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Physico-chemical and immunological properties and partial amino acid sequencing of a new metalloprotease: endoprotease Thr-N

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Abstract

Previous studies have described the isolation of a new metalloprotease with a strict specificity for the amide bonds of peptide substrates having a threonine residue at the P1′ position [Biochem. Biophys. Res. Commun. 256 (1999) 307]. The present work reports the physico-chemical properties of the enzyme which enable the optimal conditions for the digestion of proteins by the protease to be determined. At pH 8.2 and up to 37 °C, the enzyme possesses a good proteolytic activity and is stable for at least 12 h. The protease is sensitive to detergents and dithiol-reducing agents so that these chemicals must be eliminated after treatment of the protein substrate when this needs to be denatured and reduced before its hydrolysis by the enzyme. An increase in the enzymatic activity is observed in the presence of urea up to a 2.0 M concentration, beyond which the activity decreases. The enzyme can also be used in the presence of organic solvents such as acetonitrile, isopropanol or dioxane (10%, v/v) without loss of activity. Studies performed with antibodies raised against the purified endoprotease Thr-N indicated the absence of cross-immunoinactivation and cross-immunoprecipitation with all tested proteases. Also, no homology of sequence was found with the proteases indexed in the databases. Thus, our results show that endoprotease Thr-N not only represents an original protease by its unique specificity but also by its immunological and molecular properties.

Keywords: Metalloprotease; Endoprotease Thr-N; Enzymatic specificity; Digestive enzyme; Snail (*Achatina*)

1. Introduction

To date, a very limited number of proteases possess a narrow specificity [1–9]. Recently, we have purified from the digestive juice of a gastropod, *Archachatina ventricosa*, such an enzyme that displays a novel and unique specificity [10]. This enzyme, identified as a metalloendoprotease, catalyzes the hydrolysis of only the amide bonds involving the N-terminal side of threonine residues (i.e. at the P1′ position) in peptides such as leucopyrokinin, insulin B-chain or glucagon [10]. However, it is well known that structured polypeptides like the globular proteins are hydrolyzed slowly and to only a limited extent by proteases [11]. Hence, most protein substrates require the cleavage of their disulfide bonds and a treatment by denaturing reagents before their enzymatic digestion.

In this study, we report the behavior of the protease in the presence of ions, detergents, dithiol-reducing agents and organic solvents. The effects of pH and temperature on the activity and stability of the enzyme are also presented and lead to the definition of the optimal conditions to digest the proteins by the enzyme. Immunological studies and partial amino acid sequencing are also reported to evaluate the structural relatedness of endoprotease Thr-N with the known proteases.

2. Materials and methods

2.1. Enzymatic source and purification

The protease was purified from the digestive juice of the giant snail, *A. ventricosa* (formerly named *Achatina balteata*), by chromatography on columns of Sephacryl S-300, DEAE-Sepharose and phenyl-Sepharose as reported previously [10].

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2.2. Enzyme assays

Under the standard test conditions, the enzyme activity was measured at 37 °C for 5 min in 70 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 0.5 mM Val-His-Leu-Thr-Pro as the substrate. The final reaction volume was 60 μl and the reaction was stopped by heating the mixture at 100 °C for 10 min. The substrate was cleaved into Val-His-Leu and Thr-Pro by the protease and its hydrolysis was easily monitored by capillary electrophoresis [10].

2.3. Effect of pH and temperature on activity and stability

In these experiments, Val-His-Leu-Thr-Pro was used as the peptidic substrate. The highest measured activity was set at 100%. For determination of the pH optimum, protease activity was measured by performing the assays at various pH values in the following buffer systems: sodium acetate buffer (70 mM) from pH 4.5 to 5.5, sodium phosphate buffer (70 mM) from pH 5.7 to 8.0, Tris–HCl buffer (70 mM) from pH 7.2 to 9.0 and glycine–NaOH buffer from 8.8 to 10.0. All the buffers contained 100 mM NaCl. For the pH-stability study, the enzyme solutions were preincubated at 37 °C for 1 h in the Tris–HCl buffer at various pH values between 7.2 and 9.0. After adjusting the mixtures to pH 8.0, the residual activity was measured under the standard assay conditions.

