Mammalian Peptide Isomerase: Platypus-Type Activity Is Present in Mouse Heart

by **Jennifer M. S. Koh**, **Stephanie J. P. Chow**, **Ben Crossett**, and **Philip W. Kuchel***

School of Molecular Biosciences, Building G08, University of Sydney, NSW 2006, Australia (phone: +61 2 9351 3709; fax: +61 2 9351 4726; e-mail: philip.kuchel@sydney.edu.au)

Male platypus (*Ornithorhynchus anatinus*) venom has a peptidyl aminoacyl L/D-isomerase (hereafter called peptide isomerase) that converts the second amino acid residue in from the N-terminus from the L- to the D-form, and *vice versa*. A reversed-phase high-performance liquid chromatography (RP-HPLC) assay has been developed to monitor the interconversion using synthetic hexapeptides derived from defensin-like peptide-2 (DLP-2) and DLP-4 as substrates. It was hypothesised that animals other than the platypus would have peptide isomerase with the same substrate specificity. Accordingly, eight mouse tissues were tested and heart was shown to have the activity. This is notable for being the first evidence of a peptide isomerase being present in a higher mammal and heralds finding the activity in man.

Introduction[1]). – A peptidyl aminoacyl L/D-isomerase (hereafter called peptide isomerase) was discovered in the venom of the male platypus (*Ornithorhynchus anatinus*) [1][2]. This enzyme interconverts L- and D-amino acid residues at the second position in from the N-terminus of a small series of different peptides [3]. The discovery of the peptide isomerase occurred after finding two pairs of platypus venom peptides that showed ^{1}H-NMR-spectral differences primarily at the second amino acid residue; these peptides were defensin-like peptides (DLP-2/DLP-4) and *Ornithorhynchus* venom C-type natriuretic peptides (OvCNPa/OvCNPb). DLP-4 and OvCNPa have all the amino acid residues in the normally occurring L-form, while DLP-2 and OvCNPb have the second amino acid residue in the D-form [4]. As the protein synthesis 'machinery' on ribosomes only generates peptides from L-amino acids, it is generally accepted that DLP-2 and OvCNPb are post-translationally modified from DLP-4 and OvCNPa, respectively. And it is the peptide isomerase that catalyses this L- to D-conversion [1].

Isomerases that catalyse the conversion of L- to D-amino acid residues have already been reported in spider venom [5], lobster nervous system [6], and frog skin secretion [7]. All the isomerases that have been characterised to date, including platypus venom peptide isomerase, isomerise amino acids at different positions in peptides either near the N- or C-terminus of the respective peptide. However, platypus venom peptide

[1]) **Abbreviations.** – MeCN: acetonitrile; DAO: D-amino acid oxidase; DLP-2: defensin-like peptide-2; DLP-4: defensin-like peptide-4; DEPC: diethylpyrocarbonate; EDTA: ethylenediaminetetraacetic acid; MALDI-TOF-MS: matrix-assisted laser desorption/ionisation-time of flight mass spectrometry; Nle: norleucine; NMDA: *N*-methyl-D-aspartate; NMR: nuclear magnetic resonance; OvCNPa: *Ornithorhynchus* C-type natriuretic peptide A; OvCNPb: *Ornithorhynchus* C-type natriuretic peptide B; PBS: phosphate buffered saline; RP-HPLC: reversed-phase high-performance liquid chromatography; TFA: CF_3COOH.

isomerase is the only one which is of mammalian origin; thus, it is the first such enzyme reported in mammals [3].

One proposed outcome of peptide isomerases is that the modified peptides are less prone to peptidase-mediated degradation in the circulation or in tissues, so they persist longer *in vivo* [1][2]. It is also possible that the D-amino acid-containing peptides are more potent than their L-counterparts as is the case with dermophin [8].

