Cathepsin B carboxydipeptidase specificity analysis using internally quenched fluorescent peptides

Maria Helena S. CEZARI, Luciano PUZER, Maria Aparecida JULIANO, Adriana K. CARMONA and Luiz JULIANO[1]

Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Três de Maio, 100, São Paulo 04044-020, Brazil

We have examined in detail the specificity of the subsites S_1, S_2, S_1' and S_2' for the carboxydipeptidase activity of cathepsin B by synthesizing and assaying four series of internally quenched fluorescent peptides based on the sequence Dnp-GFRFW-OH, where Dnp (2,4-dinitrophenyl) is the quenching group of the fluorescence of the tryptophan residue. Each position, except the glycine, was substituted with 15 different naturally occurring amino acids. Based on the results we obtained, we also synthesized efficient and sensitive substrates that contained *o*-aminobenzoic acid and 3-Dnp-(2,3-diaminopropionic acid), or ϵ-amino-Dnp-Lys, as the fluorescence donor–receptor pair. The higher kinetic parameter values for the carboxydipeptidase compared with the endopeptidase activity of cathepsin B allowed an accurate analysis of its specificity. The subsite S_1 accepted preferentially basic amino acids for hydrolysis; however, substrates with phenylalanine and aliphatic side-chain-containing amino acids at P_1 had lower K_m values. Despite the presence of Glu^{245} at S_2, this subsite presented clear preference for aromatic amino acid residues, and the substrate with a lysine residue at P_2 was hydrolysed better than that containing an arginine residue. S_1' is essentially a hydrophobic subsite, and S_2' has particular preference for phenylalanine or tryptophan residues.

Key words: fluorescent peptide, fluorogenic substrate, proteinase, thiol protease.

INTRODUCTION

Cathepsin B (EC 3.4.22.1) is a cysteine proteinase of the papain super-family, and in mammals is one of the major lysosomal cysteine proteinases, where it is involved in protein turnover [1]. It has also been implicated in several physiological processes (reviewed in [2,3]), as well as in pathological conditions, particularly in tumour invasion and metastasis [4–7]. In addition, cathepsin B seems to be involved indirectly in apoptosis by a cytochrome *c* release route [8,9] and in angiogenesis through inactivation of tissue inhibitors of matrix metalloproteinases [10]. Thus cathepsin B may play a significant role in tumour progression and could therefore be a good target for cancer treatment.

Cathepsin B is known to present both carboxydipeptidase and endopeptidase activities; however, the former activity is significantly higher [11–13]. This pronounced carboxydipeptidase activity of cathepsin B is due to an extra occluding loop in the cathepsin B structure, which is located in the primed side of the catalytic site. More specifically, the residues His^{110} and His^{111} are located in this occluding loop and form the S_2' subsite of cathepsin B. His^{110} interacts with Asp^{22} through an ion-pair-stabilizing electrostatic interaction, and His^{111} is a major determinant of exopeptidase activity by virtue of its interaction with the free carboxylate group of substrates [14–18]. Other cysteine proteases, such as cathepsin L, cathepsin K and papain, were also demonstrated to present carboxydipeptidase activity towards one internally quenched fluorescent (IQF) peptide [19]. This is a significant observation, because it demonstrates that typical endopeptidases, particularly cathepsin L and papain, can also present efficient carboxydipeptidase activities. The endopeptidase activity of cathepsin B has been previously described in detail [13], but for the carboxydipeptidase activity only the specificity of S_2' subsite was recently reported [18].

The importance of the physiological and pathological roles of cathepsin B, and the information reported to date regarding its structure and functional properties, prompted us to examine in detail the specificity of the subsites S_1, S_2, S_1' and S_2' involved in the carboxydipeptidase activity. We have synthesized four series of IQF peptides based on the peptide 2,4-dinitrophenyl (Dnp)-GFRFW-OH, where Dnp is the quenching group of the tryptophan residue fluorescence, and assayed these peptides with recombinant human cathepsin B. Based on these results we also synthesized short peptides containing *o*-aminobenzoic acid (Abz) and 3-Dnp-2,3-diaminopropionic acid [Dap(Dnp)], or ϵ-amino-Dnp-Lys [K(Dnp)], as fluorescence donor–receptor pair in order to obtain efficient and sensitive substrates for cathepsin B.

