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ORIGINAL PAPER

Characterization of an extracellular alkaline serine protease from marine *Engyodontium album* BTMFS10

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Abstract An alkaline protease from marine *Engyodontium album* was characterized for its physicochemical properties towards evaluation of its suitability for potential industrial applications. Molecular mass of the enzyme by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) analysis was calculated as 28.6 kDa. Isoelectric focusing yielded pI of 3–4. Enzyme inhibition by phenylmethylsulfonyl fluoride (PMSF) and aprotinin confirmed the serine protease nature of the enzyme. K_m, V_{max}, and K_{cat} of the enzyme were 4.727×10^{-2} mg/ml, 394.68 U, and 4.2175×10^{-2} s^{-1}, respectively. Enzyme was noted to be active over a broad range of pH (6–12) and temperature (15–65°C), with maximum activity at pH 11 and 60°C. $CaCl_2$ (1 mM), starch (1%), and sucrose (1%) imparted thermal stability at 65°C. Hg^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, Cd^{+}, and Al^{3+} inhibited enzyme activity, while 1 mM Co^{2+} enhanced enzyme activity. Reducing agents enhanced enzyme activity at lower concentrations. The enzyme showed considerable storage stability, and retained its activity in the presence of hydrocarbons, natural oils, surfactants, and most of the organic solvents tested. Results indicate that the marine protease holds potential for use in the detergent industry and for varied applications.

Keywords *Engyodontium album* · Serine protease · Detergent enzyme · Characterization · Amino acid analysis · MALDI-MS

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Introduction

New and emerging applications have led to a market-driven demand for enzymes, and the industry is responding with a continuous stream of innovative products. As a result, the world market for industrial enzymes, which was worth US $2.3 billion by the end of 2007, is expected to reach US $2.7 billion by 2012, with a compound annual growth rate (CAGR) of 4% [5]. In fact, the industrial enzyme market is divided into three application segments: technical enzymes, food enzymes, and animal feed enzymes. Amongst these, technical enzymes for detergent, pulp, and paper manufacturing have the largest segment, with approximately 52% market share, and the largest share of the enzyme market has been held by alkaline proteases. Proteases are the single class amongst all enzymes that has a wide range of applications in both physiological and commercial fields. They are hydrolytic enzymes that catalyze cleavage of peptide bonds in other proteins. Today, proteases account for approximately 60% of total enzyme sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management, and silver recovery [28, 46]. This dominance of proteases in the industrial market is expected to increase further in coming years.

Technological application of enzymes under demanding industrial conditions makes the currently known arsenal of

enzymes insufficient, and the search for new microbial sources is continuing. In fact, only 2% of the world's microorganisms have been tested as sources of enzymes [59], although microorganisms from diverse and exotic environments including extremophiles are considered as an important source of enzymes, and their specific properties are expected to result in novel process applications [24]. About 80% of commercial enzymes are produced using microorganisms which are primarily derived from terrestrial sources. Although the oceans cover more than two-thirds of the Earth's surface, knowledge on marine microbes as a source of enzymes is still very limited, and they remain an untapped source of many metabolites and biomolecules with novel properties.

We previously reported production of a novel extracellular protease from an alkaliphilic salt-tolerant marine *Engyodontium album* BTMFS10 under solid-state fermentation [11], and molecular cloning of the gene encoding the enzyme and homology modeling of the enzyme [33]. Detailed knowledge on the physicochemical characteristics of an enzyme is a prerequisite for efficient utilization of the enzyme for any application. Herein, we report the physicochemical characterization of the protease, and its stability under varying conditions, including the impact of metals, solvents, surfactants, hydrocarbons, and natural oils, with the purpose of evaluating the potential of the enzyme in the detergent industry and for other industrial applications. To the best of our knowledge, this is the first time that an alkaline protease from marine fungus *E. album* has been characterized for application purposes.

Materials and methods

Microorganism, and preparation of purified protease

Engyodontium album BTMFS10, isolated from marine sediment of Cochin coast and available as stock culture at Microbial Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology was used in the present study. Protocols for measurement of protease activity [39], estimation of protein [42], and enzyme purification were as described elsewhere [11].

