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ORIGINAL PAPER

Biochemical and Structural Characterization of a Detergent Stable Alkaline Serine Keratinase from *Paenibacillus Woosongensis* TKB2: A Potential Additive for Laundry Detergent

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Abstract A monomeric alkaline keratinolytic serine protease with a molecular weight 190.24 kDa was purified from *Paenibacillus woosongensis* TKB2 in submerged fermentation using waste chicken feather as substrate. The purified keratinase was highest activity at pH 9.0 and 50 °C, requiring Mo^{+} for increasing fourfold enzyme activities, showed substrate specificity for keratin powder. The K_m and V_{max} for the enzyme was 1.4 mg/ml and 251.1 U/ml respectively. An initial analysis of the circular dichroism spectrum in the ultraviolet range revealed that the protease is predominantly an α-helix structure. In the presence of 7 mg/ml (w/v) detergents, the protease was active and retained 40–90 % activity. Therefore, it may have a possible application in laundry formulations. The keratinase combined with detergent was able to destain blood, fruit juice and turmeric stained cloth within 30 min without damaging the fabric structure and strength.

Keywords Monomeric · Keratinase · Circular dichroism · Detergents · Febric

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Introduction

Keratinase is the most important enzymes among protease produced commercially [1]. They have extensive applications in a range of industrial products and processes including detergents, food, pharmaceuticals, leather, silk and for silver extraction from used X-ray films [1]. Bacterial keratinase was used for animal feed, fertilizer, or natural gas from poultry waste; and cleaning of certain fabrics [2, 3]. Global concern for the environment has attracted the researchers to investigate enzymes as replacement of chemical catalysts in various biochemical processes. Alkaline proteases are particularly suitable for industrial applications due to their high stability and activity under harsh conditions such as high temperature, alkaline pH and in the presence of surfactants or oxidizing agents [1]. These enzymes have been widely used in the detergent industry since their introduction in 1914 as detergent additives. Their use in detergent formulation makes up a high percentage (89 %) of their total sales [4]. Recently, their roles in synthesis of bioactive peptides and as additive in commercial detergents are gaining attention. The other prerequisites for the utilizing proteolytic enzymes in detergent formulations include their functional ability and stability at alkaline pH in presence of various surfactants and detergents. Proteases are commonly included in detergents for the removal of proteinaceous dirt, which was one of the early applications for these enzymes. In this context, alkaline proteases of microbial origin have been extensively studied however detergent stable keratinase were characterized from a few bacterial species [5] and there study as a detergent additive, prompted us to initiate the present study.

During industrial applications, the proteases are required to function under diverse environmental conditions where

surfactants, oxidants, detergents and solvents are present. Therefore, the activity of alkaline proteases in the presence of metal ions, denaturants, surfactants, oxidants, detergents and organic solvents is a highly desired characteristic for their use in industrial applications [6, 7]. Therefore, it is necessary to determine the optimal physical and chemical conditions under which an enzyme is most active and stable. Additionally, there is a growing awareness that structural studies need to be performed under the conditions in which the proteins actually operate and perform their biological functions [8]. Circular dichroism (CD) has become an increasingly valuable technique to elucidate insights into the structure of biological molecules. This is a beneficial technique because very little sample is used (<0.5 mg/ml) and the technique is non-destructive in nature, which makes it possible to re-use the sample for other applications. In addition, relative changes in protein secondary structure due to environmental influences, such as pH, temperature, and modulators, can be monitored very accurately.

Perusal for literature, in this paper we report for the first time the purification and characterization of a keratinase from *Paenibacillus woosongensis*. Central of this study aimed to purify and characterize an alkaline keratinolytic protease and to investigate the functional and structural modifications in a wide range of pH, surfactants, oxidants, detergents, modulators and metal ions. We focus mainly in the study for the possible utility of the purified enzyme as an additive for detergent formulation.

Materials and Methods

Isolation and Culture Condition of the Organism

Paenibacillus woosongensis TKB2 (GenBank JQ248575), isolated from poultry processing plant, Khirpai, Midnapore, West Bengal, India was used in this study. The bacterium produced keratinase enzyme in submerged fermentation [9]. The bacterial culture was maintained over skim milk agar slants (pH 8.5) and stored at 4 °C.

Enzyme Production Medium and Culture Condition

The enzyme production was carried out in the basal medium supplemented with chicken feather (0.75 %, w/v) of following composition (%, w/v): NaCl (5.0), K_2HPO_4 (0.05), $MgSO_4$ (0.025), $CaCO_3$ (0.02) and $FeSO_4$ (0.015). The basal medium was sterilized in Erlenmeyer flasks (250 ml) containing 100 ml of culture medium. The sterilized media was inoculated with 1 ml of 24 h old bacterial culture (36×10^{10} cfu/ml) and incubated at 30 °C for 3 days.

After fermentation the enzyme was separated from fermented broth by filtration (Whatman no. 1 paper) and was centrifuged at 5,000*g* for 10 min. The supernatant was used as crude enzyme.

Keratinase Assay and Protein Estimation

The enzyme assay was performed with keratin substrate [9]. One unit (U) of keratinolytic activity was defined as an increase of corrected A_{280} for 0.01 under the assay conditions. Protein estimation was done according to Lowry et al. [10] method.

Enzyme Purification

Keratinase obtained from submerged fermentation was purified by ammonium sulfate (80 % saturation) precipitation followed by Sephadex™ G100 column chromatography [11]. The purified enzyme fraction was concentrated by freeze-drying and this was used for study of enzyme properties like molecular weight and zymography. The estimated molecular weight was determined by SDS-PAGE in reduce and non-reducing condition, also confirmed by size exclusion-HPLC (Agilent 1100, USA). Briefly purification to homogeneity was achieved by HPLC using Shodex Protein WK 802-5 column (8 × 300 mm), pre-equilibrated with Tris-buffer pH 7.5 and previously calibrated with the standard marker proteins (Genei, Bangalore, India). Proteins were separated by isocratic elution at a flow rate of 0.8 ml/min with same buffer and detected by UV–VIS spectrophotometric detector (Knauer) at 280 nm.