Platypus venom peptide isomerase is *ca.* 55–65 kDa in size; it isomerises the second amino acid residue in from the N-terminus in both L- to D- and D- to L-directions. The enzyme remains active up to 55° [2], and it is inhibited by diethylpyrocarbonate (DEPC) [9]. In previous work, we reported that the peptide isomerase can operate on short hexapeptides with the N-terminal tripeptide sequences derived from DLP-2 and DLP-4, with a short water-solubilising chain appended at the C-terminus [9]. Some modifications have successfully been made to these synthetic hexapeptides; thus, norleucine can replace methionine as the second amino acid residue and it displays faster isomerisation kinetics. Also, the solubilising chain can be made with all D-amino acids which has the benefit of protecting the hexapeptides from L-specific carboxypeptidases [9].

Although the DLP-2/DLP-4-derived hexapeptides have identical amino acid sequences, their elution profiles on RP-HPLC are different [9]. This is because the L-/D-amino acid residues at the second position cause differences in the overall fold of the hexapeptides and thus change their binding affinity to *C18* chromatographic medium (*Fig. 1*). Therefore, a convenient isomerase assay was able to be established using RP-HPLC in which we employed the differences in the elution times of each isomeric species.

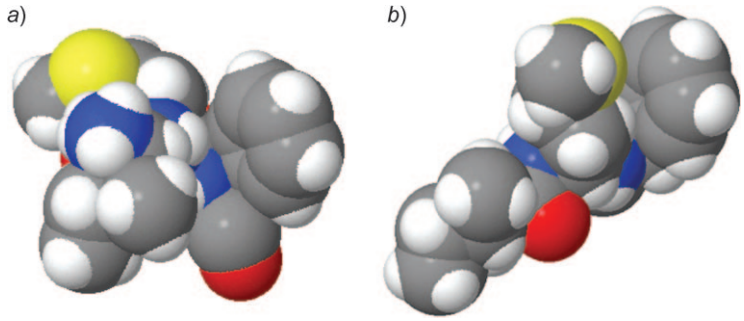

Fig. 1. *Molecular structures of the N-terminal tripeptide sequences, IMF:* a) *DLP-4, with* L-*methionine; and* b) *DLP-2, with* D-*methionine.* The third amino acid residue, phenylalanine, is aligned in almost the same direction in each structure. The different folding of the peptides is caused by the side chain of methionine being positioned on different sides of the α-C-atom depending on whether it is the L- or D-form. Such folding differences are the basis for distinctly different interactions with the RP-HPLC *C18* chromatography medium even though the medium itself is not chirally selective. Structures were drawn using Jmol [10][11].

The presence of peptide isomerase in higher mammals was postulated to be possible once it had been detected in the platypus. However, due to the lack of easily accessible techniques and convenient methods to monitor the L-/D-interconversion, the activity has not been widely studied. In this work, the HPLC-based assay developed for

platypus venom peptide isomerase was employed to test for isomerase activity in various mouse tissues. This is the beginning of a broad search for a wide range of different peptide specificities in mammals, including man, because there appears to be no *a priori* reason why only platypus-type specificity should exist and no others, such as those from the phylogenetically older frogs [7], spiders [5], and crustaceans [6].

Results and Discussion. – *Screening.* Eight mouse tissues (brain, heart, kidney, liver, lung, small intestine, spleen, and stomach) were tested for isomerase activity using the synthetic hexapeptides derived from DLP-4, *viz.*, IMFsrs and its mutant, I(Nle)Fsrs (uppercase letters denote L-amino acids; lowercase letters denote D-amino acids). When platypus-specific isomerisation occurs, the second amino acid residue of these hexapeptides is converted into the D-amino acid that is differentiated from the all-L-amino acid hexapeptides on the basis of their different elution times on RP-HPLC.

