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Purification and characterisation of a highly thermostable extracellular protease from *Bacillus thermantarcticus,* strain M1

Laura DIPASQUALE, Valeria CALANDRELLI, Ida ROMANO, Barbara NICOLAUS, Agata GAMBACORTA, Licia LAMA*

Istituto di Chimica Biomolecolare, CNR, Via Campi Flegrei 34, 80078 Pozzuoli (NA) Italy

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Abstract - A high thermostable extracellular protease was purified to homogeneity and characterised from *Bacillus thermantarcticus,* strain M1. The molecular mass was about 42 kDa. Almost total inhibition of protease by phenyl methyl sulphonylfluoride (PMSF), suggested that the enzyme belonged to the serine protease family. The enzyme was active and stable in a broad range of pH with an optimum at pH 7.0. The protease showed the highest activity at 70 °C and was stable for 24 h at 70 °C, with an increase of the enzymatic activity of about 4 times, in the presence of $CaCl_2$. The protease retained about 50% activity after 3 h of incubation in the presence of $CaCl_2$ with various commercial detergents. Purified protease was found to be stable, for one week, in presence of DMSO, methanol, ethanol, acetonitrile, isopropanol.

Key words: thermophilic *Bacillus,* extracellular proteases, purification, characterisation, stability.

INTRODUCTION

Proteases [serine protease (EC. 3.4.21), cysteine (thiol) protease (EC 3.4.22), aspartic proteases (EC 3.4.23) and metallo-protease (EC 3.4.24)] constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market (Rao *et al*., 1998). Bacterial proteases are a well-studied group of hydrolases that catalyse the total hydrolysis of proteins. Besides their physiological importance, they constitute a class of enzymes of great application in commercial fields (Rao *et al.,* 1998) i.e., detergent, protein, brewing, meat, photographic, leather, dairy and waste treatment industries, accounting for at least one quarter of the total worldwide sale of enzymes (Gupta *et al.,* 2002a, 2002b).

Enzymes synthesised by thermophiles and hyperthermophiles are known as thermozymes and are advantageous in some applications, because higher processing temperatures can be employed with the consequences of faster reaction rates, increase in the solubility of nongaseous reactants and products and reduced incidence of microbial contamination from mesophilic organisms (Sokkheo *et al.,* 2000; Chen *et al.,* 2004).

Bacillus spp. has remained major sources of enzymes of industrial and commercial value and in fact a number of thermophilic *Bacillus* spp. has been reported to produce useful enzymes (Mala *et al*., 1998; Rahman *et al*., 2003). Enzymes isolated from these microorganisms are not only thermostable and active at high temperature, but are also often resistant to and active in the presence of organic solvents and detergents (Hawumba *et al.*, 2002).
In view of the remarkable industrial and commercial value of thermoactive proteases, the search for new microbial sources for these enzymes is of continuous interest and, for this reason, in this report we describe the identification and characterisation of a proteolytic enzyme from, *Bacillus thermantarcticus* strain M1, a thermophilic microorganism isolated from Antarctic continent (Nicolaus *et al.,* 1996).

* Corresponding author. Fax +39/081-8661770;
llama@icmib.na.cnr.it

MATERIAL AND METHODS

Bacterial strain and culture conditions. *Bacillus thermantarcticus* strain M1 (DSM 9572), (named simply M1), an aerobic thermophilic Gram-positive bacterium (Nicolaus *et al.,* 1996), was routinely cultured at 60 °C on a standard medium (YN) containing: yeast extract 0.6 g/l and NaCl 0.3 g/l in tap water at pH 6.0.

The effects of culture conditions on protease production were assayed by growing M1 at 60 °C in the following media (with and without skim milk powder 0.1 g/l): YN; medium A containing (g/l): yeast extract 1.0, tryptone-soya broth 0.8, $CaSO_4 \cdot 2H_2O$ 0.36, $MgCl_2 \cdot 6H_2O$ 1.6, iron citrate 0.01 M 4.0 ml, in distilled water and the pH of the medium was adjusted to 6.0 with NaOH or H_2SO_4; medium B containing: bactopeptone 15 g, K_2HPO_4 2.9 mM and $MgCl_2 \cdot 6H_2O$ 5 mM in 1 litre of distilled water. Flasks (500-ml) containing 200 ml of medium were inoculated with 5% v/v of pre-inoculum and incubated at 60 °C for 24 h at 70 rpm. Growth was followed at different time intervals by measuring the absorbance at 540 nm and the free cell supernatant was assayed for protease activity.

