

[Gln^3]-IGF-I peptide is similar to that of Bayne *et al.* (1988) who found that [Gln^3, Ala^4]-IGF-I was only twofold less potent than IGF-I for binding to serum binding proteins. In general, those peptides which bound most strongly to the L6-myoblast binding protein were the least potent in the bioassay, consistent with the binding protein acting as an inhibitor of IGF action.

In order to develop additional and more-potent analogues of IGF-I, peptides were synthesized bearing substitutions of Arg or Gly at positions three or four with the intention of further antagonizing the interaction between the IGFs and binding proteins. Other peptides exhibiting substitutions at position 4 as well as N-terminal truncations were made. As expected, the most potent peptides were those bearing arginine substitutions (table 7.2). The high potencies of these peptides were more obvious when the biological potencies were expressed relative to the receptor binding potencies (table 7.3), suggesting that these proteins were the least inhibited by IGF-binding proteins during the course of the bioassay. Peptides bearing glycine substitutions, as well as des-(1-3)-IGF-I, were three- to fourfold less potent on the basis of their receptor-binding abilities than Arg-substituted peptides. Des-(1-3)-[Val^4]IGF-I exhibited an intermediate potency. IGF-I was approximately 30-fold less active than the most active peptides, [Gly^3, Arg^4]- and des-(1-3)-[Arg^4]IGF-I.

The peptides from synthesis BIO-3 exhibited similar potencies in the binding protein assays using either crude L6-myoblast-conditioned medium or the purified binding protein. The analogues competed for tracer binding 30- to 200-fold less well than IGF-I with des-(1-3)-[Arg^4]- and [Gly^3, Gly^4]IGF-I binding the most weakly. The estimates of potency of these analogues may not truly reflect their affinities for the binding protein since the high concentrations of peptide required in order to observe competition for tracer IGF-I binding interfere with the non-equilibrium separation of tracer and binding protein. The use of an anti-(binding protein) antibody to effect the separation may make the assay more suitable for measuring competition by weakly-binding peptides.

The results of the experiments reported in this chapter suggest that the side-chain of residue Glu^3 is important for interaction with IGF-binding proteins, especially the MDBK-cell binding protein. Analogues of IGF-I, bearing substitutions which introduce a positively-charged side-chain at postion 3, bind very weakly to binding proteins and exhibit biological potencies greater than that of des-(1-3)-IGF-I.

Table 7.1: Summary of assay data for peptides substituted at position 3

IGF-I PEPTIDE	Bioassay ED_{50} adjusted for RIA (% of rec. IGF-I)	Receptor assay ED_{50} adjusted for RIA	ratio of ED_{50} values (bioassay/RRA)	binding protein assay ratio of ED_{50} values (peptide/rec.IGF-I)
recombinant	100	100	1.0	1.0
synthetic	180	200	0.93	N.D.[1]
des-(1-3)	31	82	0.38	29
[Gly 3]	34	100	0.34	3.4
[Asp 3]	210	380	0.54	2.9
[Gln 3]	73	240	0.30	2.9
[Leu 3]	48	150	0.31	9
{Lys 3]	35	190	0.19	29
[Phe 3]	140	150	0.91	0.9

1) Not determined

Table 7.2: Summary of assay data from figures 7.8 and 7.9

IGF-I PEPTIDE	RADIOIMMUNOASSAY		BIOASSAY			RADIORECEPTOR ASSAY		
	ED 50 (ng/ml)	ED 50 % of rec.[1] IGF-I	ED 50 (ng/ml)	ED 50 based on RIA (ng/ml)	ED 50 % of rec. IGF-I	ED 50 (ng/ml)	ED 50 based on RIA (ng/ml)	ED 50 % of rec. IGF-I
from figs. 7.8a & 7.9a								
recombinant	1.3	100	13	13	100.0	9.0	9.0	100
natural des-(1-3)	1.5	120	0.68	0.59	4.5	4.5	3.9	43
synthetic des-(1-3)	3.4	260	1.5	0.58	4.5	9.0	3.5	39
des-(2-4)	3.2	250	6.4	2.6	20	37	15	170
des-(1-3)-[Arg ^{4}]	3.3	260	0.51	0.20	1.5	10	3.9	44
des-(1-3)-[Val ^{4}]	3.2	250	1.0	0.42	3.2	14	5.7	63
from figs. 7.8b & 7.9b								
recombinant	0.93	1.0	11	11	100	10	10	100
synthetic	0.70	0.75	21	27	240	15	20	190
natural des-(1-3)	0.80	0.86	0.78	0.91	8.1	4.5	5.3	51
synthetic des-(1-3)	2.5	2.6	5.7	2.2	19	31	12	110
des-(2-4)	2.2	2.4	23	9.6	86	130	53	520
des-(1-3)-[Arg ^{4}]	1.1	1.2	0.37	0.31	2.8	8.1	6.9	67
des-(1-3)-[Val ^{4}]	1.2	1.3	1.9	1.5	13	11	8.7	85
[Gly ^{3}]	0.80	0.86	2.1	2.5	22	14	16	160
[Arg ^{3}]	2.5	2.7	2.4	0.88	7.9	34	13	120
[Gly ^{3},Gly ^{4}]	1.00	1.1	2.4	2.2	20	13	12	120
from figs. 7.8c & 7.9c								
recombinant	0.57	1.00	23	23	100	10	10	100
natural des-(1-3)	0.53	0.94	1.7	1.8	8.0	4.5	4.8	47
des-(1-3)-[Val ^{4}]	1.10	1.9	2.4	1.2	5.4	11	5.8	57
[Gly ^{3}]	0.97	1.7	5.7	3.3	15	14	8.1	79
[Arg ^{3}]	1.57	2.8	2.0	0.72	3.2	34	12	120
[Gly ^{3},Gly ^{4}]	0.87	1.5	3.5	2.3	10	13	8.5	83
[Gly ^{3},Arg ^{4}]	1.73	3.1	1.4	0.44	2.0	18	5.7	56

1) Recombinant

Table 7.3: Summary of assay data for peptides from synthesis BIO-3

IGF-I PEPTIDE	mean bioassay ED_{50} (% of recombinant)[1]	mean receptor[2] assay ED_{50} (% of recombinant)	ratio of ED_{50} values (bioassay/RRA)	binding protein assay ratio of ED_{50} values (peptide/rec.IGF-I)[3]
recombinant	100	100	1.0	1.0
synthetic	240	190	1.3	1.3
natural des-(1-3)	6.6	47	0.14	N.D.[4]
synthetic des-(1-3)	9.3	66	0.14	50
des-(2-4)	42	290	0.14	N.D.
des-(1-3)-[Arg^4]	2.0	54	0.038	190
des-(1-3)-[Val^4]	6.1	67	0.091	47
[Gly^3]	18	110	0.16	35
[Arg^3]	5.0	120	0.041	27
[Gly^3,Gly^4]	14	99	0.14	150
[Gly^3,Arg^4]	2.0	56	0.036	32

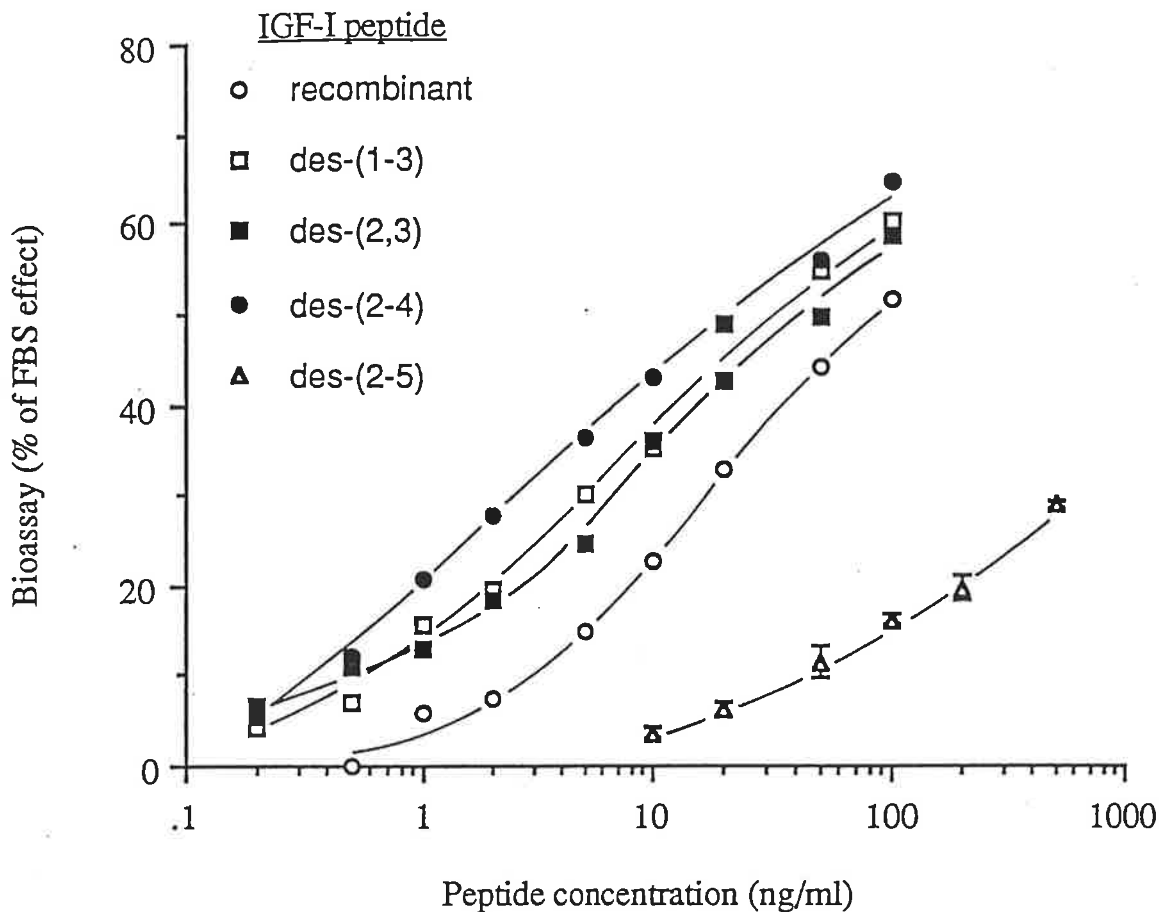
1) Geometric mean of bioassay data from table 7.2
2) Geometric mean of radioreceptor assay data from table 7.2
3) Geometric mean of binding protein assay data from table 7.4
4) Not determined

Table 7.4: Binding protein assay of peptides from synthesis BIO-3

IGF-I PEPTIDE	RIA ED_{50} (ng/ml)[1]	Assay using conditioned medium			Assay using purified protein			Mean of relative potencies[4]
		ED_{50} (ng/ml)	adjusted ED_{50} (ng/ml)[2]	Relative potency[3]	ED_{50} (ng/ml)	adjusted ED_{50} (ng/ml)[2]	Relative potency[3]	
recombinant	170	0.18	0.18	1.0	0.18	0.18	1.0	1.0
synthetic	N.D.[5]	0.22	0.22	1.2	0.25	0.25	1.4	1.3
natural des-(1-3)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
synthetic des-(1-3)	N.D.	5.4	5.4	29	16	16	84	50
des-(2-4)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
des-(1-3)-[Arg 4]	N.D.	33	33	180	38	38	210	190
des-(1-3)-[Val 4]	330	13	6.7	36	22	11	62	47
[Gly 3]	290	4.9	2.9	16	25	15	80	35
[Arg 3]	470	22	8.0	43	8.4	3.0	17	27
[Gly 3, Gly 4]	260	73	48	260	25	16	89	150
[Gly 3, Arg 4]	520	13	4.1	22	26	8.5	46	32

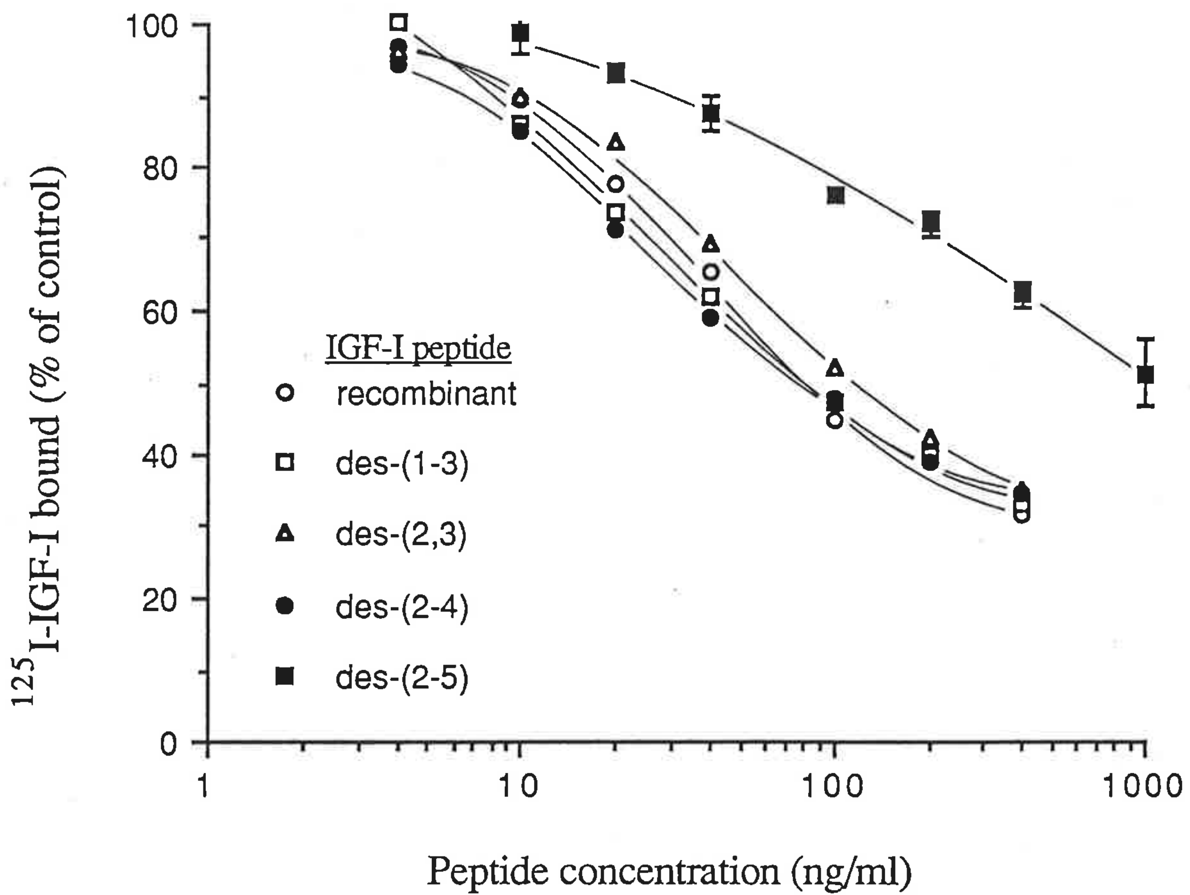
1) concentration required for 50% competition in an IGF-I radioimmunoassay
2) potency in the binding protein assay adjusted for the protein amount estimated by radioimmunoassay
3) ratio of ED_{50} values for the IGF analogue versus recombinant IGF-I
4) Geomatric mean of the relaive potencies in the two binding protein assays
5) not determined