Molecular mass determination by gel filtration chromatography

Molecular mass of the protease was determined using gel filtration chromatography of the purified active enzyme fraction, obtained after ion-exchange chromatography. Sephadex G75 (Sigma–Aldrich), used as the column matrix, was suspended in distilled water and allowed to hydrate for 3 h at 100°C in a water bath, and the fine particles were removed by decantation. The hydrated gel suspension was then degassed under vacuum to remove air bubbles. The gel suspension was then carefully poured into the column (Amersham Biosciences XK26/70 column) without trapping air bubbles and then allowed to settle under gravity while maintaining a slow flow rate through the column. Later, the column was stabilized by allowing two times the bed volume of eluent (0.1 M phosphate buffer, pH 7.0) to pass through the column bed in descending eluent flow. An aliquot of 2 ml of the sample, obtained after ion-exchange chromatography [11], with protein content of 1.94 mg/ml, was then applied to the column. After complete entry of the sample into the column, the proteins were eluted using 0.1 M phosphate buffer (pH 7.0) with flow rate of 1 ml/min. Eluent fractions (1 ml) were collected, and their protein contents were estimated by measuring the absorbance at 280 nm in an ultraviolet (UV)–visible spectrophotometer (Shimadzu, Japan). Peak fractions from the column were pooled and assayed for protease activity and protein content.

Isoelectric focusing, amino acid analysis, and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)

Isoelectric focusing of the purified protein sample was performed following the method of O'Farrells [43], and amino acid analysis of the purified protein sample was done using a Shimadzu high-performance liquid chromatograph (LC-4A) Amino Acid Analysis System [2].

The molecular mass and the peptide fingerprinting of the purified sample were determined using MALDI-MS analysis. The protease purified by ion-exchange chromatography was electrophoresed [11], and the stained protease band was used for MS analysis (Applied Biosystems-Voyager System 4263). For the peptide fingerprint, the protein bands digested with trypsin were extracted and desalted, and a mass spectrum was generated. The mass peak list obtained was submitted to Mascot (www.matrixscience.com) database for protein identification.

Determination of class specificity of the enzyme using protease inhibitors

Effect of various protease inhibitors on the purified enzyme sample was determined to classify the enzyme based on their inhibition pattern. The inhibitors evaluated included the following: 20–50 mM phenylmethylsulfonyl fluoride (PMSF), 0.1–1 μM aprotinin, 20–50 mM ethylenediamine tetraacetic acid (EDTA), 10 mM 1,10-phenanthroline, 10–50 μM L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, *N*-[*N*′-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl]-agmatine (E-64), 1–50 μM iodoacetamide, and

5–100 μM pepstatin. Enzyme inhibitors were added to the purified enzyme and then incubated at room temperature (RT, 28 ± 2°C) for 30 min. After incubation, an aliquot of 1 ml 1% casein was added to each enzyme reaction mixture, and residual enzyme activity was measured and expressed in terms of percentage.

Substrate specificity and kinetic studies

Substrate preference of the purified enzyme was evaluated with casein, gelatin, hemoglobin, and bovine serum albumin (BSA), according to the method of Kunitz [39], with 1% solution of the respective substrates prepared in carbonate-bicarbonate buffer, pH 10.0. Trichloroacetic acid (TCA) soluble fractions were measured at 280 nm, and enzyme activity was expressed in U/ml.

Kinetic studies of the purified enzyme were performed in order to determine the K_m, V_{max}, and K_{cat}. K_m, the substrate concentration at which the reaction velocity is half-maximum, and V_{max}, the velocity maximum of the enzyme reaction, were determined by incubating 0.2 ml purified enzyme in different concentrations of casein (0.01–2.0 mg), at pH 10.0, for 30 min at 40°C. The initial velocity data was plotted as a function of concentration of substrate by linear transformation and usual nonlinear curve fitting of the Michaelis–Menten equation for calculation of K_m and V_{max} of the reaction.

Stability of the protease at different pH and temperature, and effect of stabilizers on thermal stability of the enzyme

Stability of the purified enzyme over a range of pH was determined by incubating the enzyme in different buffer systems of pH 2–13 for 24 h at 4°C, and measuring the residual activity at pH 10.0. An aliquot of 0.2 ml purified enzyme was incubated in 3.8 ml aliquots of different buffer systems which included HCl–KCl (pH 2.0), citrate–phosphate (pH 3–6), phosphate (pH 7.0), Tris–HCl (pH 8.0), glycine-NaOH (pH 9.0), carbonate-bicarbonate (pH 10.0), boric acid/KCl/NaOH (pH 11.0), Na_2HPO_4/NaOH (pH 12.0), and KCl/NaOH (pH 13.0). After incubation, 0.2 ml fractions of the samples were assayed for protease activity.

Temperature stability of the purified enzyme was determined by incubating enzyme samples at various temperatures ranging from 30°C to 80°C over a period of incubation, and measuring the residual activity of the enzyme. Enzyme assay was conducted after incubation for 30 min and 1, 2, 4, 6, 8, 10, 12, and 24 h. Enzyme activity of the sample maintained at 4°C was taken as control.

Effect of stabilizers on enzyme activity at higher temperature was studied by incubating enzyme solution supplemented with various reported thermal stabilizers at 65°C and 70°C for 3 h, and estimating the residual activity at regular time intervals. Stabilizers studied included $CaCl_2$ (1, 5, and 10 mM), $CoCl_2$ (1 mM), PEG 6000, glycerol, sucrose, mannitol, sorbitol, starch, glycine, and BSA (at 1% level).

