The specificity of lysosomal tripeptidyl peptidase-I determined by its action on angiotensin-II analogues

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Abstract Tripeptidyl peptidase-I (TPP-I) is a lysosomal peptidase which cleaves tripeptides from the N-terminus of peptides. The function of the enzyme is unclear but its importance is demonstrated by the fact that mutations in TPP-I are responsible for late infantile neuronal ceroid lipofuscinosis, a lethal lysosomal storage disease. As a step towards identifying its natural substrates, we have used a series of synthetic peptides, based on angiotensin-II, to explore the effects of peptide chain length and the effects of amino acid substitutions at the P_1 and P_1' positions on the rate of catalysis. With the exception of angiotensin-(1–8) (angiotensin-II), which is a relatively poor substrate for TPP-I, the rate of catalysis increases with increasing chain length. K_{cat}/K_m values increase 50-fold between angiotensin-(1–5) and angiotensin-(1–14). TPP-I shows little specificity for the nature of the amino acids in the P_1 and P_1' positions, K_{cat}/K_m values varying only 5-fold for a range of substitutions. However, Pro or Lys in the P_1 position and Pro in the P_1' positions are incompatible with TPP-I activity. These observations suggest that TPP-I is a non-specific, but essential, peptidase involved in the latter stages of lysosomal protein degradation.

Key words: Tripeptidyl peptidase-I; Lysosomal peptide degradation; Angiotensin-II

1. Introduction

Tripeptidyl peptidase-I (TPP-I) is a lysosomal exopeptidase which cleaves tripeptides from the N-terminus of peptides although recent evidence suggests that the enzyme may also possess some endopeptidase activity [1–4] The upper size limit for peptide degradation by TPP-I appears to be about 5 kDa [2,5]. TPP-I was originally thought to be a pepstatin-insensitive carboxyl peptidase based on its homology with a family of bacterial enzymes [6] and its resistance to inhibitors of the four major classes of proteases [1,2]. However, recent experiments suggest that TPP-I belongs to the subtilisin family of serine proteinases but that the active site serine is relatively unreactive [7]. TPP-I is structurally unrelated to TPP-II, a cytoplasmic enzyme with a similar specificity and which is also a member of the subtilisin family [8].

TPP-I can completely degrade small peptides, e.g. glucagon, to tripeptides in vitro [2] and there is evidence for the involvement of an endosomal acidic tripeptidyl peptidase in the hepatic degradation of glucagon [9]. TPP-I has a ubiquitous distribution and was originally thought to be involved in the degradation of collagen [10]. The enzyme can release Gly-Pro-X triplets from synthetic collagen-like polymers, and tripeptidyl chloromethyl ketone inhibitors of TPP-I are potent inhibitors of bone resorption in an in vitro test system, where the major protein being degraded is type I collagen [1]. However, the importance of TPP-I in collagen degradation remains unresolved.

Mutations in TPP-I are responsible for the lysosomal storage disease, classical late infantile neuronal ceroid lipofuscinosis (CLN2, late infantile Batten disease) [6,11]. Children with this disease are symptom free until the age of about three. They then suffer from progressive neurodegeneration with associated seizures, increasing spasticity and blindness. Death occurs around the age of ten [12]. Autopsy reveals a dramatic loss of neuronal cells and to a lesser extent other brain cell types. Brain cells that remain and several cell types in other tissues contain cytoplasmic (lysosomal) storage vesicles although the viability of non-neuronal cells does not seem to be affected. The vesicles contain autofluorescent ceroid lipofuscin and other proteinaceous material. For reasons that are not entirely clear, a major protein which accumulates in these vesicles is subunit c of mitochondrial ATP synthase [13]. Recent evidence suggests that TPP-I cleavage may initiate the degradation of subunit c which is then completed by other lysosomal proteinases [14].

Little is known about the natural substrates or specificity of TPP-I especially with regard to the effects of the nature of the substrate amino acids on either side of the cleavage site, or at enzyme binding sites on the rate of peptide bond cleavage. We now explore how increasing the peptide chain length or replacing amino acids on either side of the cleavage site in a model substrate, angiotensin-II, alters the rate of catalysis. We have chosen angiotensin-II purely as a model substrate because its degradation by TPP-I, into two readily identifiable and quantifiable products, has been described previously [2].