Figure7.1: Bioassay of glycinyl truncated IGF-I peptides



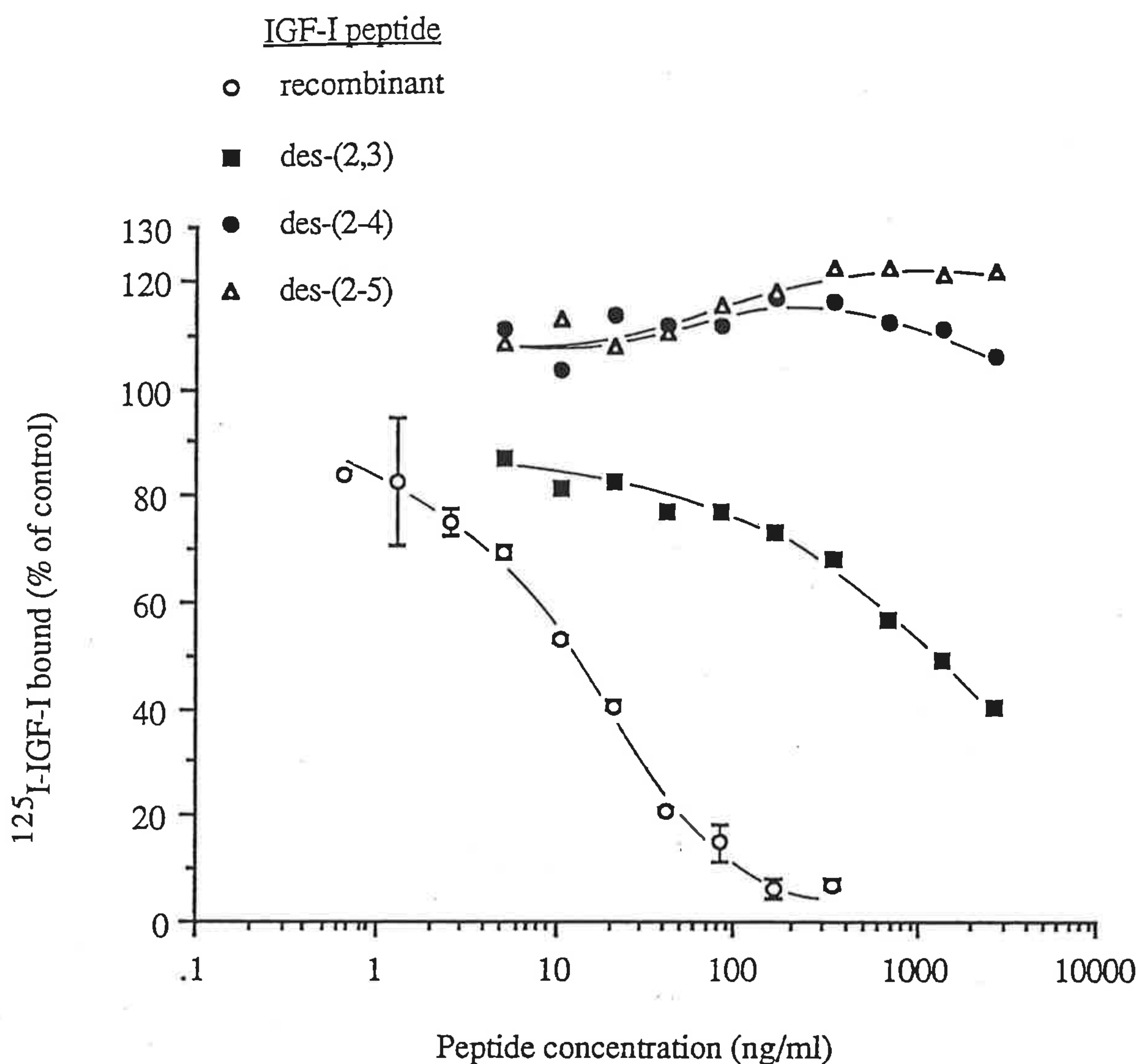
The indicated peptides were tested for their abilities to stimulate protein synthesis in L6 myoblasts. Data represent the means of triplicate determinations and are expressed as a percentage of the stimulation caused by 5% foetal bovine serum. Incorporation of radioactivity into protein in the absence of added growth factor was 0.7% of total added radioactivity and the incorporation caused by 5% foetal bovine serum was 3.0%. As an indication of assay variability, standard errors of the mean are indicated for the des-(2-5)-IGF-I data.

Figure 7.2: Radioreceptor assay of glycinyl truncated IGF-Is



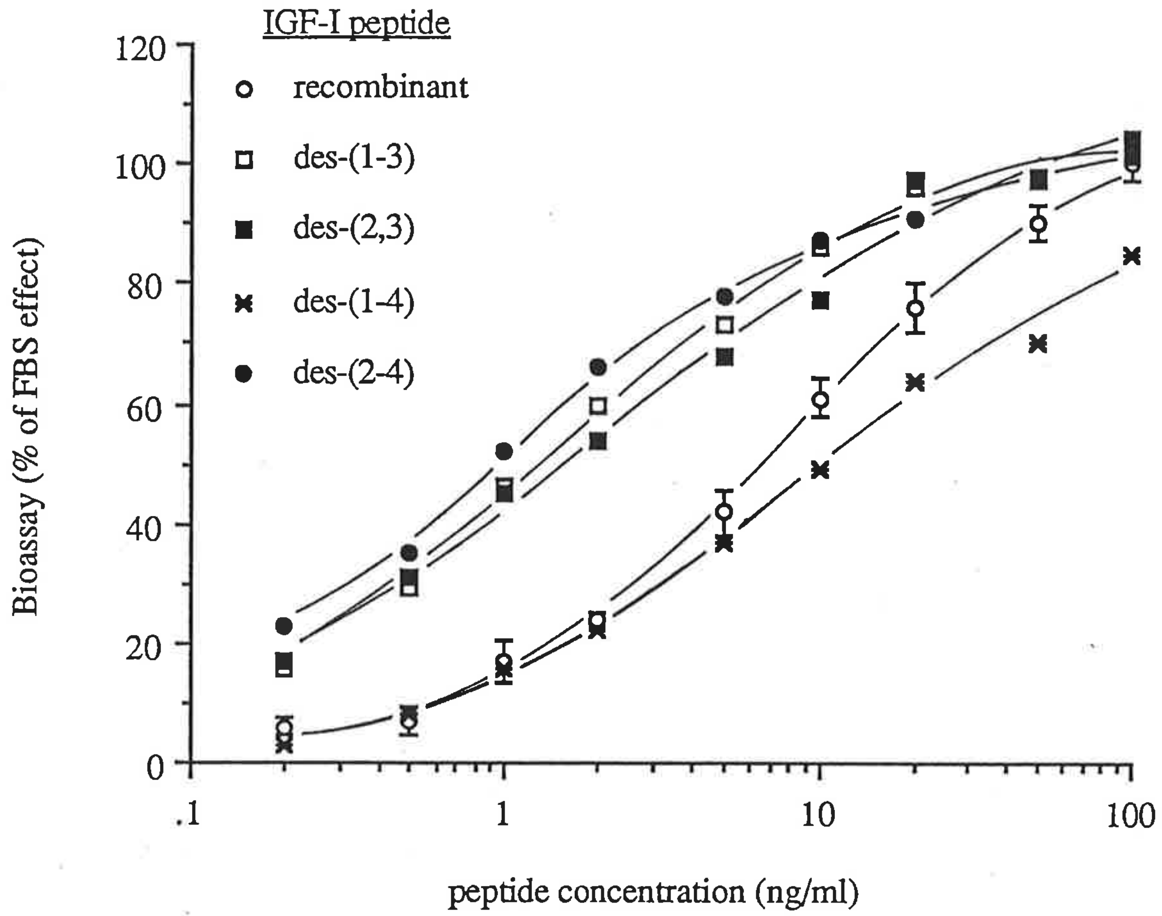
The abilities of the indicated peptides to compete with ^{125}I-IGF-I for binding to L6-cell monolayers were determined. 6.7% of added radioactivity was bound in the control wells and non-specific binding has not been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. As an indication of assay variability, standard errors of the mean are indicated for the des-(2-5)-IGF-I data.

Figure 7.3: Competitive protein binding assay of glycinyl truncated IGF-I peptides



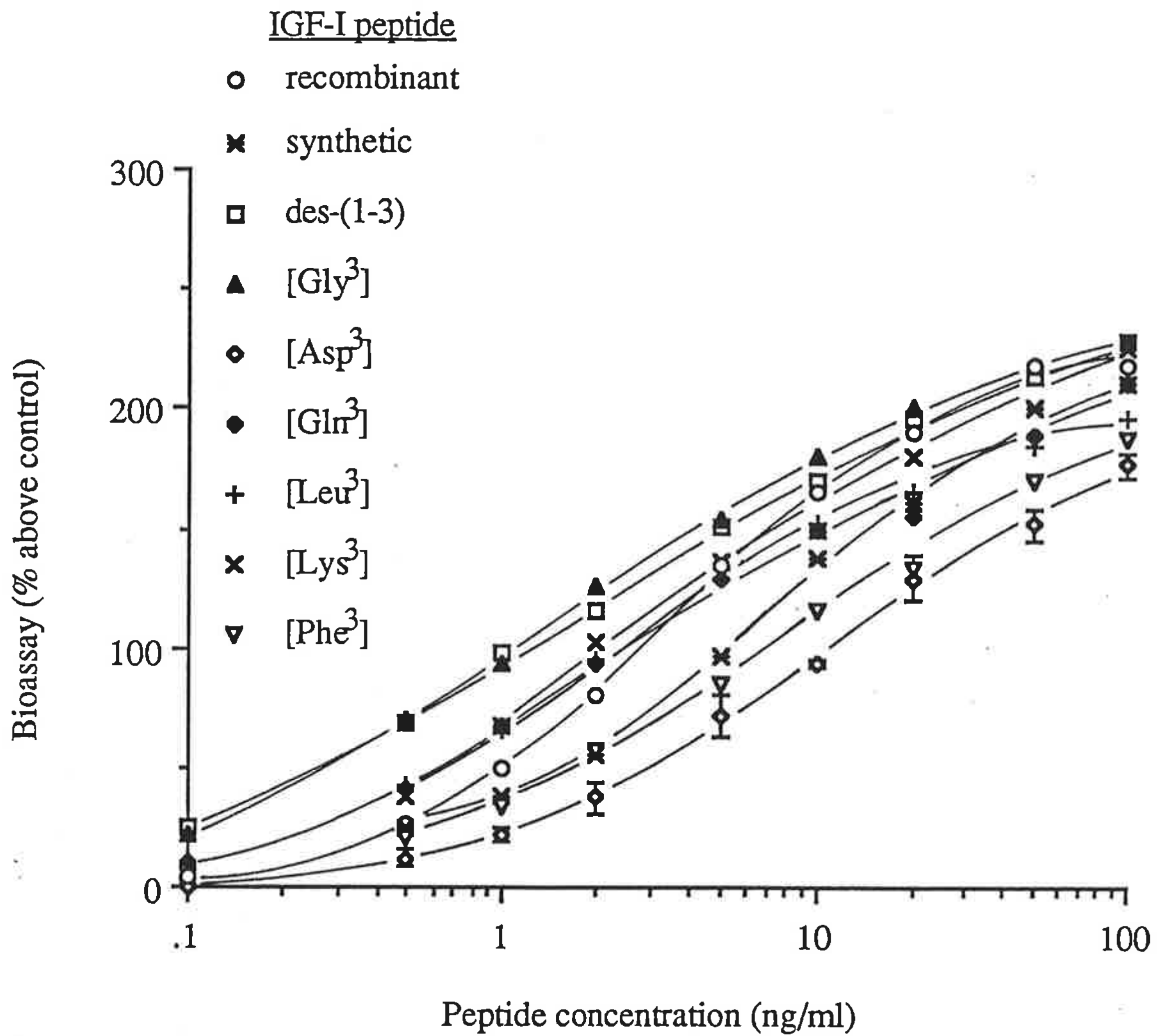
Competition for ^{125}I-IGF-I binding to MDBK-cell binding protein by IGF-I and the indicated analogues was measured. Total binding was 16% after a blank, determined in the absence of binding protein, equal to 12% had been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. As an indication of assay variability, standard errors of the mean are indicated for the IGF-I data.

Figure 7.4: Bioassay of glycinyl truncated IGFs



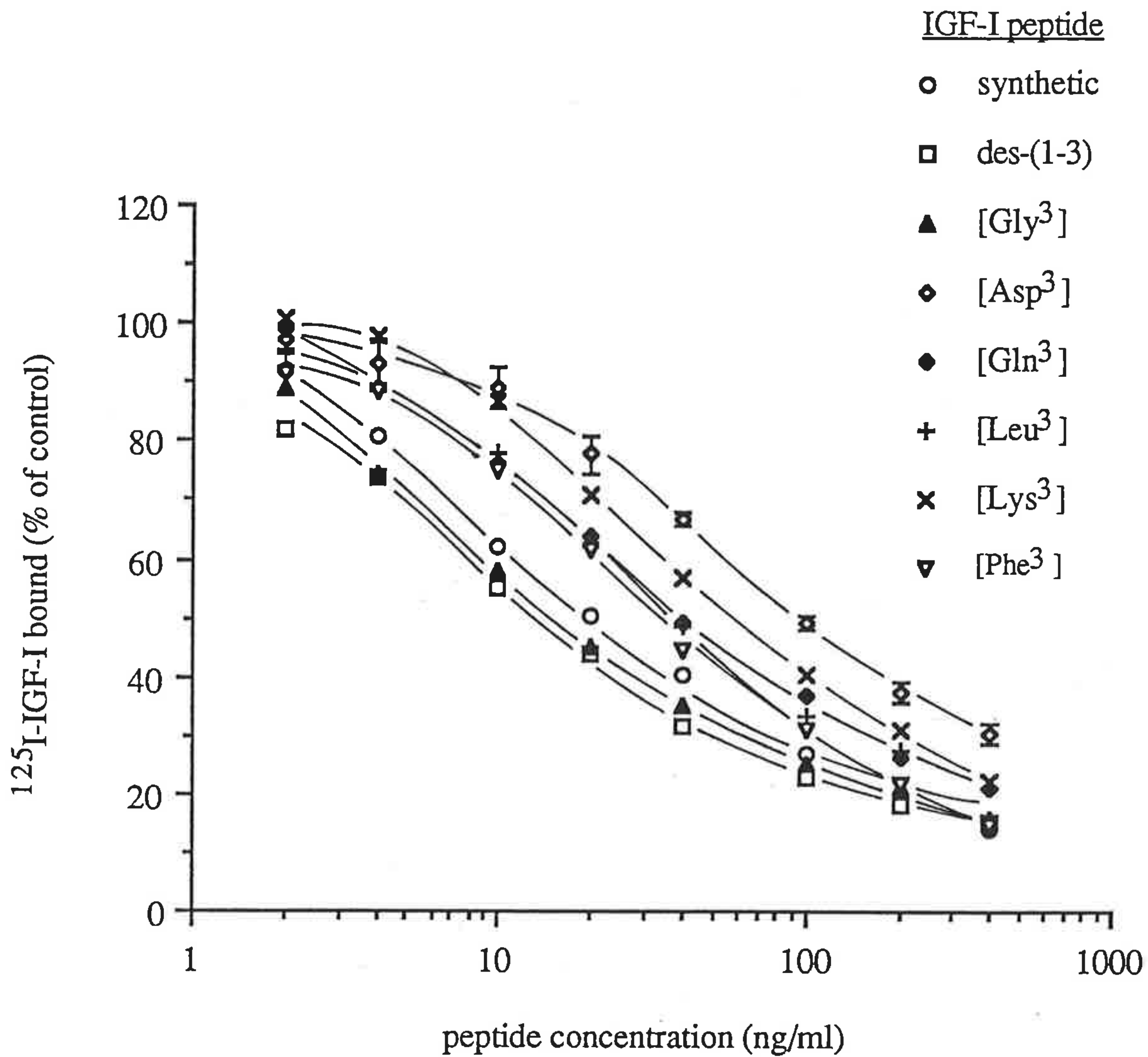
The abilities of the indicated peptides to stimulate protein synthesis in L6 myoblasts was measured. Data represent the means of triplicate determinations and are expressed as a percentage of the stimulation caused by 5% foetal bovine serum. Incorporation of radioactivity into protein in the absence of added growth factor was 0.7% of total added radioactivity and the incorporation caused by 5% foetal bovine serum was 4.0%. As an indication of assay variability, standard errors of the mean are indicated for the IGF-I data.

