Biochemistry **2001,** *40,* 4417−4425

Substrate Specificity Characterization of Recombinant Metallo Oligo-Peptidases Thimet Oligopeptidase and Neurolysin[†]

Vitor Oliveira,[‡] Marcelo Campos,[‡] Robson L. Melo,[‡] Emer S. Ferro,[§] Antonio C. M. Camargo,[||]
Maria A. Juliano,[‡] and Luiz Juliano*,[‡]

Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Três de Maio, 100 São Paulo - SP - 04044-020, Brazil, Department of Histology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, 05508-900, SP, Brazil, and Laboratory of Biochemistry and Biophysics, Instituto Butantan, São Paulo, 05503-900, SP, Brazil

Received November 28, 2000; Revised Manuscript Received January 30, 2001

ABSTRACT: We report a systematic and detailed analysis of recombinant neurolysin (EC 3.4.24.16) specificity in parallel with thimet oligopeptidase (TOP, EC 3.4.24.15) using Bk sequence and its C- and N-terminal extensions as in human kininogen as motif for synthesis of internally quenched fluorescent substrates. The influence of the substrate size was investigated, and the longest peptide susceptible to TOP and neurolysin contains 17 amino acids. The specificities of both oligopeptidases to substrate sites P_4 to P_3' were also characterized in great detail using seven series of peptides based on Abz-GFSPFRQ-EDDnp taken as reference substrate. Most of the peptides were hydrolyzed at the bond corresponding to P^4−F^5 in the reference substrate and some of them were hydrolyzed at this bond or at F^2−S^3 bond. No restricted specificity was found for P_1' as found in thermolysin as well for P_1 substrate position, however the modifications at this position (P_1) showed to have large influence on the catalytic constant and the best substrates for TOP contained at P_1, Phe, Ala, or Arg and for neurolysin Asn or Arg. Some amino acid residues have large influence on the K_m constants independently of its position. On the basis of these results, we are hypothesizing that some amino acids of the substrates can bind to different sub-sites of the enzyme fitting P−F or F−S bond, which requires rapid interchange for the different forms of interaction and convenient conformations of the substrate in order to expose and fit the cleavage bonds in correct position for an efficient hydrolysis. Finally, this plasticity of interaction with the substrates can be an essential property for a class of cytosolic oligopeptidases that are candidates to participate in the selection of the peptides to be presented by the MHC class I.

Thimet oligopeptidase (EC 3.4.24.15, TOP)[1] and neurolysin (EC 3.4.24.16) are two zinc-dependent peptidases, which contain the consensus motif HEXXH in the primary sequence (*1*−*3*). Among the metallopeptidases TOP and neurolysin belong to the M3 family so far not included in any clan (*4*, *5*). On the other hand, the third zinc-binding residue in TOP was identified by site-directed mutagenesis as a glutamic acid residue (Glu^{502}, numbering related to rat testes enzyme), as observed in the thermolysin-like peptidases from clan MA of the metallopeptidases (*6*). However, the mutations at other Asp, Glu, and His residues and also at the same Glu^{502} residue were reported to impair TOP activity; therefore, the structure of the zinc-binding site is not unequivocal (*7*). TOP is able to hydrolyze oligopeptides but not proteins (*8*, *9*), although most of the substrates for neurolysin are oligopeptides it was reported to process proproteins such as the vitamin K-dependent proteins (*10*). The mechanism that guaranties the oligopeptidase activity of these enzymes prevents incident cleavage of other proteins, particularly because TOP and neurolysin do not have a precursor inactive form and the higher amount of these soluble enzymes are found in the cytosol. Membrane associated form of TOP (*11*) and neurolysin (*12*, *13*) have been identified by microscopy approaches. The secretion of TOP has been reported in AtT20 (*14*, *15*) and MDCK cells (*16*) while neurolysin was showed to be secreted by astrocytes (*17*). Camargo and cols introduced the concept of oligopeptidase in 1979 (*18*); however, a mechanism for an oligopeptidase activity was only recently described for prolyl oligopeptidase, in which the access to the catalytic center is restricted by a gating filter mechanism (*19*−*21*) but the mechanism by which TOP and neurolysin select only oligopeptides to hydrolyze remains unknown.

TOP and neurolysin have approximately 78 kDa and an identity of about 65% between their primary sequences. They

---

[†] This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Financiadora de Estudos e Projetos (FINEP) through the PADCT-Biotecnologia III program.

* To whom correspondence should be addressed. Phone: 55-11-5575-9617. Fax: 55-11-5575-9040. E-mail: juliano.biof@epm.br.

[‡] Department of Biophysics.

[§] Department of Histology.

[||] Laboratory of Biochemistry and Biophysics.

[1] Abbreviations: Bk, bradykinin; DTT, dithiotreitol; Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance. Two numbers identify the peptides: one in roman to identify them in table and the other in arabic to indicate the table where it is presented.

10.1021/bi002715k CCC: $20.00

hydrolyze several bioactive or synthetic peptides at the same peptide bond (*22*−*27*) and both are strongly inhibited by phosphonamide (*28*) or phosphinic (*29*) peptides, suggesting that they have closely related active sites.

The initial studies of TOP specificity indicate that it preferentially cleaved peptide bonds on the carboxyl side of hydrophobic residues (*30*, *31*). Specific tightly bound phosphinic inhibitors for TOP and neurolysin were obtained using combinatorial chemistry procedures (*32*, *33*) and it was reported a preference for Arg at P_2' by TOP and for Pro at P_2 and P_2' by neurolysin. It was also reported that TOP did not hydrolyze peptides with Ile at P_1 and only cleaves peptides 3 to 6 residues from the C-terminal end (*34*, *35*) indicating that negative charged carboxylate group at P_3' or P_4' has favorable effect as suggested by TOP inhibitors (*33*, *36*). Fluorogenic-quenched peptides derived from bioactive peptide sequences were also used for TOP specificity studies (*37*, *38*), and the more outstanding observed feature of TOP was its variability in terms of cleavage sites even in substrates with very similar sequences, such as Abz-GGFL↓RRV-EDDnp and Abz-GGFL↓R↓RVQ-EDDnp or Abz-RPPGF↓SPFR-EDDnp and Abz-RPPGFSP↓FRQ-EDDnp for example. Much less information is available for neurolysin specificity although it is an important enzyme, which was found to have 90% homology with a zinc-dependent enzyme from rabbit liver that process vitamin K-dependent proproteins (*10*, *39*) and it has been shown that neurolysin is the major neurotensin-degrading-enzyme in vivo (*40*, *41*).

To make a systematic and detailed analysis of neurolysin specificity in parallel with that of TOP, we chose the Bk sequence and its C- and N-terminal extensions as in human kininogen and we used that as motif for synthesis of internally quenched fluorescent substrates. The influence of the substrate size was investigated using Bk analogues or homologues containing 6−21 amino acids. The peptide Abz-GFSPFRQ-EDDnp was taken as reference substrate and seven series were synthesized with substitutions at each position. All these series of peptides were assayed with recombinant TOP and neurolysin in order to characterize in great detail the determinants of their specificity from P_4 to P_3'.

