Purification, molecular properties and specificity of a thermoactive and thermostable proteinase from *Pyrococcus abyssi*, strain st 549, hyperthermophilic archaea from deep-sea hydrothermal ecosystem

Robert Dib[a], Jean-Marc Chobert[a,*], Michèle Dalgalarrondo[a], Georges Barbier[b], Thomas Haertlé[a]

[a] *Institut National de la Recherche Agronomique, Laboratoire d'Etude des Interactions des Molécules Alimentaires, B.P. 71627, 44316 Nantes Cedex 3, France*
[b] *IFREMER, Laboratoire de Caractérisation des Micro-organismes Marins, B.P. 70, 29280 Plouzané, France*

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Abstract A protease was isolated and purified from the supernatant of a culture of hyperthermophilic archaebacteria: *Pyrococcus abyssi* strain st 549. Purification consisted of three chromatographic steps. The enzyme purification yield was 4% and the purification factor 890. This protease is a seryl-protease hydrolyzing proteins and peptides with a preference for cleavage at the aromatic and hydrophobic residues in P1 and P′1 positions. Its activity is optimal at 95°C and at pH 9. The electrophoretic mobility of the protease observed by zymogram suggests that it can adopt several oligomer forms. Three of them predominate displaying apparent molecular masses of 150, 105 and 60 kDa. Interdependence of the observed bands was revealed by changing the denaturation conditions of the samples (temperature, SDS concentration) before electrophoresis.

Key words: Protease; Aggregation; *Pyrococcus abyssi*

1. Introduction

In the past few years, the upper temperature limit for life was considerably extended by the discovery of extraordinary bacteria whose optimum temperatures for growth approach, and in some cases exceed, 100°C. Hyperthermophilic bacteria were isolated from marine geothermal springs, including hydrothermal vents more than 2500 m below sea level under pressures exceeding 250 atm. They were also found in shallow marine environments, at terrestrial geothermal sites (such as geysers and volcanoes), and even in the boiling outflows of geothermal power plants.

These microorganisms utilize peptides and carbohydrates to satisfy their nutritional requirements [1–3]. Underlying the metabolic processes of these organisms are enzymes functioning best under extreme conditions. Several of these hydrolases were isolated from many hyperthermophilic species and biochemically characterized [4]. Otherwise, certain enzymes originating from conventional organisms were also shown to be able to function under extreme conditions, including non-aqueous solvents. As such, the biochemist is faced with the perspectives of expanding the limits of biocatalysis, and, in turn, of the range of its associated applications, made possible by the use of extremozymes. However, the exact principles of the function and stability under many extraordinary conditions are not well understood. Useful insights gained from the study of such biological systems can extend our understanding of protein chemistry in addition to increasing the potential applications of biocatalysis. An additional challenge is to determine the potential utility of these extremozymes as either alternatives of existing biocatalysts or to catalyze reactions impossible to perform by known biologically-derived catalytic systems.

Thermostable enzymes, like DNA polymerase, some proteases and α-amylases are already used in biotechnology [5], the first having opened the PCR based revolution in molecular biology. Proteases from hyperthermophiles can offer many potential uses in industries, being able to hydrolyze resistant substrates in high temperatures. Such proteases could be used for production of new peptides or as additives in formulation of washing powders. Heat stable proteases may resist also to denaturation with organic solvents, and as such could be used in organic peptide synthesis.

The aim of this work was the purification and characterization of a protease isolated from the supernatant of the hyperthermophilic archaeon *Pyrococcus abyssi*.

2. Materials and methods

Pyrococcus abyssi is a hyperthermophilic, anaerobic, sulfur-metabolizing archaeon isolated from a deep-sea hydrothermal vent in the North Fiji basin (SW Pacific) [6]. This archaeon (strain st 549) was found at a depth of 2000 m and classified by the IFREMER team (Brest, France). The PPA (Protease from *Pyrococcus abyssi*) was isolated and purified, as follows, from a culture supernatant of this microorganism.