Platypus Control. A control reaction was performed by incubating I(Nle)Fsrs with a partially purified platypus venom-gland extract at 37°. As seen in *Fig. 2*, I(Nle)Fsrs was converted to I(nle)Fsrs after incubation, based on the elution time being the same as that of the known, pure synthetic I(nle)Fsrs. To ascertain if the converted product was indeed I(nle)Fsrs, the eluted fraction was collected and MALDI-TOF-MS was performed to determine the main molecular mass. The product peak corresponded to a molecular species with a mass that was the same (within 0.2 mass units) of that of I(Nle)Fsrs; thus the isomerisation was confirmed.

Mouse Tissue Extracts. Similar results were obtained from assays using mouse heart extract. Two peptide substrates were used, *viz.*, IMFsrs and I(Nle)Fsrs. They both showed isomerisation; a peak appeared in the chromatogram at the time where the D-amino acid-containing hexapeptide was eluted (see *Figs. 3* and *4*). Again, the identities of the main constituents of the corresponding fractions were established by MALDI-TOF-MS. The fractions contained peptides with identical molecular masses as the corresponding L-amino acid-containing hexapeptides.

The assays performed with extracts of other mouse tissues revealed no isomerisation. It is likely that the substrate hexapeptides were degraded by peptidases in the tissue extracts during the assay procedure.

In this work, mouse hearts from both male and female mice were positive for the activity, and this occurred across different ages from 3 weeks to 18 months.

In the assays with platypus venom peptide isomerase, L- to D-isomerisation occurred rapidly on incubation at 37°. At *ca.* 4 h, the reaction has almost reached equilibrium (quasi-steady state; data not shown here). However, the standard reaction (same relative volumes of extract and peptide) with the mouse heart extracts was much slower, and clear evidence of the isomerisation was only seen after several hours of incubation. This occurred even though the extracts had been concentrated. This slow isomerisation by the mouse heart extracts may have resulted from either a lower absolute concentration of the enzyme in the tissue, or different kinetic parameters such as the binding affinity, *Michaelis–Menten* parameters, or susceptibility to inhibitory effector molecules, both endogenous and added to the assay medium. This aspect of the investigation is encompassed by our future plans.

Substrate Specificity. It has been proposed that isomerisation involves at least three amino acid residues, where the second amino acid residue must have a long