Figure 7.5: Bioassay of peptides substituted at position 3



The abilities of the indicated peptides to stimulate protein synthesis in L6 myoblasts was measured. Data represent the means of triplicate determinations and are expressed as a percentage stimulation above that observed with no added growth factor. Incorporation of radioactivity in the absence of added growth factor was 0.6% of total added radioactivity. As an indication of assay variability, standard errors of the mean are indicated for the[Asp^3]IGF-I data.

Figure 7.6: Radioreceptor assay of peptides substituted at position 3

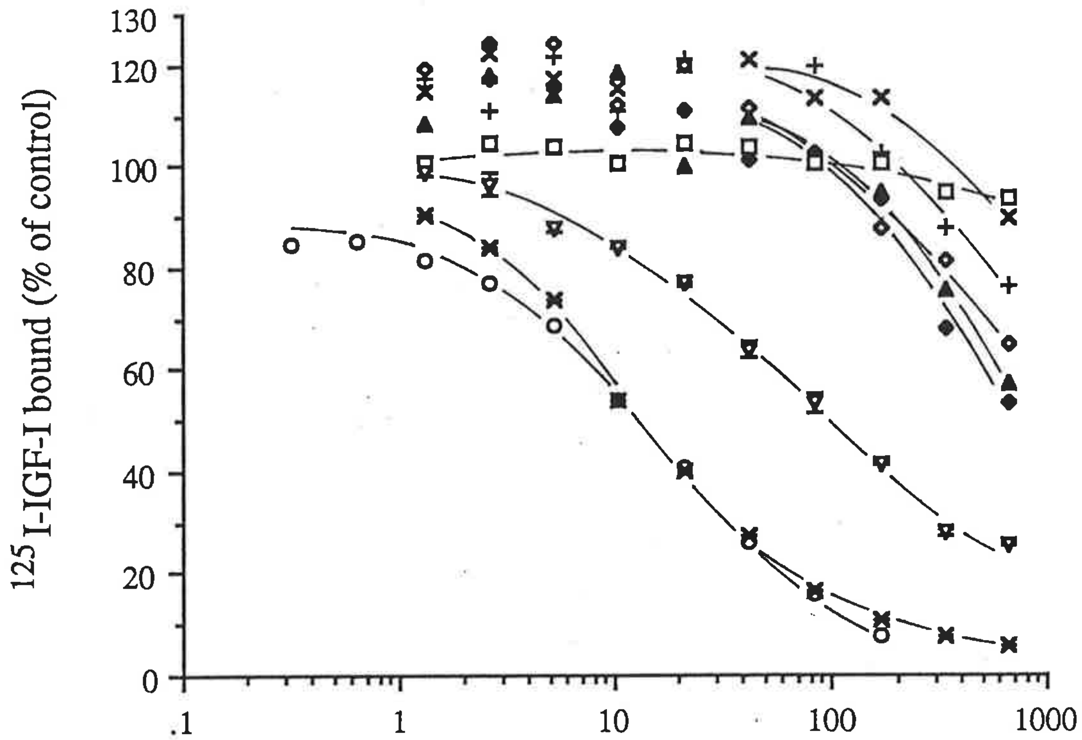


The abilities of the indicated peptides to compete with ^{125}I-IGF-I for binding to L6-cell monolayers were determined. 7% of added radioactivity was bound in the control wells and non-specific binding has not been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. As an indication of assay variability, standard errors of the mean are indicated for the [Asp3]IGF-I data.

Figure 7.7: Binding protein assays of peptides substituted at position 3

The indicated peptides were tested for their abilities to compete with tracer IGF-I binding to L6-myoblast-conditioned medium of MDBK-cell binding protein. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. As an indication of assay variability, standard errors of the mean are indicated for the [Phe^{3}]IGF-I data. b) Competition with ^{125}I-IGF-I for binding to the L6-myoblast-conditioned medium by IGF-I and analogues was measured. Total binding was 14% after a blank, determined in the absence of binding protein, equal to 16% had been subtracted. b) Competition with ^{125}I-IGF-I for binding to the MDBK-cell binding protein by IGF-I and analogues was measured. Total binding was 21% after a blank, determined in the absence of binding protein, equal to 7% had been subtracted.

a) MDBK protein



IGF-I peptide
- recombinant
- synthetic
- synthetic des-(1-3)
- [Lys3]
- [Phe3]
- [Leu3]
- [Gln3]
- [Asp3]
- [Gly3]

b) L6 myoblast conditioned medium

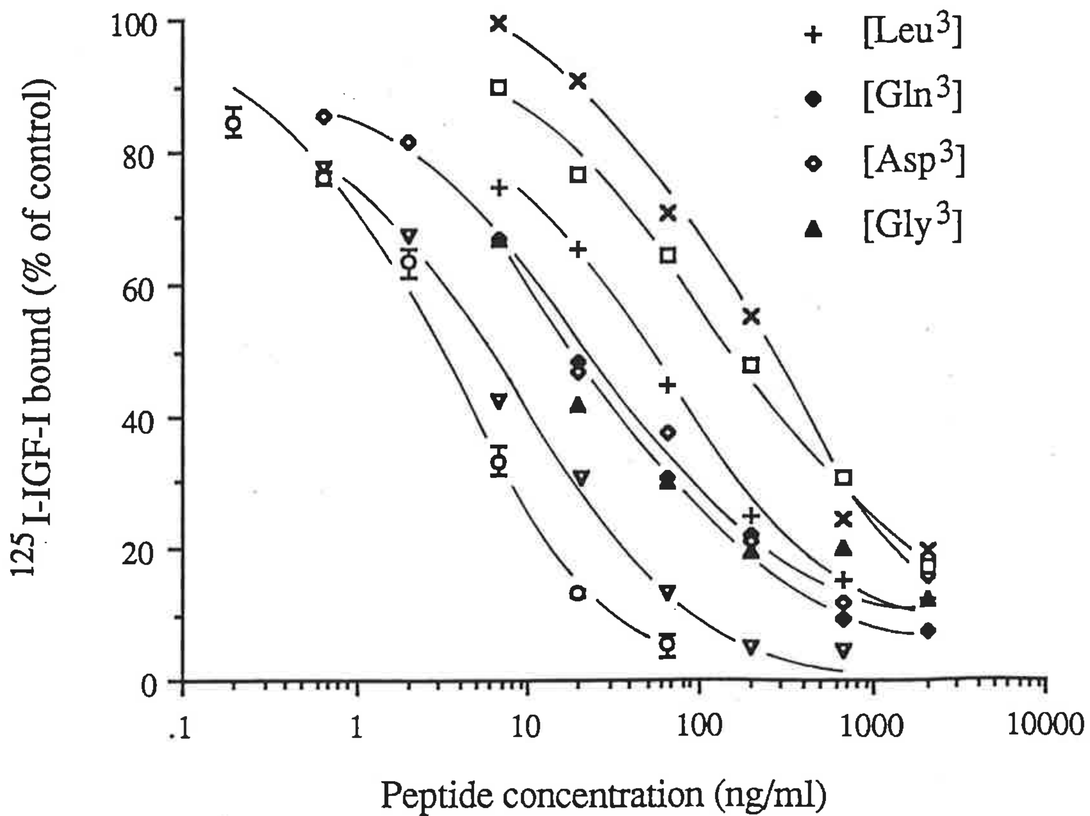


Figure 7.8: Bioassays of peptides bearing N-terminal deletions or substitutions at positions 3 or 4

The abilities of various peptides to stimulate protein synthesis in L6 myoblasts was measured. Data represent the means of triplicate determinations and are expressed as a percentage of the effect caused by 10% foetal bovine serum. *(a)* Stimulation by the indicated peptides. Incorporation of radioactivity into protein in the absence of added growth factor was 1.4% of total added radioactivity and the incorporation caused by 10% foetal bovine serum was 3.7%. As an indication of assay variability, standard errors of the mean are indicated for the des-(2-4)-IGF-I data. *(b)* Stimulation by the indicated peptides. The des-(1-3)-IGF-I, des-(2-4)-IGF-I, des-(1-3)-[Arg^4]IGF-I and des-(1-3)-[Val^4]IGF-I peptides had been reprocessed. Incorporation of radioactivity into protein in the absence of added growth factor was 1.3% of total added radioactivity and the incorporation caused by 10% foetal bovine serum was 3.5%. As an indication of assay variability, standard errors of the mean are indicated for the recombinant IGF-I data. *(c)* Stimulation by the indicated peptides. The des-(1-3)-[Val^4]IGF-I, [Gly^3]IGF-I, [Arg^3]IGF-I and [Gly^3, Gly^4]IGF-I peptides had been reprocessed. Incorporation of radioactivity in the absence of added growth factor was 1.7% of total added radioactivity and the incorporation caused by 10% foetal bovine serum was 4.4%. As an indication of assay variability, standard errors of the mean are indicated for the recombinant IGF-I data.

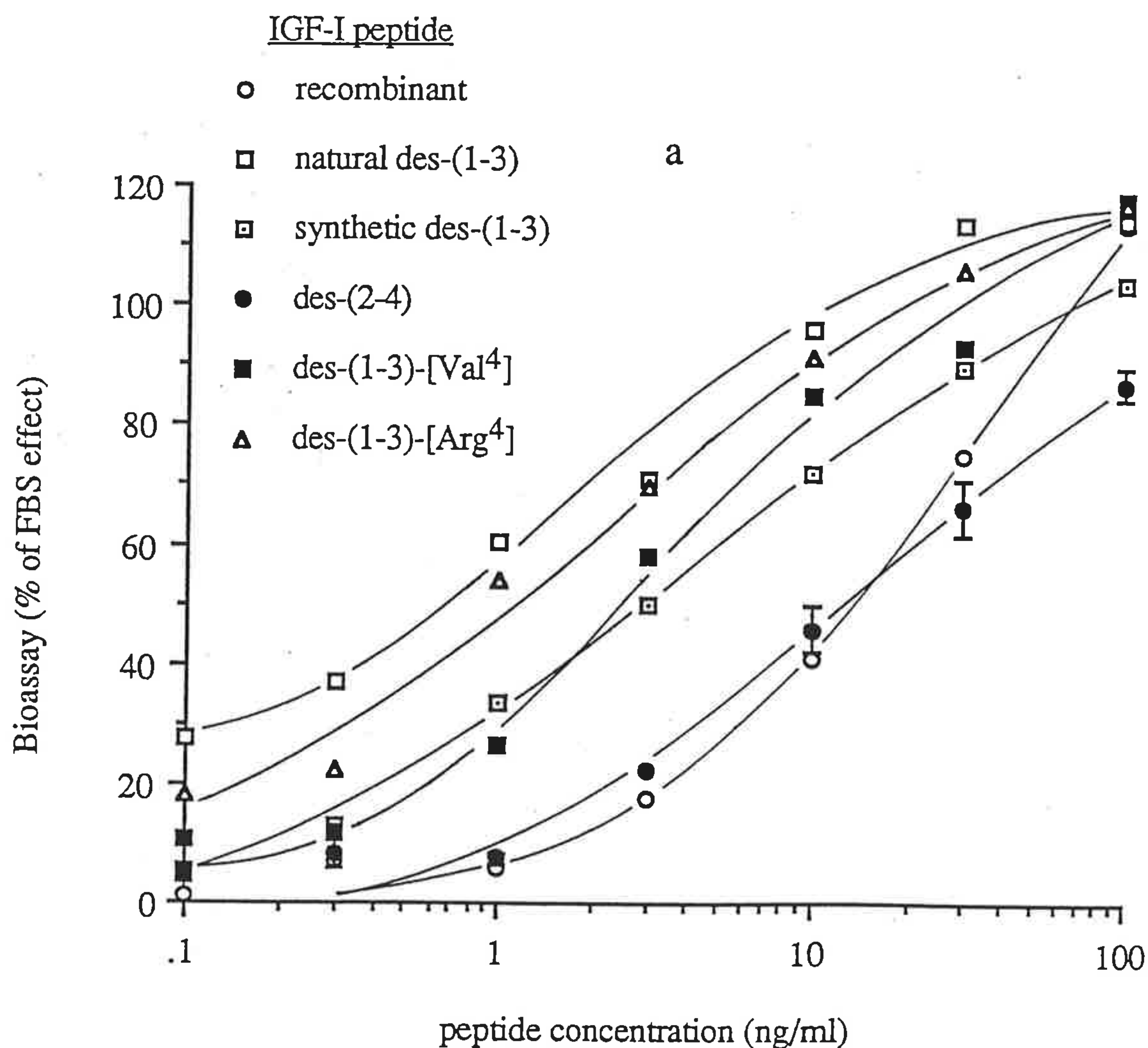
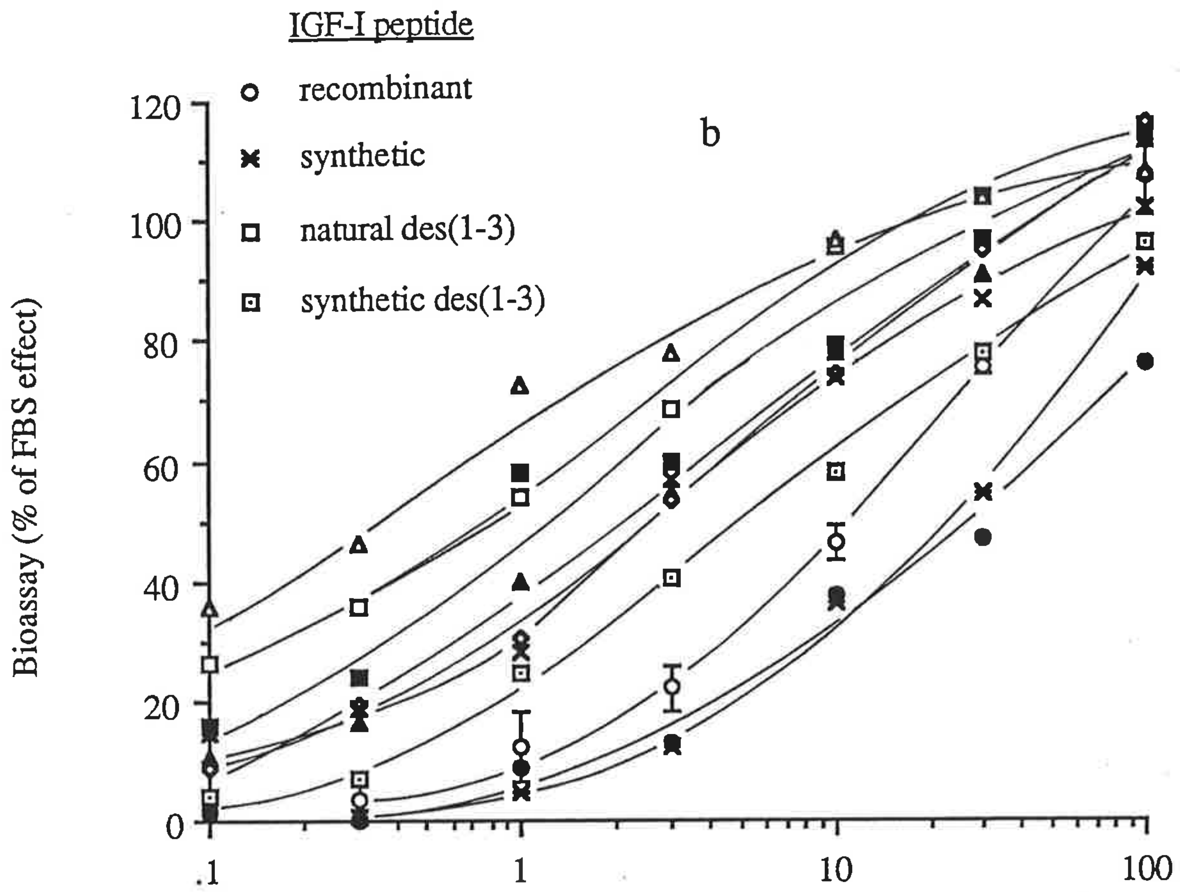


Figure 7.8 (cont.)