Determination of Optimum pH and Stability

Activity of purified enzyme preparation was measured at pH 4–10 at 50 °C using keratin powder (HiMedia, India) as a substrate. The pH stability of the purified enzyme was determined by pre-incubating the enzyme preparation in buffer solutions with different pH values for 72 h at room temperature. Aliquots were withdrawn and residual activity was determined at pH 9 and 50 °C. The following buffer systems were used at 50 mM: Glycine–HCl for pH 4–6, phosphate buffer for pH 6–8, Tris–HCl for pH 8–9, Glycine-NaOH for pH 9–10.

Determination of Optimum Temperature and Thermal Stability

The effect of temperature on enzyme activity was examined at 40–80 °C for 30 min at pH 9. Thermal stability was determined by incubating purified enzyme at 50–100 °C and pH 9 for 0–3 h. Aliquots were withdrawn at desired time intervals (20 min) to test the remaining activity under

standard conditions. The non-heated enzyme, which was left at room temperature, was considered as control (100 %).

Circular Dichroism Spectroscopy

Conformational changes of the enzyme due to pH and temperature in compare to their optimized state was established by the CD spectrophotometer JASCO-810, Japan. The samples (0.83 mg/ml) were prepared by mixing with buffer of different pH ranges (6, 8 and 10) and allowed them to incubate at room temperature for 3 h. For temperature, the enzyme solutions were incubated at 30, 50 and 80 °C for 3 h with working buffer (pH 9). The changes in CD spectrum ellipticity were recorded at 220 nm of the enzyme samples.

Substrate Specificity Determination with Kinetics

Purified enzyme activity was assayed in a reaction mixture containing various concentrations (1–4.5 mg/ml) of the individual substrates being evaluated casein, keratin, bovine serum albumin and gelatin in 50 mM phosphate buffer, pH 9. The kinetic constants (K_m and V_{max}) were estimated by double reciprocal plots of the data according to standard method of Lineweaver and Burk [12] with Enzyme kinetics software module 1.3, USA.

Effects of Inhibitors, Metal Ions and Organic Solvents on Enzyme Activity

The effects of PMSF, 8-Hydroxyquinoline, ethylene-diaminetetraacetic acid (EDTA), sodium dodesyl sulphate (SDS), β-marcaptoethanol and of various bivalent ions (1 and 5 mM concentration) and organic solvents (1 and 5 %) on keratinase activity was investigated by pre-incubating purified enzyme for 30 min at room temperature with each chemicals. The changes in activity of enzyme were studded with florescent spectrophotometer (Hitachi F-7000, Japan) and CD spectrophotometer. Enzyme assays were carried out under standard assay conditions.

Enzyme Stability with Commercial Detergents

The study evaluated the compatibility of the enzyme with commercial solid detergents. The commercial detergents such as Tide™, Nirma™, Rim™, Sunlight™, Ariel™, Wheel™, Anmol™, Safed™, Ghadi™, Ayana™, Fena™, Power™ and Surfexcel™ were diluted in tap water to give a final concentration of 7 mg/ml to simulate detergent conditions. The endogenous enzymes in the detergents were inactivated by heating the diluted detergents for 30 min at 100 °C prior to the addition of the keratinase enzyme [13]. The enzyme and detergent mixture was then incubated for 30 and 60 min at 50 °C and the residual enzyme activity was determined under optimal assay conditions.

Washing Performance

Clean cotton cloth pieces (4 cm × 6 cm) were stained with human blood, Pomegranate sauce, turmeric paste and then dried. The stained clothes were subjected to wash treatments in flasks with 100 ml either of tap water, commercial solid detergent (Nirma™) diluted in tap water at 7 mg/ml, detergent solution supplemented with purified enzyme and another was detergent supplemented with commercial protease (Sigma Aldrich, USA). Each flask was incubated at temperatures 25, 50 and 75 °C for 30 min under agitation (200 rpm). After incubation, cloth pieces were taken out, rinsed with water and dried. After drying, the resulting reflectance (%) and transmission (%) of the cloth was measured on double beam spectrophotometer (UV/VIS-2202, Japan) and UV/Vis spectrophotometer (Shimadzu, Japan) respectively in range of wavelength from 300 to 800 nm in reflectance mode. In this analysis baseline was corrected with a clean blank white cotton cloth. The optimization of enzyme concentration and washing time was carried out after performing the wash analysis with different concentrations of enzyme (37, 72 and 144 U/ml), and for different pre-washing times (5, 15 and 30 min). Visual examination of different treatment cloth pieces was also carried out to show the effect of enzyme in the removal of stains. The surface characteristics of cotton fiber after each wash were examined with scanning electron microscopy (JEOL-JSM5 800, Japan) and tensile strength was measured using Universal testing machine (Tinius Olsen, UK).

In-Vitro Hematological Analysis of Blood

The removal of blood strain was examined in-vitro analysis of blood reaction with enzyme. Briefly, prepared a blood smear on a glass slide, mixed with 10 µl of enzyme (72 U/ml) and incubated at 50 °C for 30 min. The control slide was prepared with blood smear without enzyme. After incubation the slides were observed under a light microscope (Olimpus CX22, USA) at 40× magnification.

Statistical Analysis

Data presented in this article show the mean of three replicates with their standard deviation (mean ± SD) using Microsoft Excel 2003.

Results and Discussion

Micro-Organisms and Enzyme Production Medium

For the present study, the previously isolated strain of *P. woosongensis* TKB2 was selected for their ability to degrade chicken feathers in submerged fermentation and produced optimum 71.9 U/ml of keratinase protein in 48 h fermentation period at 30 °C and pH 8.5 in a basal medium containing 5 % (w/v) NaCl however the bacteria tolerate salinity up to 10 % (w/v) [9]. Jain et al. [14] and Shivanand et al. [15] reported optimum production of protease from *Bacillus* sp. at 3.5 % and 0.6 M NaCl respectively. Therefore the bacterium and its extracellular keratinase with salt tolerance signify their potential applicability in laundry industry in a better way than the other reported surfactant and detergent stable proteases from *Bacillus* sp. [5, 16]. This property of this bacteria and its enzyme facilitates their use in saline environment.

Purification of Keratinase

In the present investigation, keratinase was purified from culture filtrate (total activity 7,200 U) by 80 % ammonium sulfate precipitation, then dialyzed this precipitate against Tris buffer (pH 9) and size exclusion chromatography and gel filtration (Fig. 1a). Table 1 summarizes the purification procedures. The isolated enzyme was homogeneous, as seen by a single protein both in native and SDS–PAGE with reduced and non-denaturing condition. The apparent molecular mass of the purified enzyme was estimated to be 190.24 kDa by SDS–PAGE with Gel-Documentation system (Vilber-Lourmart, Germany) (Fig. 1a). Purification to homogeneity was achieved by HPLC using a Shodex Protein WK 802-5 column and a unique elution symmetrical peak at RT = 10.109 min corresponding to a protein of nearly 205 kDa (RT = 10.089) on size exclusion HPLC (Fig. 1b). These results indicate that purified keratinase is monomeric in nature.