Purification of the enzyme. For protease production a Biostat-D (B. Braun Biotech, Germany) was used. The growth medium A with skim milk powder 0.1 g/l pH 6.5 was used, the operating temperature was 60 °C. After 12 h the cells were harvested by centrifugation and the supernatant was concentrated and diafiltered by continuous ultrafiltration (Sartorius) equipped with a 10000 Da cut-off membrane. The resulting sample was precipitated by ammonium sulphate at 80% saturation and dialysed overnight against Tris-HCl buffer, 50 mM, pH 7.0 at 4 °C. Sample was loaded onto a column of Q-Sepharose Fast Flow (1.6 x 27 cm, Pharmacia) preequilibrated in Tris-HCl buffer, 20 mM, pH 7.0 (buffer A). Bound proteins were eluted at a flow rate of 3 ml/min by applying a linear NaCl gradient (0-0.6 M) in buffer A and fractions of 12 ml were collected. The active fractions were pooled, concentrated and dialysed against buffer A by ultrafiltration (Amicon, YM 30). The concentrated sample was applied to a Superdex 200 (1.5 x 41 cm, Pharmacia), equilibrated in Tris-HCl buffer 50 mM, pH 7.0, 0.15 M NaCl. The elution was performed with the same buffer, at a flow rate of 0.5 ml/min and fractions of 1 ml were collected. The fractions with proteolytic activity were pooled and used for further characterisation.

Enzyme assay and estimation of protein. Proteolytic activity was determined by the hydrolysis of azocasein (Sigma) under the following standard conditions. The reaction mixture consisted of 250 µl of 1% (w/v) azocasein, 230 µl of Tris-HCl pH 7.0, 20 mM and 20 µl of enzyme solution. The mixture was incubated at 70 °C for 30 min and the reaction was initiated by addition of the enzyme solution. The enzyme reaction was stopped by addition of 10% (v/v) TCA and was kept on ice for 15 min. The reaction mixture was then centrifuged for 10 min at 9000 *x g* and the absorbance of the supernatant was measured at 366 nm against a blank (complete reaction mixture stopped before incubation) (Lama *et al.,* 2005). One unit of protease activity was defined as the amount of the enzyme giving an absorbance change at 366 nm of 0.1 OD in 30 min in the standard assay conditions. The characterisation of enzyme was carried out by incubating the purified enzyme 2.0-4.0 U/ml final concentration, as following described. Protein concentration was determined by the method of Bradford (1976) using the Biorad protein assay with bovine serum albumin as standard.

Molecular mass determination. The apparent molecular mass of the enzyme was determined by gel filtration chromatography on a Sephacryl S-100 16/60 connected to a Pharmacia FPLC system, using a standard gel filtration calibration kit (Pharmacia). The molecular mass was also analysed by SDS-PAGE by the method of Laemmli (1970) in 10% polyacrylamide slab gel. The molecular masses were calibrated by using a low molecular weight calibration kit (Pharmacia Biotech).

For zymogram analysis, protease was separated in a 10% SDS-polyacrylamide gel containing 0.5% skim milk powder as substrate, which copolymerised with resolving gel. The samples were not heated prior to electrophoresis, which was performed at 4 °C at a constant current of 8 mA/gel. Then the gel was washed with 2.5% Triton X-100 for 30 min and incubated overnight in Tris-HCl buffer, 50 mM, pH 7.0 at room temperature. Finally, the gel was stained with Coomassie brilliant blue R-250 (0.1%) for 30 min and then destained in distilled water/methanol/acetic acid (50:40:10), until clear bands, resulted of substrate hydrolysis, were visible against a dark background.