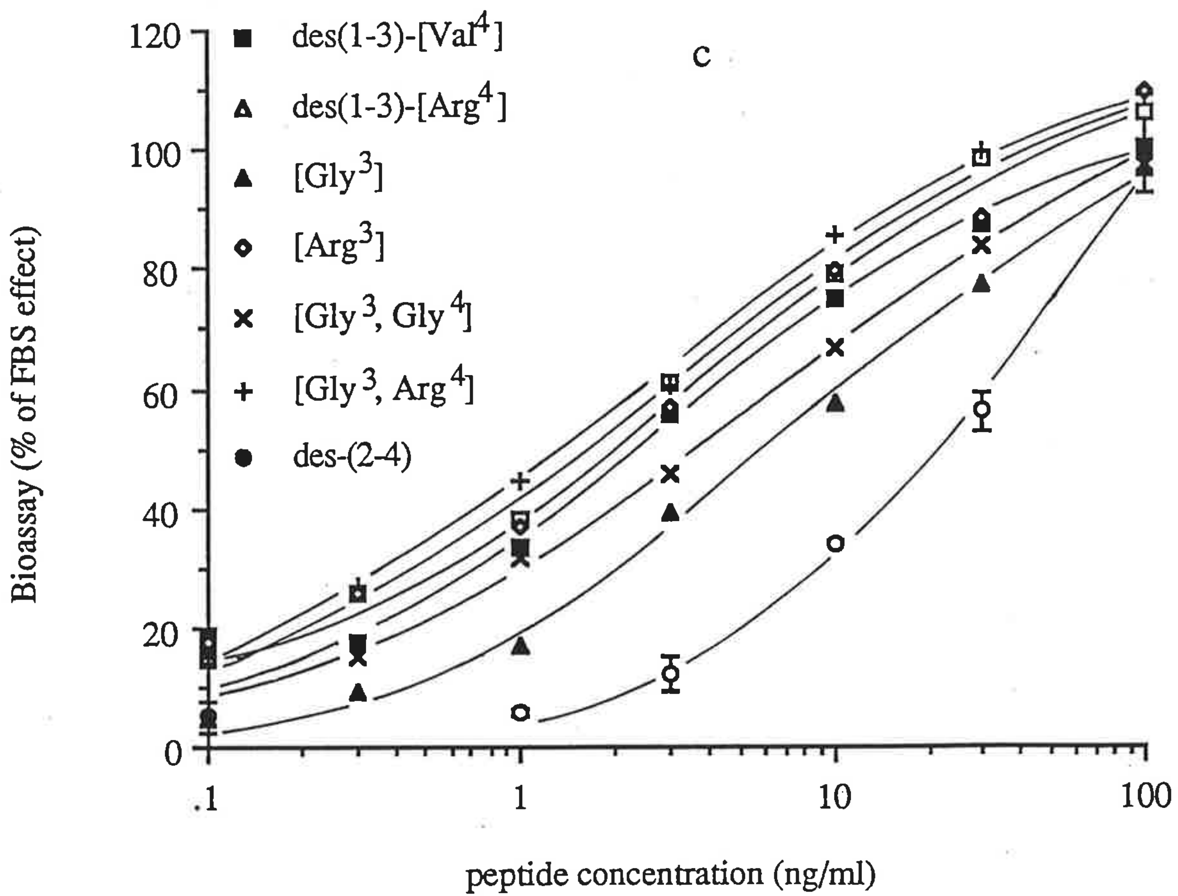


Figure 7.9: Radioreceptor assay of peptides bearing N-terminal deletions or substitutions at position 3 or 4

The abilities of various peptides to compete for binding of ^{125}I-IGF-I to L6 cell-monolayers were determined. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand. Non-specific binding has not been subtracted. *(a)* Competition by the indicated peptides. 18% of added radioactivity was bound in the control wells. As an indication of assay variability, standard errors of the mean are indicated for the des-(2-4)-IGF-I data. *(b)* Competition by the indicated peptides. The des-(1-3)-IGF-I, des-(2-4)-IGF-I, des-(1-3)-[Arg^4]IGF-I and des-(1-3)-[Val^4]IGF-I had been reprocessed. 17% of added radioactivity was bound in the control wells. As an indication of assay variability, standard errors of the mean are indicated for the des-(2-4)-IGF-I data. *(c)* Competition by the indicated peptides. des-(1-3)-[Val^4]IGF-I, [Gly^3]IGF-I, [Arg^3]IGF-I and [Gly^3, Gly^4]IGF-I peptides had been reprocessed. 20% of added radioactivity was bound in the control wells. As an indication of assay variability, standard errors of the mean are indicated for the natural des-(1-3)-IGF-I data.

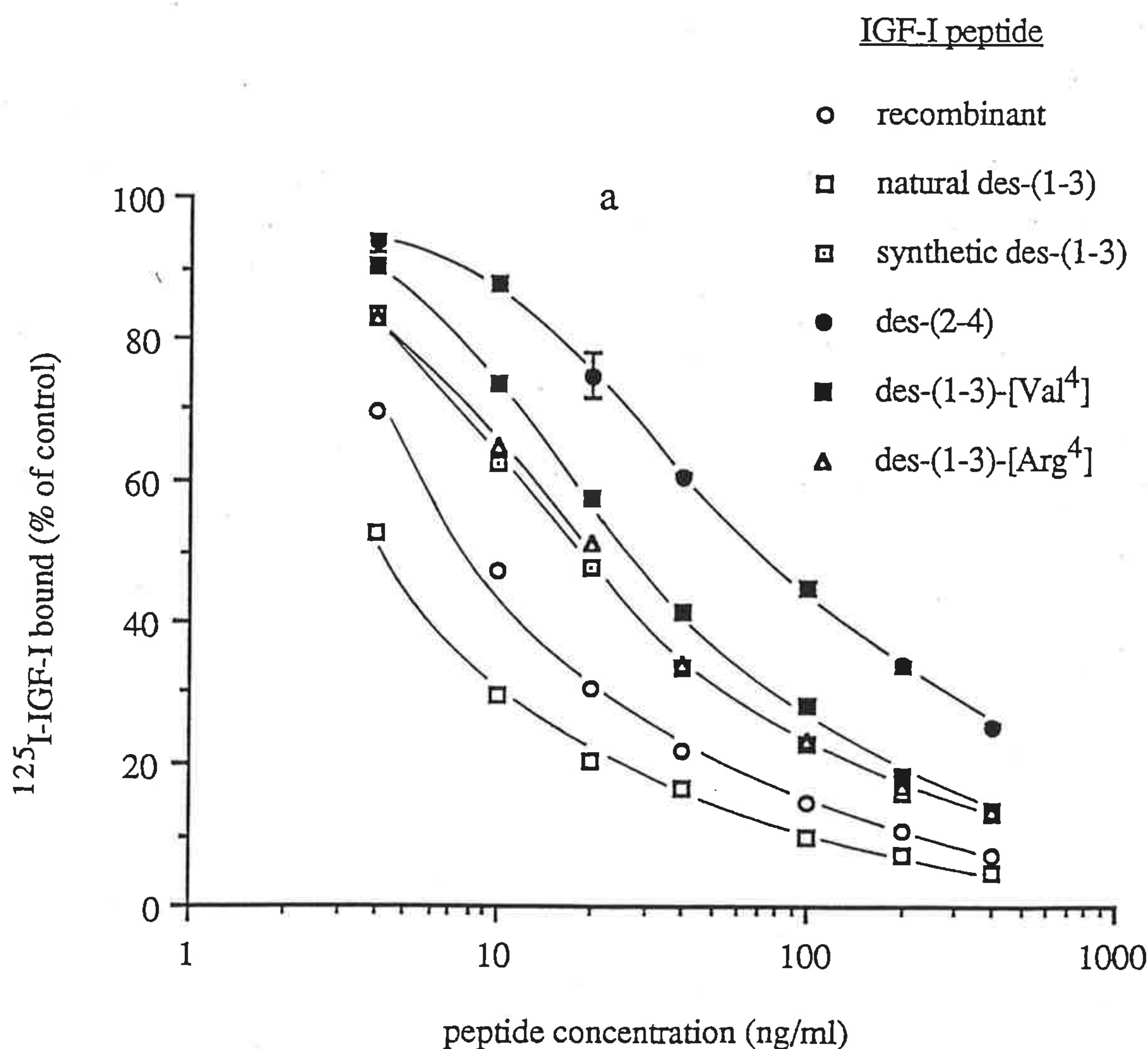
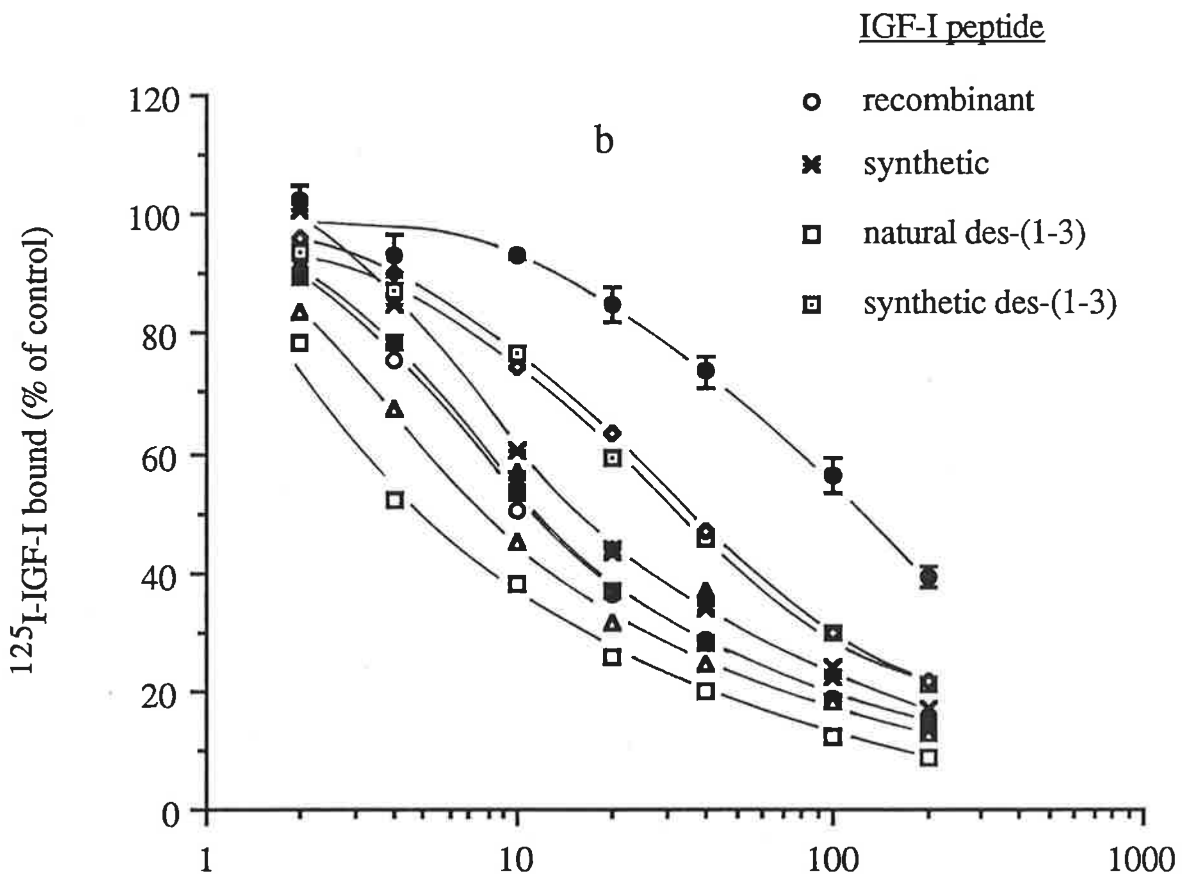


Figure 7.9 (cont)