Effect of various metal ions on enzyme activity

Effect of various metal ions on enzyme activity was evaluated by incubating enzyme along with different concentrations (1, 5, 10, 15, and 20 mM) of various metal ions in the enzyme reaction mixture for 30 min, followed by measuring residual enzyme activity.

Effect of various surfactants, organic solvents, reducing agents, oxidizing agents, and NaCl on enzyme activity

Impact of various surfactants and organic solvents on enzyme activity was evaluated by incubating enzyme with the respective surfactant and solvent for 30 min and then assaying the residual activity. The nonionic and ionic surfactants studied were Triton X-100, SDS, Tween 80, Tween 20, and Brij 35 (w/v) at 0.2%, 0.4%, 0.6%, 0.8%, 1%, and 5% level. Organic solvents studied included dimethyl sulfoxide (DMSO), isopropanol, acetonitrile, ethanol, petroleum ether, acetone, and ethyl ether [1%, 5%, and 10% (v/v)]. Effect of reducing agents and oxidizing agents on the activity and stability of the enzyme was studied by incubating enzyme in the presence of respective reducing and oxidizing agents in solution for 30 min and measuring the residual activity. Reducing agents studied included dithiothreitol, β-mercaptoethanol, and sodium thioglycolate [0.2%, 0.4%, 0.6%, 0.8%, 1%, and 5% (v/v)]. Hydrogen peroxide [1%, 2%, 3%, 4%, 5%, and 8% (v/v)] was used as the oxidizing agent. Effect of NaCl on the enzyme activity was tested by measuring residual enzyme activity after incubating the enzyme assay mixture incorporated with various ionic concentrations of the same (1, 2, 3, 4, and 4.5 M) for 30 min.

Stability of the enzyme in the presence of hydrocarbons and natural oils

Stability of the enzyme in the presence of the hydrocarbons was evaluated by incubating the enzyme with the various hydrocarbons (petrol, kerosene, diesel, grease, used machine oil, and used engine oil at 1% and 5% concentrations) for 30 min and then assaying the residual activity. Stability of the enzyme in the presence of natural oils was determined using coconut oil, gingelly oil, palm oil, mustard oil, sunflower oil, vegetable oil, olive oil, castor oil, dalda, and ghee each at 1% and 5% concentrations. The enzyme was incubated for 30 min in the presence of various natural oils, and then residual activity was estimated.

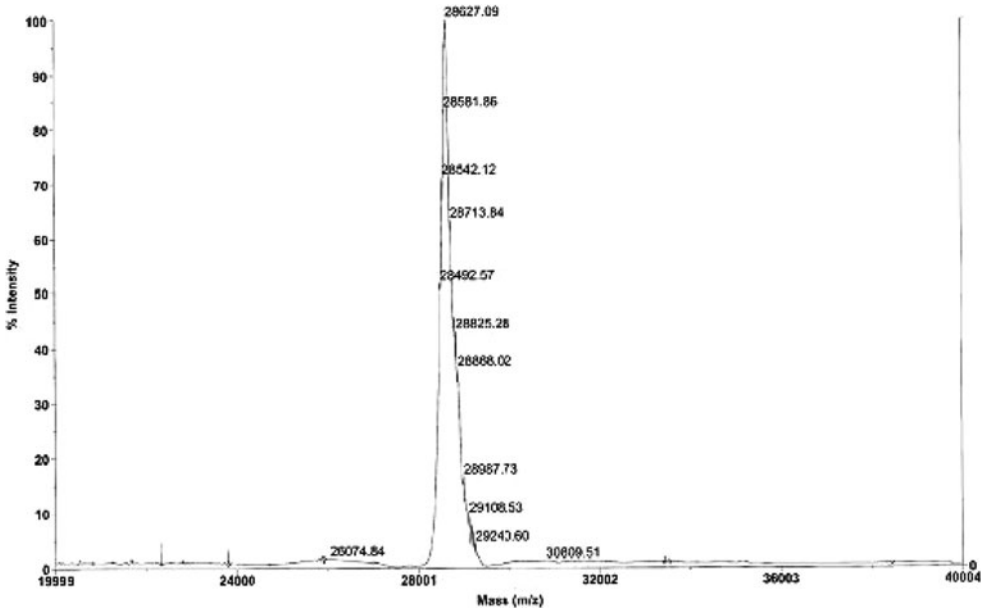

Fig. 1 MALDI mass spectrum for the purified protease enzyme

Storage stability of the protease

Storage stability of the lyophilized and liquid samples of the partially purified enzyme (i.e., 40–60% ammonium sulfate precipitate dialyzed against phosphate buffer pH 7.0) was evaluated after storage at RT, 4°C, and −20°C for a period of 1 year, followed by estimation of residual enzyme activity. Periodically, samples were drawn, properly diluted, and assayed for enzyme activity, protein content, and specific activity.