Effects of pH and Temperature on Activity and Stability of the Enzyme

The optimal pH of keratinase activity was determined by varying the pH of reaction buffer at 50 °C. The purified enzyme has a maximum activity at pH 9.0. These finding were also observed with *Bacillus pumilus* A1 that shows a maximum activity at pH 9.0 with keratin as substrates [17].

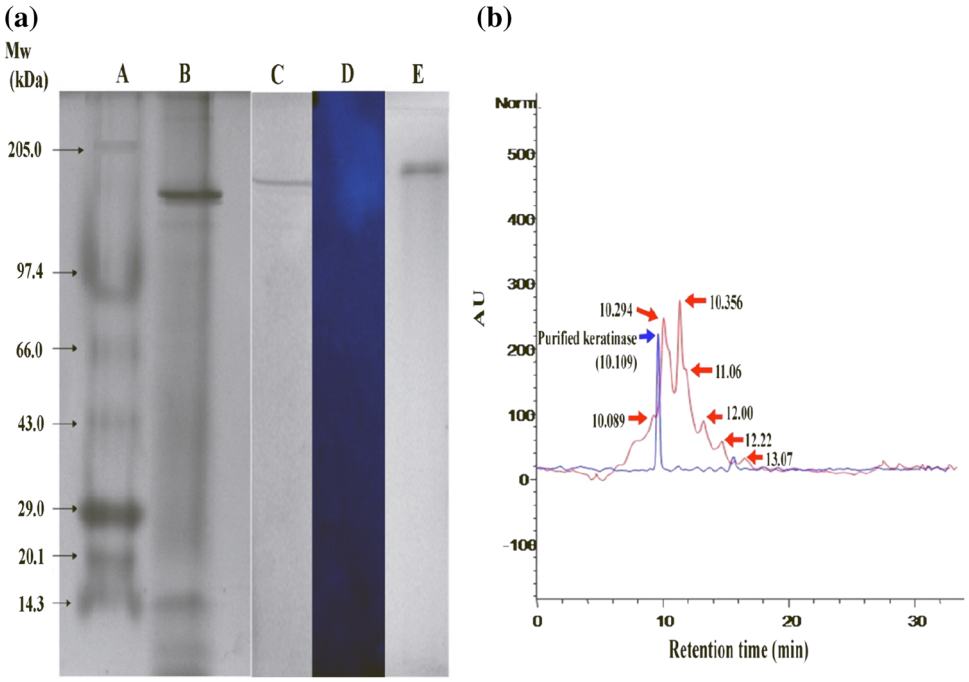

Fig. 1 SDS-PAGE analysis of the purified protease (**a**). *Lane A* molecular weight markers (kDa): Myosin, rabbit muscle—205.0; phosphorylase b—97.4; bovine serum albumin—66.0; ovalbumin—43.0; carbonic anhydrase—29.0; soyabean trypsin inhibitor—20.1 and lysozyme—14.3; *Lane B* 80 % ammonium sulfate precipitate of protease; *Lane C* protease after Sephadex G-100 size exclusion chromatography; *Lane D* zymography of purified protease; *Lane E* protease in reducing gel-electrophoresis. Superimpose the chromatogram of size exclusion HPLC filtration profile of purified protease (*blue*) and native protein markers (*red*) **b** (kDa) of 205.0 (RT = 10.08 min), 97.4 (RT = 10.29 min), 66.0 (RT = 10.35), 43.0 (RT = 11.06 min), 29.0 (RT = 12.0 min), 20.1 (RT = 12.22 min), 14.3 (RT = 13.07 min) on Shodex Protein WK 802-5 column: purified keratinase shows a single and symmetrical peak of approximately 190.24 kDa (RT = 10.10 min). (Color figure online)

Table 1 Summary of purification of keratinase

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	100 ± 0.02	157 ± 0.31	7,200 ± 1.32	45.81 ± 0.20	1 ± 0.01	100 ± 0.02
80 % $(NH_4)_2SO_4$ precipitation	49.76 ± 0.58	58.06 ± 0.16	4,525.23 ± 0.32	78.04 ± 0.06	1.69 ± 0.02	62.73 ± 0.16
Sephadex G-100 size exclusion chromatography	10.06 ± 0.11	8.3.06 ± 0.05	2,211.97 ± 0.15	266.56 ± 0.20	5.67 ± 0.19	30.76 ± 0.04

The relative activity of purified enzyme at pH 8 and 10 were 72 and 68 % respectively (Fig. 2a). Keratinase maintained 90 % of its activity when exposed to 7.5–10 pH range for 3 h (Fig. 2a). This characteristic would allow application of this enzyme in detergent formulations since laundry detergents generally operate at a pH of 7–11 [18]. The pH stability is similar to the reported for the most often used detergents proteases, subtilisin BPN and Savinase™, which have a pH optimum of 10.5 and pH stability at 8–10 [19]. Enzyme was found to be more stable at high pH values. In lower pH value the enzyme lost its catalytic activity quickly. In pH 6, enzyme retain its catalytic activity was 50 %. Change in pH can protonate or deprotonate a side group, thereby changing its conformation. In conclusion, this conformation could cause a decrease in substrate affinity. A more drastic pH change can change the protein folding; thereby completely deactivating the enzyme. The Circular dicroism spectra support this phenomenon (Fig. S1). Based on the CD spectra, it is estimated that the keratinase conformation contained α-helix, β-sheet, β-turn and random coil (Table 2). This result strongly suggested that the keratinase from *P. woosongensis* TKB2 is predominantly an alpha rich protein (46 %). CD-based conformational evaluation of the protease after incubation at different pH values, temperature and Mo^{+} metal ion, demonstrated that deviation in the β-content was directly correlated with the altered (increased/decreased) protease activity (Table 2). The secondary structure of keratinase, treated with three different pH and temperature values, slightly changes in the α-helix, random

