Biologically Active Peptides from Australian Amphibians

A thesis submitted for the Degree of Doctor of Philosophy

by

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from the

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In loving memory of John C.H. Williams.

Contents

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Statement of Originality

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

I give consent to this copy of my thesis, when deposited in the University Library, to be made available for loan and photocopying.

Rebecca Jo Jackway

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Abbreviations

1D	one-dimensional
2D	two-dimensional
3D	three-dimensional
$\Delta\delta$	secondary shift
ϕ	backbone torsion angle
ψ	side chain torsion angle
Å	angstroms
Ach	acetylcholine
ACTH	adrenocorticotropic hormone
apoCaM	calcium-free calmodulin
ANOVA	analysis of variance
AOP	angular order parameter
AP-1	activator protein-1
ARIA	Ambiguous Restraints for Iterative Assignment
BH_4	tetrahydrobiopterin
bp	base pairs
Ca^{2+} CaM	calcium-bound calmodulin
Ca_4CaM	calcium-saturated calmodulin
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CCK-8	cholecystokinin fragment 26 - 33
CCK-8-NS	non-sulfated cholecystokinin fragment 26 - 33
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CID	collision induced dissociation
CMC	critical micelle concentration
CNS	central nervous system
CNS Solve	Crystallography and NMR Systems
COSY	correlation spectroscopy
Da	Daltons
DAG	diacylglycerol
DC	direct current
derazepide	N-[(3S)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-1*H*-indole-2-carboxamide
DNA	deoxyribonucleic acid
DPC	dodecylphosphocholine
DQF	double-quantum filtered
DSS	sodium 2,2-dimethyl-2-silapentane-5-sulphonate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ESI	electrospray ionisation
ESI-MS	electrospray ionisation mass spectrometry

eNOS	endothelial nitric oxide synthase
FAB	fast atom bombardment
FAD	flavin adenine dinucleotide
FID	free induction decay
FMN	flavin mononucleotide
GI	gastrointestinal
GPI	guinea pig ileum
G protein	guanine nucleotide-binding regulatory protein
GRP	gastrin releasing peptide
heme	iron protoporphyrin IX prosthetic group
HPLC	high performance liquid chromatography
HSQC	heteronuclear single-quantum coherence
Hz	hertz
I	nuclear spin quantum number
IFN-γ	γ interferon
IL-2	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
LB media	*Luria-Bertani* media
L-NNA	N_ω-nitro-L-arginine
mAchR	muscarinic receptor
MALDI	matrix-assisted laser desorption ionisation
MIC	minimal inhibitory concentration
MLCK	myosin light chain kinase
MOPS	4-morpholinepropanesulfonic acid
M_r	relative molecular mass
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mRNA	messenger ribonucleic acid
MW	molecular weight
m/z	mass-to-charge
NADPH	nicotinamide adenine dinucleotide phosphate
NMB	neuromedin B
NMR	nuclear magnetic resonance
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
PCR	polymerase chain reaction
PDB	Protein Data Bank
pGlu	pyroglutamate
pI	isoelectric point

PIP_2	phosphatidyl inositol biphosphate
PLC	phospholipase C
ppm	parts per million
$polyA^+$	polyadenylated
rf	radiofrequency
RMD	restrained molecular dynamics
RMSD	root-mean-square derivation
RNA	ribonucleic acid
SA	stimulated annealing
SDS	sodium dodecylsulfate
SEM	standard error mean
Taq	*Thermus aquaticus*
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TOCSY	total correlated spectroscopy
TOF	time of flight
TRH	thyrotropin-releasing hormone
tris base	tris(hydroxymethyl)aminomethane
UMP	universal primer mix
UV	ultraviolet
VIP	vasoactive intestinal peptide
YM022	(*R*)-*N*-[2,3-dihyro-1-[2-(2-methylphenyl)-2-oxoethyl]-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-*N*`-(3-methylphenyl)-urea

Abstract

Amphibians secrete potent host defence compounds from dorsal glands onto the skin when stressed, sick or under attack by predators and microbials. Many of these defence compounds, such as biologically active peptides, provide potential targets for new biotechnological and therapeutic investigation. The research presented in this study focuses on the isolation and investigation of peptides from Australian frogs of the genera *Litoria* and *Crinia* and endeavours to determine the biological activity and important structural and mechanistic features of these biological compounds.

Isolation and identification of the skin peptide profile of the Eastern Dwarf Tree Frog *Litoria fallax* has revealed a number of novel peptides named fallaxidins. This frog species is quite unique in that it does not secrete a peptide that displays potent broad spectrum antimicrobial activity nor a peptide that inhibits nitric oxide formation through the enzyme neuronal nitric oxide synthase. Instead it secretes several narrow spectrum antimicrobial peptides, including fallaxidin 3.1. In addition, there are numerous small peptides displaying unique primary structures with unknown biological function. Interestingly, *L. fallax* produces a skin peptide profile that is quite distinct from the skin peptide profiles of other related *Litoria* species.

The majority of anurans from the *Litoria* genus contain at least one peptide in their skin secretion that inhibits the enzyme neuronal nitric oxide synthase. These peptides exert this action by preventing the association of the regulatory cofactor Ca^{2+} calmodulin to the enzyme binding site. The non-covalent binding of the potent neuronal nitric oxide synthase inhibitor dahlein 5.6 (*L. dahlii*) to calmodulin in the presence of Ca^{2+} is confirmed by electrospray ionisation mass spectrometry. A peptide-protein complex was observed in the gas-phase with a 1:1:4 calmodulin/dahlein 5.6/Ca^{2+} stoichiometry. In addition, the structure and binding interactions have been investigated by means of nuclear magnetic resonance spectroscopy. These experiments illustrated that upon binding dahlein 5.6, Ca^{2+} calmodulin undergoes a substantial conformational transition towards a globular complex with the helical dahlein 5.6 engulfed in a hydrophobic channel.

Typically, the granular secretion of amphibians contains numerous peptides that exert activities in the central nervous system, termed neuropeptides. The biological activities, in

particular smooth muscle action, proliferation of lymphocytes and opioid action are investigated to provide insight into the role of these peptides in the host defence. The structure activity relationships of disulfide peptides, caerulein peptides, tryptophyllins, rothein 1 and its related synthetically modified peptides has identified several important structural features essential for their corresponding biological function.

Peptides from the granular secretion of anurans are synthesized within and released from larger precursors molecules. The genes that encode for the skin peptides of *Crinia riparia* and several *Litoria* species were isolated and identified. The cDNA sequence of the precursors provides a mechanism by which the evolution of amphibian species can be traced and information about the relationships existing among closely or distantly related species be obtained. All prepropeptides isolated from the *Litoria* species illustrated sequence homology to those isolated from numerous ranid and hylid frogs and demonstrate that the skin prepropeptides originated from a common ancestral gene. The precursors of peptides from *C. riparia* are significantly diverse and suggest that these prepropeptides either originated from the same common ancestral gene but have undergone substantial divergent evolution relative to the ranid and hylid frogs or that they have originated from distinct ancestral genes.