2.1. Anion exchange purification

The supernatant of the culture had the same saline concentration as sea water (0.4 M). In order to allow the binding of the protease to the DEAE resin (Pharmacia), the supernatant was diluted 3 times prior to application on the column. At a concentration of 0.13 M NaCl, the PPA was retained on the column. Four hundred and twenty litres of diluted supernatant were incubated with 1 l of DEAE in several steps. The purification was performed at pH 8 with 50 mM Tris-HCl buffer. Two fractions were collected. The first was obtained by washing the gel with 50 mM Tris-HCl, pH 8, 0.25 M NaCl buffer, the second by washing the gel with the same Tris-HCl buffer but containing 1.5 M NaCl. Between multiple purification steps, the gel was regenerated by successive washing with 2 M NaCl solution and with a neutral detergent (0.5% Triton X-100).

2.2. Cation exchange purification

The active fraction obtained from DEAE purification was dialyzed against distilled water and concentrated by filtration on a membrane with a 10-kDa cut off (Millipore, cassette: 5.0-SQ.FT). The dialyzed fraction was subsequently brought to pH 3 with HCl and passed through 900 ml of SP Trisacryl M resin (Sepracor) equilibrated with

*Corresponding author. Fax: (33) (2) 40675084.
E-mail: chobert@nantes.inra.fr

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25 mM glycine buffer, pH 3. After application, the first fraction was eluted with the same glycine buffer containing 1 M NaCl. The second fraction was eluted with 0.1 M Tris-HCl, 1 M NaCl, pH 8.5.

2.3. Fast performance liquid chromatography (FPLC) on HQ column

The FPLC apparatus used was a Sprint Biocad model (PerSeptive Biosystems) and the column was a strong anionic exchanger, Poros HQ (4.6×100 mm, 20 μ; PerSeptive Biosystems). The column was equilibrated with 50 mM citrate buffer, pH 4. Elution was done at a flow rate of 10 ml/min. Purification was performed in two steps by varying two parameters, ionic strength and pH: (1) elution (50 mM citrate buffer, pH 4) with a NaCl gradient from 0 to 1.5 M including a step at 1 M. (2) Rapid change of pH from 4 to 8 with 50 mM Tris-HCl buffer containing 1.5 M NaCl.

2.4. Electrophoresis and zymogram analysis

Electrophoresis and zymogram were performed according to Laemmli [7] on a Mini Protean II (Bio-Rad) apparatus at constant voltage (200 Volts) on 70×80×0.5-mm plates. Stacking and running gels, containing 4.5 M urea, were 3.75% and 7.5% in acrylamide, respectively. For zymogram, 0.1% gelatin was copolymerized in the gel.

The enzyme solution diluted with the sample buffer (v/v) containing 4% SDS was boiled for 3 min. After migration, the gel was colored with Coomassie blue or stained with silver nitrate according to Nesterenko et al. [8]. During the detection of proteolytic activity by zymogram, the gel was placed under agitation for 45 min in a solution of 2.5% Triton X-100. Then, it was rinsed and washed 3 times with distilled water for 10 min in order to wash away SDS and Triton. It was finally incubated overnight at 80°C or for 2 h at 90°C in 50 mM phosphate buffer, pH 7, to allow the hydrolysis of gelatin.

2.5. Amino acid analysis

PPA was hydrolyzed under vacuum in the presence of constant boiling 6 N HCl for 24 h at 110°C in a Pico-Tag station (Waters). After acid hydrolysis, the amino acids were derivatized with phenyl isothiocyanate (PITC) according to Bidlingmeyer et al. [9] and quantified by RP-HPLC on a Pico-Tag C_{18} column (3.9 mm i.d.×15 cm). Speed-Vac dried samples were dissolved in 95% 2 mM Na_2HPO_4, pH 7.4/5% acetonitrile. The column was equilibrated in solvent A (94% 0.14 M CH_3COONa, 0.5 ml TEA/l, pH 6.4/6% acetonitrile); the elution was performed with a gradient from solvent A to solvent B (40% H_2O/60% acetonitrile) according to Dalgalarrondo et al. [10]. Both the column and solvent were maintained at 38°C. The flow rate was 1.0 ml/min and absorbence was recorded at 254 nm.