MATERIAL AND METHODS

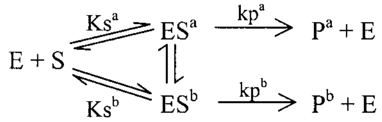
Thimet Oligopeptidase (TOP). The purified recombinant rat testes TOP (rTOP) was obtained as previously described (*42*). The amino acid composition of the purified enzyme [Asp, 57; Glu, 97; Ser, 32; Gly, 46; His, 16; Arg, 38; Thr, 35; Ala, 58; Pro, 34; Tyr, 18, Val, 43; Met, 18; Ile, 18; Leu, 71; Phe, 27; Trp (not determined); Lys, 41] reproduced the theoretical composition values of the rat testes TOP (*9*). The homogeneity of the enzyme preparation was confirmed by MALDI-TOF mass spectrometry (TofSpec-E, Micromass, Manchester, U.K.). Calculated MW, $MH^+ = 78\,315.51$, Obtained MW, $MH^+ = 79\,748.33$. A difference of approximately 2% is a reasonable error for the low accuracy condition for the external calibration employed. The purified enzyme was aliquotted in vials containing about 1 μg of enzyme in 2% serum albumin and 30% glycerol. After an initial reduction in 15%, the enzyme activity remained constant for at least 2 months at −70 °C.

The enzyme concentration was determined by amino acid analysis corrected for the 15% enzyme activity decrease. We also performed an active site titration, with a tightly biding inhibitor [*Z*-PheΨ(PO_2CH_2)-Ala-Lys-Ile], with a $K_i \approx 2$ nM (*27*) that was a kind gift from Dr. Vincent Dive (Department d'Ingénierie et d'Etudes des proteins, DSV, CE-Saclay 91191 Gif Yvette Cedex, France). For this titration, aliquots of rTOP were preincubated at different inhibitor concentrations, during 24 h at 4 °C, in a final volume of 100 μL in the TBS buffer, containing glycerol 30% and albumin 1 μg/mL, after this, the reminiscent activity was measured with 9 μM of the substrate Abz-GFSPFRQ-EDDnp, at a final volume of 1 mL (dilution of 10 times) in TBS buffer at 37 °C. DTT was added 5 min before the preincubation and 5 min before the reminiscent activity determination; however, DTT concentration was kept constant (0.5 mM) regardless of the dilution employed. The enzyme concentration and the Ki were calculated accordingly the method described by Knight et al. (*43*). All the obtained data were fitted to nonlinear least-squares equations using Grafit v 3.0 from Erithacus Software [Leatherbarrow, R. J. (1992) Grafit Version 3.0, Erithacus Software Ltd., Staines, U.K.].

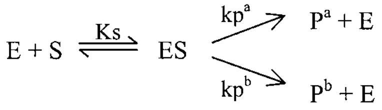
Neurolysin. The recombinant cDNA of porcine kidney Neurolysin (citosolyc form) was a kind gift from Drs. Shigehisa Hirose and Akira Kato (Department of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midoriku, Yokohama 226, Japan). Details concerning the expression system including plasmid contructions and vectors used were described elsewhere (*44*). The procedures for expression and purification of recombinant porcine kidney neurolysin were performed as previously reported for the recombinant rat testes TOP (*42*). Purified recombinant enzyme (0.1 mg/mL) was aliquotted and the enzyme activity remained constant for at least 6 months at −20 °C. Protein concentration was determined using the method described by Bradford (*45*) using bovine serum albumin as standard. The enzyme concentration was calculated using neurolysin molecular weight (MW = 78 106 g mol^{-1} calculated from amino acid sequence) and the protein concentration. Homogeneity of the recombinant enzyme preparation was analyzed by polyacrilamide gel electrophoresis (*46*) after silver staining.

Peptide Synthesis. The intramolecularly quenched fluorogenic peptides containing *N*-[2,4-dinitrophenyl]-ethylenediamine (EDDnp) attached to glutamine were synthesized by solid-phase strategy and the details of which are provided elsewhere (*47*). An automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the Fmoc-procedure. The final deprotected peptides were purified by semipreparative HPLC using an Econosil C-18 column (10 μm, 22.5 × 250 mm) and a two-solvent system: (A) trifluoroacetic acid (TFA)/H_2O (1:1000) and (B) TFA/acetonitrile (ACN)/H_2O (1:900:100). The column was eluted at a flow rate of 5 mL/min 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 from Shimadzu with a SPD-10AV Shimadzu UV-vis detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μm, 4.6 × 150 mm) which was eluted with solvent systems A and B at a flow rate of 1 mL/min and a 10 to 80% gradient of B over

Scheme 1



Scheme 2



20 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm following excitation at 320 nm. The molecular weight and purity of synthesized peptides were checked by MALDI-TOF mass spectrometry (TofSpec-E, Micromass) and/or peptide sequencing using a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan).

The series of peptides derived from Abz-GFSPRX-EDDnp were synthesized in solution, using the following strategy: N^{α}-Boc-X-(CO$^{\alpha}$-EDDnp), where X stands for Ala, Ile, Leu, Phe, Ser(OBzl), Asn, Gln, Glu(OBzl), Arg (Tos), and Pro, were obtained by coupling the corresponding N^{α}-Boc-amino acid with EDDnp·HCl by mixed anhydride procedure, using isobutyl chlorocarbonate as previously described (*48*). All the amino acid derivatives were purified in silica gel chromatography using as solvent chloroform−methanol mixtures and characterized by thin-layer chromatography and mass spectrometry. The amino acids Boc-Arg(Tos)-OH, Boc-Pro-OH, Boc-Ser(OBzl)-OH, Boc-Phe-OH, Boc-Gly-OH, and Boc-Abz-OH were successively coupled by mixed anhydride procedure intercalated by TFA deprotection. The final peptides were deprotected by treatment with anhydrous HF, purified and characterized as described above for the peptides obtained by solid-phase procedure.

Kinetic Assays. Hydrolysis of the fluorogenic peptidyl substrates at 37 °C in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl, was followed by measuring the fluorescence at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm in a Hitachi F-2000 spectrofluorometer. The 1 cm path-length cuvette containing 2 mL of the substrate solution was placed in a thermostatically controlled cell compartment for 5 min before the enzyme solution was added (for TOP an additional preincubation time of 5 min with 0.5 mM of DTT were applied after the enzyme addition) and the increase in fluorescence with time was continuously recorded for 5−10 min. The slope was converted into moles of hydrolyzed substrate per minute based on the fluorescence curves of standard peptide solutions before and after total enzymatic hydrolysis. The concentration of the peptide solutions was obtained by colorimetric determination of the 2,4-dinitrophenyl group (17 300 M^{-1} cm^{-1} extinction coefficient at 365 nm). The enzyme concentration for initial rate determination was chosen at a level intended to hydrolyze less than 5% of the substrate present. The inner-filter effect was corrected using an empirical equation as previously described (*49*). The kinetic parameters were calculated according Wilkinson (*50*) as well as by using Eadie-Hofstee plots. All the obtained data were fitted to nonlinear least-squares equations, using Grafit, version 3.0 from Erithacus Software (*51*).

The hydrolysis of the peptides cleaved at F−S or P−F bond could be represented as showed in Schemes 1 and 2.