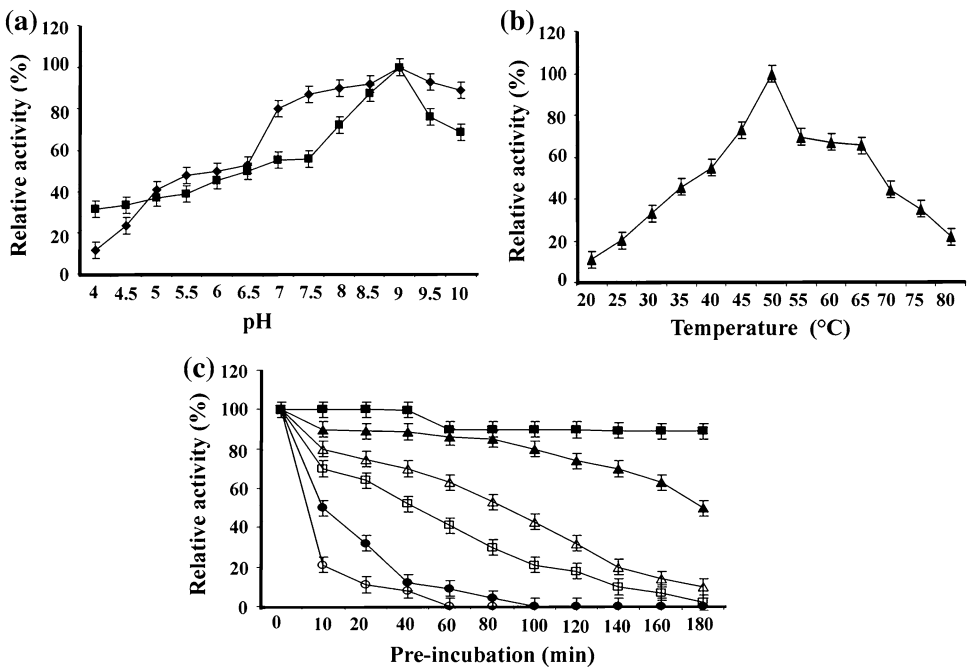

Fig. 2 Effect of pH and temperature on the activity and stability of the purified enzyme. **a** Effects of the pH on the activity and stability of keratinase. The pH optimum profile (*filled square*) was determined at 50 °C in different buffers. The pH stability (*filled diamond*) of the enzyme was determined by incubating the protease for 3 h at 50 °C in different buffers and the residual activity was measured under standard conditions. The activity of the enzyme at pH 9 was taken as 100 %. **b** Effect of the thermoactivity of enzyme. The temperature profile was determined by assaying enzyme activity at different temperature values ranging from 40 to 80 °C (*filled triangle*). The activity of the enzyme at 50 °C in the presence of buffer pH 9 was taken as 100 %. **c** Effect of the thermostability of keratinase. The residual keratinase activity was determined from 0 to 180 min at 10 min intervals. The activity of the non-heated enzyme was considered as 100 %. Each point represents the mean ($n = 3$) ± standard deviation

coil and β-turn but significantly alteration was observed in β-sheet appeared in all cases (Table 2). This result showed that β-sheet play an important role in keratinase structure and activation, implying that keratinase is a globular protein. The similar result showed by Bhattacharyya et al. [20] and Singh et al. [21].

The purified keratinase exhibit maximum activity at 50 °C and showed activity over range of temperature 40–80 °C (Fig. 2b).The stability of the enzyme was tested by incubation at 50–100 °C for 3 h. The enzyme retained 63 and 40 % enzyme activity at 70 and 80 °C for 1 h. The half life of keratinase was determined to be 80 min at 70 °C in presence of 50 mM Tris-buffer pH 9. This indicated that the enzyme have a stabilizing effect in higher temperature (Fig. 2c). In 80 °C and above the protein coagulates and its tertiary structure break down, especially the catalytic domain can changed so substrate affinity decreased. The literature supported that keratinase from other bacteria showed temperature optima in the range of 40–70 °C [22–24].

Effect of Modulators, Solvents and Metal Ion(s)

Cu^{+}, Zn^{+2}, Pb^{+2}, As^{+2}, Na^{+} and Ba^{+} had completely inhibited the enzyme activity and stability. Ca^{+2}, K^{+} and Mg^{+} suppressed the activity of protease by 0.41 (mg/ml), 3.08 (mg/ml) and 2.2 (mg/ml) respectively. In contrast the higher concentration of these three divalent cations, totally destroy the enzyme activity (Table 3). However Fe^{+2}, Mn^{+2} and Mo^{+} had positive effect on enzyme activity and stability. Mo^{+}, Mn^{+2} and Fe^{+2} increase 4, 2.53 and 3.6 fold enzyme activity respectively. The result theorized that the molybdenum played as cofactors of the keratinase enzyme. Previously it has been observed that Mn^{+2} and Fe^{+2} stimulated the keratinase activity [1]. It is first time noticed that Mo^{+} has significantly induced keratinase activity. It was obviously detected by the CD data and florescent spectroscopic analysis. Mo^{+} played conformational changes in the active site which are essential for increasing the catalytic function of the enzyme. Keratinase treated with Mo^{+} was composed of 26.9 % α helix, 9.2 % β-sheet, 27.1 % β-turn, and 36.8 % random coil (Table 2). Calculation of secondary structures revealed an increase in the content of β-sheet and a decrease for α-helix and random coil, which may be related to the active site of the enzyme.

But in case of Mn^{+2}, lower concentration (1 mM) had no effect on enzyme activity. Protease inhibitors like SDS, β-marcaptoethanol and PMSF were inhibited the enzyme activity. Complete inhibition of protease activity by PMSF, even at 1.0 mM, indicates that the enzyme is a serine alkaline protease with a serine residue in its active site. PMSF blocks the active site of proteases by sulfonating the essential serine residue, resulting in complete inhibition of protease activity [25]. The slightly decrease in activity by EDTA reveals the requirement of metal ion(s) for this protease activity because EDTA removes metal ion(s) through chelation [1].

The enzyme had negative effect with all solvents tested in Table 3, except acetaldehyde, acetonitryle and ethyl acetate. The keratinase gave 2.5 fold higher activities in addition with acetaldehyde. The observed activity and stability in certain organic solvents may allow use of the enzyme for peptide synthesis reactions in low water systems [26].