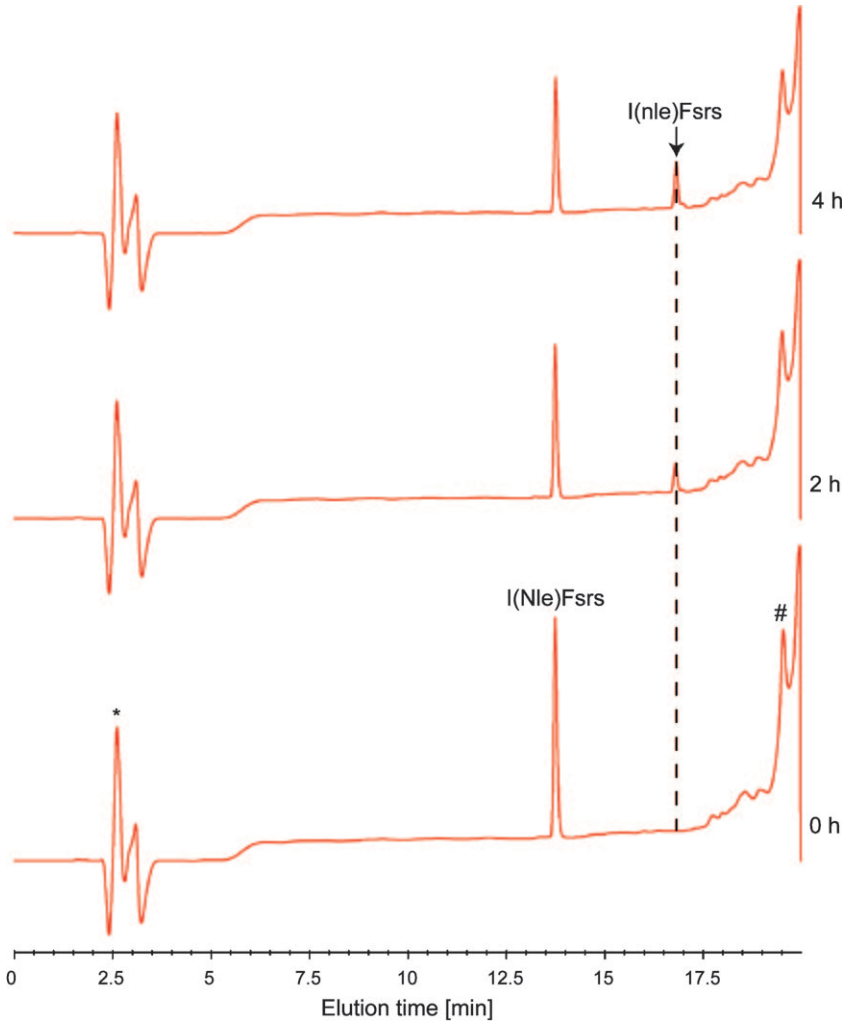

Fig. 2. *RP-HPLC Chromatograms from the assay of peptide isomerase activity with the synthetic hexapeptide I(Nle)Fsrs, catalysed by platypus venom peptide isomerase.* The peptide substrate was incubated with a partially purified platypus venom-gland extract at 37°; HPLC Analysis was carried out at 0, 2, and 4 h. The elution of I(nle)Fsrs is indicated by the arrow; peak complexes marked with * corresponded to the elution of salts, and # coincided with the programmed abrupt change in elution-solvent concentration.

hydrophobic chain and the third amino acid residue has to be aromatic [9]. Peptide isomerase does not act on short peptides derived from OvCNPa and OvCNPb. This forms an important basis of our isomerase assays by using synthetic short peptides as substrates because they can be purchased cheaply or easily made in the laboratory. However, it does mean that other peptide isomerases could be missed if they require larger peptide substrates.