For determination of the temperature optimum, the incubations were performed for 7 min in 70 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl at temperatures ranging from 25 to 75 °C. The thermal inactivation of the protease was studied at 37, 40 and 55 °C by prewarming the enzyme solutions in 70 mM Tris–HCl buffer (pH 8.2) containing 100 mM NaCl. Aliquots were removed at different times and the residual activity was measured at 37 °C under the standard conditions.

2.4. Effect of ions and denaturing agents

Ions were incubated with the enzyme for 15 min at room temperature, then the protease activity was measured under the standard test conditions. The final concentration of ions in the reaction mixture was 1 mM. Studies with denaturing agents were performed under the same conditions except for a preincubation time of 1 h with the enzyme.

2.5. Analytical isoelectric focusing

IEF-PAGE was performed using 0.3-mm-thick precast gels (Servalyt Precotes, 12.5 × 12.5 cm; pH gradient range: 3–10) according to the instructions given in the Serva IEF application note 7-90. The isoelectric point of the protease was determined with reference to standard protein markers (Amersham Biosciences) ranging from p*I* 3.5 to 9.3.

2.6. Sedimentation coefficient and Stokes radius determinations

Sedimentation analyses were performed in 4–20% (w/v) linear sucrose gradients prepared in 10 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl. Samples were mixed with sedimentation markers (alkaline phosphatase, 6.1 S; catalase, 11.3 S; β-galactosidase, 16.0 S) carefully layered onto gradients and centrifuged for 15 h at 119,000 × *g* and 4 °C in a Beckman SW40 Ti rotor. Gradients were fractionated from top to bottom and fractions of 0.250 ml were collected. The Stokes radius determination was carried out by chromatography on a Sephacryl S-300 HR column as described by Siegel and Monty [12].

2.7. Amino acid analysis

The purified protease preparation was thoroughly dialyzed against 0.5% (v/v) acetic acid, freeze-dried and hydrolyzed at 110 °C for 24 h in a sealed tube under nitrogen in 6 M HCl containing 0.02% (v/v) 2-mercaptoethanol and crystals of phenol to protect tyrosine from oxidation. Free amino acids were derivatized with phenylisothiocyanate (PITC). Separation and identification of the resulting phenylthiocarbamyl-amino acids were carried out at 50 °C according to the method of Bidlingmeyer et al. [13] on a Pico-tag column (0.39 × 15 cm) from Waters.

2.8. Cyanogen bromide cleavage, separation of peptide fragments and N-terminal amino acid sequence analysis

The protease preparation (2 nmol, 400 μg) was dialyzed against distilled water for 12 h, then denatured and reduced with 8 M urea and 14 mM 2-mercaptoethanol at room temperature for 4 h in the dark and finally *S*-carboxymethylated with 14 mM iodoacetate for 15 min. After dialysis against distilled water and lyophilization, the carboxymethylated protease was incubated in the presence of a 2000-fold molar excess of cyanogen bromide in 70% formic acid at 4 °C in the dark with constant stirring. After 24 h, the same amount of reagent was added for a further 24-h incubation. After lyophilization, the resulting peptides were dissolved in 0.1 M Tris–Tricine buffer and were separated by Tricine SDS-PAGE according to the method of Schägger and von Jagow [14] using 16.5% acrylamide gels. Peptides were then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon Q, Millipore) using a Bio-Rad Mini Trans-Blot Cell. Electroblotting was performed at room temperature for 90 min at 350 mA using a 50 mM Tris–HCl/50 mM borate buffer (pH 8.3) as transfer solution. After transfer, the peptides were stained with a 0.25% (w/v) Coomassie blue solution containing 40% (v/v) methanol.

The amino acid sequence of the peptides was determined by performing Edman degradation on an Applied Biosys-

tems liquid–gas phase protein sequencer (model 477A) equipped with on-line HPLC detection [15].