Determination temperature optimum and thermal stability. To determinate influence of temperature on enzymatic activity, the purified enzyme (2 µg) was incubated at temperatures from 40 to 90 °C with and without $CaCl_2$ (2 mM) under the standard assay conditions. Thermostability was investigated preincubating the purified enzyme at 60, 70, 80, and 90 °C both with and without $CaCl_2$ (2 mM). After various time intervals, samples were withdrawn and residual activity was determined at the standard assay conditions.

Determination of pH optimum and pH stability. The effect of pH on protease activity was deter-

mined by hydrolysis of azocasein, incubating the purified enzyme (2 µg), in various buffers from pH 3.0 to pH 10.0 at 70 °C, under the standard assay conditions. The pH stability of the enzyme was determined by incubating the enzyme in buffers 20 mM of different pH in the range 7.0-10.0 for 1 week at 4 °C. The proteolytic activity was determined under the standard assay condition

Kinetic parameters and substrates specificity. Kinetic parameters were determined by incubating the purified enzyme (2 µg) with different amounts of azocasein (0.05 to 1.5%) in Tris-HCl pH 7.0, 20 mM, at 70 °C. The values of Michaelis-Menten constant were determined from Lineweaver-Burk plots. Proteolytic activity with the following natural substrates: bovine serum albumin, turkey egg albumin (ovalbumin), hemoglobin from bovine blood and gelatin powdered, was assayed by mixing purified enzymatic solution (2 µg) and 950 µl of Tris-HCl buffer, 50 mM, pH 7.0 containing the protein substrate (1%). After incubation at 70 °C for 30 min, the reaction was stopped by adding 500 µl of 10% TCA and was kept in ice for 20 min. Undigested proteins were removed by centrifugation (13000 rpm for 3 min) and the released peptides were assayed at 280 nm against Tris-HCl buffer, 50 mM, pH 7.0.
The purified enzyme was also tested for its ability to hydrolyze synthetic substrates as: *N*-succinyl-*L*-phenylalanine-*p*-nitroanilide, *N*-succinyl-ala-ala-ala-*p*-nitroanilide, *L*-alanine-*p*-nitroanilide, *L*-leucine-*p*-nitroanilide, *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (*DL*-BAPNA), *N*-α-benzoyl-*L*-arginine-*p*-nitroanilide (*L*-BAPNA), *N*-benzoyl-Gly-Arg (Hippuryl-Arg), *N*-benzoyl-Gly-Phe (Hippuryl-Phe), *N*-CBZ-*D*-alanine, *N*-CBZ-Glu-Tyr and *N*-CBZ-Phe-Ala.

Protease activity was assayed by incubating purified enzyme solution (2 µg) and each substrate (5 mM) in Tris-HCl buffer, 20 mM, pH 7.0 in a final volume 1 ml, at 70 °C for 30 min (for chromogenic *p*-nitroanilides) and for 5 min (for Hippuryl). The substrate hydrolysis was monitored spectrophotometrically at 420 nm (for the *p*-nitroanilides) and 254 nm (for Hippuryl). Instead, the hydrolysis of *N*-CBZ-*D*-alanine, *N*-CBZ-Glu-Tyr and *N*-CBZ-Phe-Ala was monitored by thin-layer chromatography (TLC) (Kato, 1976).

Effect of organic solvents on protease stability. Various water miscible organic solvents (25% v/v) were tested for their effect on enzyme stability. An aliquot of 0.3 ml enzyme solution and 0.1 ml organic solvent were incubated at 30 °C with constant shaking at 160 rpm. The residual activity was estimated, after 1 week, by the assay procedure described above.

RESULTS AND DISCUSSION

Effect of culture conditions on protease production

Proteolytic activity was found in the supernatant free-cells and its maximum yield was detected after 12 h, at the end of exponential growth phase (data not shown), in according to most extracellular proteases secreted by *Bacillus* sp. (Debadov, 1982). The synthesis of the extracellular protease was significantly enhanced when the microorganism was cultivated on a medium with a high concentration of proteins, suggesting that the presence of proteins induced the enzyme activity. In fact, the highest protease production (ca.15 times more with respect to the medium YN) was obtained when the microorganism was grown on medium A containing skim-milk 0.1%. Medium B, with and without skim-milk 0.1% gave a moderately increase (ca. 2 times) on protease production with respect to the medium YN.