The intrinsic fluorescence of protein is mainly related to Trp, Tyr, and Phe residue. With excitation at 280 nm, the fluorescence emission spectra of protein is provided by Trp and Tyr residue, but with excitation at 295 nm the emission spectra is provided by Trp residue. Conformational changes can be estimated by measuring the fluorescence intensity of keratinase. The UV excitation wavelength was set at 278 nm. The fluorescence emission spectra keratinase in the presence and absence of 5 mM Mo^{+} following an excitation wavelength at 278 nm are presented in Fig. 3. The characteristic spectroscopy of keratinase with Mo^{+} exhibited a maximum intensity at 334 and 331 nm when the keratinase was excited at 278 nm, suggesting that the keratinase contained Trp and Tyr residue. Compared with the Trp residues of keratinase, free Trp residue show a maximum wavelength at 350–353 nm [26]. A decrease in fluorescence intensity of keratinase at maximum

Table 2 Secondary structural changes due to Physico-chemical parameters

Secondary structural component	Test conditions							
	Control (purified enzyme)	Purified enzyme + Mo (5 mM)	pH-6.5	pH-8.5	pH-10.5	Temp.-20 °C	Temp.-50 °C	Temp.-80 °C
Alpha helix	46.13 ± 0.08	26.96 ± 0.12	33.03 ± 0.07	31.8 ± 0.06	32.3 ± 0.12	28.2 ± 0.09	29.7 ± 0.11	27.4 ± 0.23
Beta sheet	4.5 ± 0.07	9.2 ± 0.05	1.1 ± 0.03	7.1 ± 0.06	1.4 ± 0.03	2.2 ± 0.01	7.7 ± 0.03	1.7 ± 0.02
Turn	33.6 ± 0.03	27.1 ± 0.02	34.0 ± 0.17	29.4 ± 0.09	34.9 ± 0.06	35.2 ± 0.11	29.3 ± 0.14	36.5 ± 0.21
Random coil	18.1 ± 0.12	36.8 ± 0.21	33.0 ± 0.14	38.8 ± 0.04	32.8 ± 0.12	36.5 ± 0.11	33.3 ± 0.14	36.1 ± 0.20

Table 3 Effects of metal salts, chemicals and solvents on purified enzyme

Salt	Concen-tration (mM)	Relative activity (%)	Activation/ inhibition (%)	Chemical	Concent-ration (%, v/v)	Relative activity (%)	Activation/ inhibition (%)
Control	–	100	–	Control	–	100	–
$FeSO_4$	1	272.28	▲ (172.28)	Acetaldehyde	1	40.91	▼ (59.09)
	5	360.08	▲ (260.08)		5	251.07	▲ (151.07)
$CaCO_3$	1	41.00	▼ (59.00)	2- propanol	1	0	▼ (100)
	5	0	▼ (100)		5	0	▼ (100)
K_2HPO_4	1	30.76	▼ (69.24)	Triethanolamine	1	0	▼ (100)
	5	0	▼ (100)		5	0	▼ (100)
$CuSO_4$	1	0	▼ (100)	Acetone	1	57.62	▼ (42.38)
	5	0	▼ (100)		5	98.53	▼ (1.47)
$MgSO_4$, $7H_2O$	1	21.90	▼ (78.1)	Isoamyle alcohol	1	0	▼ (100)
	5	0	▼ (100)		5	0	▼ (100)
$ZnSO_4$, $7H_2O$	1	0	▼ (100)	Methanol	1	0	▼ (100)
	5	0	▼ (100)		5	0	▼ (100)
Pb_3O_4	1	0	▼ (100)	Ethanol	1	0	▼ (100)
	5	0	▼ (100)		5	0	▼ (100)
As_2O_3	1	0	▼ (100)	Toluene	1	41.81	▼ (58.19)
	5	0	▼ (100)		5	0	▼ (100)
Na_2CO_3	1	0	▼ (100)	Tween 80	1	51.10	▼ (48.90)
	5	0	▼ (100)		5	0	▼ (100)
$Ba(OH)_2$, $8H_2O$	1	0	▼ (100)	1-propanol	1	37.19	▼ (62.81)
	5	0	▼ (100)		5	0	▼(100)
$KMnO_4$	1	0	▼ (100)	Acetonitrayle	1	99.06	▼ (0.04)
	5	253.36	▲ (153.36)		5	97.00	▼ (3.00)
Ammonium molybodate	1	337.90	▲ (237.90)	Ethyle acetate	1	103.29	▲ (3.29)
	5	400	▲ (300.00)		5	0	▼ (100)
EDTA	1	92.19	▼ (7.81)	Xylene	1	42.67	▼ (57.33)
	5	87.10	▼ (12.90)		5	18.00	▼ (82)
Sodium dodesyle sulfate (SDS)	1	0	▼ (100)	Triton X100	1	69.72	▼ (30.28)
	5	0	▼ (100)		5	0	▼ (100)
β marcaptoethanol	1	0	▼ (100)	DMSO	1	0	▼ (100)
	5	0	▼ (100)		5	0	▼ (100)
PMSF	1	0	▼ (100)				
	5	0	▼ (100)				

▼ inhibition, ▲ activation

wavelength was observed after addition of EDTA when excited at 278 nm. These variations in the fluorescence spectrum indicate that the microenvironment around Trp residue was changed, which may be the result of a conformation change caused by interaction between Mo^+ and EDTA with amino acid residues [26].

Substrate Specificity of the Purified Keratinase

The efficacy of the purified keratinase to degrade various native proteins. The kinetics of the purified enzyme on different substrates was estimated at various concentrations of protein substrates. The protease activity obeyed Michaelis–Menten type kinetics when various protein substrates were used (Fig. 4). Kinetic parameter (V_{max} and K_m) values for the hydrolysis of various protein substrates by the keratinase were illustrated in Table 4. According to the result reveled that keratin gave lowest K_m (1.4 mg/ml) and highest V_{max} (251.1 U/ml) showing that the keratinase has higher affinity for keratin than others.