EXPERIMENTAL

Peptides

All the intramolecularly quenched fluorogenic peptides were synthesized by the solid-phase technique, using the fluoren-9-ylmethoxycarbonyl methodology, and all protected amino acids were purchased from Calbiochem–Novabiochem (San Diego, CA, U.S.A.) or from Neosystem (Strasbourg, France). The synthesis was carried out in an automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system; Shimadzu, Tokyo, Japan). The final de-protected peptides were purified by semi-preparative HPLC using an Econosil C-18 column (10 μm particle size, 22.5 mm × 250 mm) and a two-solvent system: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, by vol.). The column was eluted at a flow rate of 5 ml · min^{-1} with a 10 or 30 to 50 or 60% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system (Shimadzu) with a SPD-10AV UV/vis detector (Shimadzu) and a fluorescence detector (RF-535: Shimadzu), coupled to an

Abbreviations used: Abz, *o*-aminobenzoic acid; Dap(Dnp), 3-Dnp-(2,3-diamino propionic acid); Dnp, 2,4-dinitrophenyl; EDDnp, *N*-ethylenedianine-Dnp; IQF, internally quenched fluorescent; K(Dnp), ϵ-amino-Dnp-Lys.

[1] To whom correspondence should be addressed (e-mail juliano.biof@epm.br).

Ultrasphere C-18 column (5 μm particle size, 4.6 mm × 150 mm), which was eluted with solvent systems A1 (H_3PO_4/water, 1:1000, v/v) and B1 (acetonitrile/water/H_3PO_4, 900:100:1, by vol.) at a flow rate of 1.7 ml · min^{-1} and a 10–80 % gradient of B1 over 15 min. The HPLC column-eluted materials were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm, following excitation at 320 nm for the peptides containing the Abz–Dnp pair, or emission at 360 nm followed by excitation at 280 nm for the peptides containing the Trp–Dnp pair. The molecular mass and purity of synthesized peptides were checked by matrix-assisted laser-desorption ionization–time-of-flight MS (TofSpec-E; Micromass, Manchester, U.K.) and/or peptide sequencing using a protein sequencer (PPSQ-23; Shimadzu). The concentrations of the substrate solutions were found by colorimetric determination of the Dnp group (molar absorption coefficient at 365 nm was 17300 $M^{-1} \cdot cm^{-1}$).

Enzymes

Human recombinant cathepsin B was obtained as described previously [12]. The molar concentrations of this enzyme was determined by active-site titration with the proteinase inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane according to [20].

Enzyme assays

The hydrolysis of the IQF peptides containing the Trp–Dnp pair were quantified using a Hitachi F-2000 spectrofluorimeter by measuring the fluorescence at 360 nm, following excitation at 280 nm. For the hydrolysis of the IQF peptides containing Abz–Dnp, the fluorescence emission was measured at 420 nm, following excitation at 320 nm, using a Hitachi F-2000 spectrofluorimeter. The concentration of the enzymes varied from 0.5 nM, for the best substrates, to 2.0 nM, for the less susceptible ones. The enzymic hydrolysis by cathepsin B of the IQF peptides were carried out in 100 mM sodium phosphate/1 mM EDTA, pH 6.0. All determinations were made at 37 °C, and the enzyme was pre-activated for 5 min with 5 mM 1,4-dithioerythritol. The kinetic parameters K_m and k_{cat} were calculated by the non-linear regression data analysis GraFit version 3.0 program (Erithacus Software, Horley, Surrey, U.K.). The k_{cat}/K_m values were calculated as the ratio of these two determined parameters. The errors for determination of the kinetic parameters were lower than 7 % for the hydrolysis of IQF peptides containing the Trp–Dnp pair and lower than 5 % for those with Abz–Dnp pair. The bonds cleaved were identified by isolation of the fragments by HPLC, followed by amino acid analysis.