Purification of the extracellular protease

A single protease was purified from the culture supernatant of M1 by ammonium sulphate precipitation, Q-Sepharose F.F. and Superdex 200. The protease was purified about 169-fold with a specific activity of about 4326.5 U/mg and a final yield of 5%. The purification is summarised in Table 1.

TABLE 1 - Purification of extracellular protease from M1

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Ammonium sulphate	65.25	1670.4	25.6	1	100
Q-Sepharose F.F.	2.73	174.7	64	2.5	10.4
Superdex 200	0.0196	84.8	4326.5	169	5

The purification was performed with 3 l supernatant (about 3 g of wet cells).

Molecular mass determination

SDS-PAGE analysis of the molecular mass of the purified enzyme revealed a single band of 42 kDa (Fig. 1A). A zymogram, shown in Fig. 1B, revealed the high-level activity of the purified protease. A zymogram, carried out under native conditions, also showed a single band indicating that protease is a monomer (lane 1), no activity was detected in lane 2 by the total inhibition of protease in presence of phenyl methyl sulphonylfluoride (PMSF). The relative molecular mass of the purified protease, determined by gel filtration chromatography on a Sephacryl S-100 16/60 (Pharmacia), was about 42 kDa (data not shown).

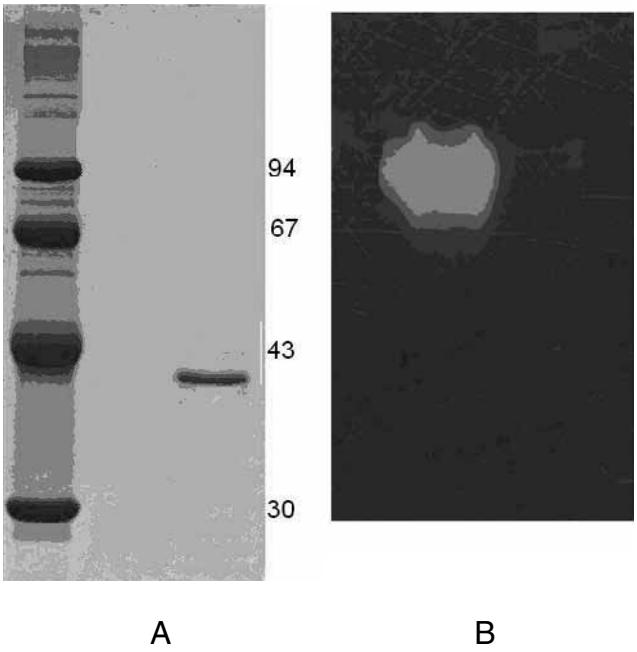

FIG. 1 - A: SDS-PAGE in 10% polyacrylamide slab gel. Lane 1: low molecular weight standard calibration kit (Pharmacia Biotech), lane 2: purified protease (2 µg). B: For zymogram analysis, protease was separated in a 10% SDS-polyacrylamide gel containing 0.5% skim milk powder as substrate, which copolymerised with resolving gel. The samples were not heated prior to electrophoresis, which was performed at 4 °C at a constant current of 8 mA/gel. Then the gel was washed with 2.5% Triton X-100 for 30 min and incubated overnight in Tris-HCl buffer, 50 mM, pH 7.0 at room temperature. Finally, the gel was stained with Coomassie brilliant blue R-250 (0.1%) for 30 min and then destained in distilled water/methanol/acetic acid (50:40:10), until clear bands, resulted of substrate hydrolysis, were visible against a dark background. Lane 1: purified protease without PMSF, lane 2: purified protease (2 µg) with PMSF.

Effect of temperature on proteolytic activity

The protease excreted from M1 exhibited maximum activity at 70 °C; in the presence of $CaCl_2$ the enzyme showed higher activity at all temperatures tested (about 10% more). The enzyme retained 60% of the optimal activity at 80 °C, while without $CaCl_2$ this value was 40% (data not shown).