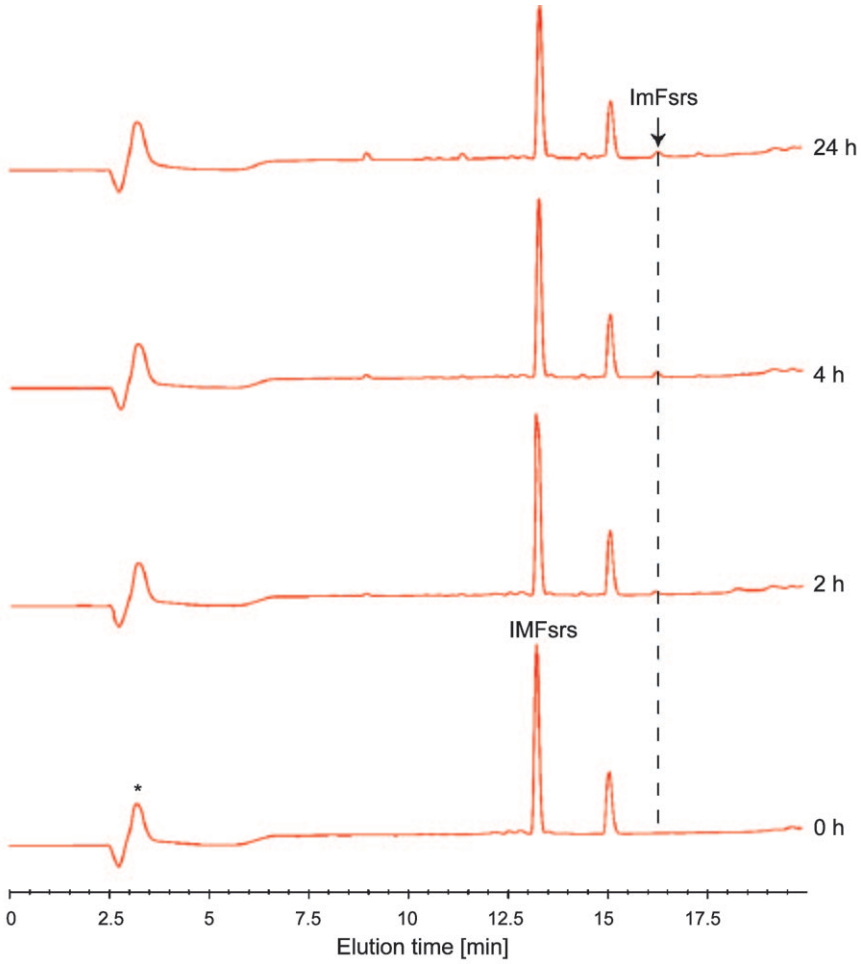

Fig. 3. *RP-HPLC Chromatograms from the assay of peptide isomerase activity in mouse heart extract with the synthetic hexapeptide IMFsrs, incubated at 37°*. HPLC Analysis was carried out at 0, 2, 4, and 24 h. The appearance of the ImFsrs peak is indicated by the arrow; peak complexes marked with * corresponded to the elution of salts.

Protease and Peptidase Inhibition. In the assays using I(Nle)Fsrs as the substrate, the isomerisation to I(nle)Fsrs by platypus venom peptide isomerase was seen to be faster than the isomerisation of IMFsrs to ImFsrs [9]. As for all enzymes, peptide isomerase catalyses the reaction in both directions, and the fact that the L- to D-isomerisation took a shorter time to reach the equilibrium (in reality a quasi-steady state) implies a bias of the equilibrium in favour of the D-form. On the other hand, since the conversion of the D-form to the L-form yields a product that is more susceptible to attack by aminopeptidase(s) in the tissue extract, this impression may be artefactual. In other words, the assay medium contains a 'cocktail' of protease and peptidase inhibitors, but it is known that at least one of the inhibitors (amastatin) is an inhibitor of

