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Mass Spectrometric Identification of Catabolic Pathways in the Selected Amphibian Skin Peptides with the Saliva and the Major Salivary Protease, Kallikrein

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Abstract- As nature has selected amphibian skin defensive peptides for inter-species delivery through the oral route in the recipient, structural stabilisation modifications may have occurred to facilitate this and such information would be most useful and could potentially provide new insights to the design of orally-active and selectively-targeted peptide therapeutics. The purposes of this study were to study catabolic pathways in saliva for selected but commonly occurring bioactive peptide types belonging to the protease inhibitor (PI) and bradykinin-related peptide (BRP) families, namely pLR (LVGRCWTKS-YPPKPCFVR), HV-BBI (SVIGCWTKSIPPRPCFVK) and I-11-R (IRPPGFSPLR), and to extend this study by determining catabolic pathways with kallikrein – the major salivary protease. These data will aid in the establishment of a database of peptide stabilities that may be useful in the design of future orally-delivered peptide therapeutics.

Keywords: amphibian; peptide; kallikrein; metabolism; catabolic; mass spectrometry.

I. INTRODUCTION

The diverse compounds stored in the granular glands of amphibians have various pharmacological effects, such as cardiotoxic, myotoxic and neurotoxic activities. Even just one single species is able to produce a large number and variety of host-defence compounds reflecting the wide range of potentially pathogenic microorganisms in their living habitats as well as various species of predators including mammals, birds, snakes, etc. [1-3]. Under natural selective pressures, the amphibians have to develop and maintain effective anti-predator defence systems such that the compounds produced in the skin secretions not only conserve bioactive core sequence but also induce structural modifications for optimisation of chemical structures required for survival [4-6]. Once amphibians are attacked, the peptides secreted from skin glands are mostly delivered into the oral cavities of attacking predators where they play roles in several

bioactive processes ranging from the noxious to the toxic and in some instances, to the fatal. Noxious or toxic sequelae serve to make the predators uncomfortable therefore saving the amphibians lives and leaving the predator with a bad memory that may serve to save individual amphibians of that species in the future [5-6]. Killing ones assailant is of course then ultimate in anti-predator defence but aeons of natural wisdom renders this rare as such end-points may serve ultimately to create worse problems. This successful scheme of molecular evolution and adaptation of molecular structures for unusual purposes will without doubt supply the scientist with valuable insights into the stabilising/protease resistance modifications required for the peptide to access and activate/block its target.

It is quite difficult for the scientist to design therapeutic peptides to be given orally because they are so readily degraded by the plethora of proteases in the gut which results in a short duration of action if any action results at all. Also, even after injection for certain conditions of the nervous system, the peptides cannot readily access their targets due to the fact that they are not able to cross the blood-brain barrier. However, the defensive peptides secreted by frog skin, synthesised as parts of large inactive precursors and subsequently generated by selective proteolytic cleavages [7-8], are able to be delivered effectively via the oral route as they have been to predators for millions of years. Kallikrein, the major protease secreted by salivary gland, causes a trypsin-like cleavage in a wide range of proteins. It participates in the reactions that release lysyl-bradykinin from kininogen, which has the effect of increasing the permeability of blood vessels and capillaries of the salivary gland to generate some responses to the bioactive processes, such as vasodilatation and inflammatory [8-11]. Kallikrein is also important in cleavages of peptides and that recognises Lys-Lys, Lys-Arg, and Arg-Arg motifs as processing signals at either COOH-terminals or between pairs of basic amino acids in addition to cleavage at single basic residues [9-13]. Accordingly, kallikrein, a serine protease, is one of the most frequently-used enzymes in protein structure analysis [12, 14-15].

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Here, we describe the application of this method of studying the oral stability and catabolism of several widely-distributed amphibian skin peptides belonging to the protease inhibitor (PI) and bradykinin-related peptide (BRP) families, namely pLR and HV-BBI-A (PIs) and I-11-R (BRP). The two PIs have similar sequences with subtle differences but have been isolated from different species frogs. We have studied their biotransformation by whole saliva and then by the major salivary protease, kallikrein, in isolation, and have subsequently structurally-characterised metabolite oligopeptides and plotted the time courses of their generation/disappearance. The kallikrein experiments were performed to determine the overall contribution of this protease to the peptide catabolism observed in whole saliva and in addition, to determine if this protease could cleave the Arg residue at the C-terminus of I-11-R to supply additional evidence to support the previous observation by our previous study that kallikrein can not only cleave after the C-terminal side of Arg but can also cleave the bond at the N-terminal side of Arg when this residue occupied the C-terminus [4].