2.9. Immunological methods

Polyclonal antibodies against endoprotease Thr-N were raised in two rabbits by subcutaneous dorsal injections (about 10 shots into the back of the animal). For the first injection, each rabbit received an emulsion containing 200 μl of complete Freund's adjuvant and 200 μl of the enzyme (200 μg) solution. After 2 weeks, a second injection was performed with an incomplete Freund's adjuvant, the emulsion containing the same amount of enzyme (200 μg). Three booster injections were performed at 3-week intervals. One week after each booster, a blood sample was taken via the ear vein to control the titer in antibodies by ELISA. The final bleeding was intracardiac. The blood was allowed to clot for 8 h at room temperature. Finally, the serum was collected by centrifugation at $20{,}000 \times g$ for 15 min and 500-μl aliquots were frozen in liquid nitrogen and stored at −80 °C. Control serum (pre-immune serum) was also prepared 1 day prior to the first injection.

Rocket immunoelectrophoresis was performed as described by Axelsen et al. [16]. Ten milliliters of 1% (w/v) agarose gel in 37.5 mM Tris–100 mM glycine buffer (pH 8.6) containing 0.8% (v/v) emulfogen (polyoxyethylene 10-tridecyl ether) was layered on glass plates (10 × 10 cm). The same buffer but without emulfogen was placed in the electrode compartments. Immunoelectrophoresis was carried out at 1 V/cm for 18 h and the immuno-plates were then washed in phosphate-buffered saline (PBS) for 24 h and in distilled water for another 24 h. Finally, the gels were dried, stained with a 0.25% (v/v) Coomassie blue solution for 5 min and destained.

3. Results

3.1. pH and temperature dependence of the activity of endoprotease Thr-N

The effect of pH on the proteolytic activity of the enzyme was studied at 37 °C by measuring the hydrolysis of Val-His-Leu-Thr-Pro between pH 4.5 and 10.0 in various buffers. The pH curve displayed a maximal activity at around pH 8.0 in Tris–HCl buffer, but endoprotease Thr-N retained more than 80% of its activity in the range pH 7.5–8.5 (Fig. 1). A maximum of activity was also found at pH 8.0 in phosphate buffer but, in this case, the activity was reduced by about 20% when compared to that obtained in Tris–HCl buffer (data not shown). The effect of the nature of the buffer was also sensitive at pH 9.0 since at this pH the enzyme displayed a higher activity in the glycine–NaOH buffer than in the Tris–HCl buffer (ratio of 1.7).

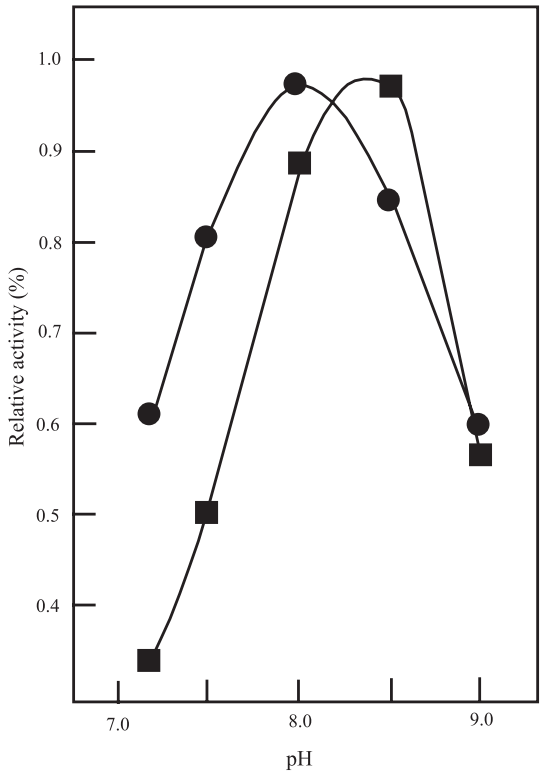

Fig. 1. Effect of pH on the activity (●) and the stability (■) of endoprotease Thr-N. The experiments were carried out at 37 °C with Val-His-Leu-Thr-Pro as substrate and using 70 mM Tris–HCl buffers containing 100 mM NaCl; for further details, see Materials and methods.

The activity of endoprotease Thr-N was also studied at various temperatures ranging between 25 and 75 °C. The incubations were performed for 7 min according to the standard assay procedure. Under these conditions, the activity was optimal at 55 °C (Fig. 2) and the value of the temperature coefficient (Q_{10}), calculated between 35 and 45 °C, was found to be 1.3. The latter is much lower than that observed for most enzymes (Q_{10} around 2.0). From the Arrhenius plot (inset, Fig. 2), a value of 21.6 kJ/mol was calculated for the activation energy.

