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ORIGINAL PAPER

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Purification and characterization of a serine alkaline protease from *Bacillus clausii* GMBAE 42

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Abstract An extracellular serine alkaline protease of *Bacillus clausii* GMBAE 42 was produced in protein-rich medium in shake-flask cultures for 3 days at pH 10.5 and 37°C. Highest alkaline protease activity was observed in the late stationary phase of cell cultivation. The enzyme was purified 16-fold from culture filtrate by DEAE-cellulose chromatography followed by $(NH_4)_2SO_4$ precipitation, with a yield of 58%. SDS-PAGE analysis revealed the molecular weight of the enzyme to be 26.50 kDa. The optimum temperature for enzyme activity was 60°C; however, it is shifted to 70°C after addition of 5 mM Ca^{2+} ions. The enzyme was stable between 30 and 40°C for 2 h at pH 10.5; only 14% activity loss was observed at 50°C. The optimal pH of the enzyme was 11.3. The enzyme was also stable in the pH 9.0–12.2 range for 24 h at 30°C; however, activity losses of 38% and 76% were observed at pH values of 12.7 and 13.0, respectively. The activation energy of Hammarsten casein hydrolysis by the purified enzyme was 10.59 kcal mol^{-1} (44.30 kJ mol^{-1}). The enzyme was stable in the presence of the 1% (w/v) Tween-20, Tween-40,Tween-60, Tween-80, and 0.2% (w/v) SDS for 1 h at 30°C and pH 10.5. Only 10% activity loss was observed with 1% sodium perborate under the same conditions. The enzyme was not inhibited by iodoacetate, ethylacetimidate, phenylglyoxal, iodoacetimidate, *n*-ethylmaleimidate, *n*-bromosuccinimide, diethylpyrocarbonate or *n*-ethyl-5-phenyl-iso-xazolium-3′-sulfonate. Its complete inhibition by phenylmethanesulfonylfluoride and relatively high k_{cat} value for *N*-Suc-Ala-Ala-Pro-Phe-*p*NA hydrolysis indicates that the enzyme is a chymotrypsin-like serine protease. K_m and k_{cat} values were estimated at 0.655 μM *N*-Suc-Ala-Ala-Pro-Phe-*p*NA and 4.21×10^3 min^{-1}, respectively.

Keywords *Bacillus clausii* · Serine alkaline protease · Enzyme purification · Kinetic properties · Enzyme characterization

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Introduction

Alkaline proteases are one of the most important groups of industrial enzymes, accounting for nearly 60% of total worldwide enzyme sales [26]. Alkaline proteases are widely used in the detergent, food, and leather tanning industries. Alkaline proteases can also be used in the hydrolysis of fibrous proteins such as horn, feather, and hair, converting them to useful biomass. Other potential industrial applications of alkaline proteases include their use in peptide synthesis, in resolution of racemic mixtures of amino acids, and in hydrolysis of gelatin layers of X-ray films for silver recovery [3, 23]. *Bacillus* strains are one of the most important producers of extracellular commercially applicable proteases among various microorganisms. Some *Bacillus* strains secrete significantly large amounts of alkaline proteases with good stability at higher pH and temperature values, which can be considered as compatible additives in laundry detergents [5–7, 14, 16, 17, 20, 27]. The alkaline protease of the facultative alkalophilic *Bacillus clausii* is known as Savinase, and is used as a detergent additive for removal of tenacious stains in laundries [8]. Joo et al. [20] recently

reported an extremely oxidant- and SDS-stable alkaline protease from *B. clausii* I-52.

We have also recently isolated and identified a new type of *B. clausii* from mushroom compost capable of growing under highly alkaline conditions. This strain, designated as *B. clausii* GMBAE 42 [10], produces an extracellular alkaline protease that is highly active at around pH 11.0. Extensive research has been carried out on the purification and characterization of alkaline proteases from various *Bacillus* species. However, few such studies on strains of *B. clausii* have appeared in the literature. This paper reports on the detailed purification and characterization of alkaline protease from *B. clausii* GMBAE 42.

Materials and methods

Chemicals

Chemicals used in the cultivation of microorganisms were supplied by Oxoid (Hampshire, UK) and Merck (Darmstadt, Germany). All other chemicals used were of analytical grade and were supplied either by Merck or Sigma (St Louis, MO).

Alkaline protease production

The alkaline protease of *B. clausii* GMBAE 42 was produced using the same medium and culture conditions as described previously [10].

Alkaline protease purification

After 72 h cultivation, the culture medium was centrifuged (11,000 *g*, 30 min, 0–4°C) to remove *B. clausii* cells. The dissolved proteins in the supernatant were precipitated by the addition of solid ammonium sulphate to 75% saturation. The precipitate was collected by centrifugation (15,000 *g*, 30 min, 0–4°C), dissolved in 50 mM glycine–NaOH buffer, pH 10.5, dialyzed against the same buffer, and applied to a DEAE-cellulose column (25×2.5 cm diameter). Elution was carried out with the same buffer at a 15 ml h^{-1} flow rate. The alkaline protease activity eluted as the first major protein peak. Fractions with a specific activity greater than 3,000 U mg^{-1} were collected and pooled.

Determination of alkaline protease activity

Alkaline protease activity was determined according to the method described by Takami et al. [35], with one unit of activity being defined as the amount of enzyme able to produce 1 μg tyrosine in 1 min under assay conditions. All activity assays were carried out independently five times, and the results were calculated as mean values. The standard errors of mean values were lower than 3%.

