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ORIGINAL PAPER

Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW

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Abstract Biotreatment of feather wastes and utilization of the degraded products in feed and foodstuffs has been a challenge. In the present study, we have demonstrated the degradation of feather waste by *Bacillus cereus* DCUW strain isolated during a functional screening based microbial diversity study on East Calcutta Wetland Area. A high molecular weight keratinolytic protease from feather degrading DCUW strain was purified and characterized. Moreover, utilization of degraded products during feather hydrolysis was developed and demonstrated. The purified keratinolytic protease was found to show pH and temperature optima of 8.5 and 50 °C, respectively. PMSF was found to inhibit the enzyme completely. The purified enzyme showed molecular weight of 80 kDa (from SDS-PAGE). The protease was found to have broad range substrate specificities that include keratin, casein, collagen, fibrin, BAPNA and gelatin. The protease was identified as minor extracellular protease (Vpr) by RT-PCR and northern blotting techniques. This is the first report describing the characterization of minor extracellular protease (Vpr) and its involvement in feather degradation in *B. cereus* group of organisms.

Keywords Feather degradation · Serine protease · *Bacillus cereus* DCUW · Reverse transcription PCR(RT-PCR) · Minor extracellular protease (Vpr)

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Introduction

Shortage of protein in food and feed necessitates the search for a food source which is both economic as well as high in protein content. Waste management seems to be the only possible option answering both the needs. Recently different research groups around the world have given lot of emphasis by finding ways to recover proteins out of waste products in Poultry industry [26, 27]. The major waste produced in poultry industry is feather that contains mostly keratin (80–90% on dry mass basis). But the utilization of feather waste as a dietary protein supplement for animal feedstuffs has been carried out only on a limited basis due to its poor digestibility. The poor digestibility of native feather protein is due to the constituent amino acid composition and configuration that provides structural rigidity. The mechanical stability of keratin and its resistance to microbial degradation depend on tight packing of the protein chains in α-helix (α-keratin) and β-sheet (β-keratin) structures [31]. Moreover, cross-linking of protein chains by cysteine bridges confers high-mechanical stability and resistance to proteolytic degradation. Previously, steam pressure-cooked treatment was used to make the feather more digestible [9], although that process required significant energy and resulted in the deleterious destruction of some essential amino acids. Therefore biological treatment is another choice to improve the nutritional value of feather waste [14]. Keratinolytic enzymes find use in environment-friendly biotechnological processes that utilize keratin-containing wastes from poultry industry [29]. Moreover, keratinolytic enzymes could be interesting for

pharmaceutical and cosmetic industries. These enzymes are produced by some species of saprophytic and parasitic fungi [33], a few actinomycetes [36], some *Bacillus licheniformis* strain [13, 24, 32] and the thermophilic *Fervidobacterium pennavorans*. The use of keratinolytic protease to nutritionally upgrade feather meal has been described. A comparable growth rate was observed between chicken fed isolated soybean and a methionine-supplemented feather meal fermented with *Streptomyces fradiae* [10]. The utilization of a *B. licheniformis* feather lysate with amino acid supplementation produced a growth curve identical to that of soybean meal [38]. The use of crude keratinolytic protease significantly increased the amino acid digestibility of raw feathers and commercial feather meal [23].

In our laboratory, 38 different bacterial strains were isolated from Eastern Wet-Land, Kolkata, India, using a functional screening approach [12]. In this report, we describe the purification, characterization, and identification of a novel keratinolytic protease from a soil isolate *B. cereus* DCUW. A possible utility of the bacterium in a biosafe method to upgrade the nutritional values of the feed and food stuffs has also been described [6].

Materials and methods

Reagents

Azocasein, Cycloheximide, phenylmethylsulphonyl fluoride (PMSF), Ethylenediaminetetraacetic acid (EDTA), trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64), and Pepstatin A were purchased from Sigma Chemical Co. (St Louis, USA). All other reagents were of analytical grade. Yeast extract was purchased from Difco (Detroit, MI, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco, Life Technologies Ltd. All other tissue culture reagents were from Sigma Chemical Co.

Microorganism and enzyme production

The bacterium used in this study was strain of *B. cereus* DCUW isolated in our laboratory and identified as a keratinolytic bacterium previously [21]. The organism was grown (inoculum was used at 0.5% of the medium volume) in native feather-supplemented media containing; 0.05% NaCl; 0.03% K_2HPO_4; 0.04% KH_2PO_4; 0.024% $MgCl_2$, 6 H_2O; 0.01% yeast extract and 1% of either raw feather or finely divided feather meal. The culture was grown at pH 7.5 at 37 °C for 4 days on an orbital shaker at 140 revolution/minute (rev/min). The supernatant was used to determine the proteolytic activity on azo-casein substrate at regular intervals.

Enzyme purification

The feather supplemented medium culture supernatant was filtered through cheese cloth to remove residual undigested feathers. The filtered culture supernatant was then centrifuged at 6,000×*g* for 15 min to remove bacterial cells and the supernatant obtained after centrifugation was used as the source of crude enzyme. The crude enzyme was then precipitated by slow addition of Ammonium sulfate up to 80% saturation at 4 °C and then the total protein was separated by centrifugation (8,000×*g*, 15 min). The pellet was re-suspended in minimum volume of 100 milliMolar (mM) Tris–HCl buffer, pH 7.5. The re-suspended total protein was then dialyzed against the same buffer overnight and then lyophilized.