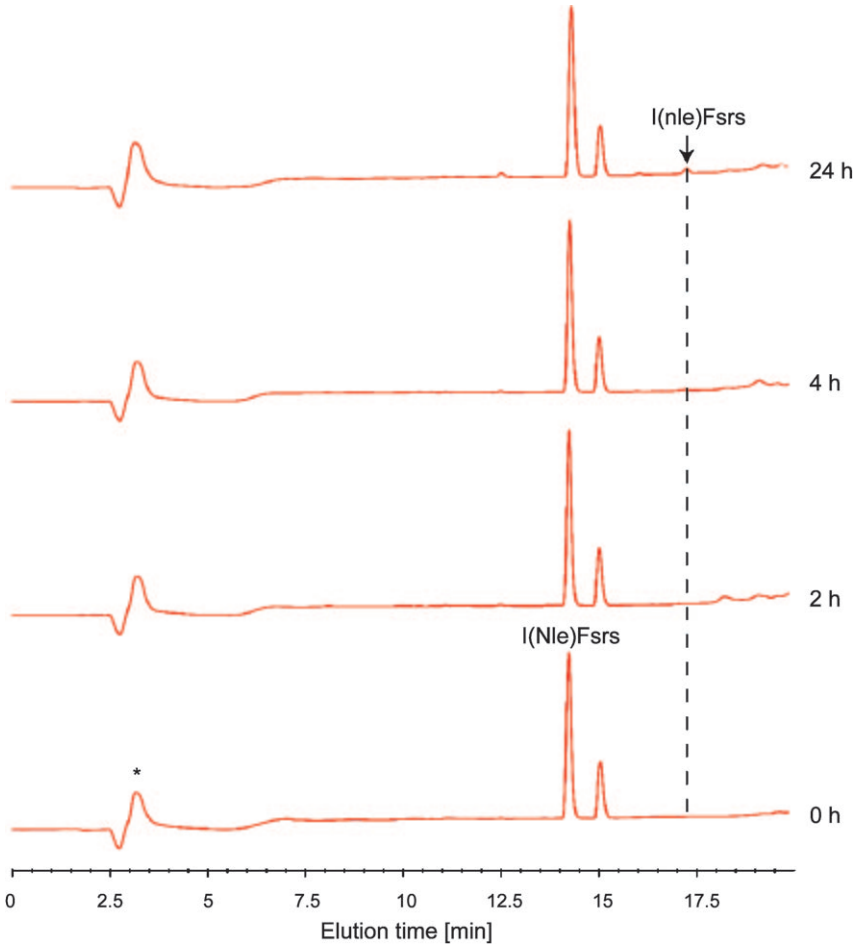

Fig. 4. *RP-HPLC Chromatograms from the assay of peptide isomerase activity in mouse heart extract, with the synthetic hexapeptide I(Nle)Fsrs incubated at 37°*. HPLC Analysis was carried out at 0, 2, 4, and 24 h. The appearance of the I(nle)Fsrs peak is indicated by the arrow; peak complexes marked with * corresponded to the elution of salts.

the platypus isomerase [2]. Therefore, there was a trade-off between invoking sufficient inhibition of the peptidases and proteases and not inhibiting the peptide isomerase. It may be true that in tissues other than the heart the peptidases and proteases 'win the race' to the probe peptide and degrade it before significant amounts are isomerised.

Peptide Isomerase Size. The tissue extracts used in the assays were not purified extracts; they were fractionated by centrifugal ultrafiltration. The fractions with molecules >30 kDa were used as the source of peptide isomerase because the molecular size of the enzyme has been estimated at *ca.* 55–65 kDa [2]. Most of the peptidases and proteases should have been separated from the fractions >30 kDa

because of their smaller molecular sizes but presumably there would have been some retention with the peptide isomerase retentate.

D-*Amino Acids in Mammals.* D-Amino acids in mammals are not rare although their occurrence was thought to be a result of nonenzymic processes. D-Serine and D-aspartic acid have long been known to be present at detectable concentrations in the mammalian nervous system [12][13]. In mouse brain, high concentrations of D-serine are detected in the forebrain [14][15]; it acts as an agonist for the *N*-methyl-D-aspartate (NMDA) receptor [16]. Serine racemase synthesises D-serine from L-serine in mouse brain; it is highly specific for L-serine and does not act on any other amino acid [17]. D-Serine in mouse is degraded by D-amino acid oxidase (DAO) that is involved in the oxidative deamination of neutral amino acids such as D-serine, D-proline, and D-alanine [18]. The distributions of serine racemase and DAO correlate with the level of D-serine in the system [15]. However, the presence of DAO is not restricted to the nervous system, and it has been reported in mouse kidney, but not in mouse liver or heart [19]. It is not clear whether DAO has other physiological functions than degrading D-amino acids [15].

Role of Peptide Isomerase. It is possible that peptide isomerase in mouse heart has a similar function to serine racemase in the nervous system. However, instead of synthesising free D-amino acids from L-amino acids, peptide isomerase may catalyse the L- to D-conversion of particular amino acid residues in peptides. In turn, such modified peptides could be involved in intracellular signalling pathways, which imply their binding to D-specific receptors, or alternatively their non-binding when the L-form does.

Thus, our finding of peptide isomerase activity in mouse heart opens up a new area of investigation of mammalian biochemistry. It is not known what role peptide isomerase plays in mouse heart. The heart of the adult mouse weighs only *ca.* 0.2 g, and yet it is a highly specialised structure composed of four chambers built up from involuntary striated cardiomyocytes, connective tissues, blood vessels, and electrically conductive *Purkinje* fibres [20].