The result of the relationship between substrate concentration and enzyme activity of different protein substrates reveal that the activity of the enzyme increased concomitantly with an increase in substrate concentration. But the enzyme activity stopped to increase when the concentration exceeded 4 mg/ml indicating that substrate

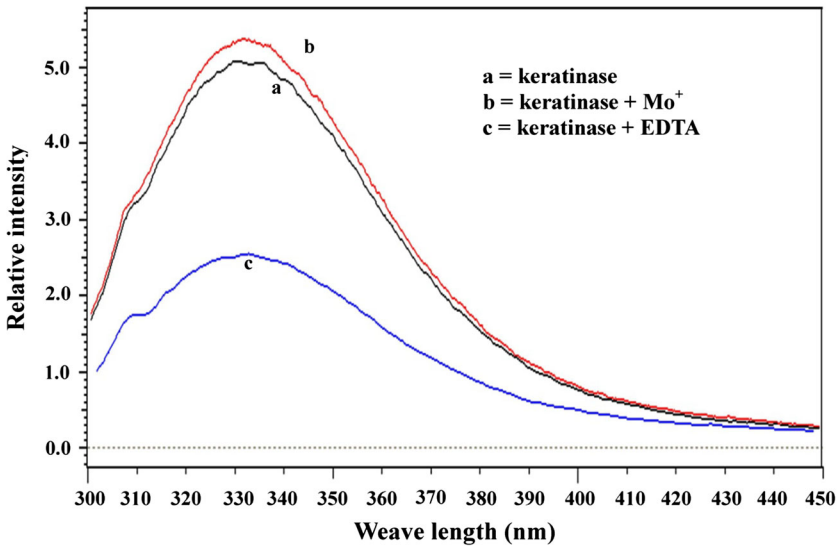

Fig. 3 Fluorescent spectra of purified keratinase (**a**), pre-incubation with Mo^{+} (**b**) and EDTA (**c**); excitation wavelength: 278 nm

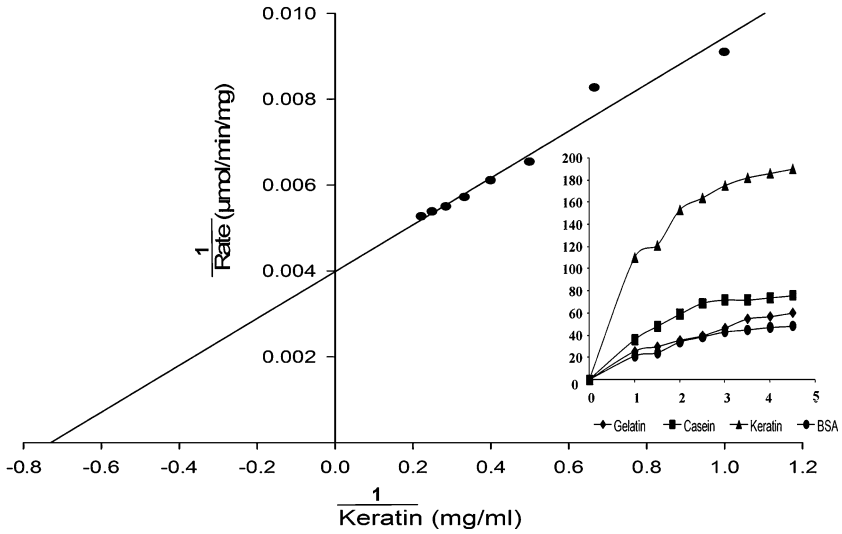

Fig. 4 Effects of different organic nitrogen substrate concentrations on the activity of keratinase (*inset*). Lineweaver–Burk plot for determinination of K_m and V_{max} of the protease on keratin

Table 4 Kinetic parameters of keratinase

Substrate	K_m (mg/ml)	V_{max} (U/ml)	K_{cat} ($\times 10^4$/min)	K_{cat}/K_m ($\times 10^4$ mg/min)
Casein	1.81 ± 0.01	110.3 ± 0.41	22.5 ± 0.21	12.5 ± 0.05
Gelatin	4.6 ± 0.02	122 ± 0.42	24.8 ± 0.11	5.39 ± 0.01
Keratin	1.4 ± 0.01	251.1 ± 0.11	51.2 ± 0.32	36.57 ± 0.21
Bovine serum albumin (BSA)	3.1 ± 0.03	83 ± 0.08	16.9 ± 0.09	5.45 ± 0.02

saturation was taking place. The result of the kinetic parameters (V_{max} and K_m) indicates that the purified keratinase hydrolyzed a broad range of protein substrates like gelatin, keratin, casein and BSA. The result showed that keratin had the lowest K_m and highest V_{max} values of 1.4 mg/ml and 251.1 U/ml, this was followed by casein, gelatin and then BSA. The kinetic parameter (V_{max} and K_m) values confirmed that the purified keratinase possessed high affinity and degradability potential for keratin than casein, gelatin and BSA. According to Lineweaver–Burk plot (Fig. 4), the highest K_{cat} (51.2×10^4 min^{-1}) and K_m (1.4 mg/ml) values of keratinase were comes from the enzymatic reaction with keratin, revealed that the remarkable hydrolysis of disulfide bond between two adjacent cystein residue. The k_{cat}/K_m ratio (36.57×10^4/mg/min) of the keratinase, reaction with keratin was higher than the others, which theorized that keratinase showed higher catalytic efficiency. It could be deduced from the great difference between the four substrate kinetic parameters that the conformation of substrate binding sites was

different. Shankar et al. [27] has reported protease with K_m value of 5.15 mg/ml with casein as substrate from *Beauveria* sp and lower K_m was 242 μM reported with soluble feather keratin as substrate from *Kocuria rosea* [28].

Effect of Commercial Detergents

The keratinolytic protease from *P. woosongensis* TKB2 was remarkably stable (40–90 %) in the presence of 0.7 % (w/v) commercial detergents at an incubation time 30 and 60 min (Fig. 5). The keratinolytic protease showed higher stability with Nirma^{TM} (89.9 %) followed by Anmol^{TM} (89.50 %). Such a remarkable stability of our protease in the presence of higher detergent concentrations reveals its usefulness as a detergent additive. Detergent stability of a protease enzyme is an important trait for its industrial application. Generally, detergent powders are used at <0.9 % (w/v) concentrations for washing clothes. Because our protease exhibited 40–90 % stability in the presence of 0.7 % (w/v) commercial detergents, it is highly suitable for use in detergents. However, our purified keratinolytic protease is slightly inhibited by the chemical chelating agent EDTA. According to New Zealand Ecolabelling Trust-2011, the global permissible limit of EDTA in the detergent is 0.6 g/l. So, this condition is not satisfactorily inhibiting the enzyme activity due to lower permissible limit of EDTA in detergent formulation, indication the enzyme is a profitable additive of laundry detergent. Detergent-stable proteases with variable stability in the presence of different detergents have been studied by several other researchers [1]. These finding is superior to the earlier repot of Singh et al. [18] where alkaline protease from *Bacillus* sp. SSR1 lost 63 % and *B. cereus* SIU1 lost 76 % of its initial activity when incubated in commercial detergent at a lower concentration of 5 mg/ml for 60 min at