Results and discussion

Molecular mass determination and peptide fingerprinting

Gel filtration chromatography of the purified enzyme protein yielded a single peak with protease activity (data not shown). From the K_{av} value obtained for the protease, molecular mass was calculated to be 30 kDa. MALDI mass spectrum obtained for the purified protease enzyme is presented in Fig. 1, indicating that the protease is pure with a single peak having molecular mass of 28,627.09 Da. MALDI-MS is a more precise method with mass accuracy of 0.05–0.1% [34], and thus the molecular mass of *E. album* protease can be considered as 28.6 kDa. This observation correlates with our own inference on molecular mass of the mature protease after cloning and homology modeling [33]. Protease enzyme isolated from the most closely related genera of *E. album* such as *Beauveria* [13] and *Tritirachium* [51] were reported to have molecular masses of 32 and 30 kDa, respectively. The molecular mass of most of the reported serine proteases was in the range between 15 and 30 kDa [27, 30, 38, 57]. Of course, there are a few exceptions such as the serine protease from *Blakeslea trispora*, which has a molecular mass of 126 kDa [25], and the enzyme from *Kurthia spiroforme* with an extremely low molecular mass of 8 kDa [55].

Table 1 Amino acid composition of *E. album* protease

Amino acid	Concentration (μmol)
Glycine	0.670
Alanine	0.507
Glutamic acid	0.442
Leucine	0.229
Proline	0.212
Aspartic acid	0.200
Valine	0.191
Serine	0.156
Threonine	0.147
Isoleucine	0.147
Lysine	0.128
Arginine	0.076
Histidine	0.075
Phenyl alanine	0.068
Methionine	0.027
Tyrosine	0.027
Cysteine	0.013

The amino acid composition of the purified enzyme protein sample (Table 1) indicated the presence of high amount of glycine in the protein (0.670 μmol per sample) and low amount of cysteine (0.013 μmol per sample). Since the glycine residues lack a side-chain, they can avoid

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  1 EFIEQDAVVT ISATQEDAPW GLARISSQEP GGTTYTYDDS AGTGTCAYII
 51 DTGIYTNHTD FGGRAKFLKN FAGDGQDTDG NGHGTHVAGT VGGTTYGVAK
101 KTSLFAVKVL DANGQGSNSG VIAGMDFVTK DASSQNCPKG VVVNMSLGGP
151 SSSAVNRAAA EITSAGLFLA VAAGNEATDA SSSSPASEES ACTVGATDKT
201 DTLAEYSNFG SVVDLLAPGT DIKSTWNDGR TKIISGTSMA SPHVAGLGAY
251 FLGLGQKVQG LCDYMVEKGL KDVIQSVPSD TANVLINNGE GSA
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Fig. 2 *E. album* protein identification by MALDI peptide fingerprinting: matched sequences (*bold underlined*) with *Tritirachium album* precursor protease T

Fig. 3 Isoelectric focusing of purified enzyme

steric clashes encountered by other amino acids, which leads to an increase in the number of accessible conformations in the unfolded states, giving stability to the protease [16]. In the present study, it was noted that the enzyme was stable under varied conditions, as discussed below, and probably the stability of the enzyme could be attributed to the high amount of glycine in the enzyme protein among other determining factors.

Peptide fingerprint profile, computed by MALDI-MS analysis for the purified *E. album* protease enzyme, showed presence of three internal peptide sequences which revealed homology to *Tritirachium album* precursor Protease T (Fig. 2). It was also noted that one of the sequences showed homology to the conserved stretch assigned to subtilisin class serine protease.

Isoelectric focusing

The enzyme appeared as a single band on isoelectric focusing, confirming the purity of protease, with pI value between 3 and 4 (Fig. 3). This observation indicated that the enzyme is an anionic protein in the neutral to alkaline pH range. In related species of *E. album*, i.e., *Beauveria* and *Tritirachium*, the pI value was reported to be 7.5 and 4.5, respectively [51, 57]. In some cases, the pI values were identical, or nearly in the range of the optimal pH of the enzyme [30].

Protease enzyme inhibition studies using various inhibitors

Results presented in the Table 2 suggest that, amongst all the inhibitors tested, PMSF, an irreversible inhibitor of serine protease, was noted to significantly inhibit protease activity at all investigated concentrations, and at 50 mM the residual activity was found to be almost nil (98% inhibition). Reduced level of inhibition was detected in the presence of aprotinin (a reversible serine protease inhibitor), EDTA (metalloprotease inhibitor), and E-64 (cysteine protease inhibitor). Activity was not inhibited by 1,10-phenanthroline (metalloprotease inhibitor) or pepstatin (aspartic protease inhibitor). Iodoacetamide, another cysteine protease inhibitor, at its higher concentration was observed to cause slight inhibition of enzyme activity.