II. MATERIALS AND METHODS

a) Solid-phase peptide synthesis

Replicates of peptides were synthesised by solid-phase Fmoc chemistry using a PS3 automated peptide synthesiser (Protein Technologies, Tucson, AZ, USA). When the synthesis cycles were completed, the peptides were cleaved from the resins using 95/2.5/2.5 (v/v/v) TFA/TIPS/water for 6h, precipitated in ether over the next 24 h, washed exhaustively in six changes of ether and then allowed to completely dry over a further 24 h. The peptides were then dissolved in a minimal quantity of 0.05/99.5, v/v, TFA/water, snap frozen in liquid nitrogen and lyophilised. The degrees of purity and authentication of structures of the synthetic peptides were determined using MALDI-TOF MS as previously described.

b) Protease biotransformations

Kallikrein—synthetic peptides were dissolved in sodium phosphate buffer (BioReagent, pH 7.4, for molecular biology, Sigma) at a concentration of $1\mu\text{g}/\mu\text{l}$ and incubated with $1\mu\text{g}/\mu\text{l}$ bovine pancreatic kallikrein (Sigma) at an enzyme to substrate ratio 1:50 (w/w) at 37°C . Samples of $2\mu\text{l}$ were taken at 10min intervals in the first hour then subsequently at 60min intervals. The degradations were directly analyzed by MALDI-TOF-MS.

Human saliva--1 ml samples of human saliva (collected from author X.C and H.W) were incubated separately with $1\mu\text{g}/\mu\text{l}$ of respective synthetic peptides in a 37°C incubator for 48h. Samples were removed and treated as described above.

c) Mass spectrometric identification

Two microlitres of each sample from the kallikrein and saliva incubation experiments were placed

in separate wells of a MALDI-TOF sample plate, mixed with one microlitre of matrix solution (α -cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA in water 30/70 (v/v)), air dried and subjected to analysis on a Perseptive Biosystems DE MALDI-TOF instrument (Perseptive Biosystems, Framingham, MA, USA).

III. RESULT

a) Synthesis and structural characterisation of replicates of natural peptides

pLR (LVKGCWTKSYPPKPCFVR), HV-BBI (SVIG-CWTKSIPPRPCFVK) and I-11-R (IRRPPGFSPRLR) were successfully solid-phase synthesised and their levels of purity were established by a combination of reverse phase HPLC followed by subjection to MALDI-TOF mass spectrometric analysis, where observed molecular ions consistent with predicted molecular masses of replicates of peptides indicated successful syntheses and confirmed high degrees (>95%) of purity of the expected products in each case.

b) Protease biotransformations

Incubation of pLR with saliva resulted in the rapid generation of a series of metabolites that are shown in Table1. The metabolites produced by kallikrein could be found in the series of metabolites produced by saliva (Table 2). Thus, it appeared that pLR, which is an antibacterial and trypsin-inhibitor peptide secreted by frog skin, could readily be cleaved by salivary proteases and that the major effective protease appeared to be kallikrein. A most unexpected finding was that a major catabolite, des-arginyl18-pLR (pLR 1-17) was produced. This observation was consistent with previous reports using bradykinins as substrates but this is the first demonstration of such a cleavage in peptide belonging to another structural family. Moreover, as the reaction time proceeded, this des-arginyl18-pLR (pLR 1-17) became the major metabolite (Figure1).

HV-BBI, which is likewise a trypsin inhibitor peptide from frog skin secretion and has a similar structure to pLR but with subtle differences, was also selected for study in these experiments. The results showed surprisingly that HV-BBI-A was not as readily degraded by saliva as its structural analogue, pLR, and no catabolism was detected in saliva under the same degradation conditions as pLR, although the peptide could be cleaved by kallikrein (Table 3), albeit rather slowly. The fragments (SVIGCWTKSIPPRPC, 1643 Da) and (KSIPPRPCFV, 1142 Da) were the major catabolic products (Figure 2). A further fragment, 5-17 (CWTKSIPPRPCFV, 1532 Da) was observed after 24h indicating that the X-Lys bond at the carboxyl terminus could be cleaved by kallikrein.

The BRP, I-11-R, which has an Arg residue as its C-terminus, was employed to assess yet again if this residue could be cleaved by kallikrein (Table 4), to confirm previous observations. As anticipated, this

residue was removed rapidly and the subsequent des-arginyl11-peptide generated represented a stable major catabolite (Figure 3).