RESULTS

Carboxydipeptidase specificity of cathepsin B

Dnp-GFXFW-OH series

Table 1 shows the kinetic parameters for the hydrolysis of this series of IQF peptides with different amino acids at the P_1 position. Most of the amino acids were accepted at this position, but with different susceptibilities to cathepsin B. The highest k_{cat}/K_m values were obtained with the residues arginine and lysine, but hydrophobic amino acids, such as phenylalanine, leucine and valine, were also hydrolysed with relatively high k_{cat}/K_m values. The K_m values of the substrates with phenylalanine, isoleucine and valine were the lowest in the series, in contrast with the substrates with arginine and lysine that exhibited both higher K_m and k_{cat} values. However, the peptide containing isoleucine also showed relatively low k_{cat} and k_{cat}/K_m values. These data indicate that hydrophobic amino acids at the P_1 position result in substrates with higher affinity, in contrast with the peptides with basic amino acids, which are more susceptible to hydrolysis. Acidic amino acids, particularly aspartic acid, are the poorest accepted residue at S_1 subsite. The polar amino acids, glutamine and serine, were hydrolysed with intermediate k_{cat}/K_m values. The presence of a side chain at the P_1 position is not essential for the cathepsin B activity, since the peptide with a glycine residue at this position is significantly hydrolysed. The only resistant peptide was that containing a proline residue. This clear discrimination at the S_1 subsite of cathepsin B was not observed in IQF peptides of the general structure Abz-KLXFSKQ-EDDnp (where EDDnp is *N*-ethylenedianine-Dnp), in which endopeptidase hydrolysis could occur only at the Xaa–Phe bond [21]. Therefore, the peptides with a free C-terminal carboxy group, which allows the carboxydipeptidase activity of cathepsin B to occur, resulted in better and more discriminative substrates for the S_1–P_1 interaction.

Table 1 Kinetic parameters for hydrolysis of the peptide series Dnp-GFXFW-OH by recombinant human cathepsin B for characterization of the carboxydipeptidase S_1 subsite specificity

Conditions for hydrolysis were 100 mM sodium phosphate/1 mM EDTA, pre-activated with 5 mM 1,4-dithioerythritol, at 37 °C, pH 6.0. The errors for determination of the kinetic parameters were lower than 7 %. N.H., no hydrolysis occurred until the enzyme concentration reached 25 nM.

Xaa	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Phe	0.5	0.47	940
Tyr	1.1	0.15	136
Ala	1.6	0.51	319
Val	0.4	0.26	650
Leu	1.7	1.47	864
Ile	0.5	0.03	60
Gly	1.4	0.58	414
Pro	N.H.		
Gln	0.6	0.32	533
Ser	1.2	0.57	475
Glu	0.8	0.13	162
Asp	3.4	0.15	44
Lys	2.1	2.70	1285
Arg	1.2	2.07	1725
His	0.7	0.39	557

Dnp-GXRFW-OH series

Table 2 shows the kinetic parameters for the hydrolysis of this IQF peptide series with variations at the P_2 position. The carboxydipeptidase activity of cathepsin B on the substrates containing phenylalanine and tyrosine residues have the highest k_{cat}/K_m values in this series, followed by the substrates with lysine, valine, alanine, isoleucine, proline and leucine residues. It is noteworthy that the K_m values for the hydrolysis of the substrates with alanine and leucine residues were significantly higher than for the other aliphatic amino acids, and that the k_{cat} value for the hydrolysis of the peptide with an alanine was the highest in the series. Furthermore, besides the restrictions of a proline residue to the substrate flexibility, its presence at the P_2 position resulted in a peptide significantly susceptible to hydrolysis and with low K_m value. The substrates containing amino acids with negatively charged side chains were hydrolysed with significantly high K_m values, particularly those containing aspartic acid and glutamic acid residues. Possibly, this is a consequence of the presence of Glu^{245} in the S_2 subsite of cathepsin B, which would repulse the negatively charged amino-

Table 2 Kinetic parameters for hydrolysis of the peptide series Dnp-GXRFW-OH by recombinant human cathepsin B for characterization of the carboxydipeptidase S_2 subsite specificity

Conditions for hydrolysis are as described in Table 1. N.H., no hydrolysis occurred until the enzyme concentration reached 25 nM.