Closing Comments. In the present work, we showed peptide isomerase activity in mouse heart that converts the second L-amino acid residue in from the N-terminus into the D-form. How peptides that contain D-amino acids are involved in maintaining normal heart function requires further investigation. On the other hand, free D-amino acids have been found in the heart [21], and it remains to be determined whether they are formed from the products of peptide isomerisation coupled with peptidase-mediated degradation, or from other racemisation pathways. The absence of DAO in heart suggests that these free D-amino acids, no matter how they arise, will be transported to other tissues for degradation.

The RP-HPLC method for assaying platypus venom peptide isomerase provides a quick and reliable way to monitor L-/D-amino acid residue interconversion in other systems whether animate or inanimate; and the general approach is ready to 'go fishing' for peptide isomerase activities with isomerising substrates that are present in species other than the platypus.

This work was funded by an *ARC Discovery Project Grant* to *P. W. K.* and Assoc. Prof. *J. I. Vandenberg*. Dr. *A. Torres*, and Dr. *P. Bansal* are thanked for vital contributions to the work on platypus venom peptide isomerase. Mr. *A. Hallen* is thanked for valuable discussions on enzyme purification. *J. M. S. K.* was supported by an Australian Postgraduate Award.

Experimental Part

Synthetic Hexapeptides. Customer-specified synthetic hexapeptides were purchased from *EZBiolab* (Indiana, USA). To make up the peptide soln., 1 mg of peptides was dissolved in 1 ml of 50 mM phosphate buffered saline (PBS; 100 mM NaCl, 50 mM $Na_2H_2PO_4$, pH adjusted with NaOH to 7.4).

Mouse Tissue Extracts. Eight tissues were dissected from wild type C57/BL6 mouse; these tissues were brain, heart, kidney, liver, lung, small intestine, spleen, and stomach. Each tissue was finely sliced and then homogenised in 5 ml of ice cold 1% (*w*/*v*) trehalose soln. with a *Bamix* cutter using short bursts to prevent enzyme denaturation by heating. The homogenate was then centrifuged at 3000*g* at 4° for 30 min. After centrifugation, the solid material was separated and discarded while the supernatant was kept and filtered through a 0.2 μm *Minisart* filter (*Sartorius*, D-Göttingen). The filtrate was concentrated by centrifugal ultrafiltration using a *Macrosep* 30-kDa molecular weight cut-off membrane (*Pall*, New York, USA) and then frozen for later use.

Isomerase Assays. Frozen tissue extract was thawed and further concentrated by centrifugal ultrafiltration using a *Nanosep* 30 kDa molecular-weight cut-off membrane (*Pall*). It was spun at *ca.* 5000*g* at 4° for 10 min and then washed with 50 mM PBS (pH 7.4) and 100 mM EDTA (pH 7.0). The retentate (MW >30 k) was resuspended in 50 mM PBS, pH 7.4. For each assay, 40 μl of the concentrated tissue extract was mixed with 40 μl of peptide soln. and 40 μl of *Complete* protease inhibitor cocktail, 5 × concentration (*Roche*, D-Mannheim). The mixture was incubated at 37°; aliquots of mixture were withdrawn for RP-HPLC assays at 0, 2, 4, and 24 h.

RP-HPLC. Isomerase assays mixtures were analysed using RP-HPLC with an *Agilent Eclipse XDB-C18* column (4.6 mm × 250 mm) on an *Agilent Technologies 1200* series system (California, USA). The solvent system was made up of H_2O containing 0.1% (*v*/*v*) F_3CCOOH (TFA; solvent *A*) and 90% MeCN containing 0.1% (*v*/*v*) TFA (solvent *B*). The solvent gradient for the assay was 5–45% *B* for 15 min, 45–60% *B* for 1 min, 60–5% *B* for 1 min, 5–0% *B* for 2 min and 0% *B* for 1 min, at the rate of 1 ml min^{-1}.