Protein measurement

Protein was measured by the Coomassie Blue G-250 binding method [30, 34] using bovine serum albumin as the standard.

Electrophoretic analysis and zymogram

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [24], using a 5% (w/v) stacking gel and a 12% (w/v) resolving gel. Electrophoresis was performed at a constant 100 V for 90 min in Tris–glycine buffer, pH 8.3 (25 mM Tris-HCl, 192 mM glycine, 1 g L^{-1} SDS). Enzyme samples were denatured by boiling for 3 min in the presence or absence of 5 mM phenylmethanesulfonylfluoride (PMSF) before loading onto the stacking gel. After electrophoresis, proteins in the separating gel were visualized by silver staining [9]. Protein molecular weight markers containing seven proteins covering the molecular weight range between 14.4 and 116.0 kDa were used for molecular weight determination (Protein Molecular Weight Marker SM0431; MBI Fermentas, St. Leon Rot, Germany).

To prepare a zymogram, SDS-PAGE was carried out according to a method modified from that of Ferrero et al. [12]. A 12% (w/v) resolving gel containing 1% skim milk was used in electrophoresis. The gels were then rinsed for 1 h in 0.25% Triton-X100, and were further incubated for 1 h at 35°C in 0.1 M Glycine–NaOH buffer, pH 10.0 for proteolysis of skim milk proteins. Afterwards, the gels were stained with 0.2% Coomassie Brilliant Blue G250 solution. A clear zone on the destained gel indicates the presence of alkaline protease activity.

Effect of temperature and pH on alkaline protease activity profiles

The temperature profile of the enzyme (9,882 U mg^{-1} specific activity, 0.152 mg mL^{-1} protein) was estimated at temperatures between 30 and 70°C in the presence and absence of Ca^{2+} ions at pH 10.5. The pH profile of the enzyme (4,470 U mg^{-1} specific activity, 0.050 mg mL^{-1} protein) was estimated at 30°C and pH values between 8.0 and 13.0. Enzyme activity at each temperature and pH value was measured as described above.

Temperature and pH stability of alkaline protease

The thermal stability profile of the enzyme (9,882 U mg^{-1} specific activity, 0.152 mg mL^{-1} protein) was estimated at

temperatures between 30 and 65°C. The enzyme was incubated at different temperatures for 2 h and residual activity at each temperature was then measured. The pH stability profile of the enzyme (4,470 U mg^{-1} specific activity, 0.050 mg mL^{-1} protein) was estimated at pH values between 8.0 and 13.0. The enzyme was incubated for 4 days at room temperature, and residual activity at each pH value was then measured. The temperature and pH stability were expressed as percent residual activity, taking the initial enzyme activity at each temperature and pH value studied as 100%.

Effect of metal ions on alkaline protease activity

The effects of Ca^{2+}, Ba^{2+}, Zn^{2+}, Mg^{2+} and Cu^{2+} ions on alkaline protease (4,470 U mg^{-1} specific activity and 0.05 mg mL^{-1} protein) were investigated by adding these ions to the reaction mixture at a concentration of 5 mM. Relative enzyme activities were measured at 30°C.

Effect of hydrogen peroxide as an oxidizing agent on alkaline protease activity

The effect of 5% hydrogen peroxide on alkaline protease activity (4,470 U mg^{-1} specific activity, 0.050 mg mL^{-1} protein) was investigated by incubating the reaction mixture for 30 and 60 min at 30 and 40°C. Percent residual activity was measured.

Effect of active-site-directed irreversible inhibitors on alkaline protease activity

Iodoacetate, diethyl-pyrocarbonate (DEPC) *N*α-*p*-tosyl-L-lysine chloro-methyl ketone (TLCK) and *Np*-tosyl-L-phenyl alanine chloro-methyl ketone (TPCK) were used for the modification of histidine residues. Serine, lysine, arginine, tryptophan and aspartate residues were modified with PMSF, ethylacetimidate, phenylglyoxal, *N*-bromosuccinimide and *N*-ethyl-5-phenyl-iso-oxasolium-3′-sulfonate, respectively. Iodoacetic acid, iodoacetimidate and *N*-ethylmaleimidate were used for modification of cysteine residues. PMSF, *N*-bromosuccinimide and TPCK were freshly prepared in absolute ethanol, acetone and methanol, respectively, as stock solutions, and were diluted to appropriate concentrations with 50 mM NaOH–glycine–NaCl buffer, pH 10.5. *N*-Ethylacetimidate was dissolved in 50 mM NaOH–glycine–NaCl buffer, pH 10.5. Other active site inhibitors were dissolved in 50 mM NaOH–glycine–NaCl buffer, pH 10. Enzyme solution (4,470 U mg^{-1} specific activity and 0.05 mg mL^{-1} protein concentration) was mixed with each inhibitor at concentrations of 1 and 10 mM, and incubated at 30°C for 2 h. The percent residual activity of each enzyme solution was measured.