Xaa	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Phe	1.2	2.07	1725
Tyr	1.1	1.47	1336
Ala	16.1	7.39	459
Val	2.3	1.20	521
Leu	10.6	1.74	164
Ile	1.9	0.84	442
Gly	N.H.		
Pro	2.2	0.68	309
Gln	N.H.		
Ser	3.8	1.22	321
Glu	12.0	0.54	45
Asp	9.6	0.51	53
Lys	4.1	2.92	712
Arg	6.6	0.55	83
His	8.6	2.00	232

Table 3 Kinetic parameters for hydrolysis of the peptide series Dnp-GFRXW-OH by recombinant human cathepsin B for characterization of the carboxydipeptidase S_1' subsite specificity

Conditions for hydrolysis are as described in Table 1. N.H., no hydrolysis occurred until the enzyme concentration reached 25 nM.

Xaa	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Phe	1.2	2.07	1725
Tyr	3.6	1.20	333
Ala	5.2	3.22	619
Val	1.8	0.57	316
Leu	2.1	1.44	685
Ile	3.3	1.70	515
Gly	7.7	0.61	79
Pro	N.H.		
Gln	4.5	1.23	273
Ser	9.5	10.2	1073
Glu	9.6	0.18	18
Asp	5.3	0.27	50
Lys	0.8	0.13	162
Arg	5.0	0.18	36
His	4.5	0.24	53

acid side chains. Indeed, the substrate with the positively charged lysine residue is hydrolysed well, with a significantly high k_{cat} value; however, the low k_{cat}/K_m value for the hydrolysis of the peptide with arginine residue is quite surprising, even when compared with the substrate with a histidine residue, in which the imidazole side chain is only half-protonated at the pH of the enzymic reaction. The peptides containing glycine and glutamine were resistant to hydrolysis until the enzyme was at a concentration of 25 nM. These results show the very determinant role of the S_2–P_2 interaction for the cathepsin B affinity and hydrolytic activity, which was not evident with substrates that allowed only endopeptidase activities to be studied for this protease [13].

Dnp-GFRXW-OH series

Table 3 shows the kinetic parameters for the hydrolysis of this IQF peptide series with variations at the P_1' position. The highest k_{cat}/K_m values in this series were obtained with the substrates with phenylalanine and serine residues. For the hydrolysis of the former peptide, the low K_m value was the main component in the k_{cat}/K_m parameter, whereas for the hydrolysis of the latter, the k_{cat} value was the highest of the series. All the peptides with aliphatic side chain amino acids were well hydrolysed, except the peptide with a proline residue, which was resistant. The peptide with a tyrosine residue was significantly hydrolysed; however, its lower k_{cat}/K_m value when compared with the hydrolysis of the peptide with a phenylalanine residue, or even leucine or isoleucine residues, indicated the unfavourable effect of a hydroxy group at the *p* position of the tyrosine side chain. The absence of a side chain, as in the peptide containing a glycine residue, resulted in poor hydrolysis with relatively a high K_m and a low k_{cat}, indicating that the S_1'–P_1' interaction is also relevant for the carboxydipeptidase activity of cathepsin B. Indeed, the peptides with a charged amino acid side chain, either acid or basic, were poorly hydrolysed in comparison with the hydrophobic residues.

Table 4 Kinetic parameters for hydrolysis of the peptide series Dnp-GFRWX-OH by recombinant human cathepsin B for characterization of the carboxydipeptidase S_2' subsite specificity

Conditions for hydrolysis are as described in Table 1. N.H., no hydrolysis occurred until the enzyme concentration reached 25 nM.

Xaa	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Phe	2.2	2.76	1254
Tyr	6.6	1.16	175
Ala	9.0	0.06	6
Val	22.3	2.36	105
Leu	4.2	0.53	126
Ile	12.0	1.93	160
Gly	N.H.		
Pro	16.2	0.50	30
Gln	12.4	0.26	21
Ser	12.8	0.22	17
Glu	10.6	0.07	6
Asp	5.5	0.05	9
Lys	12.6	0.44	34
Arg	11.5	0.85	73
His	N.H.		