2.6. Determination of the catalytic activity of PPA

The protein concentration was measured according to Bradford [11] or using the BCA method according to Smith et al. [12]. Bovine serum albumin was used as a standard.

The amidasic activity of PPA was monitored spectrophotometrically at 405 nm by the hydrolysis of synthetic substrates (succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide) and by the hydrolysis of a natural substrate, insulin (oxidized chain B). Insulin (0.25 mg/ml) was solubilized in a 50-mM borate buffer, pH 8.5. Hydrolysis by PPA was realized at 80°C and aliquots were taken at intervals from 30 min to 6 h. The resulting peptides were separated by RP-HPLC on an MN Nucleosil 300-5 C_{18} column by using a gradient from solvent A (0.1% TFA) to solvent B (acetonitrile-H_2O, 40–60%) in 80 min. Their amino acid composition was determined as previously described.

The esterasic activity of PPA was measured at 50°C with 0.1 mM benzyloxycarbonyl-amino acid-*p*-nitrophenyl esters solutions in 0.1 M Tris-HCl buffer, pH 7, containing 25% acetonitrile [13]. The absorbance of obtained *p*-nitrophenol was measured at 400 nm.

Temperature dependent activity of PPA was followed at pH 8 in 50 mM Tris-HCl buffer by hydrolysis of gelatin (0.1%) or of succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide in the range of temperature 20–95°C.

The pH dependence of PPA was determined at 95°C in the case of gelatin and 60°C in the case of succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide. Buffers used were: 50 mM citrate (pH 4–5), 50 mM phosphate (pH 6–7), 50 mM borate (pH 8–9), 50 mM glycine-NaOH (pH 9–11).

In order to find the enzyme's protease class, different inhibitors were tested at pH 7.5 and 60°C, with succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide as substrate.

The effect of salts of monovalent (NaCl, KCl; 20–450 mM) and divalent ($MgCl_2$, $CaCl_2$; 1–100 mM) cations, and urea (0.5–4.0 M) on the activity of PPA was measured by hydrolysis of succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, in a 50-mM Tris-HCl buffer, pH 8.

Thermic inactivation of PPA was studied in the range of temperature 90–115°C in several conditions: pH 9 (50 mM glycine buffer); pH 9 in the presence of 0.45 M NaCl, 0.45 M KCl, 0.1 M $MgCl_2$ or 0.1 M $CaCl_2$; pH 7 (50 mM PIPES buffer); pH 7 in the presence of 35 mM (1%) SDS. The residual activity was measured at 75°C by following the hydrolysis of succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide.

3. Results and discussion

3.1. Purification

Protease from *Pyrococcus abyssi* (PPA) was purified in 3 steps. The first and second steps were successively performed in batches on an anionite-DEAE and a cationite-SP resin. The last step was carried out by FPLC on a strong anionite column (HQ). Fig. 1 shows chromatographic profiles of the three steps.

The active fraction was obtained during chromatography on DEAE-trisacryl, after elution with 1.5 M NaCl at pH 8. PPA was eluted after chromatography on SP-trisacryl, by radically changing the pH of the buffer from 3 to 8. Finally, during FPLC on HQ column, enzymatic solution was applied at pH 4 but active fraction was eluted at pH 8 in the presence of 1.5 M NaCl. The final purification yield was 4% and the purification factor was 890. One unit of enzymatic activity