3.2. Stability of endoprotease Thr-N

3.2.1. Storage

The enzyme preparations could be stored for several weeks at 4 °C in a 15 mM Tris–HCl buffer (pH 8.2) without appreciable loss of activity. Repeated cycles of freezing and thawing affected the activity of the protease resulting in a loss of 10% to 15% of the activity after each cycle.

3.2.2. pH stability

The profile of the pH dependence of the stability of the enzyme showed a maximal stability at pH 8.4 when endoprotease Thr-N was preincubated for 1 h at 37 °C in 70 mM Tris–HCl buffer containing 100 mM NaCl (Fig. 1).

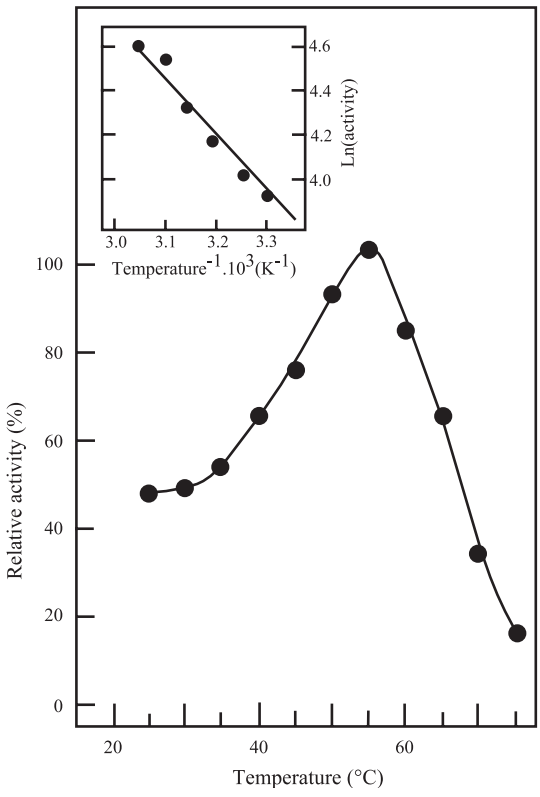

Fig. 2. Effect of temperature on the activity of endoprotease Thr-N. The experiments were carried out at the indicated temperatures for 7 min in 70 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 0.5 mM Val-His-Leu-Thr-Pro. Inset: Arrhenius plot of the data in the range 30–55 °C.

However, the protease exhibited a significant stability (more than 85%) at the pH optimum (pH 8.0) of its proteolytic activity.

3.2.3. Thermal stability

Fig. 3 shows the thermal inactivation kinetics performed at 37, 40 and 55 °C. After a 12-h incubation at 37 °C, the enzyme retained 90% of its initial activity. At 40 °C, an activity loss of about 30% was observed after 90 min while the half-life of the protease was 10 min at 55 °C.

3.3. Effect of metal ions on the activity of endoprotease Thr-N

The enzyme activity was completely inhibited after treatment of endoprotease Thr-N with the metal-chelating EDTA (1 mM) and 1,10-phenanthroline (0.5 mM). The activity could be almost totally restored (90%) by addition of 10 μM $ZnCl_2$m, suggesting a direct participation of a zinc ion in the catalytic process [10].

The activator or inhibitor effects of mono- and divalent cations on the enzyme activity were also studied (Table 1). Li^+, K^+ and Mg^{2+} slightly activated the enzyme. Cu^{2+} and Fe^{2+} strongly inhibited the activity whereas Mn^{2+}, Hg^{2+} and Co^{2+} had a much more limited inhibitory effect.