IV. DISCUSSION

Amphibian skin gland secretions play key roles in everyday survival. As they contain a plethora of biologically-active molecules, they are a useful and unique source of material for scientific researchers, such as those involved in biophysical research, evolutionary studies and those searching for new pharmaceutical leads [1,16]. As many potent bioactive peptides continue to be isolated and identified from the skin secretions of amphibians, more and more attention is being paid to these special and amazing little animals. Caerulein first isolated from the Australian green tree frog, *Litoria caerulea*, is able to affect blood pressure at a very low concentration and epibatidine, a heterocyclic compound from the skin of *Epipedobates tricolor*, is non-addictive and 2000 times more active than morphine. Magainins 1 and 2 from the African clawed frog, *Xenopus laevis*, exhibit potent antibiotic activity against both Gram-negative and Gram-positive bacteria as well as fungi, and they possess anticancer activity at micromolar concentrations. Caerin 1, obtained from frogs of the genus *Litoria*, is not only a wide-spectrum antibiotic but also displays strong antiviral activity against enveloped viruses that include HIV and Herpes simplex 1 [1-3]. The bioactive peptides isolated from the secretions of amphibians where they have evolved as defensive bio-weapons, are considered to be new pharmacological agents that may serve to overcome serious clinical problems in humans as well as contribute to the study of aspects of delivery and stability of peptide therapeutics.

Over vast time periods of natural selection, amphibians have developed unique defence systems against the predators. These defensive secretions can induce some extraordinary behaviour, such as oral dyskinesias in predators involving small snakes, *Thamnophis ordinoides*, buying sufficient time for the amphibians to escape [1,16]. As the defensive peptides from the amphibian skin surface are firstly delivered into the oral cavities of attacking predators where they mix with saliva, it is quite logical to study the effects of such on the stability of these agents. Saliva has long been known to be one of the first lines of innate immune defence in both humans and animals. Histatins for example, are a class of peptides found in saliva that contain a very high proportion of histidine residues (18-29%) and are thus highly-cationic. Of no surprise, they also display potent antimicrobial activity [17-18]. Saliva is rich in proteins, enzymes and peptides that not only help animals lubricate and digest their food but can also reduce the effects of some toxic food components to protect the individual from damage [19-20]. Due to molecular wisdom accumulated over vast aeons that

leads to biochemical modifications, some amphibian skin peptides were found to retain their high-selectivity at cognate receptors after exposure to salivary proteases, with some catabolites even exhibiting enhanced bioactivity. Maximakinin, an N-terminally extended bradykinin obtained from the skin secretion of a Chinese toad, *Bombina maxima*, is a very good example of such a phenomenon [4]. The primary structures of amphibian peptides thus serve as good models to provide new insights into the study of peptide-stabilising and/or protease-resistance modifications to satisfy the requirements for oral therapeutic peptide delivery.

Kallikrein, as one of the most abundant proteolytic enzymes in the saliva, has been found in various organs including salivary glands, plasma and kidneys in mammals. In addition, kallikrein-like enzymes have been discovered in cells/tissues and biological fluids of other mammals and in other species and secretions such as some snake venoms [21]. To date, some conserved serine proteases which have sufficient chemical similarities to kallikrein, occur in multiple forms in human tissues and are described as the kallikrein-related peptidase family. Some of these kallikreins, such as 3 and 6, can act as potential biomarkers of disease and also have effects on various physiological processes through participating in the corresponding cleavages of propeptides and these include such as things as inflammation induction and maintenance and blood pressure control by releasing bradykinin from kininogens[7-10]. Kallikrein is predicted to recognize Lys-Lys, Lys-Arg, and Arg-Arg as processing signals either at the carboxyl-terminal or between pairs of basic amino acids of inactive propeptide substrates and cleave these peptide bonds to generate biologically-active peptides [7-9,12].

pLR, which was originally isolated from the skin of the Northern Leopard frog, and was identified as a histamine-releasing peptide with no haemolytic activity. pLR displays diverse biological functions involving antimicrobial activity, histamine release induction, mast cell activation and granulocyte macrophage colony formation inhibition without the induction of neutrophil apoptosis [22]. After pLR was incubated with salivary proteases and mammalian kallikrein, it was quite surprising to find that the degradation reactions observed, generated a spectrum of metabolites with the same discrete bioactivity as the parent compound. Kallikrein produced metabolites, as predicted, arising from cleavage of the Arg-Lys doublet, with cleavage at Arg-X predominating. An unusual observation however, was the removal of Arg18 generating des-arginyl18 pLR. This X-Arg cleavage has only been once reported for kallikrein as it is generally accepted that this enzyme cleaves at Arg-X bonds. Although kallikreins from different tissue sources may exhibit different site specificities, both bovine pancreatic and human/canine

salivary kallikreins were found to remove the C-terminal arginyl residues from pLR.