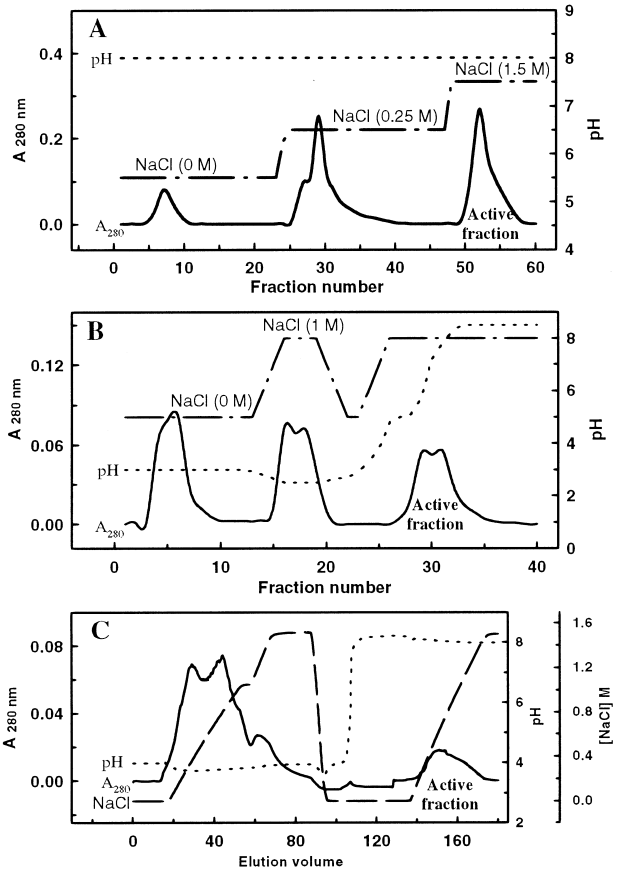

Fig. 1. Purification of PPA by chromatography on (A) DEAE-, (B) SP- and (C) HQ-column. Conditions described in Section 2. The elution volume corresponds to the number of total volumes of column.

was defined as a variation of absorbance of 10^{-3} unit per minute of hydrolysis of the tetrapeptide synthetic substrate.

3.2. Characterization of PPA by zymogram

The proteolytic activity of PPA was revealed in the culture supernatant by the presence of three main bands (A, B and C). The addition of 4.5 M urea to the gel significantly improved the resolution. Molecular masses of these bands estimated by SDS-PAGE with 10.5% acrylamide were around 220, 170, and 110 kDa for bands A, B and C, respectively (Fig. 2B). When using 7.5% acrylamide, the apparent molecular masses were lower, being estimated at 145, 105 and 60 kDa for bands A, B and C, respectively (Fig. 2C). Three bands could be observed on SDS-PAGE without preliminary boiling of the samples. After 10 min of boiling, only the C band was detected. The addition of 0.5% β-mercaptoethanol and 7 M urea did not modify the electrophoretic patterns (Fig. 2D). Relationships between these three enzymatic bands could be established by changing the SDS concentration in the sample buffer or by changing thermic treatment of the samples before electrophoresis (results not shown).