The equations for velocity related to the Schemes 1 and 2 are the eqs 1 and 2, respectively. V_t is the sum of the product (P^a and P^b) formation velocities. $V_{max}{}^a$ is $kp^a \times [E]$ and $V_{max}{}^b$ is $kp^b \times [E]$ and [E] is the total enzyme concentration in the assay.

$$V_t = \frac{[S]\left(\frac{kp^a}{Ks^a} + \frac{kp^b}{Ks^b}\right)}{1 + [S]\left(\frac{1}{Ks^a} + \frac{1}{Ks^b}\right)} \tag{1}$$

$$V_t = \frac{[S](V_{max}{}^a + V_{max}{}^b)}{K_s + [S]} \tag{2}$$

All the obtained data with the peptides cleaved at two bonds fitted to nonlinear least-squares plot of eq 2, and no-substrate inhibition was observed till 5−10 times the K_m value. This kind of inhibition would be expected, if two enzyme−substrate complexes were formed with large difference in its equilibrium constant formation. The overall V_{max} was obtained from eq 2, whereas the separate values for $V_{max}{}^a$ and $V_{max}{}^b$ were calculated using the ratio of the areas taken from the integrated HPLC chromatogram analysis.

K_i Determination for TOP and Neurolysin of Peptides Resistant to Hydrolysis. The resistant peptides obtained throughout the systematic modifications study were assayed as competitive inhibitors over the hydrolysis of Abz-GFSPFRA-EDDnp ($K_m = 4.8$ μM for TOP and $K_m = 1.1$ μM for neurolysin) by the enzymes. The K_i values for competitive inhibition assays of the peptides were determined according to (*52*).

Determination of Cleaved Bonds. The cleaved bonds were identified by isolation of the fragments by HPLC and the retention times of the products fragments were compared with authentic synthetic sequences and/or by molecular weight, which was determined by MALDI-TOF mass spectrometry and/or by peptide sequencing, using a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan).

Amino Acid Analysis. The amino acid compositions, the concentration of the peptides and the purified rTOP were determined as follows: the samples were digested for 22 h at 110 °C in 6 N HCl containing 1% phenol in a vacuum sealed tubes and then subjected to amino acid analysis using a pico-Tag station (*53*).

RESULTS AND DISCUSSIONS

1. Effect of Substrate Size on Susceptibility to TOP and Neurolysin. Table 1 shows the relative rate of hydrolysis and the cleavage sites by TOP and neurolysin of internally quenched fluorescent peptides derived from Bk and its C- and N-terminal extensions as in human kininogen. As previously observed with TOP (*38*), neurolysin also hydrolyzed Abz-GFSPFR-EDDnp and Abz-RPPGFSPFR-EDDnp (peptides I-1, II-1) at F−S bond, while the introduction of Gln at the C-terminal side of these peptides, which was a consequence of the solid-phase synthesis method, displaced the cleavage to P−F bond (peptides III-1, IV-1).

Table 1: Relative Hydrolysis by TOP and Neurolysin of Peptides Derived from Bradykinin and Human Kininogen[a]

#	Substrate	Hydrolysis rate %	
		24.15	**24.16**
I-1	Abz - G F⇩S P F R - EDDnp	72	6
II-1	Abz - R P P G F⇩S P F R - EDDnp	33	7
III-1	Abz - G F S P⇩F R Q - EDDnp	100	20
IV-1	Abz - R P P G F S P⇩F R Q - EDDnp	85	23
V-1	Abz - G F S P F R↓S↑S R Q - EDDnp	8	100
VI-1	Abz - R P P G F S P F R↓S↑S R Q - EDDnp	23	38
VII-1	Abz - G F S P F R S↑S↓R↓I G E I K E E Q - EDDnp	14	10
VIII-1	Abz- L G M I S L M K R P P G F S P F R S S R I -NH$_2$	Resistant	

[a] Cleavage sites of (open downward facing arrow) TOP and neurolysin, (↓) only TOP, (↑) only neurolysin. Cleavage sites of peptides I-1, II-1, III-1, and IV-1 were determined by comparison of the retention times of reaction products with authentic synthetic sequences: Abz-GF; SPFR-EDDnp; Abz-RPPGF; Abz-GFSP; FRQ-EDDnp; and Abz-RPPGFSP. Cleavage sites of peptides V-1, VI-1, VII-1, and peptide III-1 were determined by mass spectrometry analyses and by sequencing (four cycles) of the products without fluorescence signal, which were isolated by HPLC.

Abz-GFSPFRQ-EDDnp (peptide III-1) was the most susceptible substrate to TOP but the addition of three amino acids at its C-terminal side (peptide V-1) significantly reduced its hydrolysis rate and the cleavage point moved to the R−S bond. In contrast, neurolysin hydrolyzed peptide V-1 with the highest rate but at S−S bond. Similar effects were observed with the peptides IV-1 and VI-1 that contain the complete sequence of Bk. Peptide VII-1, with 17 amino acids, which resulted of a further C-terminal extension of peptide V-1, was poorly hydrolyzed by both TOP, at S−R or R−I bonds with a ratio of about 50% for each cleavage, and by neurolysin only at S−S bond. The reaction products of hydrolysis by TOP of the peptides V-1, VI-1, and VII-1 (Abz-GFSPFR or Abz-RPPGFSPFR) or by neurolysin (Abz-GFSPFRS or Abz-RPPGFSPFRS) were further hydrolyzed at the F−S bond. This cleavage at F−S bond did not seem to be dependent only on the presence of free C-terminal carboxyl group, but also from the nature of the C-terminal amino acid, because the peptide Abz-GFSPFRQ-OH was hydrolyzed only at P−F bond by both enzymes. Therefore, neurolysin and TOP removed C-terminal segment of the Bk and human kininogen derived substrates located 3−5 residues from the C-terminus, which activity was also previously reported for hydrolysis by TOP of (GPL)$_n$ oligomers (*34*).

The peptide VIII-1, which contains Bk sequence extended to both, N- and C-terminal sides is the longest peptide assayed, and was completely resistant to TOP and neurolysin. Therefore, peptide containing 17 amino acids is the maximum size still hydrolyzed by TOP with human kininogen sequence, what is in accordance to the previously described resistance to hydrolysis by endooligopeptidase A from rabbit brain of the peptide formed by two Bk molecules attached by succinic acid (*18*). Endooligopeptidase A was partially cloned and confirmed to be distinct from TOP (*54*) but with similar hydrolytic activities. The substrate size selectivity of neurolysin using human kininogen derived peptides (Table 1) is in accordance to the previously described susceptibility of opioid and neurotensin derived peptides (*55*). Regarding the longest peptide accepted for hydrolysis, neurolysin behaved like TOP (Table 1).

2. Systematic Study of TOP and Neurolysin Activities on Series of Peptides Derived from Abz-G^{1}F^{2}S^{3}P^{4}F^{5}R^{6}Q^7-EDDnp. Tables 2−8 show the k_{cat}, K_m and k_{cat}/K_m values determined for the hydrolysis by TOP and neurolysin of the peptide series derived from Abz-GFSPFRQ-EDDnp, which was taken as reference and modified in each position with natural amino acids. All the susceptible peptides to TOP and neurolysin were hydrolyzed at the peptide bond corresponding to P^4−F^5 in the reference substrate with only one exception the peptide IX-7, which was hydrolyzed only at F−S bond.

Some peptides of the series modified at Gly^1 (Abz-XFSPFRQ-EDDnp), Phe^5 (Abz-GFSPXRQ-EDDnp), and Arg^6 (Abz-GFSPFXQ-EDDnp) were also cleaved by both enzymes at F−S or P−F bond (P−X in the modifications at Phe^5). For these peptides the plots of V_t versus [S] result hyperbolic curves as showed in Figure 1A. Due to this hyperbolic kinetics obtained we have adjusted the data to eq 2 resulting for both cleavages indistinguishable K_m values. In addition the HPLC analysis of all these double-cleaved substrates demonstrated that the ratio between the cleavages remained exactly the same regardless of the duration of the reaction and of substrate concentration (Figure 1, panels B−D). Thus, such behavior permits the evaluation of the kinetic parameters for the hydrolysis reactions of these peptides for each cleavage, using the eq 2 and the relation between the products determined by HPLC analysis.