2. Materials and methods

TPP-I was purified from pig kidneys as described previously [2]. The enzyme appeared to be homogeneous by polyacrylamide/SDS-

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Abbreviations: TFA, trifluoroacetic acid; TPP-I, tripeptidyl peptidase-I; MALDI-TOF-MS, matrix-assisted laser desorption ionisation-time of flight-mass spectrometry; -NHMec, -methylcoumarylamide

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gel electrophoresis and the specific activity was 18 milliunit mg^{-1} using Ala-Ala-Phe-methylcoumarylamide (Ala-Ala-Phe-NHMec) as a substrate [1]. Peptides were purchased from Bachem (St Helens, UK) or were synthesised by The Molecular Genetics Facility, University of Georgia, Athens, GA, USA.

Peptides (0.1 μmol) were digested with TPP-I (7 μg) in 0.1 M sodium acetate pH 4.0 for 1–5 h in a total volume of 200 μl. Reactions were terminated by adding trifluoroacetic acid (TFA) to 0.1%. Digests were subjected to reversed phase HPLC on a Dynamax C18-300 column (4.6×250 mm) using a 0% isocratic (10 min), 0–37.5% (60 min), 37.5–60% (15 min) and 60–0% (5 min) acetonitrile/0.1% TFA gradient. Peaks were identified by comparison with standards or by matrix-assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS) [15]. A comparison of the peak areas of the reaction products, measured at 214 nm, with those of standards allowed quantitation of the reaction rates. The values of kinetic parameters were calculated using the program ENZFITTER (Biosoft, Cambridge, UK).

3. Results and discussion

An HPLC assay was developed to measure the kinetics of angiotensin-(1–8) degradation by TPP-I (Fig. 1A). TPP-I degradation of angiotensin-(1–8) (DRVYIHPF) produces two fragments (DRV and YIHPF) after cleavage of the peptide bond between Val^3 and Tyr^4. The identity of these two fragments was determined by the elution position of standards and mass determination by mass spectrometry. The rate of degradation was linear with respect to time and enzyme concentration up to about 20% degradation of the substrate. When the reaction between TPP-I and angiotensin-(1–8) was performed in the presence of AAF-chloromethyl ketone, an inhibitor of TPP-I, the production of the two degradation products was greatly reduced (90% at 2.5 μM inhibitor) (Fig. 1B). The pH optimum for the degradation of angiotensin-(1–8) was pH 4 consistent with the lysosomal localisation of the enzyme (Fig. 2).

The effect of chain length on the rate of degradation of angiotensins was investigated using the HPLC assay (Table 1). K_m varied little between the different peptide substrates except for angiotensin-(1–14) which was hydrolysed with a K_m 3–4-fold lower than the other substrates. The main effect of varying the chain length was clearly on V_{max}. K_{cat} and K_{cat}/K_m values generally increased with chain length except for angiotensin-(1–8) where the value was considerably lower than those for both smaller and larger peptides. K_{cat}/K_m values increased 50-fold between angiotensin-(1–5) and angiotensin-(1–14). This result suggests that TPP-I has an extended binding site which for maximum catalytic activity requires occupancy of substrate binding sites beyond S_7'. Our previous results indicated that only peptides without tertiary structure are substrates for TPP-I [5]. In general, peptides with an M_r below 5 kDa have little tertiary structure. For example, glucagon (M_r 3.5 kDa, extended structure) is a substrate for TPP-I whereas IGF-I (M_r 7.5 kDa, globular structure) is not degraded by TPP-I [5]. Similarly, both individual A and B chains of insulin are substrates for TPP-I but the disulphide-bonded dimer is resistant to hydrolysis possibly because it adopts a globular conformation which restricts access to the active site [5]. TPP-I is likely to play a role in the degradation of small peptides released during lysosomal proteolysis. Pep-