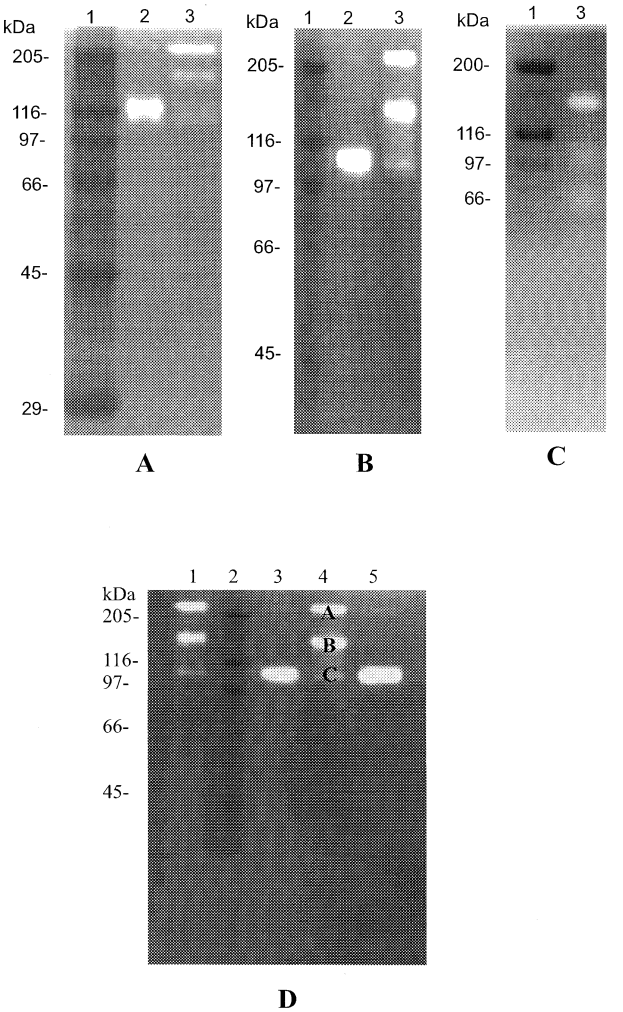

Fig. 2. SDS-zymogram pattern of PPA by gel electrophoresis (A: 11.2%; B: 10.5%; C: 7.5% acrylamide) containing 4.5% urea. 1, Molecular weight marker; 2, enzyme boiled in the presence of 2% SDS; 3, enzyme not boiled, in the presence of 2% SDS. D: Same conditions as B. 1, Enzyme not boiled, in the presence of 2% SDS; 2, molecular weight standard; 3, the same as 1, after 3 min boiling; 4, enzyme in the presence of 2% SDS, 0.5% β-mercaptoethanol and 7 M urea, not boiled; 5, the same as 4, after 10 min boiling.

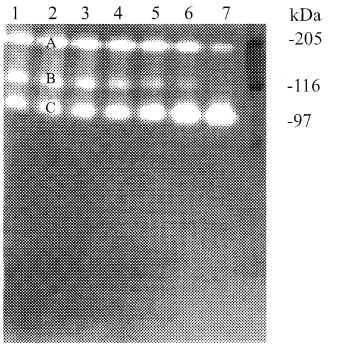

Fig. 3. Evolution of PPA 'enzymatic bands' as a function of temperature of denaturation prior to electrophoresis in 9% acrylamide gel. 1, 2, 3, 4, 5, 6 and 7: incubation for 5 min at 50, 60, 70, 80, 90 and 100°C, respectively.

The results obtained by zymogram show that apparent molecular weight of PPA varies according to the degree of gel reticulation. Data obtained with 7.5% acrylamide gel concentration (145, 105 and 60 kDa for bands A, B and C, respectively) are the most rational. Lower percentages of acrylamide gels were impossible to apply because of gel melting during incubations revealing proteolytic activities. Apparently, as can be seen in Fig. 3, protease aggregates at ambient temperature. SDS alone is not sufficient to dissociate this oligomer. After heating of the sample in the presence of SDS, zymogram revealed single molecular form with an apparent molecular mass of about 60 kDa. Nevertheless, this value seems to be still too high for a protease. Studies performed on the PfPI (a protease extracted from the hyperthermophilic archaebacteria *Pyrococcus furiosus*) showed that PfPI protease has a behavior similar to PPA [14,15]. PfPI protease has a monomeric form of 18.8 kDa oligomerising into a form of 200 kDa. The monomer and dimer of PfPI are proteolyticaly inactive, hence not detected by zymogram but 66 kDa and higher forms are. Consequently, it is plausible that the 60-kDa form of PPA is an aggregate of a sub-unit undetectable by zymogram.

3.3. Amino acid composition

The amino acid composition of the fraction issued from HQ chromatography (Table 1) indicates a high level of amino acids with short side chain (Gly, Ala) and the absence of histidine. It was not possible to determine the N-terminal sequence of PPA possibly because of N-terminal blocking.

3.4. Characterization of the proteolytic activity of PPA

3.4.1. Temperature dependence. The temperature dependence of PPA activity was studied with natural and synthetic

Table 1
Amino acid composition of PPA (%)

Asx	3.73	Tyr	7.27
Glx	3.92	Val	7.03
Ser	8.99	Met	2.75
Gly	11.82	Cys	1.22
His	0	Ile	5.62
Arg	2.49	Leu	7.70
Thr	6.94	Phe	4.44
Ala	13.40	Trp	0.81
Pro	4.82	Lys	7.04