Hydrolysis of the Abz-XFSPFRQ-EDDnp Series. Table 2 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-XFSPFRQ-EDDnp. TOP and neurolysin hydrolyzed all the substrates of this series at P−F or F−S bonds at different rates with preference for the former. TOP hydrolyzed 100% at the P−F bond the substrates containing Phe, His, Arg, and Gly (peptides IV-2, X-2, XI-2, and III-1) while those with Ile and Leu (peptides II-2 and III-2) the cleavages at F−S bond were almost 40%. Neurolysin hydrolyzed 100% at the P−F bond the substrates containing His and Gly (peptides X-2 and III-1) and approximately 25% at F−S bond the substrates with Ala, Leu, and Gln(peptides I-2, III-2, and VII-2).

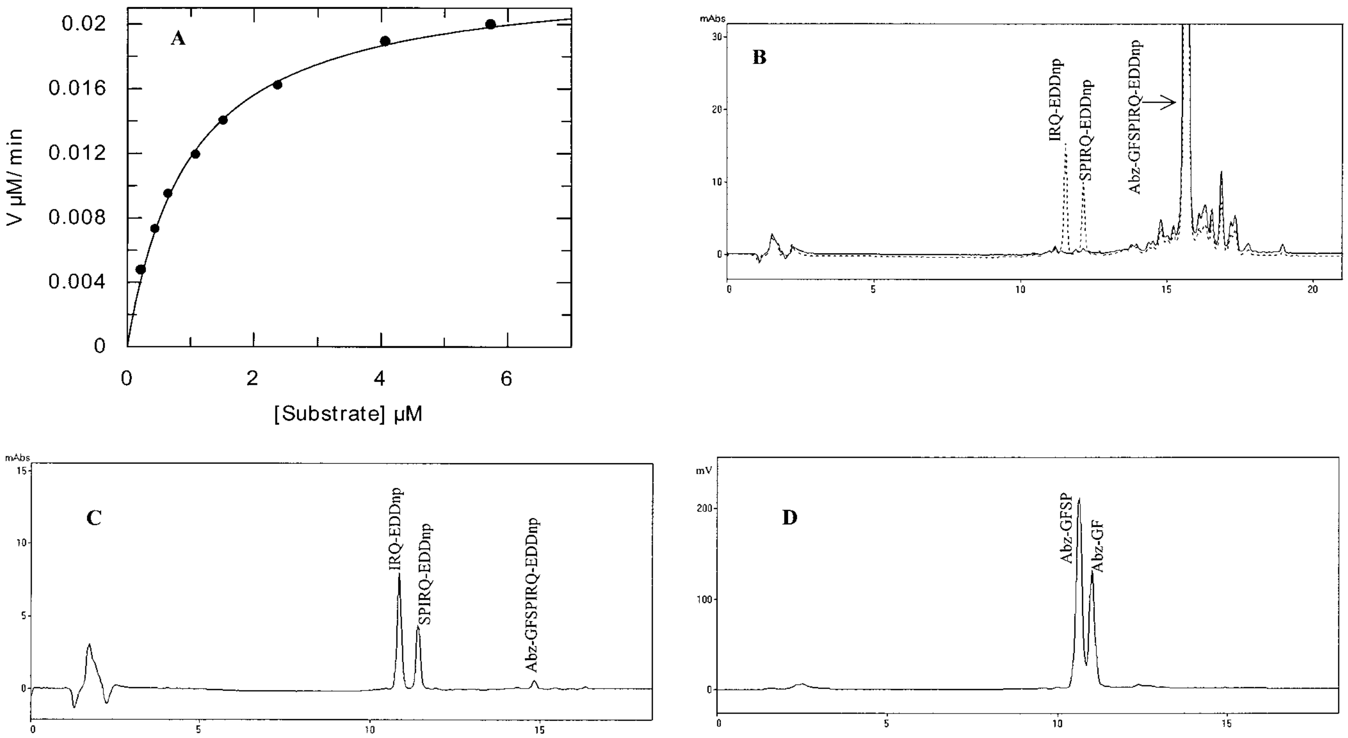


FIGURE 1: Reaction carried out with neurolysin and the fluorogenic-quenched substrate Abz-GFSPIRQ-EDDnp that is cleaved at F−S and P−I bonds (Table 6). (A) Data fitting to eq 2 of the overall initial velocities measured in the spectrofluorometer at each substrate concentration and 0.3 nM of Neurolysin with a substrate consumption of less than 5%. (B) HPLC chromatogram of the reaction with a substrate concentration of 10 times the K_m (9.1 μM) and the substrate consumption of less than 5%; solid line represent a blank and the dashed line the reaction containing 0.3 nM of neurolysin. (C, D) HPLC chromatogram of the reaction with a substrate concentration of K_m/10 (0.1 μM) and 2 nM Neurolysin with a substrate consumption of almost 100% (pseudo-first-order conditions). (C) Chromatogram obtained with the UV detection at 365 nm and (D) chromatogram obtained with the fluorescence detection with λ_{ex} = 320 nm and λ_{em} = 420 nm.

Table 2: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-**X**FSPFRQ-EDDnp[a]

		TOP (24.15)				neurolysin (24.16)			
		k_{cat} (s^{-1})		K_m	k_{cat}/K_m	k_{cat} (s^{-1})		K_m	k_{cat}/K_m
no.	X	P↓F	F↓S	(μM)	(μM^{-1} s^{-1})	P↓F	F↓S	(μM)	(μM^{-1} s^{-1})
I-2	Ala	9.8	2.6	4.5	2.8[b]	0.27	0.10	1.4	0.3[b]
II-2	Ile	1.5	0.94	0.16	15[b]	0.45	0.05	1.2	0.4[b]
III-2	Leu	3.8	2.2	0.82	7.3[b]	0.35	0.13	1.7	0.3[b]
IV-2	Phe	1.6		1.0	1.6	0.23	0.03	0.83	0.3[b]
V-2	Ser	4.1	0.67	1.4	3.4[b]	0.77	0.12	0.48	1.9[b]
VI-2	Asn	3.0	0.75	0.78	4.8[b]	1.0	0.16	1.3	0.9[b]
VII-2	Gln	4.7	1.1	1.5	3.9[b]	0.39	0.12	0.33	1.5[b]
VIII-2	Glu	1.4	0.53	1.2	1.6[b]	0.08	0.01	0.64	0.1[b]
IX-2	Asp	4.7	1.0	3.0	1.9[b]	0.12	0.02	2.3	0.1[b]
X-2	His	6.0		1.7	3.4	1.2		0.33	3.6
XI-2	Arg	4.3		1.5	2.8	0.27	0.02	0.32	0.9[b]
III-1	**Gly**	**6.3**		**2.2**	**2.9**	**0.31**		**0.58**	**0.5**

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. [b] $k_{cat}/K_m = (k_{cat}^{PF} + k_{cat}^{FS})/K_m$. P↓F and F↓S indicate the cleavage sites, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequences (SPFRQ-EDDnp and FRQ-EDDnp) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

Therefore, amino acids with aliphatic side chain favored the hydrolysis at F−S bond by both enzymes.