The presence of $CaCl_2$ increased thermostability of M1 protease.

After 24 h of preincubation at 60 °C, in absence of ions Ca^{2+}, residual activity was about 50%; instead, with the addition of $CaCl_2$ (2 mM), the enzyme still preserved about 90% of activity.

At 70 °C (optimal temperature of proteolytic activity), not only a greater termostability was observed but also a greater enzymatic activity. In particular, after 24 h of preincubation, in the presence of $CaCl_2$, the enzymatic activity was about 4 times, such increase was lower without ions Ca^{2+}.

Protease from M1 retained 80% original activity after 1 h heat treatment at 80 ºC, however in the presence of 2 mM Ca^{2+}, the enzyme retained 90% of the original activity. Instead at 90 °C after 1 h of preincubation the residual activity was of 70 and 75% with and without Ca^{2+}, respectively (data not shown).

As other *Bacillus* serine proteases, the termostability of enzyme became enhanced in the presence of 2 mM $CaCl_2$ at all temperatures tested. Moreover, at 70 °C after 24 h of preincubation, the enzyme showed an increase of activity (66%) without $CaCl_2$ and a strong increase in the presence of this divalent cation (Ward, 1983). This can be due to a rearrangement of the protein structure which would make the catalytic site more exposed to the substrate attack.

The thermostability of M1 protease increased always in the presence of $CaCl_2$. Generally, the role of the calcium ions seemed to be linked to the stabilization of the enzyme more than needed for its activity: in fact the binding of calcium at specific sites increased the stability of these enzymes reducing the flexibility of the molecule and hence the denaturation and autolysis (Steele *et al.,* 1992; Donaghy *et al.,* 1993; Adinarayana *et al.,* 2003; Catara *et al*., 2003).

Effect of pH on proteolytic activity

The protease showed activity over a broad pH range 4.0-10.0 with an optimum of pH 7.0 using Tris- HCl buffer. At pH 5.0 and 10.0 the enzyme showed about 70% of the optimal pH activity (data not shown). The enzyme was stable in the pH range 7.0-10.0 for 1 week (data not shown).

Effects of different inhibitors and denaturating reagents on the proteolytic activity

On the basis of inhibition assays the protease from M1 was defined as a serine protease. In fact, enzyme activity was totally inhibited by PMSF, a common inhibitor of this class of enzymes (Gold and Fahrney, 1964; Ward, 1983).

Chymostatin and PEFABLOC caused an inhibition

about 70%. The enzyme was inhibited to a moderate extent by incubation with ZPCK, TPCK, but not by TLCK. The partial inhibition of protease by TPCK (chymotrypsin inhibitor) but not TLCK (trypsin inhibitor) suggested that the enzyme may have some preference for hydrophobic residues rather than positively charged residues adjacent to the peptide bond to be cleaved (Dow *et al.,* 1990).

Enzyme activity was inhibited about 50% by the metal-ion chelator EDTA and by Bestatin-HCl. PCMB, E-64, Leupeptin-hemisulphate, affect the protease activity moderately (Table 2). The partial inhibition of EDTA on protease may be attributed to destabilization of the enzyme structure by removal of calcium ions and therefore the loss of activity was due to enzyme denaturation rather than inhibition. The reversibility of the inhibition from EDTA confirmed that the metallic ions have an important role in the maintenance of the active conformation of the protease. Generally, the serine proteases were not inhibited by metal chelating agents; however there were examples of serine proteases that are affected by EDTA (Kato *et al.,* 1974; Gnosspelius, 1978; Izotova *et al.,* 1983; Singh, 2001).

The enzyme retained more than 90 and 80% of activity in the presence of urea and guanidine-HCl, respectively, indicating that hydrogen bonds played only a very small role in protein stabilization. While, was strongly inhibited by DTT (Table 3) suggesting that disulfide-like bonds are involved in preserving the enzymatic structure. Moreover, the protease produced by M1 was resistant to tensioactives, as SDS, DBS and Triton X-100. In particular, an increase of the enzymatic activity was observed in the presence of SDS (302%). This increased could be attributed either to the sensitivity of the substrate to SDS, making it more susceptible to enzyme hydrolysis or to the exposure of the active site of the enzyme (Sako *et al.,* 1997).