HV-BBI, a synthetic replicate of the natural mature peptide which is similar to the trypsin inhibitor (pLR), has only one difference in primary structure within their respective inhibitory loops that is the substitution of the Lys (K) residue in the presumed P5' position of ORB (a pLR/ranacyclin family member) by Arg (R). This substitution would make a large increase in potency highly unlikely. HV-BBI was not readily degraded by the saliva, in stark contrast to its related peptide, pLR, though it could be cleaved quite readily by kallikrein. While a series of catabolic cleavages were observed with kallikrein, these included the removal of the C-terminal Lys residue. I-11-R was a bradykinin-related peptide (BRP), isolated originally from the skin secretion of the North American frog, *Rana palustris*. A synthetic replicate of this endogenous frog skin secretion peptide, was rapidly attacked by kallikrein causing removal of its C-terminal Arg residue by the non-canonical kallikrein cleavage of an X-Arg bond. This cleavage was fully consistent with several previous observations with kallikrein using different peptide substrates.

Comprehensive analysis of all the catabolic data generated here, revealed that these were in agreement with previously reported data from our research group regarding the specificity of kallikrein, confirming that the peptides whose amino acid sequences contain Arg and/or Lys residues at the C-terminus can be cleaved specifically by kallikrein. The present data have also proved these original observations unequivocally for a broader range of peptide substrates. The catabolite generation time profiles demonstrated that different peptide bonds were cleaved with different time courses and preferences but the peptide bonds of Arg residues at the carboxyl terminus appeared to be generally cleaved by particular enzymes preferentially and 2x to 10x faster than Lys residues occupying the same position. Furthermore, after longer periods of incubation, there were numerous minor metabolites revealed with other cleavage sites like (Cys-Phe, Gly-Leu), which are not specific to kallikrein. This probably indicates the presence of minor contaminant proteases and it is probably biologically irrelevant, the rapid modifications being more likely to be relevant in this respect. The catabolic reactions observed for the BRP would undoubtedly produce a major shift in biological activity spectra as the des-arginyl forms of the kinins are highly-selective ligands for the B1 receptor subtype and do not activate the B2 receptor subtype at all [4]. The biotransformation observed in saliva for several amphibian skin-derived defensive peptides would suggest that the modified structures are highly selective for certain receptors and some have even enhanced bioactivity compared to original peptides. This may represent a most subtle and largely unexplored aspect of the efficacy of peptide

action in the survival strategy of amphibians and could readily supply the scientist with insights into the development of novel orally-active peptide drugs.

V. CONCLUSION

In this study, we present a new catabolic cleavage bond of an X-Arg/Lys by kallikrein, where the peptides containing Arg and/or Lys residues at the carboxyl terminus can be cleaved specifically by kallikrein. Moreover the peptide bond of Arg residues at the C-terminus was observed to be preferentially cleaved by particular enzymes and faster than Lys residues occupying the same position. The catabolic generation of several amphibian skin-derived defensive peptides are highly selective for certain receptors, even exhibiting enhanced bioactivities compared to original peptides, based on their modified structures. These data will aid in the establishment of a database of peptide stabilities that may be useful in the design of future orally-delivered peptide therapeutics.

VI. ACKNOWLEDGE

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

Ethical statement

Secretions used in this study were obtained non-invasively from captive frogs.

