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Further Characterization and Kinetic Parameter Determination of a Milk-Clotting Protease from *Mucor bacilliformis*

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ABSTRACT

Further characterization of an aspartyl protease from *Mucor bacilliformis* with milk-clotting activity was performed. An extinction coefficient, $\varepsilon_{278\ cm}$ = 1.61 mL/mg/cm, a molecular mass of 35,400 Da and a p*I* of 5.2 were determined. Proteolytic activity and kinetic parameters were evaluated by using the hexapeptide Leu-Ser-pNO_2-Phe-Nle-Ala-Leu-OMe as the substrate. The effect of pH and temperature on peptide cleavage, as well as protease heat stability, was determined. Such properties, taken as a whole, indicate that the *M. bacilliformis* protease can be considered a potential substitute for bovine chymosin in cheese manufacture.

Index Entries: *Mucor bacilliformis* protease; milk-clotting enzyme; aspartyl protease; fungal protease; aspartyl protease kinetic parameters; mesophilic enzyme; cheese manufacture.

INTRODUCTION

The use of aspartyl proteases as milk-clotting enzymes in cheese manufacture is well-known. Calf chymosin fully qualifies for this purpose because it has a high clotting activity and leads to a limited general prote-

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olysis of caseins. However, the decreasing supply of young animals and the increase in cheese production and consumption have made utilization of rennet substitutes necessary.

We have purified and partially characterized a milk-clotting protease from *Mucor bacilliformis*, a mesophilic local mold strain. Its instability during heat treatment and its clotting:proteolytic activity ratio indicate that it may be considered a potential substitute for bovine chymosin *(1,2)*.

Although chymosin produced by *Escherichia coli* was accepted by the Food and Drug Administration of the United States in 1990, fungal enzymes are still used in cheese manufacture.

In this paper, we present a further characterization of such *M. bacilliformis* aspartyl protease and compare its proteolytic activity and the kinetic parameters for the cleavage of the synthetic peptide Leu-Ser-pNO_2-Phe-Nle-Ala-Leu-OMe with those of the proteinase from *Mucor miehei*, which is extensively used in the cheese industry.

MATERIALS AND METHODS

The following chemicals were used: DEAE-cellulose medium mesh and the hexapeptide Leu-Ser-pNO_2-Phe-Nle-Ala-Leu-OMe were purchased from Sigma, (St. Louis, MO), dry skim-milk powder was from Nestle, Argentina. All other reagents were AR and solvents HPLC grade.

Enzymes

M. miehei protease was obtained from Sigma and used without further purification. Enzyme concentration was determined spectrophotometrically by using the extinction coefficient $\varepsilon_{278\ nm}$ = 1.36 mL/mg/cm *(3)*. Molecular mass was assumed to be 34,000 Da *(4)*.

A crude extract from a *M. bacilliformis* culture having milk-clotting activity was obtained in the Biotechnology Laboratory (School of Pharmacy and Biochemistry, Buenos Aires University) according to Areces et al. *(1)*. The first purification step was performed as previously described *(1)*: The crude *M. bacilliformis* enzyme preparation was centrifuged at 1950*g* at 4°C for 20 min, and, after dialyzing against the starting buffer (50 m*M* sodium phosphate, pH 5.8, 50 m*M* NaCl), it was chromatographed on a DEAE-cellulose column (8.5 × 3 cm), equilibrated, and washed with the same buffer. The protein was eluted with 50 m*M* sodium phosphate buffer, pH 5.8, containing 170 m*M* NaCl. The eluate was monitored at 220 nm, and clotting activity determined. The fractions with milk-clotting activity were pooled and dialyzed against distilled water. An FPLC rechromatography step was then introduced in the purification protocol. An FPLC chromatograph and a Mono Q HR 5/5 column, both from Pharmacia, (Uppsala, Sweden), were used. Separation was performed by utilizing a 50 m*M* phos-

phate buffer with a 50–170 m*M* NaCl gradient. Flow rate was 1 mL/min and the effluent was monitored at 280 nm. The milk-clotting activity of all fractions was determined. The recovery of each step was followed by protein concentration *(5)* and milk-clotting activity *(6)* determination. Homogeneity of the enzyme preparation was further examined by SDS-polyacrylamide gel electrophoresis and N-terminal analysis.