Dnp-GFRWX-OH series

Table 4 shows the kinetic parameters for the hydrolysis of this IQF peptide series with variations at the P_2' position. The S_2' subsite of cathepsin B presented a marked preference for the substrates containing hydrophobic amino acids at P_2', particularly a phenylalanine residue for which the k_{cat}/K_m value is the highest in the series. The presence of a side chain in the P_2' position seems to be essential for hydrolysis by cathepsin B, because the peptide containing a glycine residue was resistant and that with an alanine residue was a very poor substrate. The peptides with charged side chain amino acids, either acidic or basic residues, were also poorly hydrolysed. It is noteworthy in this series that the highest k_{cat} values were observed for the hydrolysis of the peptides with a phenylalanine, valine and isoleucine residue; however, the K_m values for the last two substrates were high, particularly that with valine. In addition, it is also surprising to see the resistance to hydrolysis of the peptide with a histidine residue. Therefore, for the carboxydipeptidase activity of cathepsin B, the positioning of the P_2' amino acid residue at the S_2' enzyme subsite seems to be a very strong determinant for substrate binding and hydrolysis. Although

Table 5 Kinetic parameters for the carboxydipeptidase activity of recombinant human cathepsin B on IQF peptides containing Abz and Dnp as donor–receptor fluorescence pair

Conditions for hydrolysis were 100 mM sodium phosphate/1 mM EDTA, pre-activated with 5 mM 1,4-dithioerythritol, at 37 °C, pH 6.0. The errors for determination of the kinetic parameters were lower than 5%.

Substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Dap(Dnp) as quencher			
Abz-FR-Dap(Dnp)-W-OH	3.0	4.90	1633
Abz-FF-Dap(Dnp)-W-OH	2.1	5.40	2571
K(Dnp) as quencher			
Abz-FR-K(Dnp)-W-OH	3.8	3.9	1026
Abz-FR-K(Dnp)-P-OH	17.7	2.9	164
Abz-FRA-K(Dnp)-OH	17.9	34.8	1944
Abz-FRF-K(Dnp)-OH	32.8	59.1	1802

similar S_2' subsite preferences for aromatic and hydrophobic amino acids were reported for the endopeptidase of cathepsin B, the differences in the k_{cat}/K_m values between the substrates in the series Abz-AFRSXAQ-EDDnp were lower [13] than those presented in the present study. In addition, the results of the recently reported exopeptidase activity of cathepsin B [18] demonstrating the specificity of the S_2' subsite and the role of His^{110} and His^{111} of the occluding loop of the enzyme are in good agreement with results of the present study.

Hydrolysis by cathepsin B of substrates containing the Abz–Dnp fluorescent donor–acceptor pair

In order to obtain more sensitive substrates for the carboxydipeptidase activity of cathepsin B, we synthesized, based on the specificity data described above, IQF peptides containing a Abz–Dnp fluorescent donor–acceptor pair. The Dnp group was introduced into the peptides attached to the ϵ-amino of lysine [K(Dnp)] or at the 3-amino group of 2,3-diaminopropionic acid [Dap(Dnp)]. The substrate with Dap(Dnp) at position P_1' resulted in a substrate that was hydrolysed with a k_{cat}/K_m value 50% higher than that containing K(Dnp) [compare the substrates Abz-FR-Dap(Dnp)-W-OH with Abz-FR-K(Dnp)-W-OH in Table 5]. The peptides with a tryptophan residue at the P_2' position were hydrolysed with K_m values lower and k_{cat} higher than those containing K(Dnp), although the k_{cat}/K_m values show that all of these are efficient substrates for cathepsin B. These peptides are useful alternatives for cathepsin B substrates, if the goal of the study is the screening of inhibitors or the specific detection of enzyme activity.