Mass Spectrometry. MALDI-TOF-MS was used to determine the mass-to-charge ratio ($[M+H]^+$) of peptides on a *QSTAR XL* mass spectrometer equipped with a MALDI source (*Applied Biosystems*, California, USA).

REFERENCES

[1] A. M. Torres, M. Tsampazi, C. Tsampazi, E. C. Kennett, K. Belov, D. P. Geraghty, P. S. Bansal, P. F. Alewood, P. W. Kuchel, *FEBS Lett.* **2006**, *580*, 1587.
[2] A. M. Torres, M. Tsampazi, E. C. Kennett, K. Belov, D. P. Geraghty, P. S. Bansal, P. F. Alewood, P. W. Kuchel, *Amino Acids* **2007**, *32*, 63.
[3] A. M. Torres, C. Tsampazi, D. P. Geraghty, P. S. Bansal, P. F. Alewood, P. W. Kuchel, *Biochem. J.* **2005**, *391*, 215.
[4] A. M. Torres, G. M. de Plater, M. Doverskog, L. C. Birinyi-Strachan, G. M. Nicholson, C. H. Gallagher, P. W. Kuchel, *Biochem. J.* **2000**, *348*, 649.
[5] S. D. Heck, C. J. Siok, K. J. Krapcho, P. R. Kelbaugh, P. F. Thadeio, M. J. Welch, R. D. Williams, A. H. Ganong, M. E. Kelly, A. J. Lanzetti, W. R. Gray, D. Phillips, T. N. Parks, H. Jackson, M. K. Ahlijanian, N. A. Saccomano, R. A. Volkmann, *Science* **1994**, *266*, 1065.
[6] C. Ollivaux, D. Gallois, M. Amiche, M. Boscameric, D. Soyez, *FEBS J.* **2009**, *276*, 4790.
[7] A. Jilek, C. Mollay, C. Tippelt, J. Grassi, G. Mignogna, J. Müllegger, V. Sander, C. Fehrer, D. Barra, G. Kreil, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 4235.
[8] A. Jilek, G. Kreil, *Monatsh. Chem.* **2008**, *139*, 1.
[9] P. S. Bansal, A. M. Torres, B. Crossett, K. K. Y. Wong, J. M. S. Koh, D. P. Geraghty, J. I. Vandenberg, P. W. Kuchel, *J. Biol. Chem.* **2008**, *283*, 8969.

[10] Jmol: an open-source Java viewer for chemical structures in 3D; http://www.jmol.org/.
[11] A. Herráez, *Biochem. Mol. Biol. Educ.* **2006**, *34*, 255.
[12] D. S. Dunlop, A. Neidle, D. McHale, D. M. Dunlop, A. Lajtha, *Biochem. Biophys. Res. Commun.* **1986**, *141*, 27.
[13] A. Hashimoto, T. Nishikawa, T. Hayashi, N. Fujii, K. Harada, T. Oka, K. Takahashi, *FEBS Lett.* **1992**, *296*, 33.
[14] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, *J. Neurochem.* **1993**, *60*, 783.
[15] L.-Z. Wang, X.-Z. Zhiu, *Acta Pharmacol. Sin.* **2003**, *24*, 965.
[16] J. A. Kemp, P. D. Leeson, *Trends Pharmacol. Sci.* **1993**, *14*, 20.
[17] H. Wolosker, S. Blackshaw, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13409.
[18] M. S. Pilone, *Cell. Mol. Life Sci.* **2000**, *57*, 1732.
[19] R. Konno, M. Sasaki, S. Asakura, K. Fukui, J. Enami, A. Niwa, *Biochim. Biophys. Acta, Gen. Subj.* **1997**, *1335*, 173.
[20] A. Wessels, D. Sedmera, *Physiol. Genomics* **2003**, *15*, 165.
[21] Y. Nagata, T. Akino, K. Ohno, *Experientia* **1989**, *45*, 330.