TABLE 2 - Effect of protease inhibitors on the proteolytic activity of M1

Inhibitor	Concentration	Residual activity (%)
None	-	100
Tosyl *L*-lysine chloromethyl ketone	1 mM	100
(TLCK)	5 mM	97
Tosyl *L*-phenylalanine chloromethyl	1 mM	68
Ketone (TPCK),	5 mM	57
Chymostatin	50 µM	27
	100 µM	25
Leupeptin hemisulfate	50 µM	83
	100 µM	80
E-64	5 µM	83
	10 µM	80
p-Chloromercuric benzoic acid (PCMB)	1 mM	107
	5 mM	50
N-Carbobenzoxy-L-phenylalanine	1 mM	80
Chloromethyl ketone (ZPCK)	5 mM	77
Pepstatin A	10 µM	79
	20 µM	79
Ethylendiaminetetracetic acid (EDTA),	5 mM	47
	10 mM	43
Bestatin-HCl	50 µM	65
	150 µM	40
Soybean trypsin inhibitor	0.1%	50
	0.2%	42
4-(2-Aminoethyl)-benzenesulfonyl	2 mM	31
Fluoride hydrochloride (PEFABLOC®	5 mM	31
SC)		
Phenylmethylsulfonyl fluoride (PMSF)	1 mM	15
	5 mM	0

The purified enzyme solution (2 µg) was preincubated with the above compounds at 36 °C for 30 min. The protease activity was tested under standard assay conditions. Values shown are percentages of activity without compounds. The average of three replicates was taken, with no statistically significant differences noted in the three replicate determinations.

TABLE 3 - Effect of various denaturating reagents on proteolytic activity

Reagent	Concentration	Residual activity (%)
None	-	100
β -Mercaptoethanol	5 mM	90
	25 mM	63
DTT	1 mM	87
	5 mM	9
Triton X-100	0.1%	100
	0.2%	93
SDS	0.1%	302
	0.2%	173
DBS	0.2%	100
	0.4%	97
Urea	2 M	93
	4 M	90
Guanidine-HCl	2 M	83
	4 M	87

The purified enzyme solution (2 µg) was preincubated at 36 °C for 30 min with various chemical reagents and then the residual protease activity was measured in the standard assay conditions. Proteolytic activity assayed without inhibitors or denaturating agents was considered to be 100%. The average of three replicates was taken, with no statistically significant differences noted in the three replicate determinations.

Effects of metal ions on proteolytic activity

Metal ions such as Mn^{2+}, K^{+}, Ca^{2+} and Mg^{2+} had a stimulatory effect on protease activity, and this effect was also reported to increase the thermostability of other *Bacillus* proteases (Paliwal *et al.,* 1994; Rahman *et al.,* 1994). On the contrary, the enzyme was completely inhibited by Hg^{2+} (Table 4A). The total inhibition by Hg^{2+} suggested the presence of residues of cisteine near to the catalytic site.

Moreover, the same experiment carried out with a dialysed sample against EDTA demonstrated that this chelator halved the enzymatic activity (Table 4B). The added metal ions (2 mM) to the dialysed sample, gave rise to a partial reinstatement of the enzymatic activity in the presence of Mg^{2+}, Ba^{2+}, Mn^{2+}, K^{+} and Ca^{2+}, while the total inhibition by Hg^{2+} was confirmed.