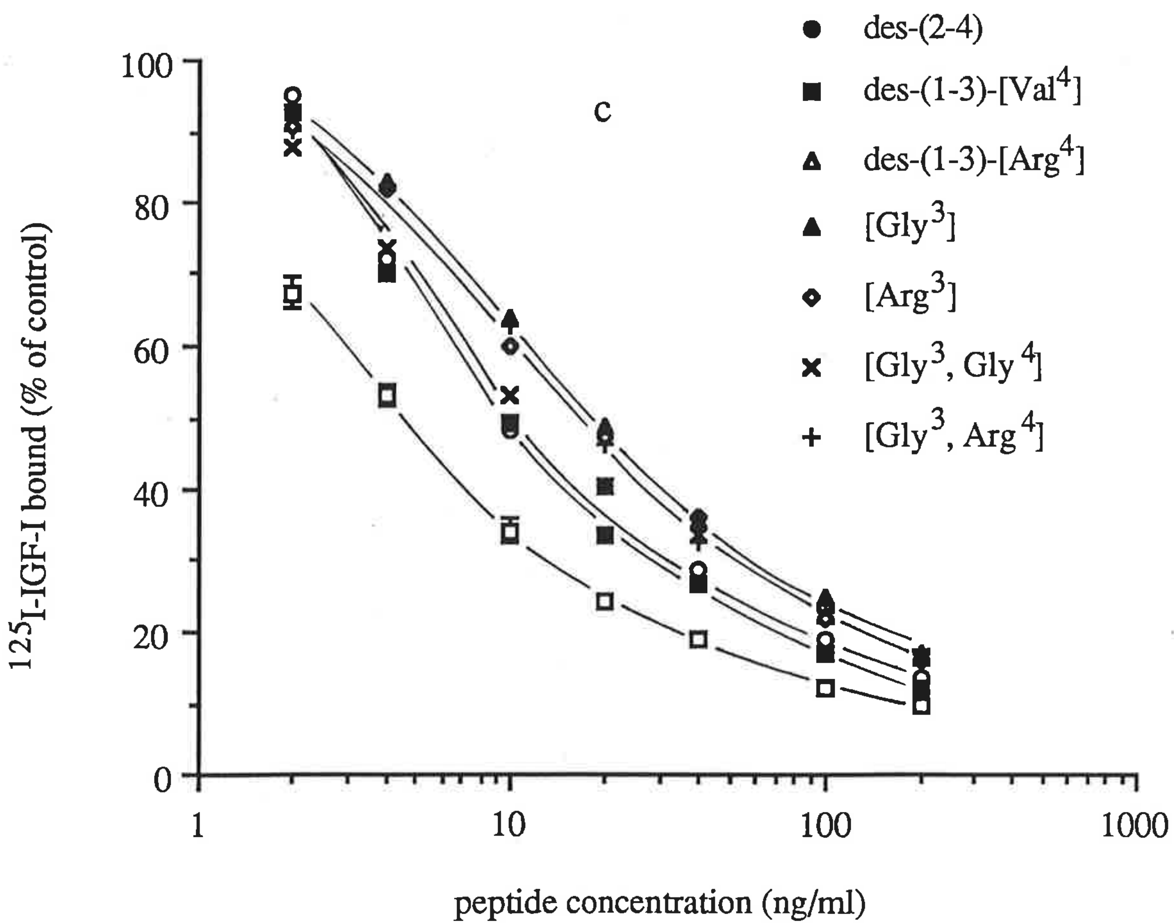
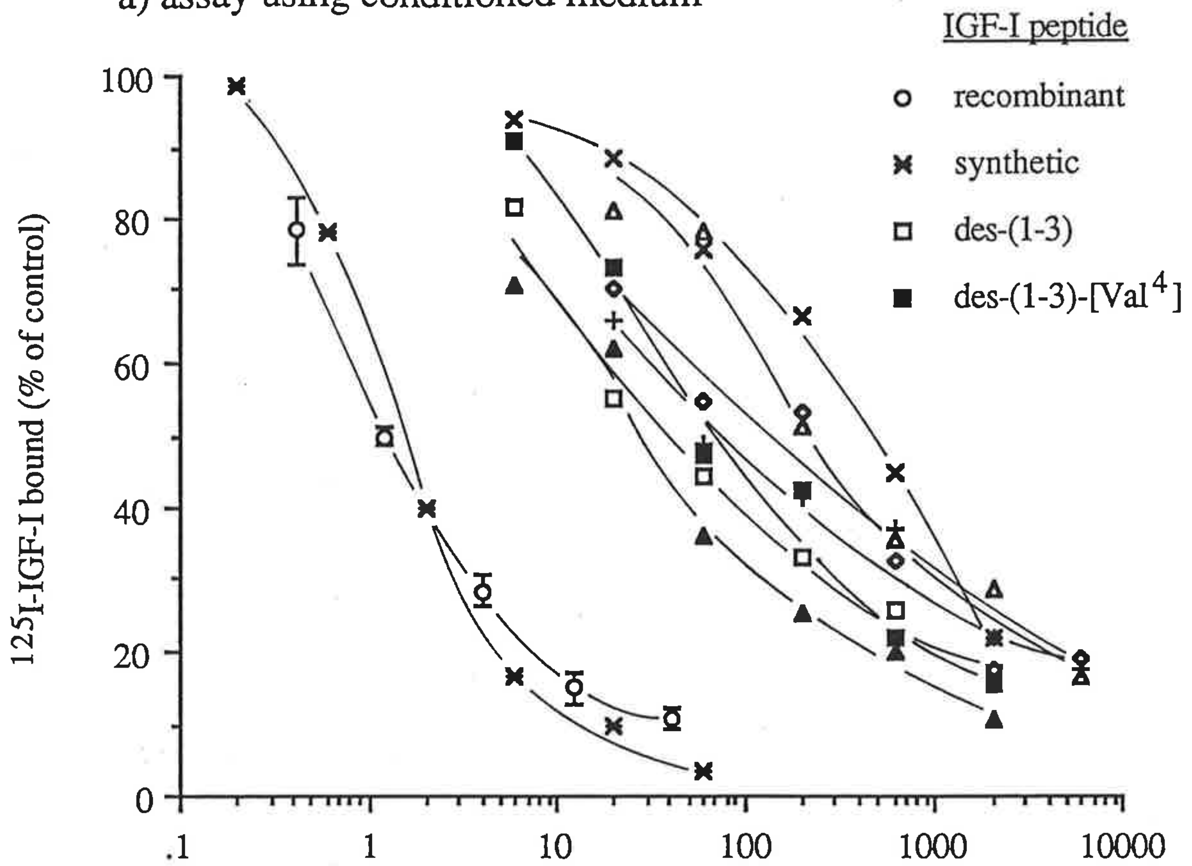
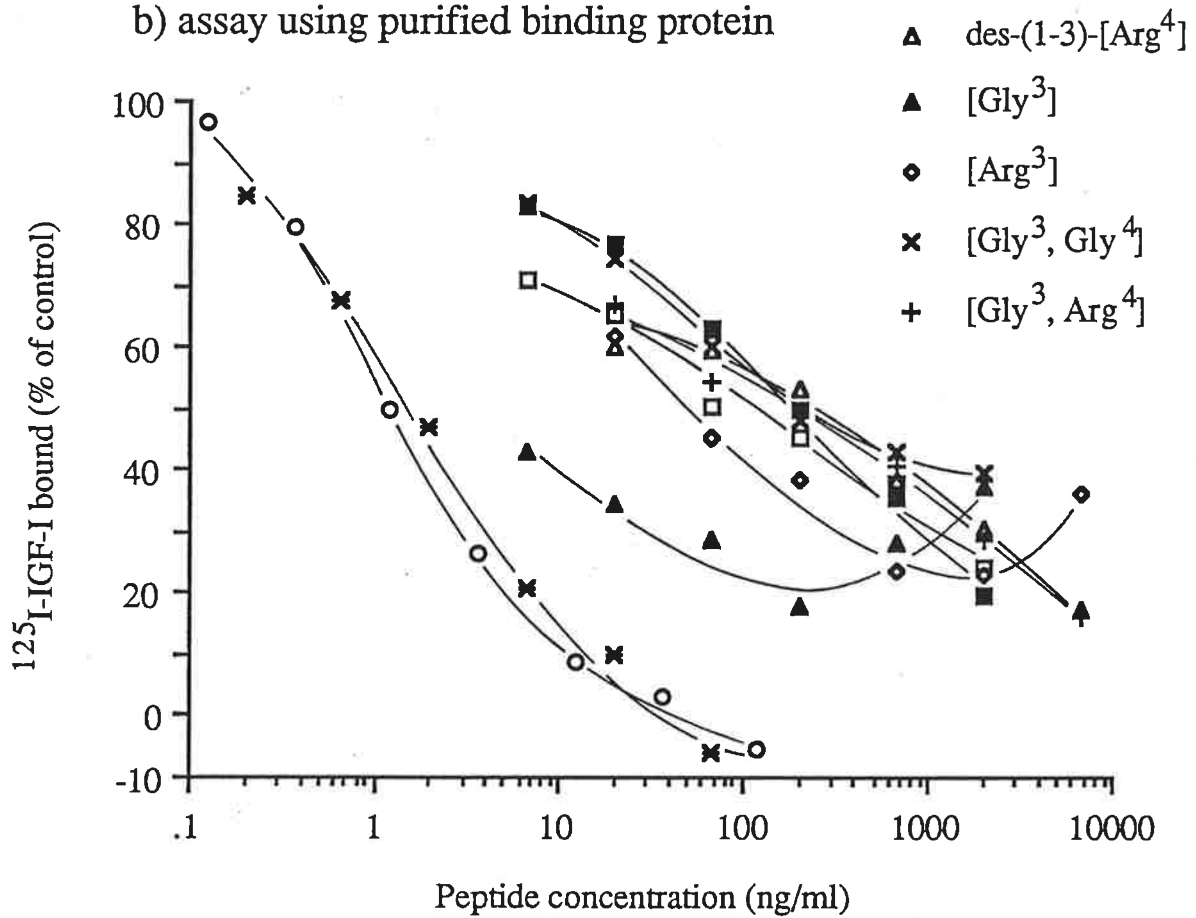


Figure 7.10: Binding protein assays of peptides bearing N-terminal truncations or substitutions

Competition for ^{125}I-IGF-I binding to purified L6-myoblast binding protein or L6-conditioned medium by the indicated peptides was measured. The competing peptides des-(1-3)-IGF-I, des-(1-3)-[Arg^4]IGF-I, des-(1-3)-[Val^4]IGF-I, [Gly^3]IGF-I, [Arg^3]IGF-I and [Gly^3, Gly^4]IGF-I had been reprocessed. *(a)* Binding assay using purified L6-myoblast binding protein. Total binding was 23% after a blank, determined in the absence of binding protein, equal to 19% had been subtracted. As an indication of assay variability, standard errors of the mean are indicated for the recombinant IGF-I data. *(b)* Binding assay using L6-myoblast-conditioned medium. Total binding was 33% after a blank equal to 18% had been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand.


a) assay using conditioned medium
IGF-I peptide
recombinant
synthetic
des-(1-3)
des-(1-3)-[Val4]
125I-IGF-I bound (% of control)
100
80
60
40
20
0
.1
1
10
100
1000
10000


b) assay using purified binding protein
des-(1-3)-[Arg4]
[Gly3]
[Arg3]
[Gly3, Gly4]
[Gly3, Arg4]
125I-IGF-I bound (% of control)
100
80
60
40
20
0
-10
.1
1
10
100
1000
10000
Peptide concentration (ng/ml)

CHAPTER 8

DISULPHIDE BOND ISOMERS OF IGF-I AND DES-(1-3)-IGF-I

CHAPTER 8: BIOCHEMICAL PROPERTIES OF IGF-I PEPTIDES CONTAINING NON-NATIVE DISULPHIDE BONDS

8.1 INTRODUCTION

When the products from a oxidation experiment were chromatographed on reverse-phase HPLC, considerable heterogeneity was observed (e.g. fig. 3.4). In the case of the oxidation of synthetic IGF peptides, much of the material which elutes at higher concentrations of acetonitrile than does native IGF-I apparently consists of failure sequence- or chemically modified peptides since this material produces very little native IGF when reduced and reoxidized (data not shown). However, even the natural IGF-I peptides exhibit a significant peak of incorrectly-oxidized IGF upon reoxidation which elutes during reverse-phase HPLC at a lower concentration of acetonitrile than does native IGF-I (denoted isomer 1 in fig. 3.4a). As reported by Saito *et al.* (1987), peptide corresponding to this peak apparently exhibits a specific bioactivity of not more than 10% that of natural IGF-I. Tamura *et al.* (1988) isolated a similar incorrectly-oxidized IGF-I peptide from their oxidation of recombinant IGF-I. This peptide was characterized by specific proteolysis and Edman degradation in order to completely assign the disulphide bonds and determined to be [6-47-, 48-52-cystine]IGF-I. [6-47-, 48-52-cystine]IGF-I was found to be 5% as potent as native IGF-I for competing with ^{125}I-IGF-I binding to Balb/c fibroblast cells and was able to stimulate the incorporation of [^{3}H]thymidine into these cells with only 8% the potency of native IGF-I. I decided to extend these biochemical studies using assays available in this laboratory as well as investigating the properties of des-(1-3)-[6-47-, 48-52-cystine]IGF-I. In this chapter, I will report the purification of the [6-47-, 48-52-cystine] isomers of IGF-I and des-(1-3)-IGF-I and the direct comparison of these peptides with their correctly-folded homologues in the L6 myoblast IGF-I radioreceptor assay, the L6 protein synthesis bioassay and in the binding protein assay. The structures of the isomer 1 peptides were not directly determined but were assumed to possess the non-native 6-47 and 48-52 disulphide bonds from their chromatographic properties.

8.2 MATERIALS AND METHODS

IGF-I radioreceptor assays and protein synthesis bioassays were performed as

described in sections 2.4b and 2.4c respectively. IGF-binding protein competitive binding assays were performed as described in section 2.4d.

Synthetic des-(1-3)-IGF-I (from synthesis BIO-3) was prepared as described in section 4.3d. Preparative reverse-phase HPLC clearly resolved a peak of peptide of the expected chromatographic properties for the [6-47-, 48-52-cystine] peptide from a peak corresponding to native des-(1-3)-IGF-I (data not shown). Reduced recombinant IGF-I was a gift from Bresatec (Adelaide, Australia) and oxidized by the method described in table 3.4.

Peptide amounts were estimated from the absorbance profile during reverse-phase HPLC as described in section 2.2c.

8.3 RESULTS

8.3a Purification of peptides

Correctly-oxidized IGF-I and [6-47-, 48-52-cystine]IGF-I were initially purified by reverse-phase HPLC (fig 8.1a). The [6-47-, 48-52-cystine]IGF-I, which was not adequately resolved from the native peptide under these conditions, was further purified using a gradient of propan-1-ol in the presence of 0.13% heptafluorobutyric acid (fig. 8.1b), a solvent system that I have previously found useful for the purification of IGFs (Francis *et al.*, 1986). Bioassay measurements indicated that a small component of a more potent IGF species eluted after the [6-47-, 48-52-cystine]IGF-I at this step. The natural and mis-folded isomers of the des-(1-3)-IGF-I and IGF-I peptides were rechromatographed on reverse-phase HPLC and fractions were assayed using the L6 myoblast protein synthesis bioassay (fig. 8.2). The peaks of biological activity corresponded closely to the peaks of peptide in all cases except that a second peak of bioactivity eluted after the main peak in the [6-47-, 48-52-cystine]IGF-I sample. As can be seen from the chromatographic profiles, the [6-47-, 48-52-cystine]- and native peptides are clearly separated. The protein contents of these peaks were estimated by absorbance and are the basis for the amounts indicated in the subsequent assays.

8.3b IGF-I radioreceptor assay of the IGF isomers

The IGF peptides were tested for their abilities to compete with ^{125}I-IGF-I for binding to cell-surface receptors on L6 myoblasts as shown in figure 8.3. The potencies of the

peptides, determined as the concentration (ng/ml) required to cause 50% competition with tracer IGF-I were;

des-(1-3)-IGF-I,	17;
IGF-I,	19;
type 1 des-(1-3)-IGF-I,	940;
[6-47-, 48-52-cystine]IGF-I,	2400.

As I have observed previously (see sections 5.3a & 6.3b), IGF-I and des-(1-3)-IGF-I exhibited similar potency in this assay. The [6-47-, 48-52-cystine]peptides were considerably less active than their natural homologues especially [6-47-, 48-52-cystine]IGF-I which was 130-fold less potent than IGF-I. The [6-47-, 48-52-cystine]des-(1-3)-IGF-I was 2.5-fold more potent than the [6-47-, 48-52-cystine]IGF-I.

8.3c Bioassay of the IGF isomers

Samples of the IGF peptides were subjected to the L6 bioassay as shown in figure 8.4. Each of the peptides tested was able to elicit a response equivalent in magnitude to that produced by 5% foetal bovine serum indicating that the [6-47-, 48-52-cystine]peptides were full agonists of IGF-I in this assay. The potencies of the peptides, assessed by measurement of the concentrations (ng/ml) required to cause a stimulation of protein synthesis equal to 50% of that caused by 5% foetal bovine serum were;

des-(1-3)-IGF-I,	2.7;
IGF-I,	28;
type 1 des-(1-3)-IGF-I,	36;
[6-47-, 48-52-cystine]IGF-I,	250.