Extinction Coefficient Determination

Extinction coefficient determination was evaluated from the absorbance of enzyme solutions at 278 nm, and protein concentration estimated by amino acid analyses in quintuplicate.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in slab gels at room temperature under reducing conditions, as described by Laemmli *(7)*. The separating gel contained 0.1% SDS, 12.5% acrylamide in 25 m*M* Tris-192 m*M* glycine, pH 8.3. The stacking gel contained 0.1% SDS, 5% acrylamide in 80 m*M* Tris-HCl buffer, pH 6.8. Samples and mol wt markers were dissolved with an 80 m*M* Tris-HCl buffer, pH 6.8, with 2% SDS, 10% glycerol, 0.02% bromophenol blue, and 5% β-mercaptoethanol. The gel was run at 50 V in the stacking step and 150 V in the running step. The gel was stained with Coomasie brilliant blue R-250.

Determination of Molecular Mass by Gel Filtration

An approximate molecular mass was estimated by size exclusion HPLC on a Protein-Pak 125 column (7.8 mm × 30 cm) equilibrated and eluted with a 100 m*M* Tris-HCl buffer, pH 6.0. The eluate was monitored at 280 nm and the flow rate was 0.5 mL/min. The column was calibrated with the following molecular-mass markers: bovine serum albumin (66 kDa), egg albumin (45 kDa), equine myoglobin (17 kDa), and porcine insulin (5.4 kDa).

Amino Acid Analysis

Samples were hydrolyzed in vacuum-sealed tubes at 110°C for 20 h in constant boiling HCl containing phenol (1 mg/mL). The hydrolysates were analyzed in quintuplicate in a Beckman 119 CL amino acid analyzer.

Isoelectric Point Determination

Phast-System equipment (Pharmacia) and a Phast Gel IEF 3–9 were used. The following markers were utilized: glucose oxidase (4.15); soybean trypsin inhibitor (4.55); β-lactoglobulin A (5.2); bovine carbonic anhydrase (5.85); human carbonic anhydrase B (6.55); the acidic band of the horse

hemoglobin (6.85); the basic band of the horse myoglobin (7.35); the lentil-lectin acidic (8.15), medium (8.45) and basic (8.63) bands; and trypsinogen (9.30).

Determination of Optimum pH for Peptide Cleavage

Protease proteolytic activity was determined—as described below—in duplicate at predefined pH values between 2.5 and 6.5. The peptide solution pH was adjusted by adding NaOH or HCl of the appropriate molarity to 1 mL of a 0.1 m*M* peptide solution in 20 m*M* sodium acetate buffer, pH 4.7. The pH remained unchanged at the end of the reaction.

Milk-Clotting Activity Determination

Milk-clotting activity was assessed from the time taken for 10 µL of enzyme solution to coagulate 100 µL of a 10% skim-milk solution containing 10 m*M* $CaCl_2$ (substrate solution): The substrate solution was preincubated at 35°C for 10 min; the enzyme solution was then added and the mixture homogenized by gentle agitation. Each 15 s, the tube was taken out of the thermostatic bath and positioned at a 45-degree angle, so that the content spread on the tube wall and the formation of the first curd fragments could be easily visualized. The milk-clotting time is defined as the time between the sample addition and and the moment the milk starts curding. One unit of milk-clotting activity was defined as the enzyme amount capable of clotting 1 mL of substrate within 40 min at 35°C *(6)*.

Proteolytic Activity Determination

A 0.7 m*M* hexapeptide, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, stock solution in 20 m*M* sodium acetate buffer, pH 4.7, was prepared. This solution was then filtered through a 0.45-µm Millipore filter and the exact concentration was spectrophotometrically determined by using the molar extinction coefficient $\varepsilon_{max} = 8300 \pm 100\ M^{-1}/cm$ at 279.5 nm *(8)*. Enzymatic cleavage of the Phe(NO_2)-Nle bond of the hexapeptide was followed by difference spectrophotometry using a Perkin Elmer (Norwalk, CT) λ 6 uv/vis computer-assisted spectrophotometer. The UV spectrum of the hexapeptide shows an absorption maximum at 279.5 nm in the pH 1.5–12 range. Studies were performed at pH 4.7, except for the determination of the optimum pH. At that pH, the cleavage of the Phe(NO_2)-Nle bond causes an absorbance decrease in the 284–324 nm range, and the difference spectrum (cleavage products vs substrate) shows its maximum absorbance change at 310 nm with a $\Delta\varepsilon = 1000 \pm 100\ M^{-1}/cm$ at 25°C *(9)*. One millileter of substrate solution (0.2 m*M*) in the assay buffer was poured into both the reference and the sample cuvets and equilibrated at 25 ± 0.3°C for 120 s before initiating the reaction by adding 50 µL of the enzyme solution to the sample cell. The absorbance change at 310 nm was monitored for 5 min and the hydrolysis rate was determined.