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LEGENDS TO FIGURES

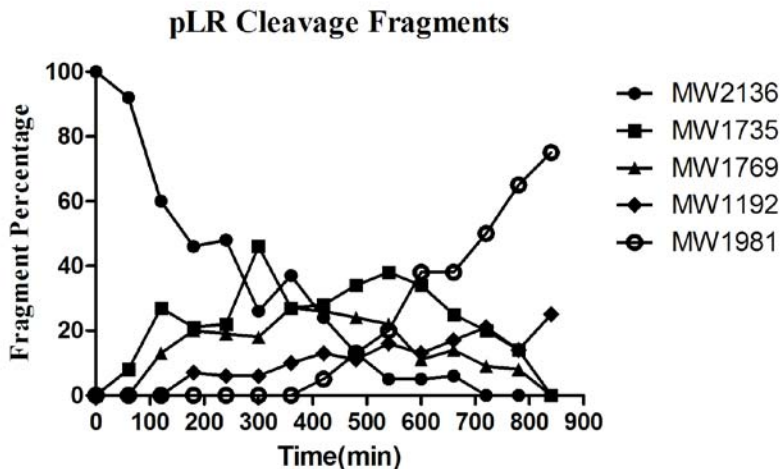


Figure 1 : The concentrations of pLR catabolism fragments with incubation time

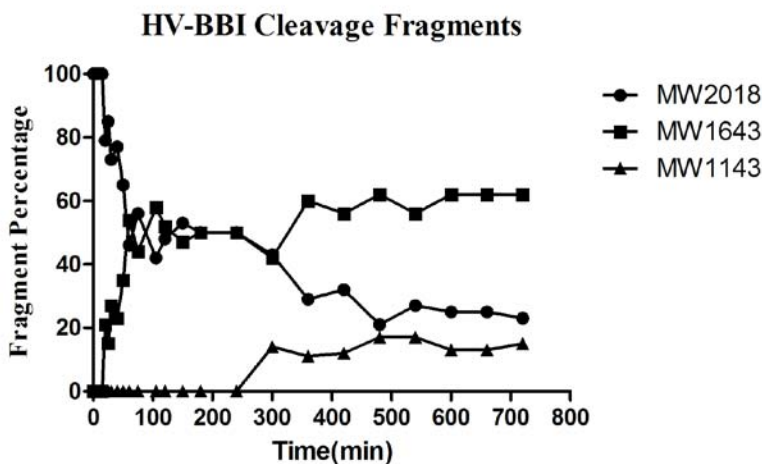


Figure 2 : The concentrations of HV-BBI catabolism fragments with incubation time

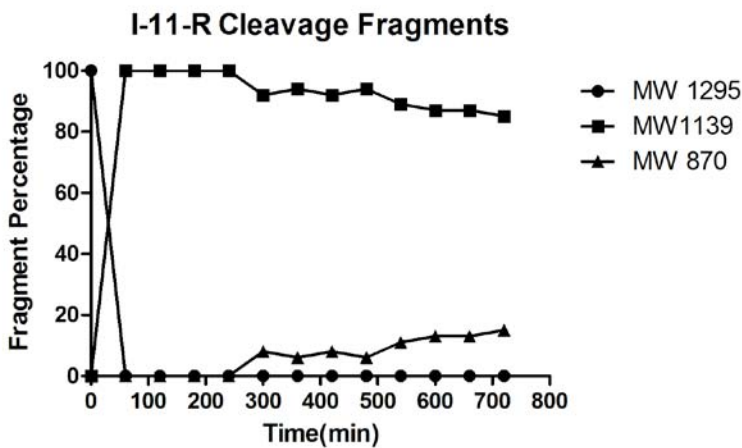


Figure 3 : The concentrations of I-11-R catabolism fragments with incubation time

Figures footnotes: The catabolite generation time figures generally show the change in relative concentration of each catabolic fragment with time. The percentage of each fragment compared with the total fragments represents their concentrations.

Table 1 : Mass spectrometric characterization of pLR fragments generated by saliva.

Cleavage time(min)	Calculated /Observed Mass (Da)	Sequence
0	2136.64/2136	LVRGCWTKSYPPKPCFVR
180	1769.14/1769	GCWTKSYPPKPCFVR
240	1192.46/1192	SYPPKPCFVR

LVR↓GCWTK↓SYPPKPCFVR
↓ = sites of saliva cleavage in pLR

Table 2 : Mass spectrometric characterization of pLR fragments generated by Kallikrein.

Cleavage time(min)	Calculated/Observed Mass	Sequence
0	2136.64/2136	LVRGCWTKSYPPKPCFVR
8	1734.14/1735	LVRGCWTKSYPPKPC
50	1769.14/1769	GCWTKSYPPKPCFVR
90	1192.46/1192	SYPPKPCFVR
120	1981.44/1981	LVRGCWTKSYPPKPCFV

LV↓R↓GCWTK↓SYPPKPC↓FV↓R
↓ = sites of kallikrein cleavage in pLR

Table 3 : Mass spectrometric characterization of HV-BBI fragments generated by Kallikrein.

Cleavage time(min)	Calculated /Observed Mass	Sequence
0	2018.53/2018	SVIGCWTKSIPPRPCFVK
10	1643.03/1643	SVIGCWTKSIPPRPC
420	1142.45/1143	KSIPPRPCFV
1620	1532.91/1533	CWTKSIPPRPCFV

SVIG↓CWT↓KSIPPRPC↓FV↓K
↓ = sites of kallikrein cleavage in HV-BBI-A

Table 4 : Mass spectrometric characterization of I-11-R fragments generated by Kallikrein.

Cleavage time(min)	Calculated /Observed Mass	Sequence
0	1295.57/1295	IRRPPGFSPLR
5	1139.38/1139	IRRPPGFSPL
300	870.03/870	RPPGFSPL

IR↓RPPGFSPL↓R
↓ = sites of kallikrein cleavage in I-11-R