Abz-IFSPFRQ-EDDnp (peptide II-2) was hydrolyzed by TOP with the highest k_{cat}/K_m value in this work, due mainly to the low K_m value. The substrates with Leu and Asn (peptides III-2 and VI-2) were also well hydrolyzed by TOP. Abz-HFSPFRQ-EDDnp (peptide X-2) was the best substrate for neurolysin in this series, followed by the substrates containing Ser and Gln (peptides V-2 and VII-2). On the other hand, the lowest k_{cat}/K_m values were obtained with substrates hydrolyzed by neurolysin containing the acid residues Glu and Asp (peptides VIII-2 and IX-2). It is noteworthy that neurolysin activity is quite sensitive to the nature of the amino acid at P_4 position relative to P−F bond cleavage, particularly to polar noncharged amino acids (His, Ser, and Gln), indicating that the side chains of the amino acids at this position make significant hydrogen bonds for enzyme activity. This contrast with TOP since His, Arg, or Gly at P_4 position resulted in substrates hydrolyzed with similar parameters. The favorable effects of Ile, Leu, and Asn could due to the occupancy of P_2 position relative to F−S bond cleavage by TOP. Indeed, in the series Abz-GFXPFRQ-EDDnp (Table 4) where these amino acids occupy the P_2 relative to P−F bond cleavage, they have a

Table 3: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-G**X**SPFRQ-EDDnp[a]

		TOP (24.15)			neurolysin (24.16)		
no.	X	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)
I-3	Ala	2.5	4.2	0.6	1.3	2.3	0.6
II-3	Ile	8.4	2.8	3.0	2.6	4.7	0.6
III-3	Leu	11.5	3.7	3.1	6.2	5.9	1.0
III-1	**Phe**	**6.3**	**2.2**	**2.9**	**0.31**	**0.58**	**0.5**
IV-3	Ser	3.2	6.0	0.5	2.0	5.8	0.4
V-3	Asn	5.2	4.5	1.2	1.3	7.5	0.2
VI-3	Gln	2.9	9.9	0.3	1.9	6.8	0.3
VII-3	Glu	5.8	22.5	0.3	0.84	31.8	0.03
VIII-3	Asp	2.2	16.6	0.1	0.34	68.4	0.005
IX-3	His	14.5	4.8	3.0	0.95	2.5	0.4
X-3	Arg	13.3	3.6	3.7	5.4	1.3	4.2
XI-3	Pro	11.5	3.7	3.1	2.2	6.3	0.4

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. All the peptides were cleaved only at P↓F bond, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequence (FRQ-EDDnp) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

Table 4: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-GF**X**PFRQ-EDDnp[a]

		TOP (24.15)			neurolysin (24.16)		
no.	X	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)
I-4	Ala	5.7	0.71	8.0	0.80	0.87	0.9
II-4	Ile	4.9	0.99	5.0	0.27	0.18	1.5
III-4	Leu	3.0	0.38	7.9	0.24	0.19	1.3
IV-4	Phe	5.3	1.4	3.8	1.28	1.1	1.2
III-1	**Ser**	**6.3**	**2.2**	**2.9**	**0.31**	**0.58**	**0.5**
V-4	Asn	2.5	0.27	9.3	0.64	0.45	1.4
VI-4	Gln	1.8	14.9	0.1	0.39	2.0	0.2
VII-4	Glu	4.0	5.2	0.8	0.07	3.1	0.02
VIII-4	Asp	3.4	6.8	0.5	0.20	10.3	0.02
IX-4	His	0.89	0.72	1.2	0.52	0.33	1.6
X-4	Arg	7.7	5.3	1.5	0.97	0.39	2.5
XI-4	Pro	1.6	6.0	0.3	0.62	13.7	0.05

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. All the peptides were cleaved only at P↓F bond, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequence (FRQ-EDDnp) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

significant and favorable effect for hydrolysis of the corresponding substrates by TOP.

Hydrolysis of the Abz-GXSPFRQ-EDDnp Series. Table 3 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-GXSPFRQ-EDDnp. All the substrates of this series are hydrolyzed only at P−F bond, therefore all the substitutions can be interpreted as being at P_3 position of the substrates. The best substrates for TOP in this series were those with hydrophobic side chain, Ile, Leu, Phe, and Pro (peptides II-3, III-3, III-1, and XI-3) or hydrophilic or charged side chain, His, and Arg (peptides IX-3 and X-3). Neurolysin is more restrictive in this position preferring Arg at P_3 position, although the substrate with Leu is hydrolyzed with high k_{cat} but with also high K_m values. The presence of negative charge residues, Glu and Asp (peptides VII-3 and VIII-3) resulted in very poor substrates for both enzymes.

Table 5: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-GFS**X**FRQ-EDDnp[a]

		TOP (24.15)			neurolysin (24.16)		
no.	X	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)
I-5	Ala	10.2	1.2	8.5	2.6	1.1	2.4
II-5	Ile	resistant $K_i = 0.4\ \mu M$			0.22	0.85	0.3
III-5	Leu	3.4	1.6	2.1	3.5	1.8	1.9
IV-5	Phe	6.7	0.69	9.7	3.4	1.5	2.3
V-5	Ser	8.8	3.7	2.4	7.5	11.1	0.7
VI-5	Asn	8.9	1.6	5.6	8.8	1.3	6.8
VII-5	Gln	6.3	1.2	5.3	3.6	1.6	2.3
VIII-5	Glu	1.5	7.5	0.2	0.67	2.4	0.3
IX-5	Asp	0.70	3.8	0.2	0.19	3.0	0.06
X-5	His	24.7	7.6	3.3	2.5	1.8	1.4
XI-5	Arg	7.0	1.1	6.4	4.8	1.3	3.4
III-1	**Pro**	**6.3**	**2.2**	**2.9**	**0.31**	**0.58**	**0.5**

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. All the peptides were cleaved at X↓F bond, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequence (FRQ-EDDnp) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

Hydrolysis of the Abz-GFXPFRQ-EDDnp Series. Table 4 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-GFXPFRQ-EDDnp. Similar to preceding series, this gave information on S_2-P_2 interaction, since for both enzymes Pro^4-Phe^5 bond was also the only cleavage site. The presence of hydrophobic amino acids, Ala, Ile, Leu, and Phe (peptides I-4, II-4, III-4, or IV-4) or Asn (peptide V-4) resulted in the best substrates for TOP in this series. It is noteworthy the 50-fold lower K_m value for the substrate with Asn compared with that with Gln (peptides V-4 and VI-4). The origin of this lowest K_m value among all the substrates for the peptide Abz-GFNPFRQ-EDDnp, could be related to previously observation (*56*), that in a variety of flanking structural contexts, more than 60% of Asn-Pro sequences adopt a turn conformation stabilized by a set of alternative hydrogen bonds among the O^{δ}−side chain and backbone C=O carbonyl oxygens of Asn (residue *i*) and the backbone NH of residues $i + 2$, $i + 3$, and in some cases $i + 4$. In contrast, the structures adopted by Ser−Pro, His−Pro, and other Xxx−Pro sequences reflect more heterogeneous hydrogen-bonding patterns. The presence of a bent at cleavage site as a requirement for hydrolysis of the peptide by TOP is in line with the previous theoretical studies performed for the substrates of this peptidase (*57*).