Table 2 Effect of protease inhibitors on enzyme activity

Inhibitor (class)	Concentration	Residual activity (%)
PMSF (serine protease)	20 mM	8.9
	40 mM	3.7
	50 mM	2.5
Aprotinin (serine protease)	0.1 μM	88.9
	0.5 μM	89.8
	1 μM	89.2
EDTA (metalloprotease)	20 mM	89.5
	40 mM	90.5
	50 mM	80
1,10-Phenanthroline (metalloprotease)	10 mM	107.9
E-64 (cysteine protease)	10 μM	90.8
	30 μM	96.3
	50 μM	93.5
Iodoacetamide (cysteine protease)	1 mM	104.6
	10 mM	108.9
	50 mM	76.9
Pepstatin (aspartic protease)	5 μM	106.1
	50 μM	115.7
	100 μM	115.0

Inhibition studies primarily give insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center [53]. Since PMSF showed 98% inhibition of protease activity, and aprotinin, a reversible inhibitor of the enzyme, caused 10% inhibition, it is concluded that the enzyme belongs to the serine protease family. PMSF sulfonates the essential serine residue in the active site of the protease, which results in complete loss of enzyme activity [22].

Generally, serine proteases are not inhibited by metal chelating agents. However, there are examples of serine proteases that are affected by EDTA [21, 31]. Detergents contain high amount of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to and chelate metal ions in the enzyme, making them unavailable in the detergent solution [6]. In this context, the stability of the protease enzyme in

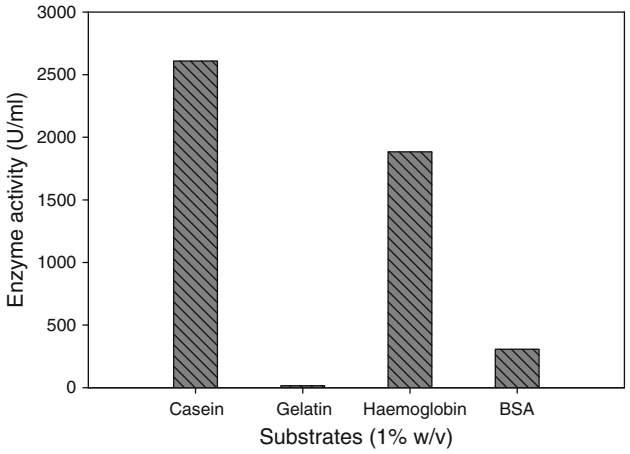

Fig. 4 Substrate specificity of *E. album* protease tested with different natural substrates

the presence of EDTA in the present study highlights the potential advantage of this enzyme for use as a detergent additive.

Substrate specificity and kinetic studies of the enzyme

Substrate specificity of protease was evaluated using various proteinaceous substrates by incubating the enzyme in 1% solution of the respective substrates. Results depicted in Fig. 4 indicated that the enzyme has highest affinity for casein followed by hemoglobin; gelatin and BSA were less preferred by the enzyme. K_m and V_{max} were estimated by plotting the initial velocity data as the function of the concentration of casein as substrate, using the Michaelis–Menten equation (data not shown). K_m and V_{max} were recorded as 4.727×10^{-2} mg/ml and 394.68 U, respectively. K_{cat} of the enzyme was estimated to be 4.2175×10^{-2} s^{-1}.

The ability to hydrolyze several protein substrates is a criterion of protease potency [26]. The highest affinity for casein as a substrate was reported earlier for the protease of the fungus *Conidiobolus coronatus* [45]. Most of the previous studies have revealed that alkaline proteases show highest activity towards casein relative to other native and modified proteins [27, 38, 45]. The substrate specificity towards hemoglobin and casein observed with *E. album* protease strongly advocates the merits of this enzyme for its possible use in detergents towards removal of blood and milk protein stains.