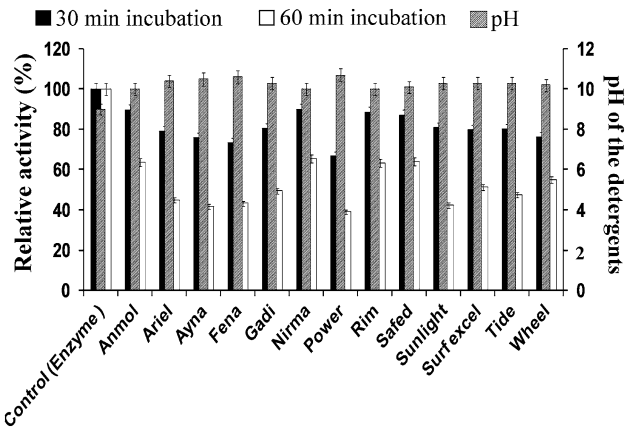

Fig. 5 Effect of commercial detergents on keratinase stability after 30 and 60 min of incubation. Each point represents the mean ($n = 3$) ± standard deviation

40 °C. However similar trend of stability with different detergent formulations was reported from proteases projected as potential candidate for laundry industry [13].

Washing Performance Analysis

Stain removal ability of purified enzyme was analyzed using cotton cloths stained with human blood, pomegranate sauce and turmeric pest. The treatment of stained cloth with detergent supplemented with purified keratinase and commercial protease gave a better stain removal over the wash performance employing detergent alone (Fig. 6). The reflectance (%) and transmission (%) (Fig. S2) of the stained cloth washed with detergent supplemented with purified keratinase and commercial protease was higher than the cloth washed with detergent alone (Table 5). The reflectance was monitored at wavelength 420, 430 and 433 nm for blood, turmeric and pomegranate sauce respectively. The effect of temperature revealed maximum reflectance (%) at 50 °C for blood, Pomegranate sauce stained, and turmeric stained cloth (Table 5). The stain removal by various protease enzymes were reported to be optimum at temperature 50–75 °C [18, 29]. The enzyme concentration of 72 U/ml resulted in maximum reflectance for all the staining agents (Table 5). However no substantial difference was observed in reflectance with incubation time for blood and pomegranate sauce and turmeric paste (Table 5). This study further involved the examination of cloth fiber after treatment with detergent and detergent supplemented with protease (in case of blood stain) using scanning electron microscope (SEM). The SEM images of cotton cloth revealed characteristic ridges and a very smooth surface was observed after washing with detergent supplemented with commercial protease (Fig. S3d) and purified enzyme (Fig. S3c) than detergent alone (Fig. S3b). Detergent residue was resided in the surface of detergent treated cloths (Fig. S3f) in comparison with untreated control cloths (Fig. S3a and e). No degradation of cloth fiber can be seen thus the enzyme can be considered for utilization as detergent additive. Both tensile strength and elongation % were measured (Table S1) to show the influence of the different wash treatments on strength fibers. As the higher numbers indicate better properties, the data affirmed the general distinction of the detergent combined commercial and purified keratinolytic protease have no significantly different to the detergent alone, also the higher estimates of the combined treatment declared its prevalence as an effective cleaner. The purified keratinolytic protease was found to be a potential digester of blood stain even in the absence of detergent (Table 5) therefore substantiated its proteolytic activity. Thus there is no significant difference in washing performance between commercial protease and our purified keratinase. However, the

Table 5 Wash performance analysis of keratinase at different, concentration, temperatures and washing time

Washing conditions	Reflectance (%)								
	Temperature (°C)			Enzyme concentration (U/ml) mixed with detergent (7 mg/ml)			Incubation time (min)		
	25	50	75	36	72	144	5	15	30
Blood strain									
Tap water	27.64 ± 0.36	30.62 ± 0.39	25.37 ± 0.27	–	–	–	30.22 ± 0.11	30.56 ± 0.22	30.58 ± 0.23
Detergent (7 mg/ml)	40.47 ± 0.20	53.17 ± 0.22	24.76 ± 0.24	–	–	–	47.85 ± 0.16	52.89 ± 0.11	53.11 ± 0.29
Enzyme + detergent	56.36 ± 0.38	59.11 ± 0.13	32.18 ± 0.22	71.64 ± 0.28	80.33 ± 0.21	68.85 ± 0.16	54.75 ± 0.18	59.00 ± 0.10	60.38 ± 0.14
Enzyme (72 U/ml)	54.41 ± 0.40	57.84 ± 0.19	31.13 ± 0.16	–	–	–	56.09 ± 0.22	57.07 ± 0.23	58.03 ± 0.05
Commercial protease + detergent	48.17 ± 0.16	55.06 ± 0.06	33.21±	72.32±	76.02±	82.72±	42.00±	48.90±	51.32±
Pomegranate sauce									
Tap water	43.26 ± 0.15	50.83 ± 0.11	44.24 ± 0.13	–	–	–	47.21 ± 0.12	50.15 ± 0.16	50.56 ± 0.10
Detergent (7 mg/ml)	50.03 ± 0.03	61.10 ± 0.10	53.28 ± 0.05	–	–	–	60.73 ± 0.16	61.00 ± 0.12	61.45 ± 0.22
Enzyme + detergent	61.21 ± 0.01	70.42 ± 0.09	64.24 ± 0.05	83.28 ± 0.06	94.42 ± 0.08	90.04 ± 0.16	69.86 ± 0.05	70.32 ± 0.08	70.54 ± 0.11
Enzyme (72 U/ml)	60.90 ± 0.10	69.03 ± 0.06	39.92 ± 0.06	–	–	–	66.77 ± 0.12	69.88 ± 0.11	70.08 ± 0.07
Commercial protease + detergent	50.03 ± 0.07	75.15 ± 0.16	40.07 ± 0.21	80.07 ± 0.34	84.39 ± 0.13	97.54 ± 0.14	62.51 ± 0.17	65.44 ± 0.11	71.10 ± 0.2
Termeric pest									
Tap water	16.64 ± 0.12	20.92 ± 0.06	25.96 ± 0.11	–	–	–	20.87 ± 0.10	20.80 ± 0.11	21.02 ± 0.02
Detergent (7 mg/ml)	30.93 ± 0.17	32.15 ± 0.17	26.45 ± 0.12	–	–	–	31.89±	31.74 ± 0.17	32.22 ± 0.21
Enzyme + detergent	45.74 ± 0.06	50.77 ± 0.11	34.85 ± 0.07	75.59 ± 0.16	83.41 ± 0.16	93.36 ± 0.17	50.07 ± 0.23	50.41 ± 0.11	50.64 ± 0.15
Enzyme (72 U/ml)	40.42 ± 0.11	46.55 ± 0.22	24.33 ± 0.21	–	–	–	45.03 ± 0.04	46.29 ± 0.02	46.61 ± 0.07
Commercial protease + detergent	28.25 ± 0.34	36.65 ± 0.14	34.74 ± 0.17	75.15 ± 0.17	80.53±	84.32 ± 0.20	51.22 ± 0.110.11	51.37 ± 0.16	52.04 ± 0.16