Similar trend was also observed with the kinetic data of hydrolysis with neurolysin, except that the values are of lower magnitude and Arg (peptide X-4) was hydrolyzed with the higher k_{cat}/K_m value. Again, as in the preceding series, negative charge residues, Glu and Asp (peptides VII-4 and VIII-4) are the poorest substrates for both enzymes.

Hydrolysis of the Abz-GFSXFRQ-EDDnp Series. Table 5 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-GFSXFRQ-EDDnp. All the peptides of this series were hydrolyzed only at X−F bond by both enzymes, except the peptide containing Ile (II-5) that was resistant to TOP, which was assayed as inhibitor and presented $K_i = 0.4\ \mu M$ in a simple competitive inhibition. This series allowed a systematic evaluation of specificity for the primary site (P_1) for TOP and neurolysin.

Table 6: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-GFSP**X**RQ-EDDnp[a]

		TOP (24.15)				neurolysin (24.16)			
		k_{cat} (s^{-1})		K_m	k_{cat}/K_m	k_{cat} (s^{-1})		K_m	k_{cat}/K_m
no.	X	P↓X	F↓S	(μM)	(μM^{-1} s^{-1})	P↓X	F↓S	(μM)	(μM^{-1} s^{-1})
I-6	Ala	18.5		4.0	4.6	0.40		0.12	3.3
II-6	Ile	5.9	3.8	8.5	1.1[b]	0.65	0.53	0.91	1.3[b]
III-6	Leu	0.74	4.2	4.5	1.1[b]	0.55	1.3	2.4	0.8[b]
III-1	**Phe**	**6.3**		**2.2**	**2.9**	**0.31**		**0.58**	**0.5**
IV-6	Ser	21.5		7.3	2.9	1.5		0.78	1.9
V-6	Asn	10.1		5.0	2.0	0.62		0.20	3.1
VI-6	Gln	12.3		4.3	2.9	2.6		1.3	2.0
VII-6	Glu	1.8	1.5	18.0	0.2[b]	0.65	0.14	0.22	3.6[b]
VIII-6	Asp	8.2	8.9	37.0	0.5[b]	0.24	0.03	3.2	0.08[b]
IX-6	His	5.3		1.5	3.5	0.19		0.21	0.9
X-6	Arg	2.7		1.8	1.5	0.42		0.03	14
XI-6	Pro	resistant $K_i = 14$ μM				0.15		1.1	0.1

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. [b] $k_{cat}/K_m = (k_{cat}^{PF} + k_{cat}^{FS})/K_m$. P↓X and F↓S indicate the cleavage sites, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequences (Abz-GF and Abz-GFSP) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

Table 7: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-GFSPF**X**Q-EDDnp[a]

		TOP (24.15)				neurolysin (24.16)			
		k_{cat} (s^{-1})		K_m	k_{cat}/K_m	k_{cat} (s^{-1})		K_m	k_{cat}/K_m
no.	X	P↓F	F↓S	(μM)	(μM^{-1} s^{-1})	P↓F	F↓S	(μM)	(μM^{-1} s^{-1})
I-7	Ala	2.3		2.0	1.2	1.40	1.3	0.95	2.9[b]
II-7	Ile	11.1		1.7	6.5	0.61		0.42	1.5
III-7	Leu	3.1		4.4	0.7	5.9		5.6	1.1
IV-7	Phe	1.1		1.9	0.6	0.40	1.4	2.0	0.9[b]
V-7	Ser	1.4	1.2	7.0	0.4[b]	1.1	1.3	1.1	2.1[b]
VI-7	Asn	4.2	7.9	131	0.09[b]	0.64	1.1	3.3	0.5[b]
VII-7	Gln	2.3	0.82	3.8	0.8[b]	0.56	0.73	6.1	0.2[b]
VIII-7	Glu	1.6	11.8	119	0.1[b]	0.29	1.6	1.1	1.7[b]
IX-7	Asp		4.0	13.9	0.3		1.8	0.23	7.8
X-7	His	3.0	1.8	12.6	0.4[b]	0.88	1.4	2.4	1.0[b]
III-1	**Arg**	**6.3**		**2.2**	**2.9**	**0.31**		**0.58**	**0.5**
XI-7	Pro	7.0	0.69	20.3	0.4[b]	1.4		0.55	2.5

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. [b] $k_{cat}/K_m = (k_{cat}^{PF} + k_{cat}^{FS})/K_m$. P↓F and F↓S indicate the cleavage sites, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequences (Abz-GF and Abz-GFSP) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

The substrate containing Phe was hydrolyzed by TOP with the highest k_{cat}/K_m value (peptide IV-5) followed by the substrates with Ala, Arg, Asn, and Gln (peptides I-5, XI-5, VI-5, and VII-5). Neurolysin was more selective, preferring Asn followed by Arg (peptides VI-5 and XI-5). The substrates containing Glu and Asp (peptides VIII-5 and IX-5) were barely hydrolyzed by the two enzymes. The resistance of the peptide II-5 that contains Ile at P_1 to hydrolysis by TOP confirmed previous observation (*34*) but the obtained K_i value indicates that the peptide has significant affinity for the enzyme. Similarly, neurolysin hydrolyzed this peptide with low k_{cat} but also low K_m values in the series. The difference in primary specificity between the two enzymes are very subtle, therefore to differentiate these enzymes through specific substrates a combination of interactions at other subsites will be necessary.

Hydrolysis of the Abz-GFSPXRQ-EDDnp Series. Table 6 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-GFSPXRQ-EDDnp. TOP and neurolysin hydrolyzed all the substrates of this series at P−X bond; however, the hydrolysis at F−S bond was also observed with the substrates containing Ile, Leu, Glu, and Asp (peptides II-6, III-6, VII-6, and VIII-6) with both enzymes. The directional effect of Leu and Asp to the hydrolysis of F−S bond was very marked particularly with TOP. On the other hand, this enzyme preferred the substrates with Ala, followed by His, Ser and Phe (peptides I-6, IV-6, and V-6) at P_1' as indicate the higher k_{cat}/K_m values for these peptides in the series, which were hydrolyzed only at P−X bond. The substrate containing Arg at P_1' position (peptide X-6) was cleaved with the lowest K_m value by neurolysin, resulting the most efficiently hydrolyzed substrate by this enzyme ($k_{cat}/K_m = 14$ μM^{-1} s^{-1}). The low value of K_m obtained for the hydrolysis of substrate with Glu (peptide VII-6) and the hydrolysis at the amino site of Pro (peptide XI-6) in the peptide XI-6 by neurolysin are noteworthy differences between this enzyme and TOP that hydrolyzed the Glu-containing substrate with K_m 80 times higher and it did not hydrolyze the peptide XI-6 with Pro at P_1'.