Substrate specificity

The substrate specificity of the enzyme was determined using *p*-nitroanilide (*p*NA)-conjugated synthetic peptide substrates: *N*-Suc-Ala-Ala-Ala-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, *N*-Suc-Ala-Ala-Val-*p*NA, *Np*-Tos-Gly-Pro-Lys-*p*NA, L-Leu-*p*NA, Nα-benzoyl-L-Arg-*p*NA (L-BAPNA) and *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. A stock solution (5 mM) of each substrate was prepared in dimethyl sulfoxide (DMSO); 25 μL of each substrate stock solution was added to 465 μL 50 mM glycine–NaOH buffer, pH 10.5, mixed and pre-incubated at 37°C for 5 min. After addition of 10 μL enzyme solution (7,019 U mg^{-1} specific activity and 0.148 mg mL^{-1} protein concentration) each reaction mixture was incubated for 10 min. The reaction was then stopped by adding 100 μL 2% acetic acid solution, and the absorbance of the mixture at 410 nm was measured. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μmol *p*NA per minute at 37°C and pH 10.5.

Kinetic parameters for *N*-Suc-Ala-Ala-Pro-Phe-*p*NA and Hammarsten casein

The initial reaction rates for *N*-Suc-Ala-Ala-Pro-Phe-*p*NA were measured in the 0.2–0.5 μM concentration range of this substrate at 37°C and pH 10.5. The initial reaction rates for Hammarsten casein were measured at the 0.25–2.00 mg mL^{-1} concentration range of this substrate at 30°C and pH 10.5. The K_m and V_{max} values of the purified enzyme for both substrates were determined according to Michaelis–Menten kinetics using a Lineweaver–Burk plot and used to calculate k_{cat} and k_{cat}/K_m values. The k_{cat} values were calculated from the equation $V_{max} = k_{cat} \times [E]_t$, where $[E]_t$ is the total alkaline protease concentration in reaction mixture.

Effect of surface-active agents on alkaline protease activity

The effects of 0.4% (w/v) SDS, 2.0% (w/v) sodium-perborate (SPB), Tween-20, Tween-40, Tween–60, and Tween-80 on alkaline protease activity (4,470 U mg^{-1} specific activity and 0.05 mg mL^{-1} protein concentration) were investigated by incubating the reaction mixtures for 1 h at 30°C. The residual activity was measured.

Results

Purification of alkaline protease from *B. clausii* GMBAE 42

Table 1 shows the purification profile of alkaline protease from *B. clausii* GMBAE 42. The elution profile of

Table 1 Purification profile of alkaline protease from *Bacillus clausii* GMBAE 42

Step	Volume (cm^3)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification yield (%)	Purification fold
Culture supernatant	900	53,991	126	429	100.00	1.0
$(NH_4)_2SO_4$ precipitation (75% saturation)—redissolved and dialyzed	33	35,301	63	556	65.39	1.3
Pooled fractions of DEAE-cellulose column eluate	30	31,243	4.56	6,852	57.86	16.0

the enzyme from DEAE-cellulose anion exchange chromatography column is shown in Fig. 1. The highest specific activity of 6,852 U mg^{-1} was obtained in the pooled fractions from the DEAE-cellulose column. The overall yield of purified enzyme was 57.86% and the purification was 16-fold. SDS-PAGE analysis of the purified alkaline protease revealed a single protein band with a molecular weight of 26,550 kDa (Fig. 2). The alkaline protease band could be visualized only when PMSF was added to the DEAE-cellulose column eluate before boiling for denaturation; no band was observed on SDS-PAGE gels with column eluate lacking PMSF.

Temperature and pH optima of alkaline protease for hydrolysis of Hammarsten casein

The optimal temperature for enzyme activity was found to be 60°C in the absence of Ca^{2+} ions; addition of 5 mM Ca^{2+} ions into the reaction mixture resulted in a shift in the optimal temperature value from 60 to 70°C (Fig. 3). Activities at other temperatures were estimated relative to these latter activities (activity at 60 and 70°C in the absence and presence of 5 mM Ca^{2+} ions, respectively, =100%). Highest enzyme activity was measured at pH 11.3; activities at other pH values were expressed relative to the activity at pH 11.3 (=100%) (Fig. 4).

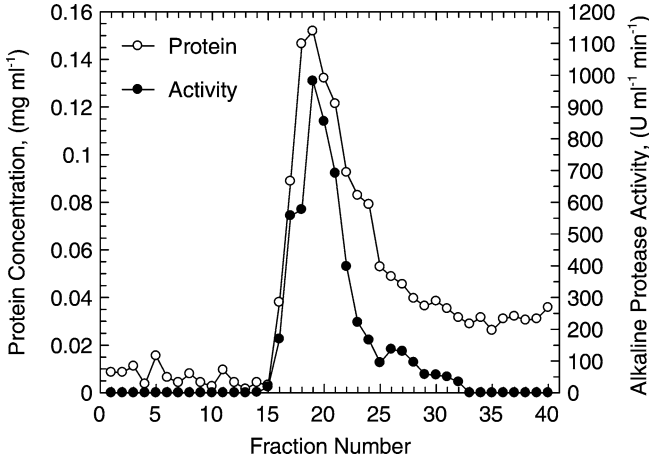

Fig. 1 Elution profile of alkaline protease from *Bacillus clausii* GMBAE 42 from a DEAE-cellulose column equilibrated and eluted with 50 mM glycine–NaOH buffer, pH 10.5