Influence of Temperature on Initial Rate of Hydrolysis

This was evaluated as described above and in the 25–65°C range. Experiments were made in duplicate and the reference and sample cuvets were equilibrated at the predefined temperatures for 120 s before initiating the reaction.

Heat Stability Evaluation

In order to determine heat stability, protease solutions in 20 m*M* sodium acetate buffer, pH 4.7, were preincubated in a water bath for 30 min at a predefined temperature in the 25–60°C range. The heat-treated enzyme solutions were rapidly cooled, and the residual proteolytic activity was immediately assayed as described under Proteolytic Activity Determination. Each result is the mean of duplicate determinations. For each experiment, a reference sample was submitted to the same treatment.

Evaluation of Kinetic Parameters

The absorbance change at 310 nm was followed for 5 min and reactions were carried out at 25 ± 0.3°C. Aliquots ranging between 5 and 15 µL of the enzyme solution (50 n*M*) were added to the substrate (0.01–0.28 m*M* solutions of the hexapeptide Leu-Ser-Phe[NO_2]-Nle-Ala-Leu-OMe in 20 m*M* sodium acetate buffer, pH 4.7) in a final volume of 1 mL. $K_m app$ and k_{cat} were estimated from the initial rate measurements carried out by utilizing 8–10 substrate concentrations. Enzyme concentration was selected to obtain initial rates in the first 3–7 min. Values obtained were averaged with those from duplicate or triplicate experiments. Slopes and intercepts were obtained by weighted linear regression analysis from two different linear plots: 1/v vs 1/[S], according to Lineweaver-Burk, and v vs v/[S], according to Eadie-Hofstee. K_i values were determined after incubating protease solutions (enzyme concentration: 50 n*M*) with pepstatin A (1- to 20-fold excess) for 5 min at 25°C.

RESULTS AND DISCUSSION

Purification Procedure for *M. bacilliformis* Protease

SDS-PAGE indicated the presence of an homogeneous material with an apparent molecular mass of 32,000 Da. Table 1 summarizes results of a typical experiment. The protease was purified from 69 mL of crude enzyme preparation and fractions were assayed for protein concentration and milk-clotting activity. The extracellular protease was purified ninefold with a yield of 3%. The introduction of FPLC in the purification procedure shortened the processing time considerably.

Table 1
Purification of *M. bacilliformis* Protease

Step	Protein (mg)	Volume (mL)	CA[a] (U/mL)	SCA[b] (U/mg)	Purification (fold)	Yield (%)
Crude enzyme preparation	129	69	6006	3264	1	100
DEAE	9	17	12012	23189	7	7
FPLC (Mono Q)	4	11	11019	30355	9	3

The enzyme was purified from 69 mL of crude extract and fractions were assayed for protein concentration and milk-clotting activity as described under Materials and Methods.

[a]CA, milk-clotting activity.

[b]SCA, specific milk-clotting activity.

Molecular Mass of Native Enzyme

Gel filtration indicated a molecular mass of 35,400 Da for the native enzyme, thus showing its monomeric nature, which agrees with that reported for all other fungal aspartyl proteases.

Isoelectric Point

Results from electrofocusing indicated that the protease has a p*I* of 5.2. This value is within the range reported for other Mucor proteases.

Extinction Coefficient

It was determined on the basis of amino acid analysis and spectrophotometric studies, yielding the following value: $\varepsilon_{278} = 1.61$ mL/mg/cm.

Proteolytic Activity

Seven different enzyme concentrations, ranging between 20 and 80 n*M*, and a 0.2 m*M* substrate concentration were used. Experiments were duplicated. Values obtained were 39.21 ± 1.63 and 29.46 ± 1.33 μmol/mg/s for *M. bacilliformis* and *M. miehei* proteases, respectively.

Effect of Temperature on Protease Proteolytic Activity

The effect of temperature on the initial rate of the hexapeptide cleavage at a 0.1 m*M* substrate concentration was determined.