Hydrolysis of the Abz-GFSPFXQ-EDDnp Series. Table 7 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-GFSPFXQ-EDDnp. TOP and neurolysin hydrolyzed all the substrates of this series at P−F bond; however, hydrophilic amino acids, particularly the negatively charged Glu and Asp (peptides VIII-7 and IX-7) strongly favored the hydrolysis at F−S bond. This is more clearly observed with the hydrolysis by neurolysin of Abz-GFSPFDQ-EDDnp (peptide IX-7) that is

Table 8: Kinetics Parameters for Hydrolysis by TOP and Neurolysin of the Peptides Derived from Abz-GFSPFR**X**-EDDnp[a]

		TOP (24.15)			neurolysin (24.16)		
no.	X	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
I-8	Ala	17.5	4.8	3.6	3.0	1.1	2.6
II-8	Ile	3.0	0.50	6.0	1.9	0.44	4.4
III-8	Leu	4.8	0.57	8.4	7.4	1.4	5.2
IV-8	Phe	7.1	0.69	10	1.8	0.22	8.4
V-8	Ser	4.3	4.3	1.0	2.1	0.65	3.2
VI-8	Asn	12.3	1.8	6.8	1.9	0.15	13
III-1	**Gln**	**6.3**	**2.2**	**2.9**	**0.31**	**0.58**	**0.5**
VII-8	Glu	3.1	1.2	2.6	1.1	0.18	6.1
VIII-8	Arg	0.77	0.19	4.0	0.45	0.21	2.1
IX-8	Pro	2.6	5.9	0.4	0.83	1.7	0.5

[a] The parameters were calculated as mean value ± SD, which was never greater than 5%. All the peptides were cleaved only at P↓F bond, which were determined, by comparison of the retention times of the reaction products with authentic synthetic sequence (Abz-GFSP) and by molecular weight obtained in the mass spectrometry analyses of the reaction mixture.

cleaved exclusively at F−S bond with the lower K_m and the higher k_{cat}/K_m values within the series. Therefore, these results strongly suggest that the S_4' subsite of neurolysin is a cationic locus that has a significant directional effect for the F−S cleavage. Similar carboxylate effect at P_4' position was observed in the substrates for TOP, although in less extent compared to neurolysin. This observation with TOP is in accordance to the favorable effect of free carboxyl group in its most potent inhibitors (*32*, *33*). The best substrates for TOP in this series are those containing Ile and Arg (peptides III-7 and III-1) while the substrate with Pro (peptide XI-7) is efficiently hydrolyzed by neurolysin, and these substrates are hydrolyzed only at P−F bond. These preferences for Arg and Pro at P_2' by TOP and neurolysin are, respectively, in accordance to the preferences found by these enzymes in the previously reported phosphinic inhibitors (*32*, *33*).

Hydrolysis of the Abz-GFSPFRX-EDDnp Series. Table 8 shows the kinetic parameters for the hydrolysis by TOP and neurolysin of the peptides from the series Abz-GFSPFRX-EDDnp, which were hydrolyzed only at P−F bond by both enzymes. The higher k_{cat}/K_m values obtained with TOP (peptides IV-8, III-8, and II-8) indicated its preference for hydrophobic amino acids at P_3', although the substrates with Asn and Arg (peptides VI-8 and VIII-8) were also susceptible to TOP. The best substrates in this series for neurolysin are those with Asn, Phe, Glu, and Leu (peptides VI-8, IV-8, VII-8, and III-8). Although these results indicate that neurolysin compared to TOP accommodates better hydrophilic or negatively charged residues at S_3', this subsite in both enzymes also fitted Arg, since the peptide VIII-8 is hydrolyzed with low K_m values. All together, these data suggest that S_3' subsite may be composed of a hydrophobic segment open to the solvent, which play a role in determining the hydrolysis of oligopeptides, since the presence of more rigid Pro (peptide IX-8) resulted in poor substrate for both enzymes.

CONCLUSIONS

The obtained results in this work indicate some clear preferences of TOP and neurolysin for amino acids at each position of the substrates that were hydrolyzed only at the position corresponding to P^4−F^5 bond. The cleavage at F−S bond depends on the nature of the amino acids that are at P_2, P_3', and P_4' position relative to this cleavage site. In this regard, particularly relevant is the strong directional effect to F−S cleavage of Asp and Glu at position P_4' relative to this cleavage (peptides VIII-7 and IX-7 in Table 7). It seems that some amino acids residues have large influence on the determination of K_m values of the substrates independently of its position in the substrate. Indeed, for TOP the presence of Ile resulted in substrates with lower K_m values in almost all series of peptides derived from Abz-GFSPFRQ-EDDnp. In contrast, the peptides containing Glu or Asp resulted in substrates with higher K_m values. For neurolysin, the presence of Arg and Asp resulted in substrates hydrolyzed with lower and higher K_m values, respectively. On the basis of these data, we are hypothesizing that some amino acids of the substrates can bind to different sites of the enzyme fitting P−F or F−S bond in a convenient position for hydrolysis. However, this requires a rapid interchange for the different forms of interaction as well as convenient conformations of the substrate in order to expose and fit the cleavage bonds in correct position for an efficient hydrolysis. According to this view, we have previously observed that the hydrolysis by TOP of the peptides Abz-GGFL↓RRV-EDDnp and Abz-GGFL↓R↓RVQ-EDDnp (arrows indicate the cleavage sites) correlated with sharp turns depicted from a detailed conformational analysis (*57*). Our data of circular dichroism and NMR with these peptides confirm the theoretical approach of conformation of these peptides as well as indicated a higher flexibility of Abz-GFSPFRQ-EDDnp (results not published). Finally, the plasticity of TOP and neurolysin interaction with the substrates can be an essential property for a class of cytosolic oligopeptidases that are candidates to participate in the selection of the peptides to be presented by MHC class I (*58*, *59*).

ACKNOWLEDGMENT

We would like to acknowledge the excellent technical assistance of Mrs. Eglelisa G. Andrade and also the contribution of Fernanda V. Portaro for providing pure recombinant neurolysin and finally Saul Jacchieri for performing theoretical analysis of the peptide conformations.

REFERENCES

1. Pierotti, A., Dong, K. W., Glucksman, M. J., Orlowski, M., and Roberts, J. L. (1990) *Biochemistry 29*, 10323−10329.
2. McKie, N., Dando, P. M., Rawlings, N. D., and Barrett, A. J. (1993) *Biochem. J. 295*, 57−60.
3. Dauch, P., Vincent, J.-P., and Checler (1995) *J. Biol. Chem. 270*, 27266−27271.
4. Rawlings, N. D. (2000) Merops database release 5.2, August 31, 2000, http://www.merops.co.uk.
5. Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (1998) *Handbook of Proteolytic Enzymes*, Academic, London.
6. Cummins, P. M., Pabon A., Margulies, E. H., and Glucksman, M. J. (1999) *J. Biol. Chem. 274*, 16003−16009.
7. Chen, J.-M., Stevens, R. A. E., Wray, P. W., Rawlings, N. D., and Barrett, A. J. (1998) *FEBS Lett. 435*, 16−20.
8. Barrett, A. J., and Rawlings, N. D. (1992) *Biol. Chem. Hoppe-Seyler 373*, 353−360.
9. Barrett, A. J., Brown, M. A., Dando, P. M., Knight, C. G., McKie, N., Rawlings, N. D., and Serizawa, A. (1995) *Methods Enzymol. 248*, 529−556.