DISCUSSION

The peptide Dnp-GFRFW-OH and its analogues modified systematically at each position, except at the glycine residue, allowed us to map in detail and with better accuracy the substrate specificity of cathepsin B carboxydipeptidase activity for P_2 to P_2' substrate sites. The S_1 subsites of cysteine proteases are described as being open to solvent, therefore making few interactions [22]; however, the 40 times higher k_{cat}/K_m value for the hydrolysis by cathepsin B of the peptide containing an arginine residue in comparison with that with aspartic acid residue in P_1 (Table 1), indicates a stabilization of the guanidine group at the S_1 subsite. In fact, in cathepsin B, Glu^{122} was described as being directed towards the active-site cleft, increasing the electrostatic negative character of the S_1 subsite [23]. In addition, the k_{cat}/K_m value of Abz-F-F-Dap(Dnp)-W-OH hydrolysis is higher than that of Abz-F-R-Dap(Dnp)-W-OH (Table 5), indicating that in these peptides phenylalanine is better accepted at S_1 subsite than arginine; however, the reverse was observed with the peptides Dnp-GFRFW-OH and Dnp-GFFFW-OH (Table 1). Therefore, the S_1 subsite specificity seems to be also modulated by the nature of the amino acids located at other positions of the substrate. The S_2 binding site of cathepsin B presented a very determinant role on the specificity of its carboxydipeptidase activity, which was not clearly observed with its endopeptidase activities [13]. In fact, the well-known hydrolysis by cathepsin B of substrates with an arginine residue at P_2 position (although, with less efficiency than phenylalanine) is due to the presence of a glutamic acid residue at position 245 [24–27], but was demonstrated only with peptidyl-7-methyl-coumarin amide substrates [1,20,28]. These substrates are not impaired by the cathepsin B occluding loop, because only 7-methyl-coumarin amide occupies the prime side of the cathepsin B catalytic groove. It is noteworthy that the large variations in the K_m values on the hydrolysis of the peptides Dnp-GXRFW-OH (Table 2) by cathepsin B indicates that the S_2–P_2 interaction seems to be essential for the carboxydipeptidase activity. This view is supported by the peptide with glycine, which was resistant to hydrolysis and is limited to interaction with the peptide main chain owing to the absence of amino acid side chain. Another interesting observation is the selectivity of the S_2 subsite for lysine in comparison with arginine and the hydrolysis of the peptides with aspartic acid and glutamic acid, despite the presence of a negative carboxy group. This behaviour could be related to a certain mobility of the Glu^{245} side chain that could be regulated by the nature of the side chain of the amino acid at the P_2 position of the substrate or inhibitor, as described for cruzain [29]. This hypothesis is further supported by the previously reported pH profile of hydrolysis by cathepsin B of benzyloxycarbonyl-Phe-Arg-*p*-nitroanilide and benzyloxycarbonyl-Arg-Arg-*p*-nitroanilide [26].

The S_1'–P_1' interaction also has a significant role on the carboxydipeptidase activity of cathepsin B, and the results with the series Dnp-GFRXW-OH (Table 3) are partially in agreement with the previously reported specificity of the S_1' subsite of cathepsin B [30], which was examined with the series of Dns-FRXWA-OH peptides. Owing to the carboxydipeptidase activity of cathepsin B, several of the peptides of this series were cleaved at two sites (Arg–Xaa and Xaa–Trp bonds), which impaired an accurate analysis of S_1' specificity. The high preference of the S_2' subsite for amino acids containing an aromatic side chain is demonstrated in the series Dnp-GFRWX-OH (Table 4), as well as in the substrates with the Abz–Dnp donor–acceptor fluorescence pair (Table 5). In addition, the negative charge of the C-terminal carboxy group of the substrate plays a role in the carboxydipeptidase activity, possibly due to an electrostatic interaction with His^{111}, as recently reported [18], and to the pH profile of k_{cat}/K_m values for hydrolysis of Abz-FR-K(Dnp)-W-OH (results not shown) that demonstrated higher cathepsin B activity on the deprotonation of C-terminal carboxy group.

Preliminary experiments with cruzain, a recombinant and C-terminal truncated form of a cysteine protease of *Trypanosoma cruzi*, demonstrated that this enzyme also presented a carboxydipeptidase activity profile quite similar to that of cathepsin B with some of the substrates presented in Table 5. Although cruzain does not have the occluding loop as in cathepsin B, the parasitic enzyme accepts basic amino acids at S_2 subsite [31,32]. This observation seems relevant for the *T. cruzi* physiology, because the same enzyme can perform both endo- and exopeptidase activities that could be involved, not only in digestive

functions, but also in the processing of oligopeptides, such as bradykinin, which appear to be very important for parasite cell invasion [33]. In conclusion, we have presented a detailed analysis of the specificity of cathepsin B carboxydipeptidase activity, and described new and efficient IQF substrates for this enzyme.