As expected, des-(1-3)-IGF-I was the most potent peptide in this assay being tenfold more potent than IGF-I. The [6-47-, 48-52-cystine]IGF peptides were approximately tenfold less potent than their corresponding native peptides.

8.3d Binding protein assay of the IGF isomers

Samples of the various IGF peptides were tested for their abilities to compete with ^{125}I-IGF-I for binding to purified L6-myoblast IGF-binding protein (fig. 8.5). Des-(1-3)-IGF-I was found to be 50-fold less potent than IGF-I in competing for binding to the L6

carrier protein while the [6-47-, 48-52-cystine]IGF-I was 500-fold less potent than IGF-I in this assay. The des-(1-3)-[6-47-, 48-52-cystine]IGF-I did not compete significantly at concentrations up to 2μg/ml. In a similar experiment, using the MDBK-cell binding protein, [6-47-, 48-52-cystine]IGF-I was found to be approximately 150-fold less potent than IGF-I.

8.4 DISCUSSION

In order to investigate the biochemical properties of the incorrectly-folded [6-47-, 48-52-cystine]IGF species, it was necessary to obtain highly purified preparations free from contaminating peptides of greater specific activity. In the case of the [6-47-, 48-52-cystine]IGF-I peptide, I introduced an additional reverse-phase HPLC step which used propan-1-ol as the organic modifier and heptafluorobutyric acid as the counterion. This allowed the removal of a small contaminant of more biologically active peptide (fig 8.1b). Further purification of the [6-47-, 48-52-cystine]IGFs was achieved by a final chromatography step using acetonitrile in the presence of 0.1%TFA. It is apparent that the preparation of [6-47-, 48-52-cystine]IGF-I contains very little native IGF-I peptide since it is at least 100-fold less active than native IGF-I in the radioreceptor assay. The [6-47-, 48-52-cystine]des-(1-3)-IGF-I was found to be 2.5-fold more active than the corresponding IGF-I peptide which may have resulted from a small (approx. 1%) contamination with native des-(1-3)-IGF-I or from true biochemical differences between the two [6-47-, 48-52-cystine]peptides.

In contrast to the results of the radioreceptor assay, both [6-47-, 48-52-cystine]peptides showed substantial activity in the protein synthesis bioassay (fig. 8.4) being only tenfold less active than their native homologues. [6-47-, 48-52-cystine]IGF-I was 14-fold more biologically active on the basis of its receptor-binding activity than IGF-I suggesting that its biological activity is only weakly inhibited by IGF-binding proteins. In support of this hypothesis, [6-47-, 48-52-cystine]IGF-I was found to be approximately 500-fold less potent than IGF-I for binding to proteins present in L6 myoblast-conditioned medium (fig. 8.5). The des-(1-3)-[6-47-, 48-52-cystine]IGF-I was fourfold more active on the basis of its receptor-binding activity than des-(1-3)-IGF-I suggesting that its biological activity is less inhibited by IGF-binding proteins than that of des-(1-3)-IGF-I. Thus des-(1-3)-IGF-I is apparently slightly inhibited by the binding proteins present in the L6 conditioned

medium, consistent with the observation that des-(1-3)-IGF-I binds weakly but significantly to the proteins in L6 cell-conditioned medium (ch. 6, fig. 8.5) whereas [6-47-, 48-52-cystine]des-(1-3)-IGF-I was not found to bind detectably (fig. 8.5).

Little is known of the IGF-I-receptor-binding site of IGF-I although it is presumed to be homologous to the site on insulin which binds to its receptor since the insulin- and IGF-I receptors are themselves homologous (Ullrich *et al.*, 1986). The major receptor-binding region of insulin resides is the carboxy-terminal pentapeptide of the B-chain and various parts of the A-chain, including the amino group of Gly^{A1} (Pullen *et al.*, 1976; Nakagawa & Tager, 1987). Similarly, residue Tyr^{24}, which is near the carboxy-terminus of the B-domain (Cascieri *et al.*, 1988), and the A-domain (Tseng *et al.*, 1987) of IGF-I have been implicated in its binding to the IGF-I receptor. However, replacement of residues 42-56 of IGF-I with the corresponding insulin sequence results in no significant change in its IGF-I receptor-binding properties (Cascieri *et al.*, 1989). Chemical labelling studies have identified the tyrosine residues at positions 24, 31 and 60 of IGF-I as being involved in the binding of IGF-I to its receptor (Maly & Luthi, 1988). Studies using mis-folded IGF-I represent an alternative method by which the receptor-binding site may be characterized. The effect of mis-folding IGF to form the [6-47-, 48-52-cystine]isomer is to cause a marked decrease in its ability to bind to IGF-I receptors and to IGF-binding proteins (figs. 8.3 & 8.5). In contrast, Sieber *et al.* (1978) found that the equivalent isomer of insulin, [A7-A11-, A6-B7-cystine]insulin, was only fourfold less potent than insulin in a variety of biological assays. In the case of insulin, for which no non-receptor binding protein exists, this potency probably reflects the receptor-binding properties of the analogue. Insulin is thought to undergo a conformational change in the N-terminal region of the A-chain on binding to its receptor with high affinity (Pullen *et al.*, 1975; Nakagawa & Tager, 1987). Apparently, this process is not greatly affected by rearrangement of the disulphide bonds involving residues A6 and A7 perhaps because the N-terminal amino group of the A-chain remains available for interaction with the receptor. IGF-I does not possess this amino group and may bind to its receptor in a manner which is more highly dependent on its conformational state. In the [6-47-, 48-52-cystine] isomer of IGF-I, the N-terminal region of the A-domain is constrained by the C-domain and may exhibit significant structural differences from the equivalent region of

insulin which is free. In support of this hypothesis, Tamura *et al.* (1988) found [6-47-, 48-52-cystine]IGF-I to exhibit less α-helix than native IGF-I.

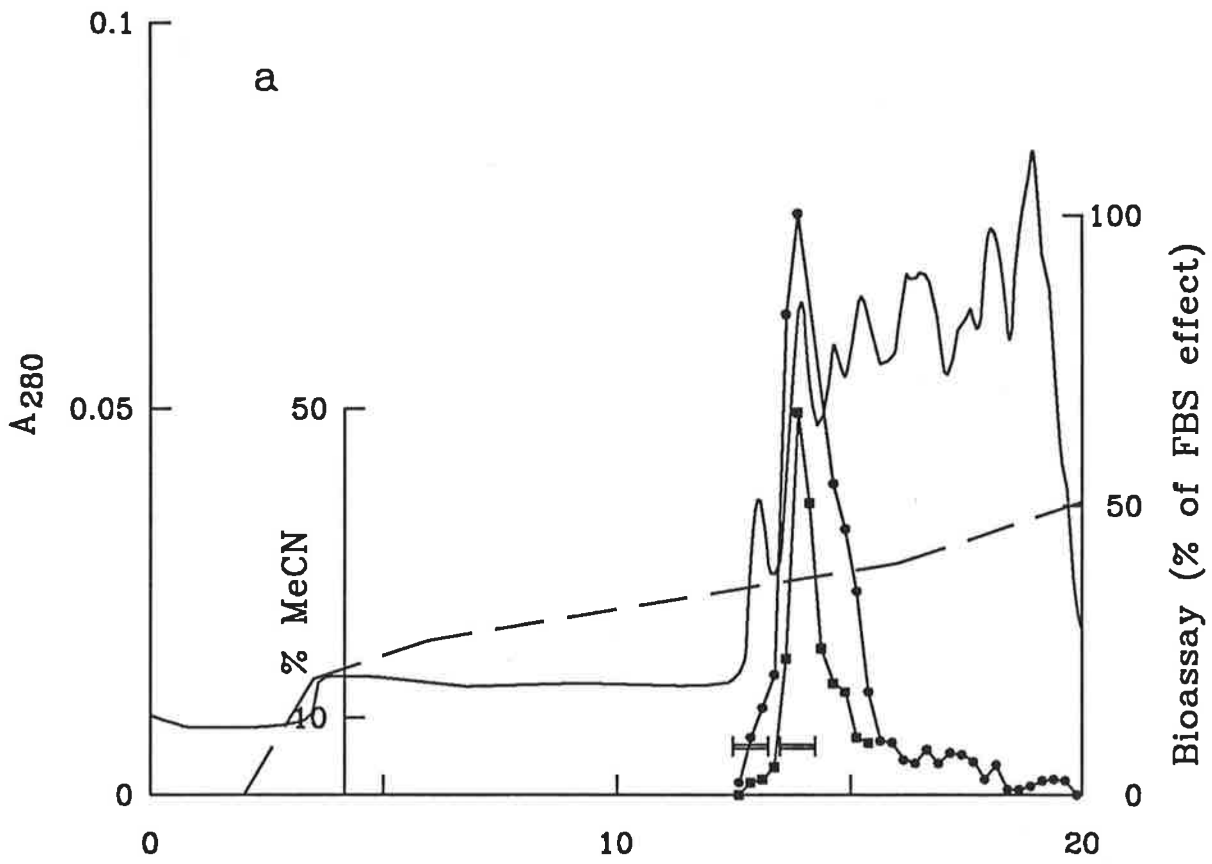
Little is known of the sites of interaction between IGFs and the various IGF-binding proteins. Bayne *et al.* (1988) found that the replacement of residues 3, 4, 15 and 16 of IGF-I by those found at the equivalent positions in insulin lead to a 600-fold decrease in affinity for serum binding proteins (mostly IBP-2). Similarly, Forbes *et al.* (1988) found that des-(1-3)-IGF-I bound poorly to human IBP-1 and I have demonstrated the critical involvement of residue 3 in binding to the MDBK-cell binding protein (chs. 6 & 7). The IGF-I analogue, which is substituted at positions 42-56 by the insulin sequence, exhibited binding to serum binding proteins equivalent to that of IGF-I (Cascieri *et al.*, 1989) suggesting that these residues of IGF-I are not important for binding to binding proteins. However, structural alteration of this region via disulphide bond rearrangement causes a significant decrease in the ability of the IGF peptide to bind to binding proteins produced by L6 myoblasts and MDBK cells. The removal of the N-terminal tripeptide and disulphide-bond rearrangement appear to independently affect the interactions of the IGF with the L6-myoblast binding protein des-(1-3)-[6-47-, 48-52-cystine]IGF-I binds less well than either des-(1-3)-IGF-I or [6-47-, 48-52-cystine]IGF-I (fig. 8.5). It should be noted that the binding proteins I have used in these studies differ from those of the Bayne and Cascieri research group since they used human IBP-2 whereas the MDBK protein that I have used is probably bovine IBP-3 (Upton, 1989) and the L6-myoblast protein may be rat IBP-1 since it weakly binds des-(1-3)-IGF-I (fig. 8.5) as does human IBP-1 (Forbes *et al.*, 1988).

In consideration of the nature of the structural perturbation found in [6-47-, 48-52-cystine]IGF-I, and its effects on the interactions with receptors and IGF-binding proteins, it is not yet possible to distinguish between local effects in the vicinity of the rearranged disulphide bonds and effects of conformational change which may be transmitted to more distant parts of the molecule. This awaits the determination of the three-dimensional structure of [6-47-, 48-52-cystine]IGF-I. The creation of altered [6-47-, 48-52-cystine]IGF-I molecules which are nevertheless able to bind to receptors or binding proteins may be instructive. In this regard, investigation of a two-chain analogue of [6-47-, 48-52-cystine]IGF-I might prove interesting.

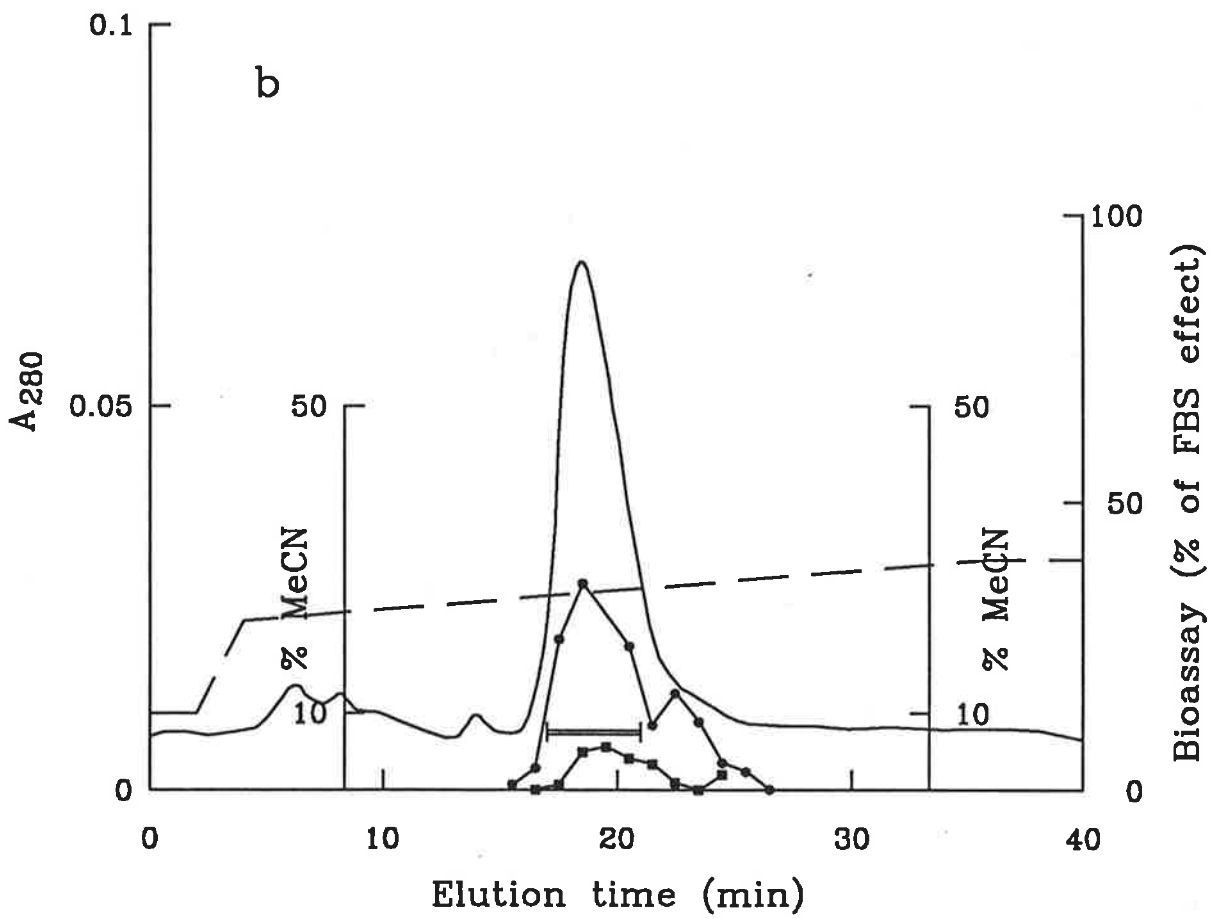
Figure 8.1: Purification of [6-47-, 48-52-cystine]IGF-I

Panel a: Oxidized recombinant IGF-I was chromatographed on a 1 5 10cm butyl silica cartridge at a flow rate of of 5ml/min. Protein was eluted with the indicated gradient of acetonitrile in the presence of 0.1% TFA (- -), the A_{215} monitored (—), fractions were collected at 0.5min intervals and samples equivalent to 0.2µl (■) or 1.0µl (●) were subjected to the protein synthesis bioassay. Data are expressed as a percentage of the stimulation caused by 5% foetal bovine serum. The incorporation of radioactivity into protein was 0.9% in the absence of added growth factors and 3.9% in the presence of 5% foetal bovine serum. The indicated fractions were pooled for the subsequent rechromatography.