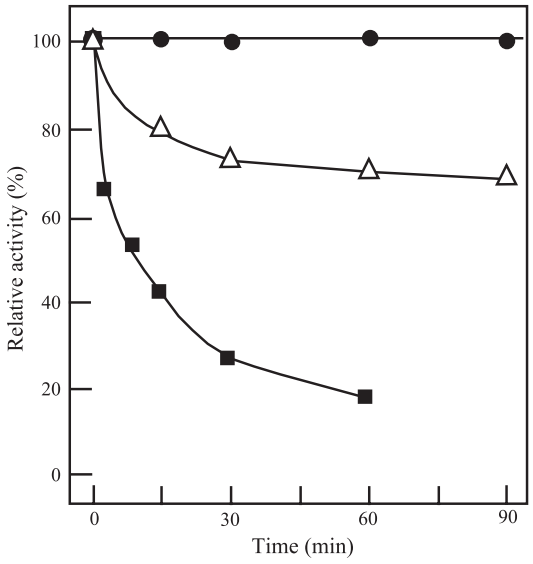

Fig. 3. Thermal inactivation of endoprotease Thr-N. The enzyme was preincubated at 37 °C (●), 40 °C (△) and 55 °C (■) in 70 mM Tris–HCl buffer (pH 8.2) containing 100 mM NaCl. At the indicated times, aliquots were withdrawn and the residual activity was measured at 37 °C under the standard assay conditions.

3.4. Behavior of endoprotease Thr-N in the presence of dithiol-reducing agents, detergents and organic solvents

3.4.1. Dithiol-reducing agents

Although the scission of disulfide bonds by performic acid oxidation is a classic technique in protein chemistry, this method leads not only to transformations of tryptophan residues but also may oxidize, under drastic experimental conditions, phenolic groups and the hydroxyl functions of serine and threonine residues [17]. As a consequence, procedures which involve reductive cleavage and subsequent blocking of the thiol groups must be preferred for quantitative digestion by endoprotease Thr-N of protein

Table 1
Effect of cations on the activity of endoprotease Thr-N

Cation (1 mM)	Proteolytic activity (percent of control)
None	100
Li^+	119 ± 2
K^+	116 ± 4
Mg^{2+}	113 ± 3
Ca^{2+}	105 ± 3
Zn^{2+}	90 ± 2
Mn^{2+}	84 ± 1
Hg^{2+}	78 ± 3
Co^{2+}	72 ± 2
Fe^{2+}	35 ± 3
Cu^{2+}	7 ± 1

All activities were measured under the conditions of initial velocity. The values reported represent the means and standard errors of four determinations.

substrates containing disulfide bonds. β-Mercaptoethanol is one of the reagents most widely used for the reduction of disulfide bonds. For this reason, its effect was tested on the activity of the protease. At a concentration of 0.1% (v/v), the β-mercaptoethanol reduced the enzyme activity by about 75% and the protease was totally inactivated when the reductive agent was present at 1% (v/v) in the reaction mixture. The other reductive agents such as cysteine and dithiothreitol displayed behaviors identical to that of β-mercaptoethanol.

3.4.2. Detergents and urea

The effect of ionic and nonionic detergents currently used for the denaturing of proteins was tested on the protease. The results reported in Table 2 show that all of them are inhibitors of the protease activity but to differing degrees. As for urea, it can be present in the reaction mixture up to a concentration of 2 M without loss of the protease activity. Below this concentration, urea even leads to an activation of the enzymatic activity (Fig. 4).

3.4.3. Organic solvents

One of the main advantages of carrying out enzyme reactions in organic media is avoiding the problems of solubility of hydrophobic substrates in water. This is why we have examined the effect of various organic solvents at different concentrations on the protease activity. For up to 10% concentrations of all the tested organic solvents, except ethanol, the enzymatic activity of endoprotease Thr-N was preserved. An activation of the enzyme was even observed with acetonitrile and dioxane (Fig. 5).

3.5. Molecular mass and molecule asymmetry

The protease, studied by sucrose gradient centrifugation, sedimented at 6.8 S and was characterized by a Stokes radius (R_s) of 6.2 nm as determined by gel-filtration. From these parameters, a relative molecular mass of 180,000 was calculated. The frictional ratio was estimated using the following relations:

$$f = 6\pi\eta R_s \text{ and } f_o = 6\pi\eta\left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3}$$

In these equations, η is the viscosity of the medium, N the Avogadro number and $\bar{v}$ the partial specific volume of the protein determined by summation of the individual values of $\bar{v}$ for the amino acids. Under these conditions, the f/f_o ratio for endoprotease was found to be 1.66, suggesting an important asymmetry of the protein molecule.