10. Kawabata, S., and Davie, E. W. (1992) *J. Biol. Chem. 267*, 10331−10336.
11. Crack, P. J., Wu, T. J., Cummins, P. M., Ferro, E. S., Tullai, J. W., Glucksman, M. J., and Roberts, J. L. (1999) *Brain Res. 835*, 113−124.
12. Woulfe, J., Checler, F., and Beaudet, A. (1992) *Eur. J. Neurosci. 4*, 1309−1319.
13. Vincent, B., Dauch, P., Vincent, J.-P., and Checler, F. (1997) *J. Neurochem. 68*, 837−845.
14. Garrido, P. A., Vandenbulcke, F., Ramjaun, A. R., Vincent, B., Checler, F., Ferro, E., and Beaudet, A. (1999) *DNA Cell Biol. 18*, 323−331.
15. Ferro, E. S., Tullai, J. W., Glucksman, M. J., and Roberts, J. L. (1999) *DNA Cell Biol. 18*, 781−789.
16. Oliveira, V., Ferro, E. S., Gomes, M. D., Oshiro, M. E., Almeida, P. C., Juliano, M. A., and Juliano, L. (2000) *J. Cell. Biochem. 76*, 478−488.
17. Vincent, B., Beaudet, A., Dauch, P., Vincent, J.-P., and Checler, F. (1996) *J. Neurosci. 16*, 5049−5059.
18. Camargo, A. C. M., Caldo, H., and Reis M. L. (1979) *J. Biol. Chem. 254*, 5304−5307.
19. Fulop, V., Bocskei, Z., and Polgar, L. (1998) *Cell 94*, 161−170.
20. Polgár, L. (1992) *Biochem. J. 283*, 647−648.
21. Polgár, L. (1992) *Biochemistry 31*, 7729−7735.
22. Orlowski, M., Michaud, C., and Chu, T. G. (1983) *Eur. J. Biochem. 135*, 81−88.
23. Checler, F., Vincent, J. P., and Kitabgi, P. (1986) *J. Biol. Chem. 261*, 11274−11281.
24. Acker, G. R., Molineaux, C., and Orlowski, M. (1987) *J. Neurochem. 48*, 284−292.
25. Dahms, P., and Mentlein, R. (1992) *Eur. J. Biochem. 208*, 145−154.
26. Tisljar, U., Knight, C. G., and Barrett, A. J. (1990) *Anal. Biochem. 186*, 112−115.
27. Dauch, P., Barelli, H., Vincent, J. P., and Checler, F. (1991) *Biochem. J. 280*, 421−426.
28. Barelli, H., Dive, V., Yiotakis, A., Vincent, J. P., and Checler, F. (1992) *Biochem. J. 287*, 621−625.
29. Vincent, B., Dive, V., Yiotakis, A., Smadja, C., Maldonado, R., Vincent, J. P., and Checler, F. (1995) *Br. J. Pharmacol. 115*, 1053−1063.
30. Orlowski, M., Reznik, S., Ayala, J., and Pierotti, A. R. (1989) *Biochem. J. 261*, 951−958.
31. Orlowski, M., Michaud, C., and Chu T. G. (1983) *Eur. J. Biochem. 135*, 81−88.
32. Jiracek, J., Yiotakis, A., Vincent, B., Lecoq, A., Nicolaou, A., Checler, F., and Dive, V. (1995) *J. Biol. Chem. 270*, 21701−21706.
33. Jiracek, J., Yiotakis, A., Vincent, B., Checler, F., and Dive, V. (1996) *J. Biol. Chem. 271*, 19606−19611.
34. Knight, C. G., Dando, P. M., and Barrett, A. J. (1995) *Biochem. J. 308*, 145−150.
35. Dando, P. M., Brown, M. A., and Barrett, A. J. (1993) *Biochem. J. 294*, 451−457.
36. Knight, C. G., and Barrett, A. J. (1991) *FEBS Lett. 294*, 183−186.
37. Juliano, L., Chagas, J. R., Hirata, I. Y., Carmona, E., Sucupira, M., Oliveira, E. S., Oliveira, E. B., and Camargo, A. C. M. (1990) *Biochem. Biophys. Res. Commun. 173*, 647−652.
38. Camargo, A. C. M., Gomes, M. D., Reichl, A. P., Ferro, E. S., Jacchieri, S., Hirata, I. Y., and Juliano, L. (1997) *Biochem. J. 324*, 517−522.
39. Kawabata, S., Nakagawa, K., Muta, T., Iwanaga, S., and Davie, E. W. (1993) *J. Biol. Chem. 268*, 12498−12503.
40. Barelli, H., Woskowska, Z., Cipris, S., Fox-Threlkeld, J. E. T., Daniel, E. E., Vincent, J. P., and Checler, F. (1995) *J. Pharm. Exp. Ther. 275*, 1300−1307.
41. Vincent, B., Jiracek, J., Noble, F., Loog, M., Roques, B., Dive, V., Vincent, J.-P., and Checler, F. (1997) *Br. J. Pharm. 121*, 705−710.
42. Glucksman, M. J., and Roberts, J. L.(1995) in *Methods in Neurociences − Peptidases and neuropeptidases processing* (Smith, I., Ed.) pp 281−285, Academic Press, New York.
43. Knight, C. G. (1995) *Methods Enzymol. 248*, 85−101.
44. Rioli, V., Kato, A., Portaro, F. C. V., Cury, G. K., Kaat, K., Vincent, B., Checler, F., Camargo, A. C. M., Glucksman, M. J., Roberts, J. L., Hirose, S., and Ferro, E. S. (1998) *Biochem. Biophys. Res. Commun. 250*, 5−11.
45. Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
46. Matsudaira, P. T., and Burgess, D. R. (1978) *Anal. Biochem. 87*, 386-390.
47. Hirata I. Y., Cesari, M. H. S., Nakaie, C. R., Boschcov, P., Ito, A. S., Juliano, M. H., and Juliano, L. (1994) *Lett. Pept. Sci. 1*, 299−301.
48. Chagas, J. R., Juliano, L., and Prado, E. S. (1991) *Anal. Biochem. 192*, 419−425.
49. Araujo, M. C., Melo, R. L., Cesari, M. H.m, Juliano, M. A., Juliano, L., and Carmona, A. K. (2000) *Biochemistry 39*, 8519−8525.
50. Wilkinson, G. N. (1961) *Biochem. J. 80*, 324−332.
51. Leatherbarrow, R. J. (1992) Grafit Version 3.0, Erithacus Software Ltd., Staines, U.K.
52. Nicklin, M. J., and Barrett, A. J. (1984) *Biochem. J. 223*, 245−253.
53. Heinrikson, R. L., and Meredith, S. C. (1984) *Anal. Biochem. 436*, 65−74.
54. Hayashi, M. A. F., Portaro, F. C. V., Tambourgi, D. V., Sucupira, M., Yamane, T., Fernandes, B. L., Ferro, E. S., Rebouças, N. A., and Camargo, A. C. M. (2000) *Biochem. Biophys. Res. Commun. 269*, 7−13.
55. Barelli, H., Vincent, J.-P., and Checler, F. (1993) *Eur. J. Biochem. 211*, 79−90.
56. Wilson, D. R., and Finlay, B. B. (1997) *Protein Eng. 10*, 519−29.
57. Jacchieri, S. G., Gomes M. D., Juliano, L., and Camargo, A. C. M. (1998) *J. Pept. Res. 51*, 452−459.
58. Portaro, F. C. V., Gomes, M. D., Cabrera, A., Fernandes, B. L., Silva, C. L., Ferro, E. S., Juliano, L., and Camargo, A. C. M. (1999) *Biochem. Biophys. Res. Commun. 255*, 596−601.
59. Silva, C. L., Portaro, F. C. V., Bonato, V. L. D., Camargo, A. C. M., and Ferro, E. S. (1999). *Biochem. Biophys. Res. Commun. 255*, 591−595.

BI002715K