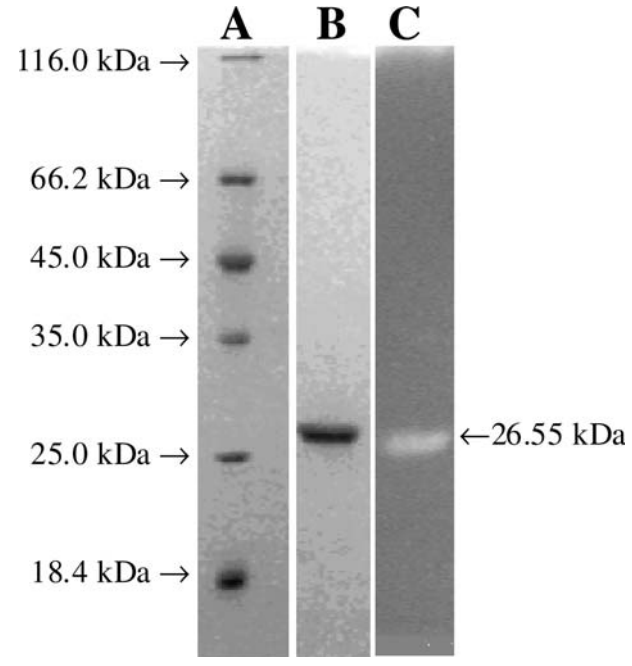

Fig. 2 SDS-PAGE of alkaline protease (12% gel, 100 V for 90 min in Tris-glycine buffer, pH 8.3). Lanes: *A* Protein molecular weight markers, *B* DEAE-cellulose column eluate treated with PMSF, *C* zymogram

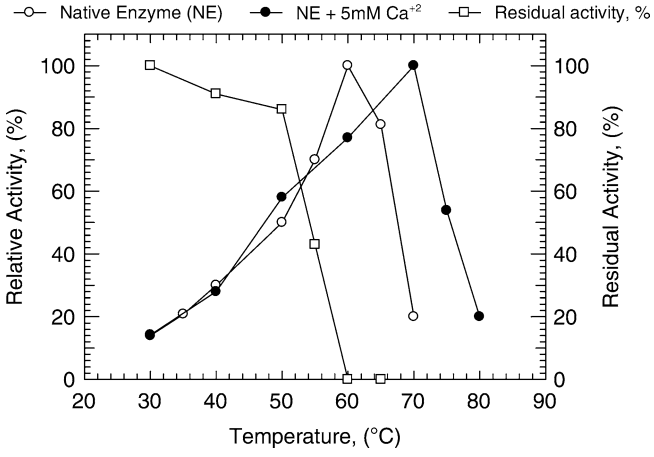

Fig. 3 Temperature stability (*open squares*) and temperature profiles of the alkaline protease from *B. clausii* GMBAE 42 in the presence (*filled circles*) and absence (*open circles*) of 5 mM Ca^{2+} ions

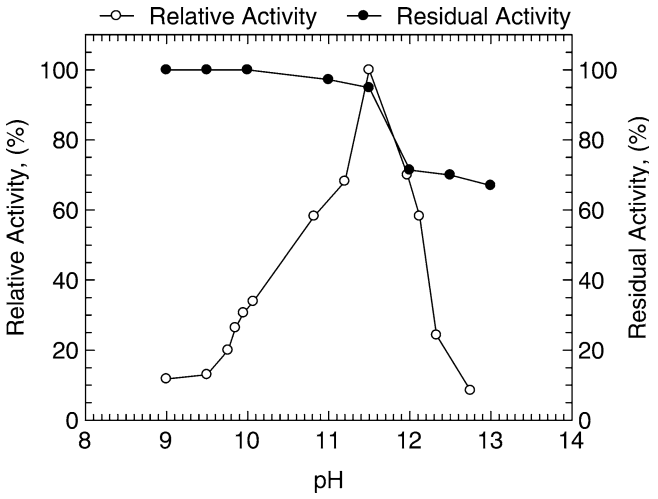

Fig. 4 pH stability (*filled circles*) and pH profiles (*open circles*) of the alkaline protease from *B. clausii* GMBAE 42

Temperature and pH stability of alkaline protease

The highly purified alkaline protease of *B. clausii* GMBAE 42 showed 100% stability at 30°C for 6 days. The enzyme was found to be stable up to 50°C after 2 h incubation. It showed 60 and 100% loss of activity at 55 and 60°C, respectively, within the same incubation time; however, 55% of activity was maintained at 60°C after 30 min incubation (Fig. 3). The enzyme was very stable over the pH range 9.0–11.5. Only 30% loss of activity was observed in the pH range 12–13 (Fig. 4).

Activation energy of alkaline protease

The activation energy of alkaline protease for Hammarsten casein hydrolysis was calculated from the slope of an Arrhenius linear plot as 10.59 kcal mol^{-1} (44.30 kJ mol^{-1}) (Fig. 5).

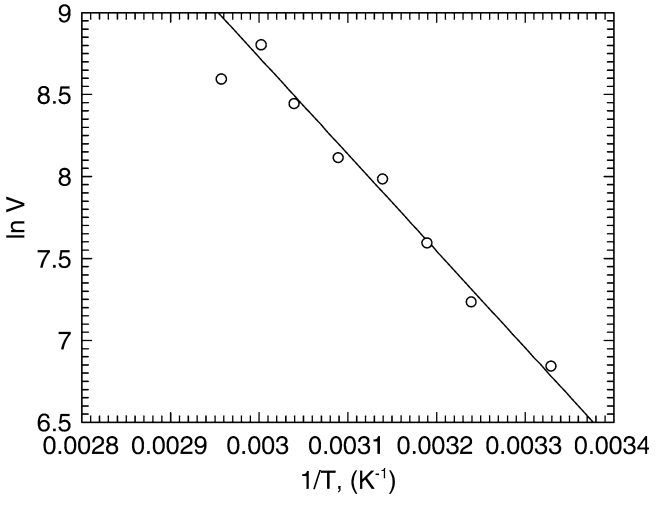

Fig. 5 Arrhenius plot to estimate the activation energy of Hammarsten casein hydrolysis by the alkaline protease from *B. clausii* GMBAE 42 ($r^2 = 0.9876$)