Effect of various metal ions on enzyme activity

Commercial detergent formulas often include water-softening "builders" [49]. Hence, it is worthwhile to evaluate the protease for its response to varying concentrations of different metal ions towards evaluating the potential of the protease for application in the detergent industry. Most of the metal ions evaluated in the present study for their effect on enzyme activity either showed no effect or exhibited a slight stimulatory effect at lower concentrations (data not shown). Of the various metals evaluated for their effect on protease activity, K^+ followed by Li^+, Pb^{2+}, Mg^{2+}, Ca^{2+}, Na^+, and Mn^{2+} did not have any negative effect and could effect marginal enhancement of enzyme activity at all tested concentrations. On the other hand, Cu^{2+}, Fe^{3+}, and Hg^{2+} showed a significant negative effect, whereas Zn^{2+}, Cd^{2+}, Mo^{6+}, and Al^{3+} showed negative effect at 5 mM and above. Cr^{3+} showed positive effect at 1 and 5 mM only. Ni^+ and Co^{2+} showed negative effect only at 15 and 20 mM. In fact, Co^{2+} effected 45% and 26% enhancement of residual enzyme activity at 1 and 5 mM, respectively, compared with any other metal tested. Next to Co^{2+}, K^+ effected 9–20% enhancement in residual activity, although 20% and 18% enhancement was effected at 15 and 20 mM, respectively. Studies have indicated that metal ions impart thermodynamic stabilization to the native state of the protein by binding to the enzyme active site [60]. According to some authors, Ca^{2+} ions are important for catalysis, and it is presumed that they stabilize the protein through specific and nonspecific binding sites, and may also allow for additional bonding within the enzyme molecule, preventing unfolding at higher temperatures, as has been demonstrated for protease from thermophilic bacteria, particularly thermolysin [32]. Ca^{2+} was reported to bind to the inner surface and autolytic sites of protein molecule, thereby strengthening the interaction inside the molecule [20]. Cu^{2+} may cause denaturation of protease [18], whereas Hg^{2+} and organomercurials interact with –SH and S–S groups of proteins in a multitude of systems, thereby causing conformational changes in proteins [58]. In *Aspergillus parasiticus*, strong stability was observed in the presence of metal ions such as Hg^{2+}, Co^{2+}, and Sn^{2+} [47].

Effect of various surfactants on enzyme activity

Most of the surfactants studied did not affect enzyme activity at their lowest concentrations tested (data not shown). However, in all cases, there was slight decrease in activity at lower concentrations of the surfactant, whereas there was enhanced activity with increasing concentration of the surfactant. In the case of Triton X-100 and Tween 80, more than 90% of activity was retained even at 1% concentration, whereas in the case of SDS, the enzyme was inactive at a lower concentration (0.2%), while 85% relative activity was recorded at 0.6% concentration. However, at higher concentrations, the enzyme lost its activity. The enzyme, with Brij 35 and Tween 20 at 5% concentration, recorded more than 70% and 80% activity, respectively. The inhibition of the enzyme at higher concentration of

SDS and other surfactants may be due to the combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and direct interactions with the protein molecule. Of course, the inhibitory effect of SDS was well documented by Creighton [14]. The increase in activity in the presence of surfactants may be attributed to increased substrate accessibility of the enzyme [9]. The property of stability towards SDS is important, because SDS-stable enzymes from wild-type microorganisms are not generally known, except for few alkaliphiles such as *Bacillus* sp. KSM-KP 43 [50] and extremophiles such as *Pyrococcus furiosus* [7] and *Thermococcus stetteri* [36]. In this context, the present results obtained with surfactants highlight the potential of this marine enzyme for detergent application.

Stability of protease enzyme at different pH and temperature, and effect of stabilizers on thermal stability

Data presented in Fig. 5 evidence that the enzyme was stable over a wide range of pH from 5.0 to 12.0. However, maximal residual enzyme activity was recorded with pH 9.0. Nevertheless, when compared with other levels of pH tested, the difference was only marginal. Optimum pH required for maximal activity was noted as pH 10.0–11.0, although the enzyme was active over pH 6.0–12.0 [11]. However, an increase in pH from 6.0 to 11.0 recorded proportionate increase in activity. Extreme pH conditions alter the structure of the surface of the enzymes, modifying the interaction between active site and substrate [29]. Optimal pH for maximal activity of alkaline protease reported earlier in the literature varied between 10.0 and 10.5 [6, 30, 47]. Even pH 11.0 and 12.3 have been reported as optima, respectively, for enzymes of *Bacillus* spp. [29, 38] and *Bacillus* sp. KSM-K16 [37]. Furthermore, it was noted that in the present study the enzyme was stable over a wide range of pH from 5.0 to 12.0, and the stability profile highlights the suitability of this protease for possible application in industrial processes carried out at alkaline or extreme alkaline conditions. The enzyme stability observed at high pH complements its potential use as an additive in washing detergents, leather processing or other applications at pH higher than 9.0.