Panel b: The indicated fractions containing [6-47-, 48-52-cystine]IGF-I were pooled and rechromatographed on a 4.6 × 30mm RP-300 cartridge at a flow rate of of 1ml/min. Protein was eluted with the indicated gradient of propan-1-ol in the presence of 0.13% HFBA (- -), the A_{215} monitored (—), fractions were collected at 0.5min intervals and samples equivalent to 2µl (■) or 1 0µl (●) were subjected to the protein synthesis bioassay. Data are expressed as a percentage of the stimulation caused by 5% foetal bovine serum. The incorporation of radioactivity into protein was 1.0% in the absence of added growth factors and 4.4% in the presence of 5% foetal bovine serum. The indicated fractions were pooled for subsequent rechromatography.



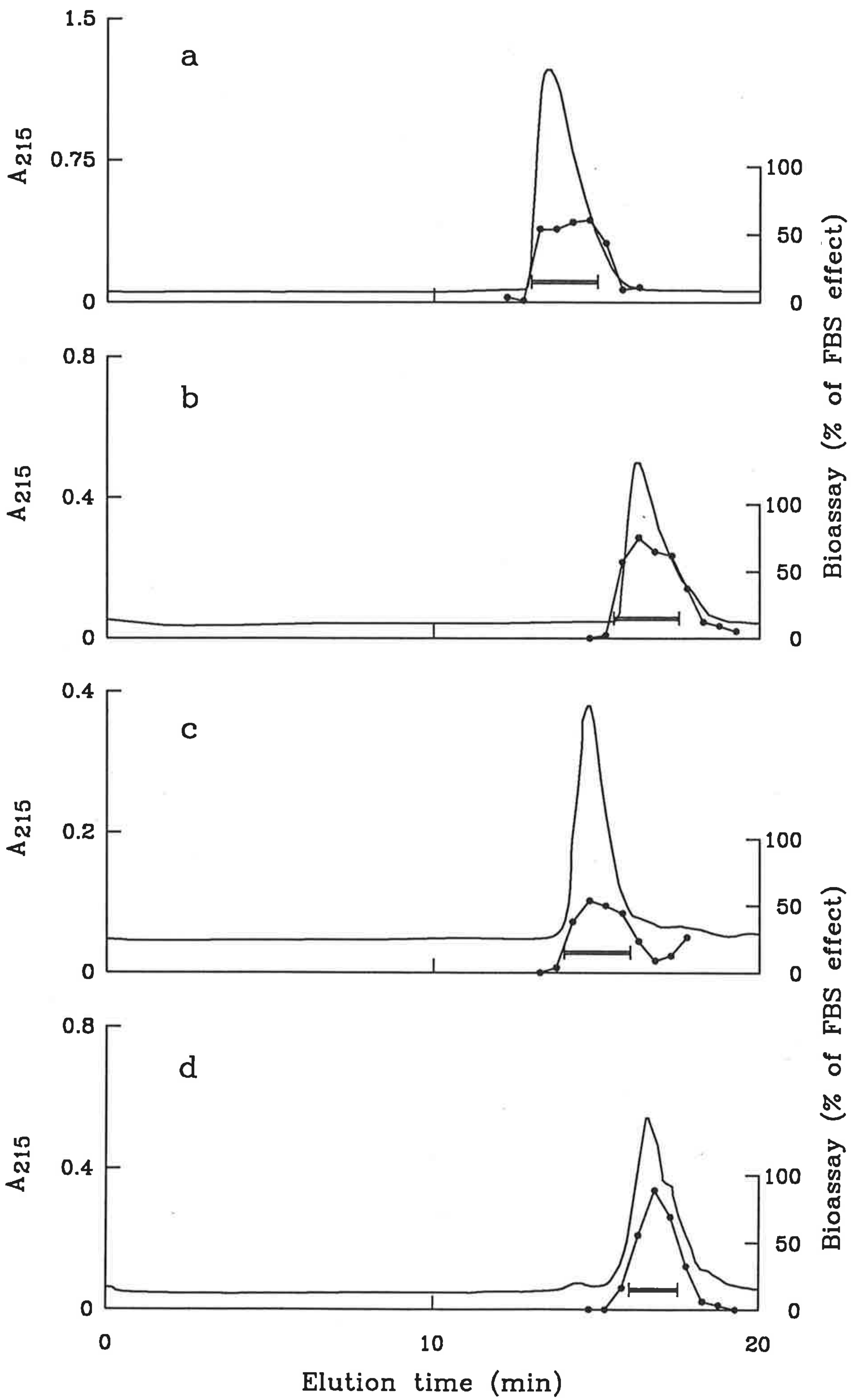
a
0.1
0.05
0
A280
50
10
% MeCN
100
50
0
Bioassay (% of FBS effect)
0
10
20



b
0.1
0.05
0
A280
50
10
% MeCN
50
10
% MeCN
100
50
0
Bioassay (% of FBS effect)
0
10
20
30
40
Elution time (min)

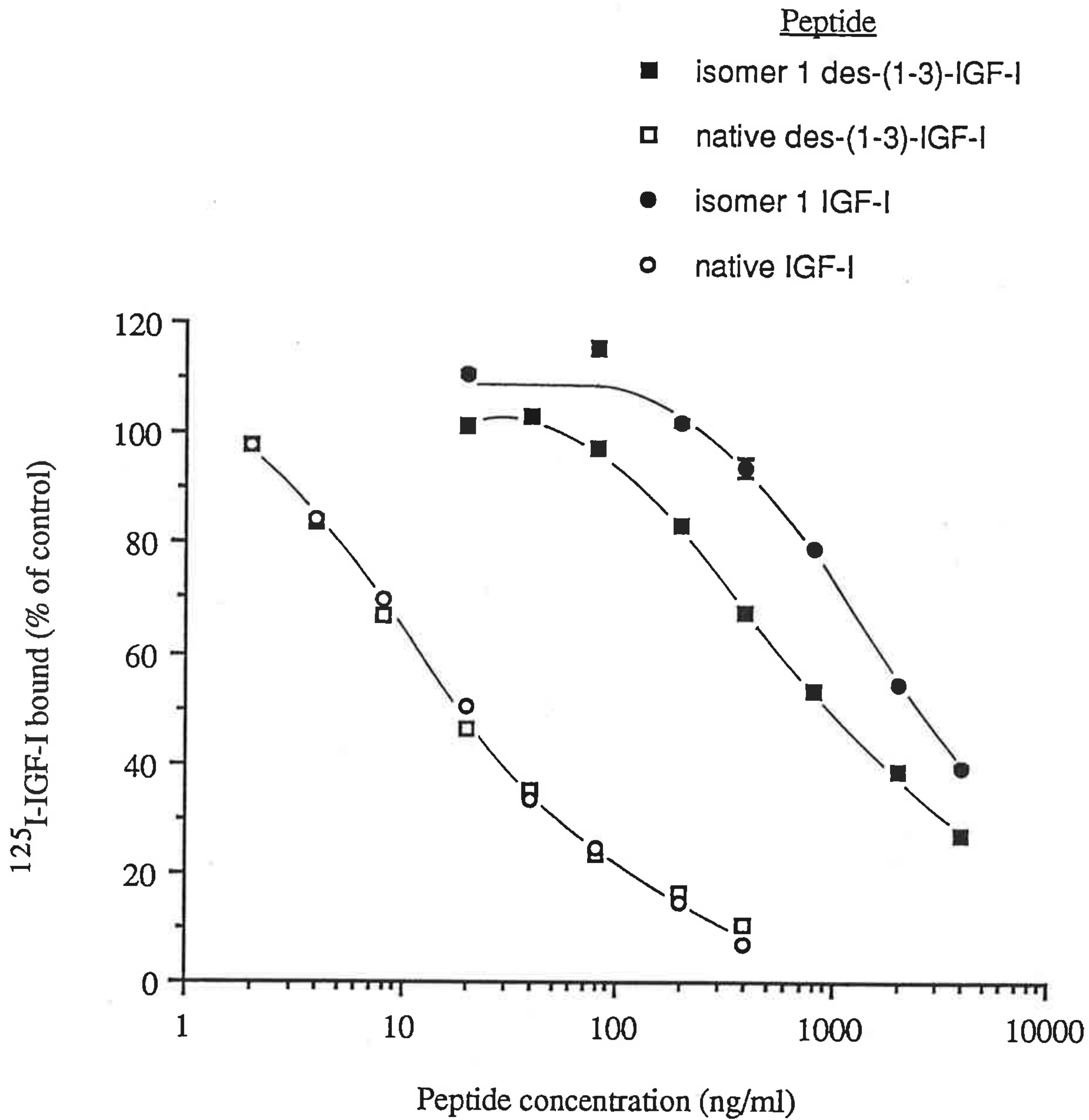
Figure 8.2: Rechromatography ofIGF-I peptides

IGF-I peptides were chromatographed on a 4.6 × 30mm RP-300 cartridge at a flow rate of of 1ml/min. Protein was eluted with the indicated gradient of acetonitrile in the presence of 0.1% TFA (- -), the A_{215} monitored (—), fractions were collected at 0.5min intervals and samples were subjected to the protein synthesis bioassay. Data are expressed as a percentage of the stimulation caused by 5% foetal bovine serum. The incorporation of radioactivity into protein was 0.9% in the absence of added growth factors and 3.9% in th presence of 5% FBS. *Panel a:* Rechromatography of des-(1-3)-[6-47-, 48-52-cystine]IGF-I. Samples equivalent to 5µl were tested in the bioassay (●). *Panel b:* Rechromatography of native des-(1-3)-IGF-I. Samples equivalent to 1µl were tested in the bioassay (●). *Panel c:* Rechromatography of [6-47-, 48-52-cystine]IGF-I. Samples equivalent to 20µl were tested in the bioassay (●). *Panel d:* Rechromatography of native IGF-I. Samples equivalent to 4µl were tested in the bioassay (●).



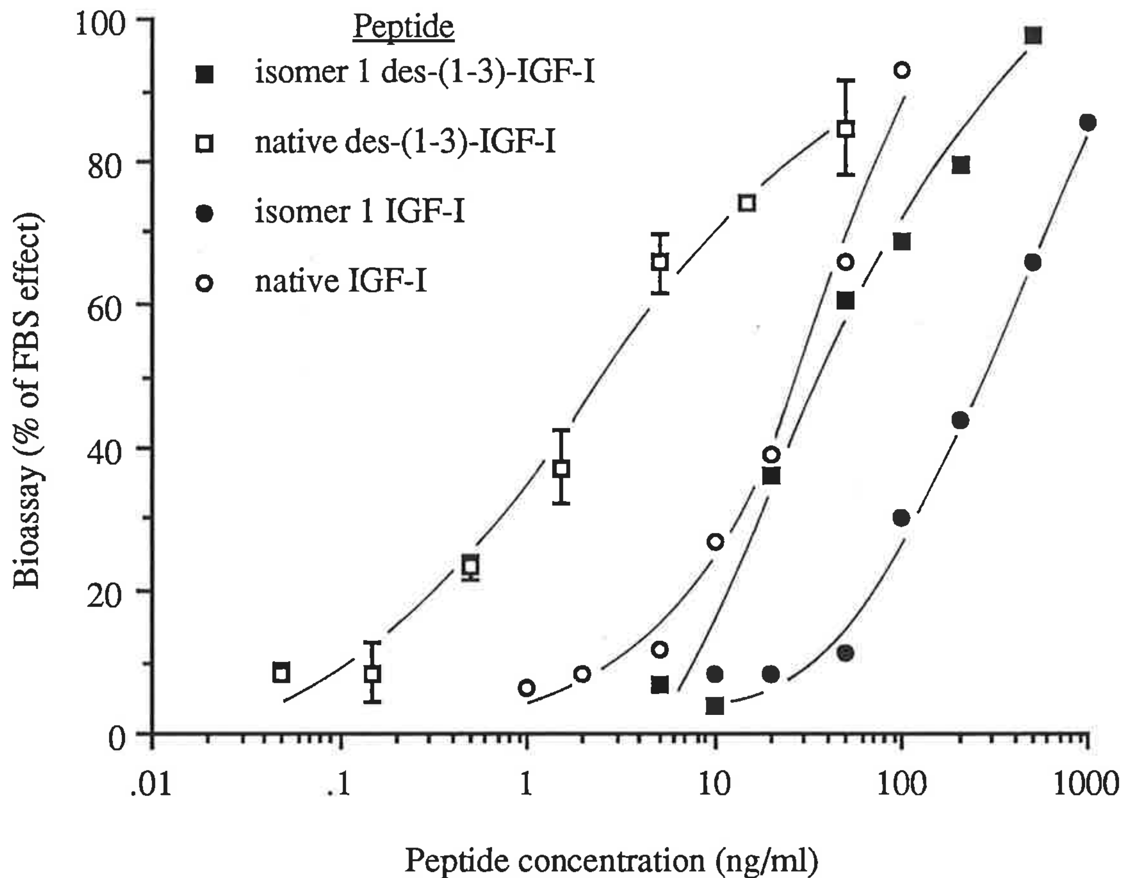
a
b
c
d
A215
1.5
0.75
0
0.8
0.4
0.2
100
50
Bioassay (% of FBS effect)
10
20
Elution time (min)

Figure 8.3: Radioreceptor assay of disulphide-bond isomers of IGF-I and des-(1-3)-IGF-I



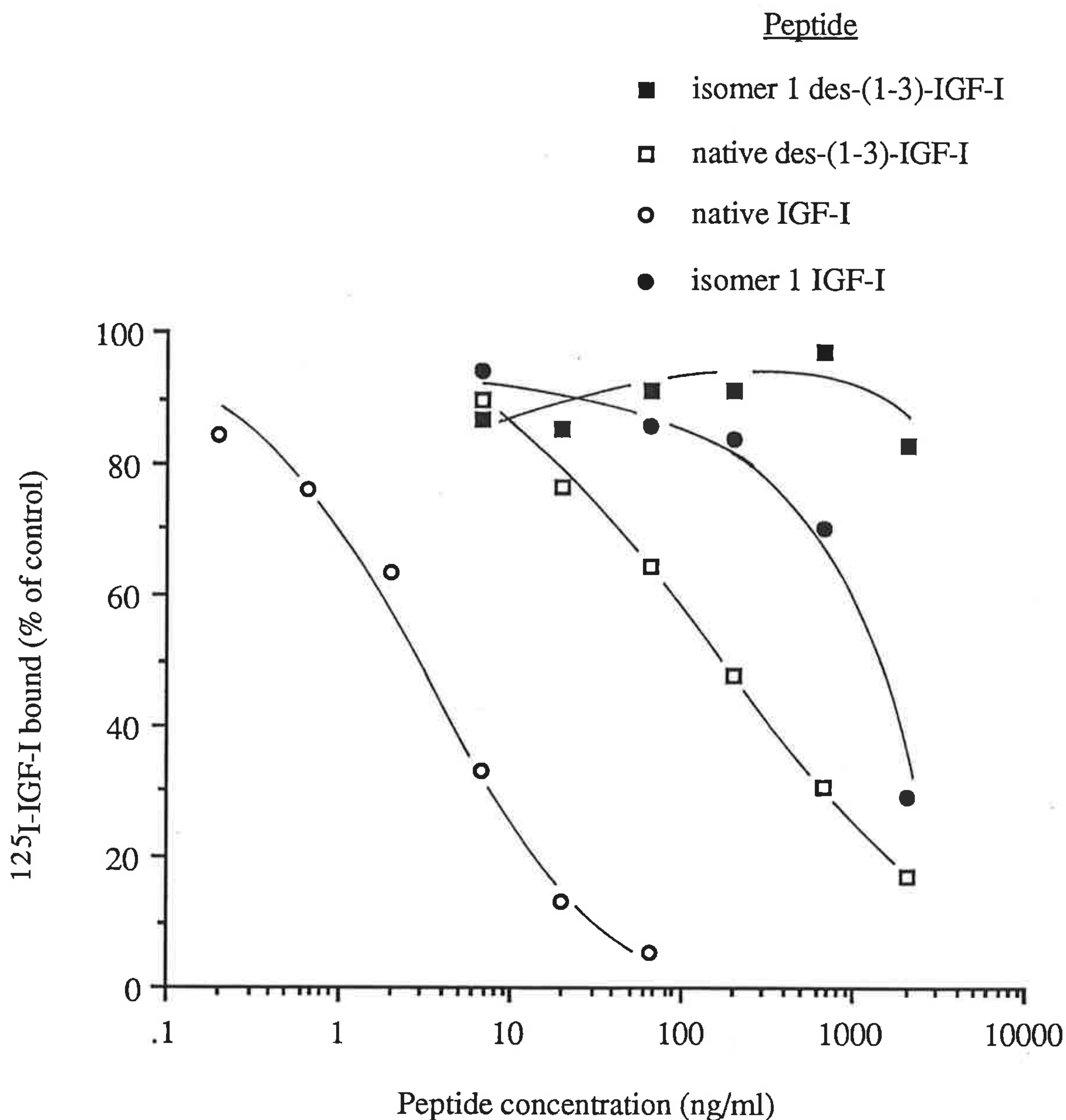
The abilities of the indicated IGF peptides to inhibit binding of ^{125}I-IGF-I to L6-cell monolayers were determined. The experiment was performed in 24 place multiwell dishes in a volume of 0.25ml. 13.1% of added radioactivity was bound in the control wells and non-specific binding has not been subtracted. Data plotted represent the means of triplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand.