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REFERENCES

1 Kirschke, H., Barrett, A. J. and Rawlings, N. D. (1995) Proteinases 1: lysosomal cysteine proteinases. In Protein Profile (Sheterline, P., ed.), vol. 2, pp. 1587–1643, Academic Press, London

2 Mort, J. S. and Buttle, D. J. (1997) Molecules in focus: cathepsin B. Int. J. Biochem. Cell Biol. **29**, 715–720

3 Mort, J. S. (1998) Cathepsin B. In Handbook of Proteolytic Enzymes (Barrett, A. J, Rawlings, N. D. and Woesner, F. J., eds.), pp. 609–617, Academic Press, London

4 Yan, S., Sameni, M. and Sloane, B. F. (1998) Cathepsin B and human tumor progression. Biol. Chem. **379**, 113–123

5 Frosch, B. A., Berquin, I., Emmert-Buck, M. R., Moin, K. and Sloane, B. F. (1999) Molecular regulation, membrane association and secretion of tumor cathepsin B. APMIS **107**, 28–37

6 Szpaderska, A. M. and Frankfater, A. (2001) An intracellular form of cathepsin B contributes to invasiveness in cancer. Cancer Res. **61**, 3493–3500

7 Sinha, A. A., Jamuar, M. P., Wilson, M. J., Rozhin, J. and Sloane, B. F. (2001) Plasma membrane association of cathepsin B in human prostate cancer: biochemical and immunological electron microscopy analysis. Prostate **49**, 172–184

8 Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingeon, P. A., Peters, C., Kaufmann, S. H. and Gores, G. J. (2000) Cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome C. J. Clin. Invest. **106**, 1127–1137

9 Stoka, V., Turk, B., Schendel, S. L., Kim, T.-H., Cirman, T., Snipas, S. J., Ellerby, L. M., Bredesen, D., Freeze, H., Abrahamson, M. et al. (2001) Lysosomal protease pathways to apoptosis: cleavage of Bid, not pro-caspases, is the most likely route. J. Biol. Chem. **276**, 3149–3157

10 Kostoulas, G., Lang, A., Nagase, H. and Baici, A. (1999) Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases. FEBS Lett. **455**, 286–290

11 Aronson, N. N. and Barrett, A. J. (1978) The specificity of cathepsin B. Hydrolysis of glucagons at the C-terminus by a peptidylpeptidase mechanism. Biochem. J. **171**, 759–765

12 Nägler, D. K., Storer, A. C., Portaro, F. C. V., Carmona, E., Juliano, L. and Ménard, R. (1997) Major increase in endopeptidase activity of human cathepsin B upon removal of occluding loop contacts. Biochemistry **36**, 12608–12615

13 Portaro, F. C. V., Santos, A. B. F., Cezari, M. H. S., Juliano, M. A., Juliano, L. and Carmona, L. (2000) Probing the specificity of cysteine proteinases at subsites remote from the active site: analysis of P_4, P_3, P_2' and P_3' variations in extended substrates. Biochem. J. **347**, 123–129

14 Musil, D., Zucic, D., Engh, R. A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and Bode, W. (1991) The refined 2.15 Å X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. EMBO J. **10**, 2321–2330

15 Cygler, M., Sivaraman, J., Grochulski, P., Coulombe, R., Storer, A. C. and Mort, J. S. (1996) Structure of rat procathepsin B. Model for inhibition of cysteine protease activity by the proregion. Structure **4**, 405–416

16 Turk, D., Podobnick, M., Popovic, T., Katunuma, M., Bode, W., Huber, R. and Turk, V. (1995) Crystal structure of cathepsin B inhibited by CA030 at 2.0 Å resolution: a basis for the design of specific epoxysuccinyl inhibitors. Biochemistry **34**, 4791–4797

17 Illy, C., Quraishi, O., Wang, J., Purisima, E., Vernet, T. and Mort, J. S. (1997) Role of occluding loop in cathepsin B activity. J. Biol. Chem. **272**, 1197–1202

18 Krupa, J. C., Hasnain, S., Nägler, D. K., Ménard, R. and Mort, J. S. (2002) S_2' substrate specificity and the role of His^{110} and His^{111} in the exopeptidase activity of human cathepsin B. Biochem. J. **361**, 613–619

19 Nägler, D. K., Tam, W., Storer, W. C., Krupa, J. C., Mort, J. S. and Ménard, R. (1999) Interdependency of sequence and positional specificities for cysteine proteases of the papain family. Biochemistry **38**, 4868–4874