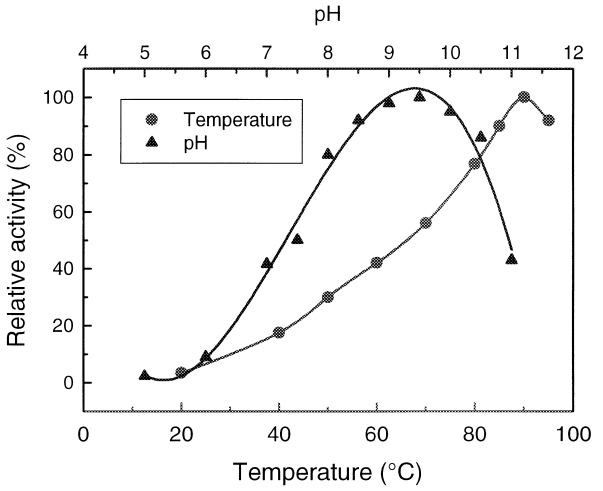

Fig. 4. Enzymatic activity as a function of temperature (measured by hydrolysis of gelatin at pH 8) and of pH (measured by hydrolysis of gelatin at 95°C). One enzymatic unit = 10^{-3} mmol of peptide bond hydrolyzed by minute and by 1 ml of enzyme. Used buffers: citrate (pH 3–6), phosphate (pH 7–8), borate (pH 9–10).

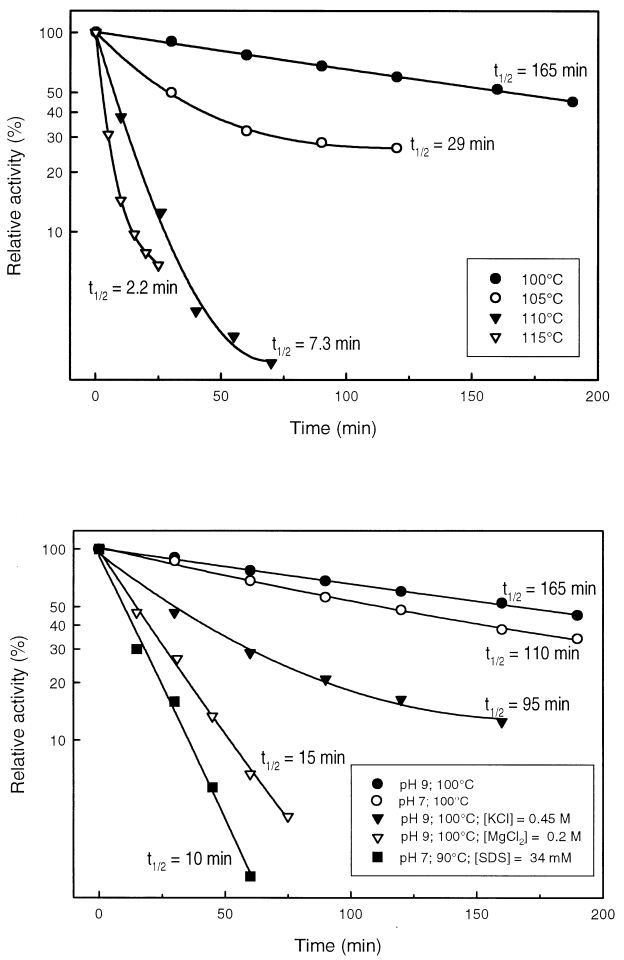

Fig. 5. Above: Thermic inactivation of PPA at pH 9. Below: Thermic inactivation of PPA at pH 9 in the presence or absence of NaCl or $MgCl_2$ and at pH 7, in the presence or absence of 34 mM SDS.