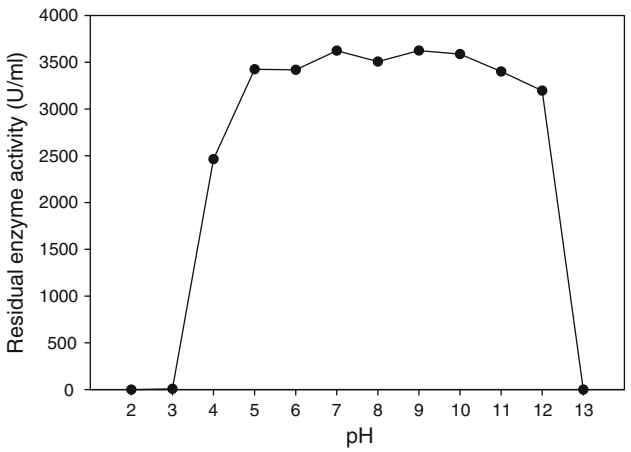

Fig. 5 Stability profile of enzyme at different pH

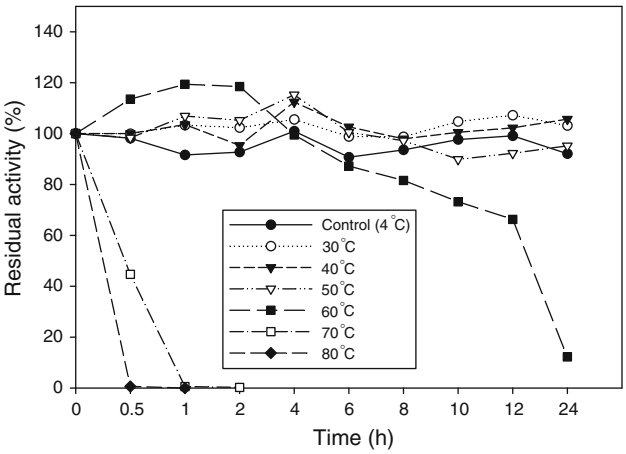

Fig. 6 Thermostability of alkaline protease at various temperatures

Results obtained for the temperature stability studies (Fig. 6) conclusively indicated that the enzyme was stable over a broad range of temperatures from 30°C to 60°C even after 24 h. At 60°C, the optimal temperature for maximal activity, the enzyme retained 66% activity after 12 h of incubation. It was observed that, at temperatures above 60°C, the enzyme was denatured and lost activity within 1 h. Among the substances tested as stabilizers, starch, glycerol, sucrose, BSA, mannitol, and sorbitol enabled enhanced thermal stability of the protease at both investigated temperatures (65°C and 70°C) compared with control (data not shown). $CaCl_2$ (1 mM), glycerol (1%), sucrose (1%), starch (1%), and BSA (1%) supported thermal stability of protease at 65°C, with more than 50% retention of activity. On the other hand, glycine (1%) and $CoCl_2$ (1 mM) did not enhance thermal stability at either investigated temperature. At 70°C, starch (1%) supported more than 60% retention of enzyme activity. Even though $CaCl_2$ at 1 mM recorded maximal residual activity at 65°C, it is inferred from the results that starch (1%) could play the role of a stabilizer promoting thermal stability at both of the higher temperatures investigated compared with the other substances tested.

In liquid formulations, physical isolation of enzymes is more difficult, and presence of solvent (water) amplifies the detrimental effect of surfactants and enhances the rate of undesirable reactions such as autolytic degradation,

oxidation, and denaturation of the enzyme [40]. This has led to the development of various enzyme stabilization strategies based on chemical additives. There are reports on enzyme stabilization using carboxylic acid salts, calcium chloride [15], and boron compounds (boric acid, borate salt), especially in conjunction with pyrrols and polyols such as propylene glycol, glycerol, mannitol, and sorbitol [3, 8, 23, 52]. The protective effect of these substances could be explained by strengthening of hydrophobic interactions inside the protein molecule and by indirect action of these compounds on water structure. Sucrose has been used to protect proteins against oxidation, aggregation, and damage during lyophilization. The stabilizing effect of sucrose is due to a preferential exclusion mechanism [56]. The improvement in protease thermal stability against thermal inactivation in the presence of Ca^{2+} may be explained by strengthening of interaction inside protein molecules and by binding of Ca^{2+} ions to autolysis sites [20]. In our case, homology modeling of *E. album* deduced protease (EAP) structure suggested three additional calcium binding sites besides the two strong calcium binding sites [33]. This could be the reason for the enhanced stability of the enzyme at 65°C in the presence Ca^{2+} ions.

Effect of reducing agents, oxidizing agents, and NaCl on enzyme activity

All the studied reducing agents led to increase in residual activity compared with control in the range of 30–36%, 15–26%, and 18–45%, in the presence of dithiothreitol, β-mercaptoethanol, and sodium thioglycolate, respectively, at concentration ranging from 0.2% to 1% (data not shown). However, β-mercaptoethanol and sodium thioglycolate at 5% led to reduction in residual activity by 28% and 60%, respectively. Oxidizing agent H_2O_2 was noted to have a drastic inhibitory effect on protease activity, since only 57% of activity was retained at 1%, and at concentrations above 2%, there was loss of enzyme activity (data not shown). It was observed that enzyme activity decreased with increasing NaCl concentration (data not shown). At 1 M NaCl, 78% residual enzyme activity was recorded, which decreased to 7% at 4.5 M NaCl. Protease activity in the presence of NaCl is a desirable property for application in the processing of dry cured meat products, as these products usually contain 1–2 M NaCl, which acts as a powerful inhibitor of endogenous proteolytic enzymes [44].