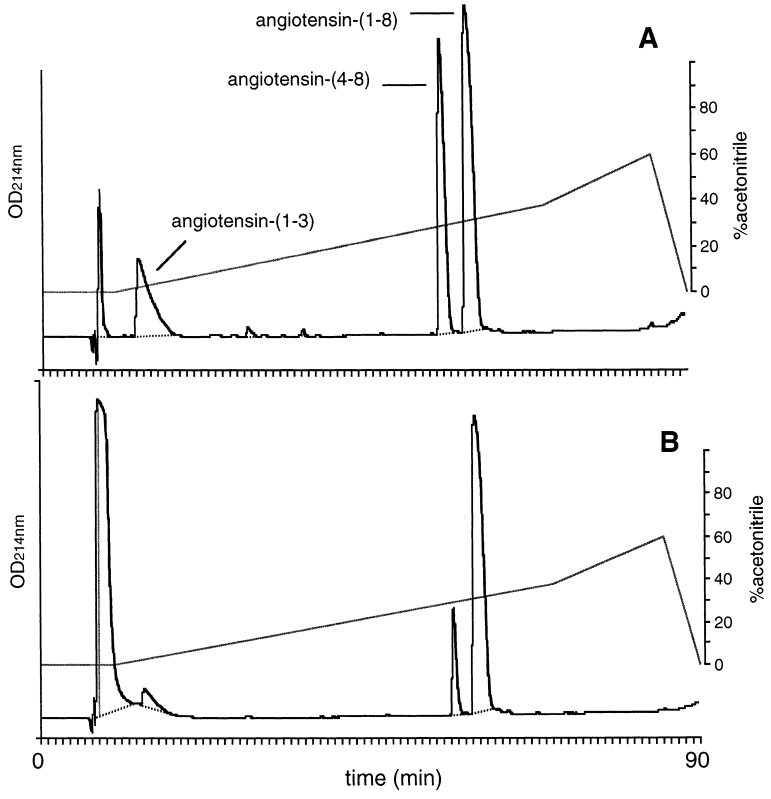

Fig. 1. Degradation of angiotensin-(1–8) by TPP-I. Angiotensin-(1–8) (0.1 μmol) was incubated with TPP-I (7 μg) for 5 h at pH 4.0 in the absence (A) or presence of 2.5 μM Ala-Ala-Phe-CH_2Cl (B). Reactions were stopped by adding TFA to 0.1% and analysed by reversed phase HPLC using a Dynamax C18-300 column (4.6×250 mm) and a 0% (10 min), 0–37.5% (60 min) and 37.5–60% (15 min) acetonitrile/0.1% TFA gradient. The elution position of fragments was compared to that of standards. Digestion of angiotensin-(1–8) (DRVYIHPF) yields two fragments, angiotensin-(1–3) (DRV) and angiotensin-(4–8) (YIHPF).

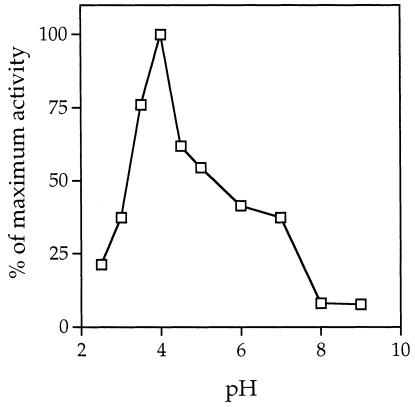

Fig. 2. pH activity profile for the degradation of angiotensin-(1–8) by TPP-I. Angiotensin-(1–8) was incubated with TPP-I over the pH range 2.5–9. Reaction mixtures were subjected to reversed phase HPLC to determine the extent of angiotensin-(1–8) degradation. The buffers used were 0.1 M sodium citrate (pH 2.5–4), 0.1 M sodium acetate (pH 4–6) and sodium phosphate (pH 6–9) all containing 0.5 M NaCl.

tides must be degraded to di- or tripeptides or amino acids before they can be transported out of the lysosomes [16]. Rapid degradation of larger peptides would provide abundant di- and tripeptides for degradation by cytoplasmic peptidases which are present in high levels in many tissues.

TPP-I requires that substrates have an unblocked N-terminus. Neither acetyl-angiotensin-(1–8) or acetyl-angiotensin-(1–8)-amide were substrates for TPP-I (Table 1). Angiotensin-(1–8)-amide was a substrate for TPP-I with a K_{cat}/K_m value 2.5-fold greater than observed for the hydrolysis of angiotensin-(1–8) but still below the K_{cat}/K_m values for the hydrolysis of the other peptides. The increased catalytic rate for the amide suggests that a carboxylic acid group at P_5' reduces the rate of catalysis. (3,5-diiodo-Tyr^4)-angiotensin-II was not hydrolysed by TPP-I possibly because of steric hindrance by the two bulky iodines.

The reasons for the comparatively slow rate of degradation of angiotensin-(1–8) by TPP-I are unclear, especially as angiotensin-(1–8) is possibly a physiological substrate for TPP-I. Evidence has been presented for the involvement of an acidic tripeptidyl peptidase in the metabolism of small peptide hormones which undergo receptor-mediated endocytosis [9]. Angiotensin-(1–8) complexed with its AT1-type receptor is rapidly internalised by endocytosis in a process which is independent of G-protein coupling [17]. A decreased sensitivity to proteolysis might prolong the generation of the intracellular signalling process which involves coupling of receptors to G-proteins. It has recently been demonstrated that signal transduction, through the JAK–STAT pathway, continues after internalisation of growth hormone–receptor complexes into endosomes [18].