Table 2 Effect of various metal ions on the alkaline protease activity purified from *B. clausii* GMBAE 42

Metal ions in alkaline protease solution	Relative enzyme activity (%)
Metal-ion-free enzyme (MFE)	100
MFE + 5 mM $CaCl_2$	88
MFE + 5 mM $CuCl_2$	140
MFE + 5 mM $MgCl_2{\cdot}6H_2O$	103
MFE + 5 mM $MnCl_2{\cdot}4H_2O$	116
MFE + 5 mM $ZnCl_2$	98

Effect of various metal ions on alkaline protease activity

Of the metal ions tested, Cu^{2+} and Mn^{2+} were found to stimulate the enzyme; however, Ca^{2+} showed a slight inhibitory effect. Enzyme activity was not significantly affected by Zn^{2+} and Mg^{2+} ions (Table 2). Ca^{2+} ions were unique in causing a shift in the temperature optimum of the enzyme from 60 to 70°C.

Effect of hydrogen peroxide as an oxidizing agent on alkaline protease activity

The enzyme was stable in 5% hydrogen peroxide for 60 min at 30°C. However, 10 and 30% activity losses were observed at 40°C for incubation times of 30 and 60 min, respectively (data not shown).

Effect of active site inhibitors on alkaline protease activity

The enzyme was not inhibited by cysteine-specific iodoacetic acid and iodoacedamide, histidine-specific diethylpyrocarbonate, lysine-specific ethylacetimidate, arginine-specific phenylglyoxal, tryptophan-specific *N*-bromosuccinimide, or aspartate-specific *N*-ethyl-5-phenylisoxazolium-3′-sulfonate at the concentrations studied. Little or no inhibition was observed by histidine-specific TLCK and TPCK at 1 mM concentration. However, the serine-specific inhibitor PMSF caused 80% and complete inhibition of the enzyme at 1 and 10 mM concentrations, respectively (Table 3). These findings indicate the absence of cysteine, histidine, lysine, arginine, tryptophan and aspartate residues at the catalytic site of enzyme. On the other hand, complete inhibition of the enzyme by PMSF indicates the existence of a serine residue at the active site. Consequently, the enzyme can be considered as a serine alkaline protease. EDTA had no inhibitory effect on enzyme activity even at 10 mM concentration (Table 3), indicating that the enzyme has no requirement for metal ions as cofactor.

Table 3 Effect of active site inhibitors on the activity of alkaline protease purified from *B. clausii* GMBAE 42

Active site inhibitor	Concentration (mM)	Residual activity (%)
Iodoacetic acid	1	100
	10	100
Iodoacetamide	1	100
	10	100
Ethylacetimidate	1	100
	10	100
Phenyl methyl sulphonyl fluoride (PMSF)	1	18
	10	0
Phenylglyoxal	1	100
	10	100
N-Bromo-succinimide (NBS)	1	100
	10	100
Diethyl pyrocarbonate (DEPC)	1	100
	10	100
Ethylene diamine tetra acetic acid (EDTA)	1	100
	10	100
N-Ethyl maleimidate	1	100
	10	100
N-Ethyl-5-phenyl isoxazolium-3′-sulfonate	1	100
	10	100
Nα-*p*-tosyl-L-lysine chloro-methyl ketone (TLCK)	1	99.50
Np-tosyl-L-phenyl alanine chloro-methyl ketone (TPCK)	1	93.6

Substrate specificity of alkaline protease

The substrate specificity of the enzyme was defined by using various peptidyl-*p*-NAs. Among the substrates studied, highest specificity was observed for *N*-Suc-Ala-Pro-Phe-*p*NA (Table 4). The enzyme has no specificity for L-leu-*p*NA or L-BAPNA. Relatively low specificity was observed for *N*-Suc-Ala-Ala-Ala-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, *N*-Suc-Ala-Ala-Val-*p*NA and *Np*-Tos-Gly-Pro-Lys-*p*NA. *N*-Suc-Ala-Pro-Phe-*p*NA is a specific substrate for chymotrypsin-like serine proteases. Thus, the alkaline protease of *B. clausii* GMBAE 42 can be considered as a chymotrypsin-like serine protease.

Kinetic parameters of alkaline protease

The K_m, k_{cat} and k_{cat}/K_m values for the hydrolysis of Hammarsten casein and *N*-Suc-Ala-Ala-Pro-Phe-*p*NA by the alkaline protease purified from *B. clausii* GMBAE 42 are shown in Table 5. The $[E]_t$ values used in kinetic measurements of Hammarsten casein and *N*-Suc-Ala-Ala-Pro-Phe-*p*NA hydrolysis were 5.4×10^{-3} and 5.5×10^{-3} μM, respectively. Comparison of the kinetic parameters for both substrates indicates a higher specificity of the enzyme for the substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA.