TABLE 4 - Effects of metal ions on proteolytic activity from purified (A) and dyalised (B) enzyme of M1 strain

Purified enzyme (A)		Dialysed enzyme* (B)	
Ions (2 mM)	Relative activity (%)	Ions (2 mM)	Residual activity (%)
None	100	Dialysed sample*	47
$MnCl_2$	141	+ $MnCl_2$	67
KCl	120	+ KCl	67
$CaCl_2$	115	+ $CaCl_2$	62
$MgCl_2$	107	+ $MgCl_2$	83
$BaCl_2$	100	+ $BaCl_2$	73
NaCl	76	+ NaCl	50
$CoCl_2$	56	+ $CoCl_2$	33
$FeCl_2$	36	+ $FeCl_2$	30
$ZnSO_4$	23	+ $ZnSO_4$	53
$CuSO_4$	17	+ $CuSO_4$	40
$HgCl_2$	0	+ $HgCl_2$	0

The enzyme solution (2 µg) was assayed in standard conditions in the presence of 2 mM of various metal ions; the activity assayed without metal ions was considered to be the reference value (100%). * Enzyme dialysed before against Tris-HCl buffer, 50 mM, pH 7.0 containing 10 mM EDTA and then against Tris-HCl buffer, 50 mM, pH 7.0 without EDTA. The average of three replicates was taken, with no statistically significant differences noted in the three replicate determinations.

K_m and substrates specificity

The K_m value for azocasein as substrate, obtained from Lineweaver-Burk plots was 0.1%.

Among natural substrates tested, egg albumin was most efficiently hydrolyzed by M1 protease. Gelatine and serum albumin were bad substrates, being hydrolyzed only 27% and 17% with respect to ovoalbumin. Haemoglobin was not substrate.

Among synthetic substrates tested, N-succinyl-L-phenylalanine-*p*-nitroanilide was efficiently hydrolyzed by protease from M1*,* followed from N-succinyl-Ala-Ala-Ala-*p*-nitroanilide, *L*-alanine-*p*-nitroanilide, *L*-leucine-*p*-nitroanilide, suggesting that the enzyme preferably cleaves hydrophobic residues at the P1 site. M1 protease hydrolyzed *N*-benzoyl-Gly-Arg (Hippuryl-Arg), while had no activity toward *N*-benzoyl-Gly-Phe (Hippuryl-Phe).

Among *N*-CBZ-*D*-alanine, *N*-CBZ-Glu-Tyr and *N*-CBZ-Phe-Ala the sole substrate used was *N*-CBZ-Phe-Ala, by cleaving the peptide bond between Phe and Ala, releasing the aminoacid alanine, as was evident from TLC (data not shown).

Effect of organic solvents and detergent on protease stability

Purified protease was found to be stable in presence of 25% of dimethylsulfoxide (DMSO), methanol, ethanol, acetonitrile, isopropanol, for one week (data not shown). Protease from M1 showed stability with a wide range of commercial detergents at 70 °C in the presence of $CaCl_2$. The enzyme retained 60% of activity with Lenor and about 50% with most of the detergents tested even after 3 h incubation at 70 °C (Table 5).

TABLE 5 - Compatibility of protease activity from M1 strain with commercial laundry detergents in the presence of 2 mM CaCl2

	Residual activity (%)						
Preincubation time (h)	Control	Lenor	ACE	Mastro Lindo	Scala	Svelto	Sofla n
0.5	100	103	59	99	76	70	99
1.5	100	86	52	79	44	29	66
3.0	100	60	49	49	35	18	53

The detergents - Lenor, ACE, Mastro Lindo (Procter & Gamble, Italy), Scala (Deco), Soflan (Colgate) and Svelto (Unilives, Italy) - were diluted in distilled water (0.5% w/v) and incubated with protease in Tris-HCl buffer, 20 mM pH 7.0, 2 mM CaCl2 for 0.5, 1.5 and 3 h at 70 °C. The residual protease activity was measured by standard assay conditions and the enzyme activity of a control sample without any detergent was taken as 100%. The average of three replicates was taken, with no statistically significant differences noted in the three replicate determinations.

CONCLUSIONS

The purified protease from M1 possessed simultaneously attributes of interest in biotechnological applications in the laundry detergents also if it was compared with analogous microbial proteases (Banerjee *et al.,* 1999; Hutadilok-Towatana *et al.,* 1999; Johnvesly and Naik*,* 2001; Zhu *et al.,* 2007). Characteristically, it possessed thermophilicity, thermostability, resistance to organic solvent, detergents and alkaline pH together with a broad activity in the pH range.

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