Effect of organic solvents on protease activity

Protease retained considerable amount of activity in the presence of most of the tested organic solvents, including DMSO, isopropanol, acetonitrile, ethanol, petroleum ether, acetone, and ethyl ether (data not shown). Ethyl ether supported retention of more than 90% of activity at all investigated concentrations (1–10%) compared with other solvents. Residual activity above 80% was recorded for concentrations up to 5% in the case of DMSO, petroleum ether, and acetone. In fact, with 10% of petroleum ether and DMSO, the enzyme showed more than 65% activity, whereas 10% isopropanol and acetonitrile supported 7% and 32% of residual enzyme activities. The nature and type of organic solvent have a strong effect on protease activity [35]. Application of proteases for production of certain oligopeptides has attracted great attention as a viable alternative to chemical approach [19, 41]. However, use of proteases for peptide synthesis is limited by the specificity and instability of the enzyme in the presence of organic solvents, since the reaction occurs in organic media. Proteases have been reported to be used successfully for synthesis of dipeptide [4] and tripeptide [54], regioselective sugar esterification [48], and diastereoselective hydrolysis of peptide esters [12]. The results obtained in the present study indicate scope for further exploration of this enzyme in peptide synthesis and other esterification reactions by virtue of its stability in solvents.

Stability of the enzyme in presence of hydrocarbons and natural oils

Hydrocarbons including petrol, kerosene, used engine oil, used lubricant oil, diesel, and grease did not influence protease activity markedly (data not shown), even though there was decrease in residual enzyme activity ($\leq$80%) in the case of petrol, kerosene, and grease at 5% concentration, whereas with 5% each of engine oil, used engine oil, used lubricant oil, and diesel, the enzyme showed marginal increase (5–12%) in activity compared with control. All the natural oils—coconut oil, gingelly oil, palm oil, mustard oil, sunflower oil, vegetable oil, dalda, olive oil, ghee, and castor oil—evaluated for their impact on protease activity enabled retention of more than 90% of initial activity even at their highest concentration. It was noted that sunflower oil, vegetable oil, olive oil, and ghee led to marginal increase (5–15%) in enzyme activity compared with others. Gingelly oil and castor oil at 5% concentration caused decline in enzyme activity by 14% and 11%, respectively. It may be said that none of the hydrocarbons or natural oils examined in this work had a drastic effect on protease activity when coexisting in the reaction mixture.

In spite of the availability of different detergents and cleaning solutions in the market, some of them are not efficient in the presence of natural oils and petroleum products. The efficiency of such detergent formulations solely relies on the stability of the detergent enzymes in the presence of oils and hydrocarbons. The present results

obtained for the stability of the enzyme in the presence of various hydrocarbon and natural oils clearly advocate the suitability of this marine protease for addition to detergent formulations.

Storage stability studies of the protease enzyme

Storage stability of purified protease was evaluated by storing the enzyme at various conditions over a period of 1 year, and estimating residual activity periodically. Lyophilized and liquid samples of partially purified enzyme were stored at RT, 4°C, and −20°C. The results indicate that the enzyme was relatively stable for the observed period of 1 year at all the storage conditions (data not shown). Protease stability is often measured in terms of inactivation over time. However, inactivation can occur via a number of different pathways such as autolysis, aggregation, oxidation, and unfolding/denaturation [10]. Tightly folded enzyme tends to be resistant to proteolysis, whereas unfolded or partially unfolded proteins are generally susceptible to proteolytic degradation. Additionally, loss of structure (unfolding) generally implies loss of enzymatic activity [1, 17]. The present results on storage stability strongly support long-term use of the enzyme in industrial applications.

Conclusions

In-depth knowledge on the characteristics of an enzyme is a prerequisite for evaluation of its biotechnological potential. The investigated protease enzyme was noted to be active over a broad range of pH (6–12) and temperature (15–65°C), with maximum activity at pH 11 and 60°C. The stability of the enzyme against a range of surfactants, reducing agents, solvents, metal ions, hydrocarbons, and natural oils, and the storage stability of the enzyme, strongly advocate the potential of this enzyme for various industrial applications. Based on the characteristics determined during the course of the present investigation, including the ability of this enzyme to retain its activity in the presence of metal chelator (EDTA), it is proposed that the extracellular alkaline protease produced by marine fungus *E. album* holds immense scope for utilization as a detergent additive. There is ample scope for further research on application studies, structure elucidation, and enzyme engineering of this protease towards improvement of the enzyme for a wide range of applications.

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