As angiotensin-(1–8) is potentially a physiological substrate for TPP-I, we investigated the effects of amino acid substitutions at positions 3 and 4 on its rate of degradation (Table 2). Substitution of either Val^3 or Tyr^4 with Pro abolished the ability of TPP-I to hydrolyse the substrate. Synthetic substrates with a Pro in the P_1 position and a form of bovine growth hormone with the N-terminus Ala-Met-Pro have been shown to be resistant to TPP-I [19]. Studies on the degradation of synthetic peptides corresponding to exposed regions of subunit c of ATP synthase have demonstrated that peptides with a Pro in the P_1 position are resistant to hydrolysis by TPP-I [14]. TPP-II is also unable to hydrolyse peptide bonds with a Pro in either the P_1 or P_1' position [20]. Substitution of Val^3 with Lys also resulted in a TPP-I resistant peptide unlike substitution of Tyr^4 with Lys which resulted in a peptide which could be hydrolysed but with a reduced K_{cat}/K_m compared to the wild-type angiotensin-(1–8). A negatively charged amino acid (glutamate) at position 3 did not impair digestion of the peptide. The inability of TPP-I to hydrolyse synthetic tripeptidyl-aminomethylcoumarin substrates with a positively charged amino acid in the P_1 position has been noted previously [1,2]. Overall, K_{cat}/K_m varied by no more than 5-fold throughout the series of peptides. A Tyr or Ser at position 3 and a Ser at position 4 gave the highest rates of catalysis.

These results are consistent with a role for TPP-I as a non-specific lysosomal peptidase which generates tripeptides from the breakdown products produced by lysosomal proteinases, e.g. cathepsins B and L. For example, the degradation of glucagon by cathepsin L produces peptides with lengths of 2, 7, 8 and 12 amino acids [21]. TPP-I might also be involved directly in the degradation of small peptide hormones brought into cells by receptor-mediated endocytosis. The tripeptides produced would be further degraded by lysosomal peptidases or transported into the cytoplasm for degradation to amino acids. TPP-I is presumably essential for lysosomal protein degradation as the absence of this activity results in a severe lysosomal storage disease.

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Table 1
Kinetic parameters for the hydrolysis of (A) angiotensin-derived peptides of various lengths and (B) derivatives of angiotensin-(1–8)

Peptide		K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} mM^{-1})
(A)				
DRVYI	angiotensin-(1–5)	0.14	4.6	32.9
DRVYIH	angiotensin-(1–6)	0.14	11.7	81.8
DRVYIHP	angiotensin-(1–7)	0.13	11.9	89.5
DRVYIHPF	angiotensin-(1–8)	0.19	0.8	4.3
DRVYIHPFHL	angiotensin-(1–10)	0.13	39.4	303
DRVYIHPFHLLYYS	angiotensin-(1–14)	0.04	61.4	1639
(B)				
Ac-DRVYIHPF		nh[a]		
Ac-DRVYIHPF-NH_2		nh[a]		
DRVYIHPF-NH_2		0.16	1.8	11.3
DRV(diiodo-Y)IHPF		nh[a]		

[a]nh = not hydrolysed.

Table 2
Kinetic parameters for the hydrolysis of angiotensin-(1–8) with amino acid substitutions at positions 3 and 4

Peptide	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} mM^{-1})
DRV-YIHPF	0.19	0.82	4.32
DR**K**-YIHPF	nh[a]		
DR**Y**-YIHPF	0.21	2.69	12.70
DR**S**-YIHPF	0.08	0.81	10.70
DR**E**-YIHPF	0.16	0.59	3.81
DR**G**-YIHPF	0.12	0.68	5.75
DR**P**-YIHPF	nh[a]		
DRV-**K**IHPF	0.19	0.62	3.36
DRV-**V**IHPF	0.19	1.55	8.13
DRV-**S**IHPF	0.15	2.49	16.70
DRV-**E**IHPF	0.10	0.76	8.03
DRV-**G**IHPF	0.13	1.22	9.49
DRV-**P**IHPF	nh[a]		

[a]nh = not hydrolysed.

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