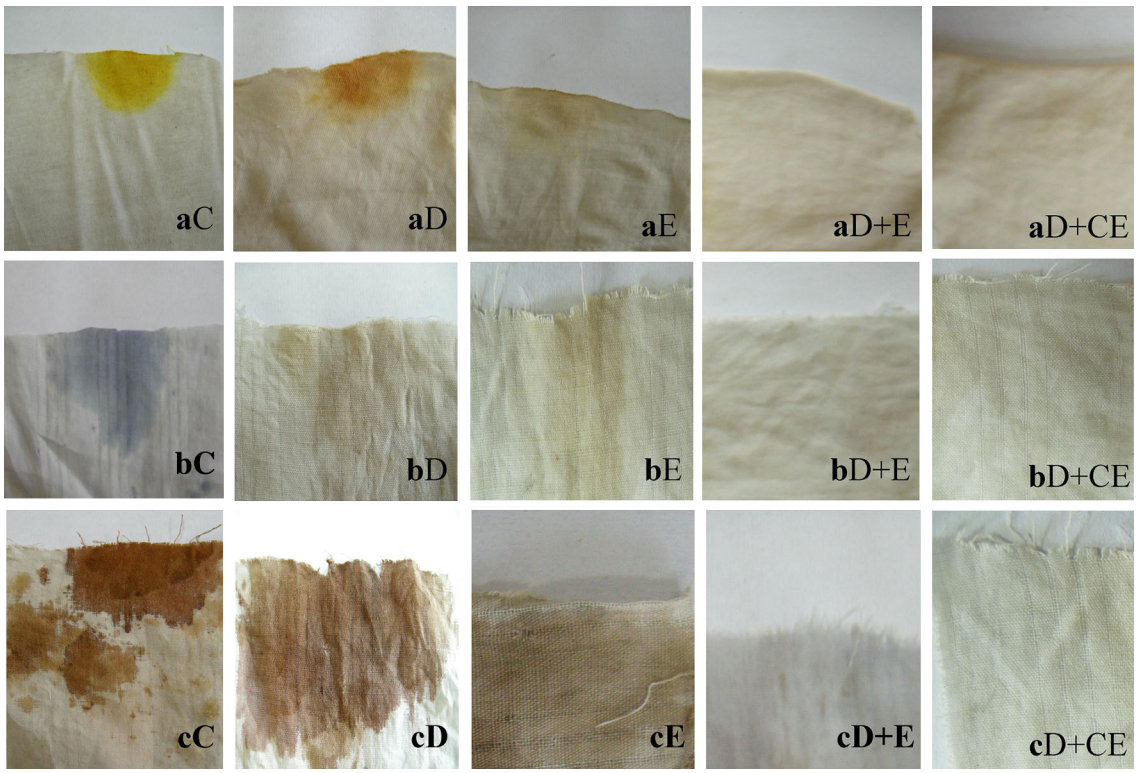

Fig. 6 Washing performance analysis of the purified enzyme preparation in the presence of the commercial detergent Nirma^{TM}. **aC** Cloth stained with termeric pest washed with tap water; **aD** termeric-stained cloth washed with detergent Nirma^{TM}; **aE** termeric-stained cloth washed with purified enzyme; **aD + E** turmeric-stained cloth washed with added Nirma^{TM} with enzyme; **aD + CE** turmeric-stained cloth washed with added Nirma^{TM} with commercial enzyme, **bC** Pomegranate sauce-stained cloth washed with tap water; **bD** Pomegranate sauce-stained cloth washed with detergent Nirma^{TM}; **bE** Pomegranate sauce-stained cloth washed with purified enzyme; **bD + E** Pomegranate sauce-stained cloth washed with added Nirma^{TM} with enzyme; **bD + CE** Pomegranate sauce-stained cloth washed with added Nirma^{TM} with commercial enzyme, **cC** Cloth stained with blood washed with tap water; **cD** blood stained cloth washed with detergent Nirma^{TM}; **cE** blood-stained cloth washed with purified enzyme; **cD + E** blood-stained cloth washed with added Nirma^{TM} with enzyme; **cD + CE** blood-stained cloth washed with added Nirma^{TM} with commercial enzyme

high costs of commercial enzymes, time consuming factor and low distain-able efficacy at lower temperature are some of the pitfalls of this method.

The hemolytic study revealed that enzyme break down the red blood cell (RBC) membrane (Fig. S4). The RBC contains 97 % hemoglobin protein in their cell. It was probably that the enzyme hydrolyzed the hemoglobin and breakdown the RBC. From this study it was conformed that the mechanism of blood stains removal from the cloths by enzymatic process.

Conclusion

This study reports a new source of keratinase having molecular weight 190.24 kDa based on SDS-PAGE, size exclusion HPLC and keratin-zymogram. The serine protease was active, stable in a broad range of pH and temperatures (50–80 °C), induced by Mo^{+}, with an optimum pH of 9.0. Determination of the secondary structure by CD revealed that α-helix structures were predominant and β-sheets were responsible for activity. The enzyme was stable in presence of commercially available detergents. Efficient removal of a blood stain, pomegranate sauce and turmeric stain proved the potential use of this protease as a laundry additive.

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