The initial velocity vs temperature plot is shown in Fig. 1. The proteolytic activity was studied at 25, 30, 35, 40, 45, 50, 55, 60, and 65°C. Δε at 310 nm is not affected by temperature within this range.

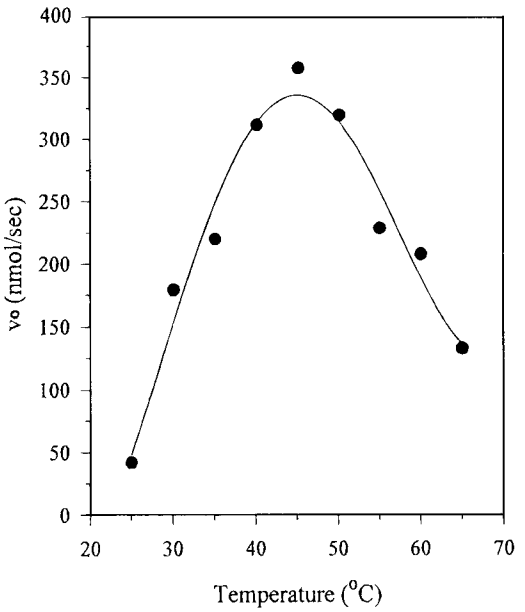

Fig 1. Effect of temperature on the initial velocity of hydrolysis of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe by the *M. bacilliformis* protease. Substrate and enzyme concentrations were 0.1 m*M* and 20 n*M*, respectively.

The optimum temperature for protease proteolytic activity was 45°C under experimental conditions; activity decreased considerably at lower or higher temperature values. This behavior resembles that of bovine chymosin, in which optimum temperature for catalytic activity, under the above experimental conditions, is 47°C *(3)*.

On the other hand, fungal proteases from thermophilic strains, such as those from *M. miehei* and *M. pusillus*, which are extensively used as chymosin substitutes in the cheese industry, show higher values: The *M. pusillus* protease exhibits its optimum temperature at 55°C and the enzyme from *M. miehei* shows a linear relationship between hydrolysis initial rate and temperature up to 60°C *(3)*.

Optimum pH for Peptide Cleavage

We have previously determined the pH activity optimum of the *M. bacilliformis* protease for protein substrates, which resulted in 3.0 and 3.5 for hemoglobin and casein, respectively *(1)*. Figure 2 shows that the optimum pH value for the hexapeptide is 5.5. This agrees with the fact that all mold acid proteases have pH optima within the 2.0–5.1 range, with lower values for protein substrates, compared with those from peptide substrates.

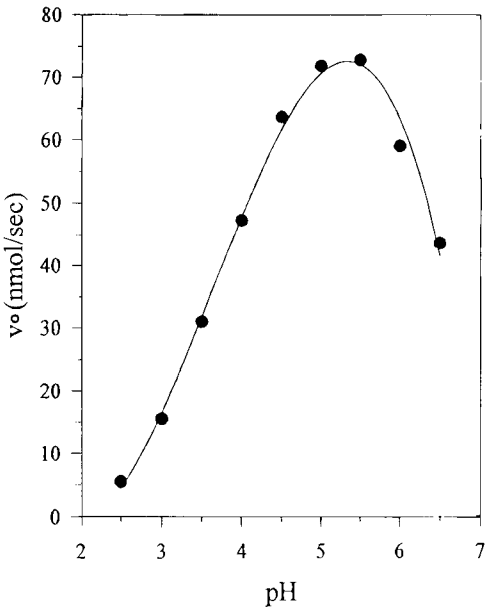

Fig 2. pH activity profile of the *M. bacilliformis* protease at 25°C. Substrate concentration was 0.1 m*M* in 20 m*M* sodium acetate buffers ranging from pH 2.5 to 6.5. Final enzyme concentration was 20 n*M*.

Heat Stability

Besides optimum pH, information involving heat stability is important for cheese storage. Figure 3 shows the effect of heat treatment on peptide cleavage for both *M. miehei* and *M. bacilliformis* proteases. The former practically retained its proteolytic activity up to 60°C, but that of the latter dropped from 98 to 36% when the temperature increased by 10°C (45–55°C). Results indicate that the *M. bacilliformis* protease, secreted to the culture medium from a mesophilic Mucor strain, has, like bovine chymosin, less heat stability, a property that is particularly useful in the manufacture of some types of cheese.