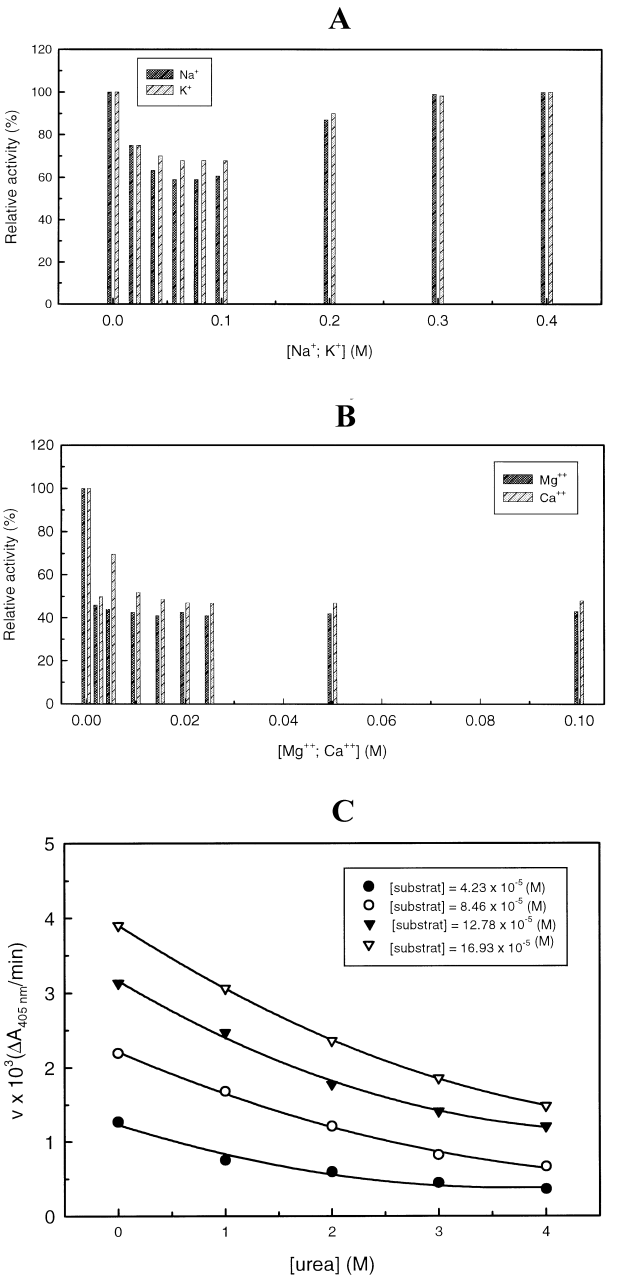

Fig. 6. Relative activity of PPA measured by hydrolysis of suc-Ala-Ala-Pro-Leu-*p*-nitroanilide, in the presence of: A: NaCl and KCl; B: $MgCl_2$ and $CaCl_2$; C: urea.

substrates by using three different methods: (1) measurement of the optical density at 405 nm for succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (data not shown); (2) measurement of the NH_2 groups with modified OPA method [16] after hydrolysis of gelatin (Fig. 4); (3) RP-HPLC following the disappearance of β-casein after 12 h hydrolysis (data not shown). In each case, PPA activity was very low at room temperature but increased quickly reaching a maximum at 95°C.

3.4.2. Influence of pH. The influence of pH on PPA activity was evaluated by using different buffers in the pH range 4–11. Measurements of enzymatic activity were performed following hydrolysis of gelatin at 95°C (Fig. 4) and hydrolysis of succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (data not shown). In