3.6. Isoelectric point and glycan detection

By isoelectric focusing gel electrophoresis, the isoelectric point of the protease was estimated to be 5.2. The periodic

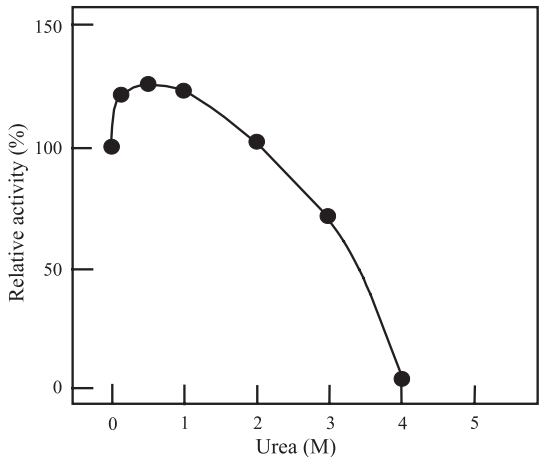

Fig. 4. Effect of urea on the activity of endoprotease Thr-N. The enzyme activity was measured at 37 °C under the standard assay conditions in the presence of urea at the indicated concentrations.

Table 2
Effect of detergents and reducing agents on the activity of endoprotease Thr-N

Reagent	Concentration	Proteolytic activity (percent of control)
None		100
Cholate	1% (w/v)	47 ± 2
Deoxycholate	1% (w/v)	37 ± 4
Tween 20	1% (v/v)	28 ± 3
Triton X-100	1% (v/v)	33 ± 2
Nonidet P40	1% (v/v)	48 ± 1
SDS	1% (w/v)	10 ± 2
β-mercaptoethanol	0.1% (v/v)	24 ± 2
	1% (v/v)	0
Cysteine	1% (w/v)	0
Dithiothreitol	1% (w/v)	0

All activities were measured under the conditions of initial velocity. The values reported represent the means and standard errors of four determinations.

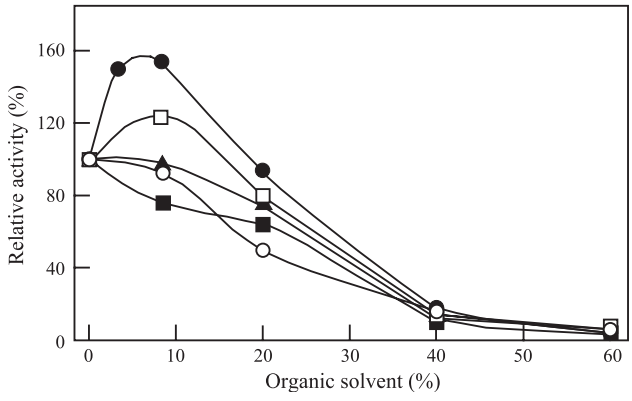

Fig. 5. Effect of organic solvents on the activity of endoprotease Thr-N. The enzyme was incubated at 37 °C in 70 mM Tris–HCl buffer (pH 8.2) containing 100 mM NaCl. The enzyme activity was measured in the presence of 1,4-dioxane (●), acetonitrile (□), isopropanol (▲), DMSO (○) and ethanol (■).

Table 3
Amino acid composition of endoprotease Thr-N

Amino acid	Number of residues (%)
Alanine	6.5
Arginine	4.2
Aspartic acid-asparagine	10.8
Cysteine	9.3
Glutamic acid-glutamine	10.5
Glycine	6.6
Histidine	3.5
Isoleucine	4.5
Leucine	6.4
Lysine	4.1
Methionine	2.9
Phenylalanine	3.7
Proline	7.6
Serine	6.9
Threonine	6.0
Tryptophan	n.d.[a]
Tyrosine	2.1
Valine	4.4

[a] n.d.: not determined.

acid-Schiff staining after electrophoresis of endoprotease Thr-N was negative. In addition, no shift of bands was observed when the protease was run in SDS-PAGE with and without treatment with the deglycosylation kit (Prozyme) containing all the enzymes (PNGase F, nonspecific neuraminidase, *O*-glycosidases) needed to completely remove all N- and O-linked carbohydrates from glycoproteins. Under the same conditions, fetuin used as the control gave a positive glycosylation.