Table 4 Substrate specificity of alkaline protease purified from *B. clausii* GMBAE 42 towards oligopeptidyl-*p*-nitroanilide (*p*-NA) substrates

Substrate	Specific activity (U mg^{-1})
N-Suc-Ala-Ala-Pro-Phe-*p*NA	45.83
N-Suc-Ala-Ala-Pro-Leu-*p*NA	8.67
N-Suc-Ala-Ala-Ala-*p*NA	1.73
N-Suc-Ala-Ala-Val-*p*NA	1.02
Np-Tos-Gly-Pro-Lys-*p*NA	0.21
L-Leu-*p*NA	0.00
Nα-Benzoyl-L-Arg-*p*NA	0.00

Effect of surface-active agents on alkaline protease activity

The enzyme was highly stable in the presence of the non-ionic surface-active agents Tween-20, Tween-40, Tween-60, and Tween-80 (all at 1.0% (w/v); Table 6). Tween-60 had a slightly positive effect on alkaline protease activity. The same level of stability was also observed in the presence of 0.2% (w/v) SDS as anionic surface-active agent. Activity was reduced only 6% by 1.0% (w/v) SPB.

Discussion

Enzyme purification

Although many alkaline proteases have been purified and characterized from different *Bacillus* species, there are few, if any, reports in the literature on the characterization of *B. clausii* alkaline protease. In this work, the extracellular serine alkaline protease of *B. clausii* GMBAE 42 was purified 16-fold with a yield of 58%. Similar procedures were applied by Singh et al. [32] to the purification of serine alkaline protease from the newly isolated *Bacillus* sp. SSR1, and by Beg and Gupta [6] to a thiol-dependent serine alkaline protease from *Bacillus mojavensis*. These latter two studies achieved almost the same level of purification, however, our

Table 5 Michaelis-Menten kinetic parameters for hydrolysis of Hammarsten casein and *N*-Suc-Ala-Ala-Pro-Phe-*p*NA by the alkaline protease purified from *B. clausii* GMBAE 42

Substrate	K_m	V_{max}	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mg mL^{-1})
Hammarsten casein	1.8 mg mL^{-1}	11.50 µmol tyrosine mL^{-1} min^{-1}	2.13×10^3	1.183×10^3
Suc-Ala-Ala-Pro-Phe-*p*NA	0.655 µM (0.41 mg mL^{-1})	23.15 µmol *p*NA mL^{-1} min^{-1}	4.21×10^3	10.268×10^3

purification yield was considerably higher than that of Beg and Gupta [6], and slightly higher than that of Singh et al. [32]. Alkaline proteases are generally positively charged enzymes [15]; consequently, they are not bound to anion-exchangers. In our work, as in the case of Beg and Gupta [6], the alkaline protease of *B. clausii* was obtained as the unbound fraction on an anion exchanger DEAE-cellulose column preequilibrated and eluted at pH 10.5. Joo et al. [20] recently reported a procedure for the partial purification of oxidant- and SDS-stable alkaline protease from *B. clausii* I-52 but no data related to this purification process was available to allow comparison with our results. Takami et al. [35] reported the 3-fold purification of an extremely thermostable alkaline protease from *Bacillus* sp. no. AH-101 with purification yield of 20%. Four-fold purification of a serine alkaline protease from *Bacillus pumilus* was reported by Huang et al. [16]. Compared with our process, the latter study included no preliminary precipitation steps in their purification procedure. The omission of an ammonium sulfate precipitation step in the purification process prior to ion-exchange chromatography may explain the low level of purification achieved. Relatively higher purification levels and yields of alkaline proteases of various *Bacillus* sp. were obtained by Kobayashi et al. [22] and Fujiwara et al. [13] with procedures consisting only of ion-exchange chromatographic steps. On the other hand, 22-fold purification of a serine alkaline protease from *Bacillus sphaericus* with 52% recovery was achieved by Singh et al. [33] by using phenyl agarose hydrophobic interaction chromatography (HIC) and Q-Sepharose ion-exchange chromatography following ammonium sulfate precipitation. Their yield was slightly higher than ours, but there was one more chromatographic step in their purification process.

When the DEAE-cellulose column eluate was analysed by SDS-PAGE, no protein band was visible after staining of the gel with Coomassie brilliant blue G250.

Table 6 Effect of surface-active agents on the alkaline protease activity purified from *B. clausii* GMBAE 42. *SPB* Sodium per borate, *SDS* sodium-dodecyl-sulfate

Surface-active agent	Residual activity (%)
Free enzyme (FE)	100.00
FE + 1.0% (w/v) SPB	93.89
FE + 0.2% (w/v) SDS	104.00
FE + 1.0% (w/v) Tween-20	103.90
FE + 1.0% (w/v) Tween-40	97.58
FE + 1.0% (w/v) Tween-60	119.20
FE + 1.0% (w/v) Tween-80	101.00

This may have been due to autolysis of the enzyme in the denaturing conditions encountered prior to loading the electrophoresis gel. To prevent such autolysis, the enzyme samples were preincubated in the presence of PMSF before denaturing; the alkaline protease was now visible by silver staining. Similar observations were reported by Kobayashi et al. [22] and Singh et al. [33] on SDS-PAGE analysis of the serine alkaline proteases of *Bacillus* KSM-K16 and *B. sphaericus*, respectively. Generally, the molecular weights of the alkaline proteases of various *Bacillus* species lie within the range 15–36 kDa [6, 16, 21, 28, 32, 33, 35]. However, the presence in some *Bacillus* species of distinctive alkaline proteases with molecular weights of 42 and 68 kDa has also been reported [33, 36]. The molecular weight of the alkaline protease of *B. clausii* GMBAE 42—26.55 kDa—places it within the 15–36 kDa range.