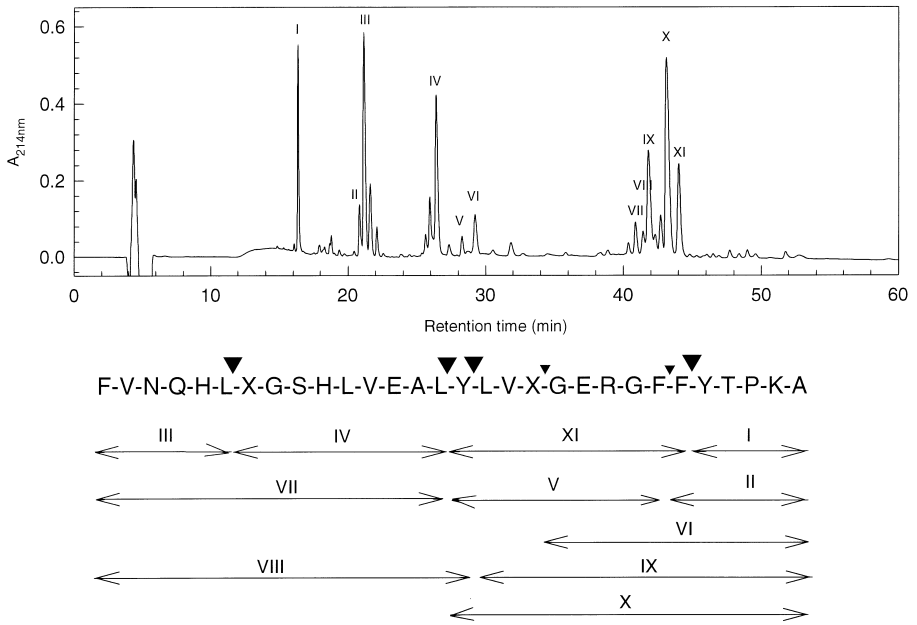

Fig. 7. Primary (large arrowhead) and secondary (small arrowhead) sites of cleavage obtained after 6 h hydrolysis at 90°C of oxidized insulin chain B by PPA.

both cases optimum of lytic activity was observed around pH 9.

3.4.3. Thermic inactivation. In order to determine thermic inactivation of PPA, the protease was incubated in the range of temperatures between 90 and 115°C and its residual activity was measured at 75°C. As shown in Fig. 5, below 100°C and at pH 9 PPA was active for a long period. The half-life ($t_{1/2}$) periods were 6 h, 165, 29, 7.3 and 2.2 min at 95, 100, 105, 110 and 115°C, respectively. The thermic inactivation was also measured in different conditions (Fig. 5). PPA was less stable at pH 7 than at pH 9 after incubation at temperatures higher than 100°C. The presence of ions increased the thermic inactivation, particularly divalent cations. In the presence of SDS the activity of PPA became very sensitive to temperature. At pH 7 and 90°C, $t_{1/2}$ was 10 h and 10 min in the absence and presence of 1% SDS, respectively. At pH 9, PPA was inactivated instantly in the presence of SDS.

3.4.4. Effect of cations and urea. The effect of cations and urea was studied at pH 9 and 75°C by measuring the hydrolysis of the substrate succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide. As shown in Fig. 6A, a 30–40% decreased activity of PPA was measured in the presence of 0.1 M NaCl and KCl whereas for higher concentrations (0.20–0.45 M) no effect was observed. Lower concentrations of divalent cations (2.5–100 mM) were more efficient to decrease the activity of PPA (Fig. 6B). Urea has an inhibitory effect on PPA activity (Fig. 6C).

3.5. Determination of type of protease

A wide range of protease inhibitors was tested with PPA. Enzymatic activity towards succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was strongly inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, 90% inhibition), phenylmethylsulfonyl fluoride (PMSF, 80% inhibition) and soybean trypsin inhibitor (100% inhibition).

These results indicate that PPA belongs to the family of seryl-proteases. It should be stated, however, that other inhibitors also inhibited the enzyme, albeit less efficiently. The poor rate of inhibition obtained with 4-hydroxymercurybenzoate (30% inhibition) can be explained by the very low specificity of this inhibitor. In the case of pepstatin, the observed 50% inhibition could be due to the hydrophobicity of this peptide.

3.6. Determination of the specificity of PPA

Protease specificity in the P1 position was tested on a wide range of peptidyl-*p*-nitroanilide substrates. Only the succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide and the succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were hydrolyzed by PPA. Additionally, PPA displayed an esterasic activity on benzyloxycarbonyl-Phe-(or Tyr-) nitrophenyl esters. The oxidized insulin B chain was hydrolyzed efficiently by PPA. Produced peptides were purified and their amino acid composition was determined. The sites of cleavage are shown in Fig. 7.

By analyzing the results obtained, specificity of the protease studied could be defined. The high affinity for aromatic (Phe and Tyr) and hydrophobic amino acids (mainly Leu) in P1 position of synthetic substrates was confirmed by the cleavage of insulin chain B. Additionally, the hydrolysis of this natural substrate suggests a specificity for aromatic moieties in P′1 position which was confirmed by an important activity of PPA towards benzyloxycarbamyl-tosyl-*p*-nitrophenyl ester, whereas it does not hydrolyze tosyl-*p*-nitrophenyl ester.

These specific features incline to consider the studied PPA as a good protease candidate for processing amide bonds in the neighborhood of highly aromatic or hydrophobic moieties.

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