20 Barrett, A. J. and Kirshke, H. (1981) Cathepsin B, cathepsin H and cathepsin L. Methods Enzymol. **80**, 535–561

21 Del Nery, E., Alves, L. C., Melo, R. L., Cezari, M. H. S., Juliano, L. and Juliano, M. A. (2000) Specificity of cathepsin B to fluorescent substrates containing benzyl side-chain-substituted amino acids at P_1 subsite. J. Prot. Chem. **19**, 33–38

22 McGrath, M. E. (1999) The lysosomal cysteine proteases. Annu. Rev. Biophys. Biomol. Struct. **28**, 181–204

23 Turk, D., Guncar, G., Podobnik, M. and Turk, B. (1998) Revised definition of substrate binding sites of papain-like cysteine proteases. Biol. Chem. **379**, 137–147

24 Jia, Z., Hasnain, S., Hirama, T., Lee, X., Mort, J. S., To, R. and Huber, C. P. (1995) Crystal structures of recombinant rat cathepsin B and a cathepsin B-inhibitor complex. Implications for structure-based inhibitor design. J. Biol. Chem. **270**, 5527–5533

25 Khouri, H. E., Vernet, T., Menard, R., Parlati, F., Laflamme, P., Tessier, D. C., Gour-Salin, B., Thomas, D. Y. and Storer, A. C. (1991) Engineering of papain: selective alteration of substrate specificity by site-directed mutagenesis. Biochemistry **30**, 8929–8936

26 Hasnain, S., Hirama, T., Huber, C. P., Mason, P. and Mort, J. S. (1993) Characterization of cathepsin B specificity by site-directed mutagenesis. Importance of Glu^{245} in the S_2–P_2 specificity for arginine and its role in transition state stabilization. J. Biol. Chem. **268**, 235–240

27 Bromme, D., Bonneau, P. R., Lachance, P. and Storer, A. C. (1994) Engineering the S_2 subsite specificity of human cathepsin S to a cathepsin L- and cathepsin B-like specificity. J. Biol. Chem. **269**, 30238–30242

28 Melo, R. L., Pozzo, R. C. B., Alves, L. C., Perissutti, E., Caliendo, G., Santagada, V., Juliano, L. and Juliano, M. A. (2001) Synthesis and hydrolysis by cathepsin B of fluorogenic substrates with the general structure benzoyl-X-Arg-MCA containing non-natural basic amino acids at position X. Biochim. Biophys. Acta **1547**, 82–94

29 Gillmor, S. A., Craik, C. S. and Fletterick, R. J. (1997) Structural determinants of specificity in cysteine protease cruzain. Protein Sci. **6**, 1603–1611

30 Ménard, R., Carmona, E., Pouffle, C., Brömme, D., Konishi, Y., Lefebvre, J. and Storer, A. C. (1993) The specificity of the S_1' subsite of cysteine proteases. FEBS Lett. **328**, 107–110

31 Judice, W. A. S., Cezari, M. H. S., Lima, A. P. C. A., Scharfstein, J., Chagas, J. R., Tersariol, I. L. S., Juliano, M. A. and Juliano, L. (2001) Comparison of the specificity, stability and individual rate constants with respective activation parameters for the peptidase activity of cruzipain and its recombinant form, cruzain, from *Trypanosoma cruzi*. Eur. J. Biochem. **268**, 6578–6586

32 Alves, L. C., Melo, R. L., Cezari, M. H. S., Sanderson, S. J., Mottram, J. C., Coombs, G. H., Juliano, L. and Juliano, M. A. (2001) Analysis of the S(2) subsite specificities of the recombinant cysteine proteinases CPB of *Leishmania mexicana*, and cruzain of *Trypanosoma cruzi*, using fluorescent substrates containing non-natural basic amino acids. Mol. Biochem. Parasitol. **117**, 137–143

33 Scharfstein, J., Schmitz, V., Morandi, V., Capella, M. M., Lima, A. P., Morrot, A., Juliano, L. and Muller-Esterl, W. (2000) Host cell invasion by *Trypanosoma cruzi* is potentiated by activation of bradykinin B(2) receptors. J. Exp. Med. **192**, 1289–1300

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