3.7. Amino acid composition

The amino acid composition of the protease is given in Table 3. The relative degree of the polar or nonpolar character of a protein depends on the proportion of both hydrophilic (H) and apolar (A) sets of amino acids. We have classified Asx, Glx, Lys, Arg, Ser, Thr and His as hydrophilic residues (H) and Val, Ile, Leu, Phe and Met as apolar residues (A). This classification follows that proposed by Hatch and Bruce [18]. The H/A ratio calculated from the data reported in Table 3 is 2.1 and corresponds to that of most soluble proteins (H/A = 2.1).

3.8. Internal amino acid sequencing

No phenylthiohydantoin amino acid was detected when the two subunits of endoprotease Thr-N were subjected to the analysis of the N-terminal residue sequence using the Edman degradation method. The data suggest that the N-terminal amino acid of each subunit is blocked. Therefore, the enzyme was cleaved by cyanogen bromide and several internal peptidic fragments were sequenced from their N-terminal extremity. The results are reported in Fig. 6. The obtained peptide sequences displayed no significant similarity to the sequences of proteases found in the protein sequence databases (BLAST).

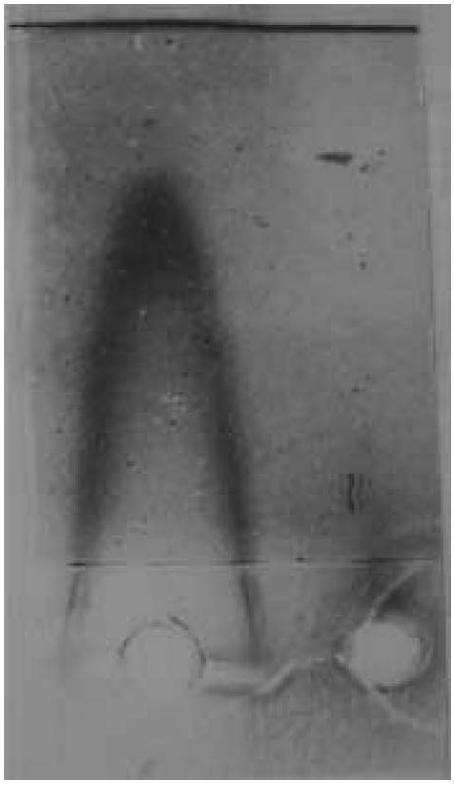

Fig. 7. Rocket immunoelectrophoresis. Anti-endoprotease Thr-N antibodies were tested with endoprotease Thr-N (left well) and trypsin (right well).

3.9. Immunological studies

Antibodies were raised in rabbits against the purified endoprotease Thr-N. These antibodies were able to inactivate the enzyme. A control experiment performed using only pre-immune serum showed no immunoinactivation. Furthermore, the antibodies gave, by the rocket immunoelectrophoresis technique, a peak of immunoprecipitation with the protease (Fig. 7). Tests of immunological cross-

Fragment	Residues	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
F3	NH_2	K	L	I	K	G	X	F	L	Q	X	G	Y	T	I	D	H	Q	R	T
F4	NH_2	I	L	I	K	G	P	L	K	H	G	F	K	A	N					
F5	NH_2	G	P	X	T	Q	V	Q	K	S										

Fig. 6. N-terminal amino acid sequences of three CNBr-fragments F3, F4 and F5 obtained from endoprotease Thr-N.

reactions were performed with these antibodies and various proteases. No cross-immunoinactivation and cross-immunoprecipitation were observed with the tested proteases whether they were metalloproteases (carboxypeptidases A and B, thermolysin, collagenase, leucine-aminopeptidase), serine-proteases (elastase, chymotrypsin A4, endoprotease Glu-C, proteinase K, trypsin), cysteine-proteases (bromelain, papain) or an aspartic-protease (pepsin). Thus, endoprotease Thr-N does not share common antigenic properties with the other proteases.