Figure 8.4: Bioassay of disulphide-bond isomers of IGF-I and des-(1-3)-IGF-I



The indicated IGF peptides were subjected to the protein synthesis bioassay. Data represent the means of triplicate determinations and are expressed as a percentage of the stimulation caused by 5% foetal bovine serum. Incorporation of radioactivity into protein in the absence of added growth factor was 1.5% and the incorporation caused by 5% foetal bovine serum was 4.6%. As an indication of assay variability, standard errors of the mean are indicated for the des-(1-3)-IGF-I data.

Figure 8.5: Competitive binding assay of disulphide-bond isomers of IGF-I and des-(1-3)-IGF-I



Competition for ^{125}I-IGF-I binding to L6-myoblast conditioned medium by the indicated IGF peptides was measured. Total binding was 14% after a blank, determined in the absence of binding protein, of 16% had been subtracted. Data plotted represent the means of duplicate determinations and are expressed as a percentage of the binding evident in the absence of competing ligand.

CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9: GENERAL DISCUSSION

At the commencement of this project, techniques for the production of IGF-I by chemical synthesis and recombinant DNA methods were poorly understood. In particular, there had been no systematic investigation of the methods by which reduced IGF peptides could be induced to form the correct disulphide bonds. I developed a protocol for the oxidation of reduced IGF peptides which was generally applicable to analogues of IGF-I bearing substitutions or deletions in the N-terminal region except those lacking residue Leu^5. The folding process also showed some dependence on the identity of the residue at position four. The involvement of Leu^5 in the formation of the native tertiary structure of IGF-I is not unexpected since this residue is highly conserved in insulin (Pullen *et al.*, 1975) and the IGFs (table 1.1) and may be involved in the hydrophobic core of the protein (Blundell *et al.*, 1983; Cooke, 1988). The yields of purified biologically active peptide, produced by chemical synthesis, were low (approx. 0.5%) due to a combination of several factors. The long synthesis (69 couplings) resulted in the accumulation of substantial amounts of failure sequence peptides despite the good average coupling yields (99.4%). The recovery of crude peptide from the trifluoroacetic acid/ trifluoromethanesulphonic acid cleavage step was low although ninhydrin analysis indicated greater than 80% loss of amine from the resin. The removal of the acetamidomethyl protecting group from cysteine residues required extended reaction times and the purified reduced peptide reoxidized significantly less well than reduced natural IGF-I (compare figs. 3.4a & 4.3). Despite these difficulties, the chemical synthetic route was able to produce 20 novel analogues of IGF-I in adequate amounts for the *in vitro* biochemical analyses reported in this thesis and for limited *in vivo* studies (not reported in this thesis). In addition, the techniques developed for the renaturation of reduced IGF peptides, have prove useful in handling IGF-I produced by recombinant DNA methods. The isolation of a disulphide-bond isomer during the preparation of IGF-I and analogues has allowed the investigation of the role of the cystine bridges in determining the ability of IGFs to interact with receptors and carrier proteins.

Chemical synthesis was chosen as the method for production of des-(1-3)-IGF-I since the presence of IGF-I, IGF-II or other non-IGF bioactive contaminants could be rigorously excluded and the means of producing the recombinant peptide were not available. I was able to demonstrate that the enhanced biological activity of des-(1-3)-IGF-I the the L6

myoblast bioassay is a true molecular property of the truncated peptide and that it is not dependent on increased potency for binding to the type 1 or type 2 IGF receptors. The previously observed higher potency of des-(1-3)-IGF-I (cf. IGF-I) at the type 2 receptor (Ballard *et al.*, 1986) probably resulted from a low level of contamination of the natural preparation by IGF-II.

Using a family of analogues of IGF-I bearing sequential deletions of between 1 and 5 residues from the amino terminus, I demonstrated that the loss of the third residue (Glu) was required in order to observe enhanced biological potency and that further truncation did not yield peptides of still greater specific activity. Using the same group of peptides, it was found that the removal of residue Glu^3 resulted in peptides with markedly decreased ability to bind to IGF-binding proteins isolated from the conditioned medium of MDBK cells or L6 myoblasts. From these results, it was postulated that the enhanced biological potency of des-(1-3)-IGF-I and des-(1-4)-IGF-I IGF-I peptides results from a lack of association with IGF-binding proteins produced by the L6 myoblasts during the course of a bioassay. Studies by Ritvos *et al.* (1988) and Rutanen *et al.* (1988) support the notion that secreted binding proteins inhibit IGF action *in vitro*. Indeed, the deliberate addition of IBP-1 to the culture medium has been shown to attenuate the biological activity of both IGF-I and IGF-II, but not that of des-(1-3)-IGF-I, in a dose-dependent manner (Ross *et al.*, 1989). These data contrast with the report by Elgin *et al.* (1987) in which IBP-1 potentiated the stimulation of DNA synthesis by IGF-I in fibroblast cell-lines although the inclusion of platelet poor plasma was required in order to observe the enhancement of IGF-I action by binding protein. Also, De Mellow & Baxter (1988) found that human IBP-2, which is only slightly related to IBP-1, enhanced the biological effect of IGF-I in the absence of any specific serum components. The mechanism by which these IGF-binding proteins can potentiate the actions of IGFs are not presently understood.

I have found that although des-(1-3)-IGF-I, which occurs naturally, was among the most biologically active peptides that I synthesized, analogues such as des-(1-3)-[Arg^4]IGF-I, des-(1-3)-[Val^4]IGF-I, [Arg^3]IGF-I and [Gly^3, Arg^4]IGF-I also show very high biological potency probbaly because they bind very poorly to the binding proteins present in the assay medium. The [Arg^3]IGF-I and [Gly^3, Arg^4]IGF-I peptides share the convenient

feature of possessing an amino-terminal Gly residue which facilitates their production by recombinant DNA methods and liberation from a fusion partner by hydroxylamine.

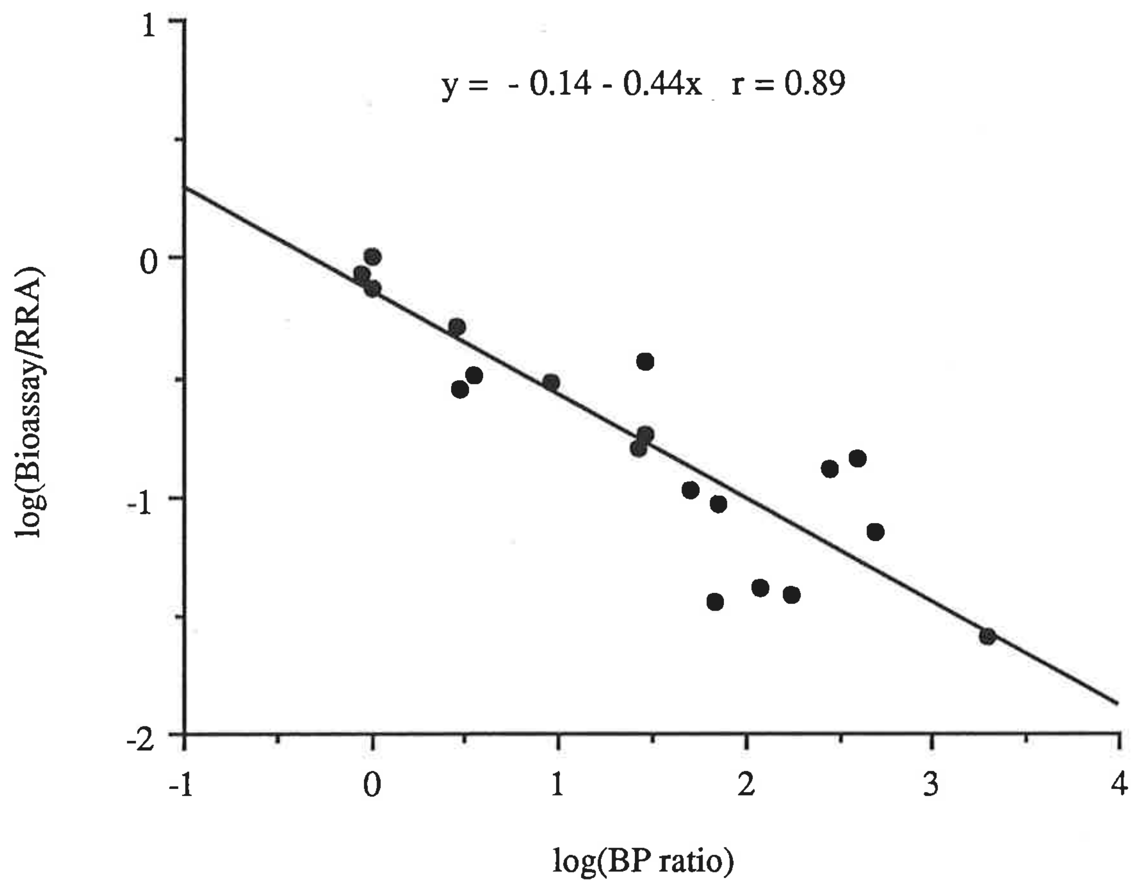
Residue Glu^3 of IGF-I was found to be very important for the binding of the peptide to the IGF-binding protein purified from the medium conditioned by MDBK cells suggesting that this Glu residue interacts specifically with a complementary residue on the binding protein. It is possible that in the normal interaction between IGF-I and the MDBK-cell-derived binding protein, an ionic bond forms between the the side-chain of residue Glu^3 and a positively-charged group on the binding protein. If this positively-charged group were situated in a hydrophobic pocket, the shorter side-chain of Asp might be unable to make the close approach required for a bond to occur resulting in an energetically-unfavourable buried charge and thus account for the weak binding of this analogue. Similarly, the binding of the other weakly-binding IGF-I analogues may tend to bury this positive charge and thus be unfavourable. The comparatively good binding of the [Phe^3]IGF-I peptide is interesting and may indicate an alternative mode of binding of the IGF to the binding protein, perhaps via hydrophobic interactions. The elucidation of the structure of the IGF-binding site on the binding protein, and the possible involvement of a positively-charged group within it, awaits the identification of the site within the primary sequence of the binding protein. At present, there are conflicting reports suggesting that, in the case of IBP-1, the IGF-binding site may be in the N-terminal region (Huhtala *et al.*, 1986), whereas it is in the C-terminal portion of IBP-3 (Wang *et al.*, 1988). The central region of the binding protein seems unlikely to be involved in IGF-binding since it is poorly conserved between the three known classes of IGF-binding protein (table 1.2). The essential role of residue Glu^3 in the binding of IGF-I to the MDBK binding protein is not seen with the L6-myoblast protein, in either the crude or purified form (fig 7.7), which was able to bind IGF-I analogues lacking residue Glu^3 suggesting that the IGF-binding site of this binding protein differs from that of the MDBK-cell protein. Similarly, human IBP-1 is able to bind weakly des-(1-3)-IGF-I and human IBP-2 binds des-(1-3)-IGF-I only two- to three-fold less well than IGF-I (Forbes *et al.*, 1988). The IGF-binding sites of the various IGF-binding proteins could be further investigated using chemical labelling techniques or by the production of analogues of the binding proteins via expression of complementary DNA clones encoding variants of these proteins.

Rearrangement of the disulphide bonds of IGF-I to form [6-47-, 48-55-cystine]IGF-I caused a large decrease in the ability of the peptide to bind to the IGF-I receptor of L6 myoblasts and to the binding proteins produced by MDBK cells and L6 myoblasts. This effect may result from either a localized perturbation of the IGF structure in the region of the altered disulphide bonds or from a more general change in the conformation of the molecule. Comparison of the solution structure of this IGF-I isomer with that of native IGF-I, using n.m.r. techniques, would help elucidate the determinants required for high-affinity binding of IGF-I to receptors and binding proteins.

There is apparently an inverse relationship between the ability of an IGF-I analogue to bind to IGF-binding proteins and its biological potency expressed on the basis of receptor binding. This relationship, which holds for analogues produced by truncation, substitution or disulphide-bond rearrangement, is illustrated in figure 9.1.

In conclusion, I have synthesized and biochemically evaluated 20 analogues of IGF-I many of which are more biologically active than natural IGF-I in the L6 myoblast bioassay. The enhanced potency of these peptides can be explained by their inability to bind to IGF-binding proteins produced by L6 myoblasts, a binding which would inhibit their ability to stimulate biological response via the type 1 IGF receptor.

Fig 9.1: Graph of the biological potency based on receptor binding versus potency for binding to L6-myoblast binding protein



Plot of data from chapters 6, 7 and 8

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PUBLICATIONS RESULTING FROM WORK REPORTED IN THIS THESIS

F.John Ballard, Geoffrey L. Francis, Marina Ross, Christopher J. Bagley, Bruce May and John C. Wallace (1987) Natural and synthetic forms of insulin-like growth factor-1 (IGF-1) and the potent derivative, destripeptide IGF-1: Biological activities and receptor binding.
Biochemical and Biophysical Research Communications, v. 149 (2), pp. 398–404, December 1987.

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1016/0006-291X(87)90380-9

Bagley, C.J., May, B.L., Szabo, L., Mcnamara, P.J., Ross, M., Francis, G.L., Ballard, F.J. and Wallace, J.C. (1989) A key functional-role for the insulin-like growth factor-1 N-terminal pentapeptide.
Biochemical Journal, v. 259 (3), pp. 665-671, May 1989.

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.