Effect of temperature and pH on enzyme activity and stability

The optimal temperature of alkaline proteases of various *Bacillus* species generally lies within the range 55–60°C [4, 6, 12, 17, 20, 22, 27]. However, the optimal temperature of some mesophilic *Bacillus* species, e.g., *Bacillus horikoshii, Bacillus mycoides* and *B. pumilus*, is between 40 and 55°C [1, 16, 19, 32], and that of some thermophilic *Bacillus* species, e.g., *Bacillus cereus* and *Bacillus stearothermophilus*, between 70 and 75°C [13, 28, 35, 36]. On the other hand, an upward shift of 5–15°C was observed upon addition of 2–5 mM Ca^{2+} to alkaline protease preparations of some *Bacillus* species [13, 17, 22, 28, 32, 36]. The optimal temperature of the alkaline protease of *B. clausii* GMBAE 42 was estimated at 60°C, and a shift in the optimal temperature from 60 to 70°C was also observed after addition of 5 mM Ca^{2+}.

The majority of alkaline proteases of various *Bacillus* sp. are thermostable for 10–15 min in the 30–60°C temperature interval [13, 22, 28, 35, 36]. Ferrero et al. [12] reported a protease from *B. licheniformis* that is stable for 10 min at temperatures up to 70°C. The serine alkaline protease of *B. pumilus* maintained 50% of its original activity after 30 min incubation at 50°C [16]. Joo et al. [19] reported a serine alkaline protease from *B. horikoshii* thermostable for 1 h at 30–45°C. They also reported a serine alkaline protease from *B. clausii* I-52 stable for 1 h at temperatures up to 55°C [20]. Beg and Gupta [6] reported a thiol-dependent serine alkaline protease from *B. mojavensis* having 100% stability up to 50°C for 3 h; only a 40% loss of activity was observed at

60°C over the same incubation period. Banerjee et al. [4] reported an alkaline protease from *Bacillus brevis* with excellent thermostability at 25–50°C over a 36-h incubation. Considering these findings, our results imply that the alkaline protease of *B. clausii* GMBAE 42 has higher thermostability at the 30–60°C temperature range than most other alkaline proteases of various *Bacillus* sp.

The optimal pH of the alkaline proteases of various *Bacillus* sp. is generally within the pH range 10.0–11.0 [4, 6, 17, 20, 22, 27, 32]. Nevertheless, the optimal pH of some *Bacillus* sp., e.g., *B. stearothermophilus* and *B. horikoshii*, is pH 9.0 [19, 28, 36], and that of some *Bacillus* sp., e.g., *B. licheniformis*, was in the range of 12.0–13.0 [12, 13, 35]. The optimal pH of *B. clausii* GMBAE 42 was found to be 11.3.

On the other hand, most of the alkaline proteases mentioned above were stable for 10–60 min at pH 6.0–12.0, and at temperatures varying from 25 to 60°C. Banerjee et al. [4] reported an alkaline protease from *B. brevis* showing 100% stability in at pH 10.0–11.0 after 1 h incubation at 60°C. Singh et al. [32] reported an alkaline protease stable for 1 h in the pH range 8.0–11.0 and at 40°C. Beg and Gupta [6] reported an alkaline protease from *B. mojavensis* stable for 2 days at room temperature at a pH between 7.0 and 11.5. Joo et al. [19] reported an alkaline protease from *B. horikoshii* stable for 16 h in the pH range 5.5–12.0. They also reported an alkaline protease from *B. clausii* I-52 showing 100% stability for 3 days in the pH range 5.0–12.0 at 30°C [20]. The alkaline protease of *B. clausii* GMBAE 42 was found to be stable for 4 days at room temperature in the pH range 9.0–11.5. Only 30% activity loss was observed for this enzyme between pH 11.0 and 13.0 under these incubation conditions. In general, all detergent-compatible enzymes currently in use have a pH optimum in the range of 9.0–12.0 and varying thermostabilities at laundry temperatures (40–70°C) [6]. Thus, the alkaline protease of *B. clausii* GMBAE 42 can be considered as a suitable candidate as an additive for commercial detergents.

Effect of metal ions on enzyme activity

Many researchers have reported the stimulatory effect of Ca^{2+}, Mg^{2+} and Mn^{2+} ions on alkaline proteases purified from various *Bacillus* sp. [2, 4, 6, 12, 16, 27, 28, 32, 35, 36]. The strong inhibitory effects of Hg^{2+} and Cu^{2+} ions on the alkaline proteases of some *Bacillus* sp. have also been reported [4, 6, 22, 27, 28]. No, or only a slight inhibitory, effect of other metal ions, such as Co^{2+}, Zn^{2+}, Na^{+}, Cd^{2+}, Al^{3+}, Fe^{2+}, Fe^{3+}, have been reported by the researchers cited above. The alkaline protease of *B. clausii* GMBAE 42 was stimulated by Cu^{2+} and Mn^{2+} ions. The stimulation of this enzyme by Cu^{2+} was interesting, because only Beg and Gupta [6] have previously reported a stimulatory effect of Cu^{2+} ions on the thiol-dependent serine alkaline protease of *B. mojavensis*. Zn^{2+} and Mg^{2+} had no significant effect on activity of the enzyme; however, Ca^{2+} ions had a slight inhibitory effect on enzyme activity in contrast to the results of many researchers. On the other hand, the fact that EDTA had no effect on the alkaline protease activity of *B. clausii* GMBAE 42 indicates that this enzyme has no requirement for a metal cofactor. An enzyme that is used as a detergent additive should not require a metal cofactor, because detergents generally contain chelating agents, which bind and chelate metal ions in detergent solutions in order to reduce water hardness.