4. Discussion

Like most gastropods, the digestive juice of *A. ventricosa* is particularly reputed as a very rich source of glycosidases [19–22]. Only a few authors have reported the presence of low protease activities from gastropods [23–25]. However, by exploiting the transglycosylation properties of glycosidases for glycosylating ester derivatives of amino acids and dipeptides [26,27], we have been led to identify several proteases with original and narrow specificities in the *A. ventricosa* digestive juice [28]. In the present study, we have reported some physico-chemical, molecular and immunological properties of one of the most remarkable of them: endoprotease Thr-N. This enzyme possesses a very narrow specificity limited to peptide bonds containing a threonine residue in the P1′ position according to the nomenclature proposed by Schechter and Berger [29].

Unlike the well-known digestive endoproteases which are monomeric and have molecular weights of 20,000–40,000, endoprotease Thr-N is unusually large. It has a native molecular weight of about 180,000 and is composed of two subunits of 90,000 and 121,000 as determined previously by SDS-PAGE [10]. Here, it is interesting to note that all enzymes characterized in the *Achatina* digestive juice have higher molecular weights than their counterparts from other sources [22,30]. Typical of metalloendopeptidases, endoprotease Thr-N is completely inhibited by chelating agents such as EDTA and 1,10-phenanthroline and can be reactivated by Zn^{2+} at low concentration [10].

Since only relatively large peptides would be liberated by the action of endoprotease Thr-N from proteins, this enzyme is expected to be of limited importance in the nutrition of the organism, but it may be useful for applications requiring the selective cleavage of proteins such as sequence analysis, peptide mapping or isolation of domains. In this context, the characterization of physico-chemical properties enables the experimental conditions for the digestion of proteins by the protease to be optimized. The activity of the enzyme is maximal at pH 8.0 but it displays a better stability at pH 8.4. So, a pH of 8.2 is a good compromise between the activity and stability of the enzyme to perform the specific hydrolysis of proteins over a long time-period. The importance of the nature of the buffer should also be noted, the enzyme activity being higher in a glycine–NaOH buffer than in a Tris–HCl buffer. The protease is sensitive to detergents and dithio-reducing agents so that these chemicals must be eliminated after treatment of the protein substrate when this needs to be reduced and denatured before its hydrolysis by the enzyme. The loss of activity by the reductive agents suggests that disulfide bonds play an essential role in the native conformation of endoprotease Thr-N. Most native proteins are rapidly unfolded by 8 M urea and tend to remain in this state even when the protein solution is later diluted to 2 M with respect to urea [11]. Thus, it is of particular interest to find that the protease is active in 2 M urea. Digestion under these conditions can be attempted for proteins that are not readily attacked by the protease under nondenaturing conditions. It should also be noted that the increase in activity in the presence of urea cannot be due in our experimental conditions to the sensitivity of the protein substrate to urea, making it more susceptible to enzyme hydrolysis, since the substrate used in this study was a small peptide (Val-His-Leu-Thr-Pro). Thus, the fact that the protease is always active in the presence of urea suggests that hydrogen bonds play a limited role in the stabilization of the protease. The enzyme shows good stability in the presence of organic solvents, an activation even being observed with acetonitrile and dioxane. The latter is particularly valuable to render soluble a certain number of proteins like, for example, the vegetable proteins [31].

The immunological studies show that no cross-immunoinactivation and cross-immunoprecipitation were observed between the anti-endoprotease Thr-N antibodies and all tested proteases from vegetable or animal origin. It therefore appears that the degree of structural homology between endoprotease Thr-N and the other known proteases is not sufficient to generate common antigenic determinants. In addition, the amino acid sequences obtained from the analysis of the N-terminal residue sequences of three internal peptides display no significant similarity to the sequences of proteases found in the protein sequence databases. In conclusion, endoprotease Thr-N not only represents an original protease by its unique specificity but also by its molecular properties.

The physiological function of the enzyme remains unknown. Considering its high specificity, it is probable that this protease does not play an essential role in the digestive process. Our first approach to determine the role of endoprotease Thr-N was to examine its cellular and intracellular localization by immunological studies. But this search failed due to the fact that polyclonal antibodies raised against the enzyme recognized not only endoprotease Thr-N but also other proteases purified to homogeneity from the same source [28]. In the same way, the detection of the endoprotease Thr-N activity in the different tissues is hindered by the presence of other proteases with wider specificities overlapping that of endoprotease Thr-N. To get round these difficulties, the preparation of monoclonal antibodies is now in progress.

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