Kinetic Parameters

Table 2 summarizes values obtained (K_i, k_{cat} K_m and k_{cat}/K_m) in parallel experiments for both *M. bacilliformis* and *M. miehei* proteases.

The best-characterized aspartic proteinases are all inhibited by pepstatin A, a pentapeptide secreted by Streptomyces species that contains two residues of the unusual β-amino acid statine *(10)*. K_i values range from $4.5 \times 10^{-11} M$ for pepsin to $5 \times 10^{-7} M$ for rennin. Susceptibility to inhibition of the *M. bacilliformis* protease by pepstatin was determined and a K_i value of 5×10^{-11} *M* was obtained.

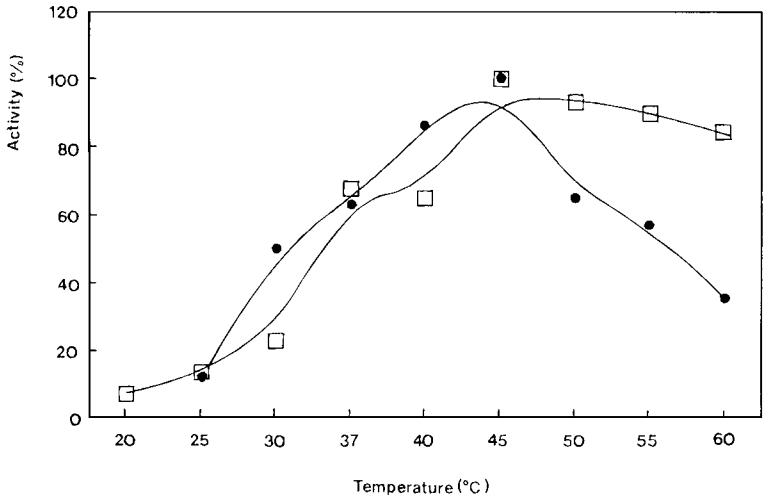

Fig 3. Heat stability of the *M. bacilliformis* (l) and the *M. miehei* (□) proteases. Enzyme solutions were incubated for 30 min at a 25–60°C range in a 20 m*M* sodium acetate buffer, pH 4.7, then cooled. Proteolytic activity was then assayed as described in Materials and Methods.

On the basis of the k_{cat}/K_m ratio for the enzymatic cleavage of the peptide used herein as the susbstrate, two kinds of aspartyl proteases have been defined: The first class, with a k_{cat}/K_m ratio higher than 1000 mM^{-1}/s, includes bovine and porcine pepsins; the second class, with a much lower ratio, includes *M. pusillus* and *M. miehei* proteases and chymosins. As shown in Table 2, the catalytic efficiency of the *M. bacilliformis* protease indicates that it belongs to the latter. This characteristic, and its heat instability, make the *M. bacilliformis* protease suitable for cheese manufacture.

Table 2
Kinetic Parameters of Cleavage of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe

Protease from *Mucor*	*n*	K_m (m*M*)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1}/s)	$K_i(M)$ (pepstatin A)
Bacilliformis	10	0.016 ± 0.002 LB	3.8 ± 0.4	239	
		0.017 ± 0.002 EH	4.2 ± 0.4	259	5×10^{-11}
		0.018 ± 0.002 MM	4.0 ± 0.3	214	
		0.165 ± 0.040 LB	14.8 ± 0.6	89.7	
Miehei	8	0.185 ± 0.025 EH	15.4 ± 0.7	83.2	5×10^{-11}

Assays were performed in 20 m*M* sodium acetate buffer, pH 4.7. Final enzyme concentration was 50 n*M* and initial substrate concentrations ranged from 0.1 to 0.28 m*M*. *n*, number of experiments; LB, Lineweaver-Burk plot; EH_2 Eadie-Hofstee plot; MM, Michaelis-Menten plot.

Extensive studies have been carried out on the structure–function relationship of these enzymes, and close similarities in their general architecture comes from crystallographic studies *(11)*. However, little is known about the molecular aspects underlying the existence of mesophilic and thermophilic proteases and their different thermal stabilities. We have undertaken the determination of the *M. bacilliformis* protease primary structure in order to contribute to the definition of the molecular differences and similarities between both types of enzymes.

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