Effect of active site inhibitors on enzyme activity

Inhibition studies can provide a first insight into the nature of the enzyme, its cofactor requirements, and the nature of the active center [30]. Most alkaline proteases from *Bacillus* sp. are completely inhibited by the serine protease inhibitor PMSF [2, 6, 12, 16, 19, 20, 22, 28, 35, 36]. The alkaline protease of *B. mojavensis* was also found to be completely inhibited by the sulphydryl protease inhibitor iodoacetic acid [6]. The alkaline proteases of *B. stearothermophilus* and *B. pumilus* are strongly inhibited by the metallo-protease inhibitor EDTA [11, 16]. The alkaline protease of *B. clausii* GMBAE 42 is also completely inhibited by PMSF. However, the enzyme was not inhibited by other active site inhibitors (Table 3). Consequently, this enzyme can also be considered as a serine alkaline protease.

Substrate specificity of the enzyme

Little work relating to substrate specificities of alkaline proteases from *Bacillus* sp. towards synthetic substrates has appeared in the literature. Yamagata et al. [37] reported the high specificity of subtilisin Sendai from an alkalophilic *Bacillus* sp. for fluorogenic peptidyl-4-methylcumaryl-7-amides (peptidyl-MCA substrates). Towatana et al. [36] reported an alkaline protease with a preference for cleavage at arginine residues on the carboxylic side of the scissile bond of the carbobenzoxy (CBZ) peptidyl-*p*-NA as substrate; their enzyme showed highest specificity against a typical tryptic substrate, *N*-CBZ-L-Arg-*p*NA with a K_m value of 0.6 mM. Studies by Singh et al. [33] on the specificity of the proteolytic activity of proteases A and B from the obligate alkalophilic *B. sphaericus* showed both proteases to be most active on *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, a substrate for chymotrypsin-like serine proteases. The K_m values of proteases A and B for this substrate were 96 μM and 116 μM, respectively. Kobayashi et al. [22] reported H and M proteases from a *Bacillus* strain most active against the oligopeptidyl substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. The K_m and k_{cat} values for this substrate were 0.60 mM and 74.7 s^{-1}, respectively, for protease H, and 0.80 mM and 139.5 s^{-1}, respectively, for protease M. Studies on the substrate specificity of the alkaline

protease of *B. clausii* GMBAE 42 on amino acid- and peptidyl-*p*-NAs showed the enzyme to be most active on *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. The K_m and k_{cat} values of *B. clausii* GMBAE 42 alkaline protease for the chymotrypsin substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA were similar to those of Kobayashi et al. [22] and Singh et al. [33]; however, comparing kinetic parameters, our enzyme showed much higher specificity against *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. According to the substrate specificity data, the alkaline protease of *B. clausii* GMBAE 42 can be considered as a chymotrypsin-like serine protease although no inhibition of enzyme by the chymotrypsin inhibitor TLCK was observed.

Effect of surfactants and oxidizing agents on enzyme activity

Besides temperature and pH stability, a good detergent protease should be stable against various surface-active agents and oxidizing agents used as detergent components. Oberoi et al. [27] reported an alkaline protease from *Bacillus* sp. RGR-14 that is stable for 1 h in 0.1% SDS, and retained more than 70% of its activity in non-ionic surfactants (Tween-40 and Tween-60), with a 30% enhancement in Tween-85. The enzyme was also stable for 1 h in 1% SPB, but showed a 40% activity loss with 1% H_2O_2, while the alkaline protease of *Bacillus* sp. KSM-KP43 was stable for 30 min in 10% H_2O_2 [29]. Jasvir et al. [17] reported an alkaline protease from *Bacillus* sp. NG312 stable for 1 h at 37°C in the presence of 0.2% SDS and 1.0% SPB. Manachini and Fortina [25] reported an oxidant- and bleach-stable alkaline protease from *B. licheniformis* having 100% stability in 0.7% SPB. This enzyme retained 45% of its activity after a 30-min treatment with H_2O_2 at 40°C. Activity losses of 40% and 5% were observed after a 1-h treatment of the alkaline protease of *Bacillus* sp. SB5 with 1% SDS and 5% H_2O_2, respectively [14]. The alkaline protease of *Bacillus* sp. JB-99 retained 75% of its activity in 0.5% SDS after a 2-h treatment at 40°C; however, the enzyme was 100% stable in 5% H_2O_2 solution under the same incubation conditions [18]. Beg and Gupta [6] reported that the alkaline protease of *B. mojavensis* showed 100% stability for 1 h at room temperature in the presence of 1% of various surfactants (SDS, Tween-20, Tween-40, Tween-60, Tween-80, and Tween-85) and some oxidising agents such as H_2O_2 and SPB. Joo et al. [20] reported an oxidant- and SDS-stable alkaline protease from *B. clausii* I-50. This enzyme retained more than 75% and 110% of its activity following a 72-h treatment with 5% SDS and 10% H_2O_2, respectively. The alkaline protease of *B. clausii* GMBAE 42 showed considerable stability towards SDS, non-ionic surfactants, and oxidising agents such as H_2O_2 and SPB, and thus can be considered as a potential additive for commercial detergents.

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