Lyophilized sample was resuspended in 100 mM Tris–Cl buffer, pH 7.5 and then loaded on to a Diethylamino Ethyl-(DEAE) sephadex column (2.5 cm × 22 cm) equilibrated with 100 mM Tris–Cl buffer, pH 7.5 and washed with the same buffer. The enzyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Fractions of 1 milliliter (ml) were collected at a flow rate of 20 ml/h. Fractions exhibiting the protease activity were pooled, dialyzed against 100 mM Tris–Cl buffer, pH 7.5, and then concentrated by using centricon of 10 kilo Dalton (kDa) cut.

Affinity chromatography was performed on α-casein agarose column (5 × 2.5 cm) (Sigma), which was equilibrated with 100 mM Tris–Cl, pH-7.5, containing 2 mM $CaCl_2$ (Buffer A). Desalted and concentrated active pooled fractions from the previous step was applied on to the column and washed with Buffer A. The bound protein was eluted from the column by two steps using Buffer A plus 1 M NaCl (Buffer B), followed by 25% (v/v) isopropanol in Buffer B, each at flow rate of 0.5 ml/min. Active fractions were pooled and concentrated by using centricon of 5 kDa cut. Finally the concentrated fraction was used for gel filtration chromatography.

The concentrated enzyme sample was loaded on to Sephacryl S-300 gel filtration column (1.5 cm × 45 cm) equilibrated with 100 mM Tris–Cl, pH 7.5, and eluted with the same buffer containing 100 mM NaCl. Fractions of 1 ml each were collected at a flow rate of 15 ml/h. The active fractions were pooled, concentrated and checked on SDS-PAGE.

SDS-PAGE electrophoresis and zymography

SDS-PAGE was performed on a slab gel (vertical) containing 10% (w/v) polyacrylamide [21]. After the electrophoresis, the gels were either silver stained or stained with Coomassie Brilliant Blue.

Gelatin, collagen, fibrin, and casein zymography was performed in 10% polyacrylamide slab gels containing

SDS and 0.2% of substrate (gelatin, collagen, fibrin, and casein) as a co-polymerized substrate [15] with some modifications. After electrophoresis, the gels were washed in Triton X-100 (2.5% v/v) for 30 min at room temperature to remove SDS, and then incubated in 100 mM Tris–Cl buffer, pH 8.0, containing 1.25% (v/v) Triton X-100, traces of $CaCl_2$ and $ZnCl_2$; overnight at 37 °C. After that the gels were stained with Coomassie Brilliant Blue. The activity band was observed as a clear colorless area depleted of substrate in the gel against the blue background.

Enzyme assay

The protease activity was assayed with azocasein as the substrate [34]. The reaction mixture contained 100 micro liter (µl) of enzyme preparation and 500 µl of 2% (w/v) azocasein in 100 mM Tris–Cl, pH-7.5. The mixture was incubated at 50 °C for 15 min and the reaction was then stopped by keeping the mixture in the freezer at −20 °C for 30 min after adding 800 µl of 10% TCA. The mixture was then centrifuged at 10,000×*g* for 5 min and the supernatant was added to 1 ml of 1 M NaOH solution. The absorbance of the resultant solution was determined at 440 nanometer (nm). One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm in 15 min at 50 °C. The proteolytic activity was also determined against chopped feather keratin, casein, fibrin, albumin, gelatin, collagen, and benzoyl-arginine-p-nitroanilide (BAPNA) [37], respectively. The keratinolytic nature was confirmed by using relative activity against casein and chopped feather keratin using method described by Anson [2].

Determination of protein concentration

The protein content was determined by Bradford method [5], by using the Bio-Rad assay reagent (Bio-Rad, Munich, Germany) and Bovine serum albumin (BSA) as standard.

Effect of pH and temperature on protease activity

The effect of pH on protease activity was determined by incubating the reaction mixture at pH buffers ranging from 4.0 to 9.5 using different buffer systems. The different buffers were 100 mM sodium phosphate, 100 mM Tris–Cl and 100 mM glycine–NaOH for pH ranges of 4.0–6.0, 6.0–9.0, and 9.0–9.5, respectively. The pH stability was determined by measuring the residual activity of the enzyme after 30 min incubation with the buffers of various pH (4.0–9.5) at 37 °C.

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 10 to 80 °C.

Determination of kinetic parameters

The V_{max} and K_m values of the purified protease were determined from a Lineweaver–Burk plot generated from increasing substrate concentrations (Azocasein) [1]. The change in absorbance at 440 nm was monitored continuously, and the initial velocity was used for calculation of the kinetic constants.

Determination of sulfhydryl group production

A volume of 100 µl 5,5′-dithiobis(2-nitrobenzoic acid) [DTNB] was added to 3 ml of the extracellular broth mixture. The extracellular broth mixture contained extra cellular broth, phosphate buffer (0.1 M, pH-8.0) and distilled water in the ratio 3:2:5. Absorbance was measured at 412 nm after 5 min stable color development. The concentration of sulfhydryl groups in the broth was then calculated using Ellman's method [11] deducing the uninoculated control value.

Disulfide reductase activity

The disulfide reductase activity was measured as described elsewhere [7] with some modifications. A measure of 1 ml the enzyme mixture [extracellular broth, phosphate buffer (0.1 M, pH-7.0), and distilled water in the ratio 3:2:5] was incubated with 1 ml of 2 mM oxidized glutathione solution (prepared in 100 mM phosphate buffer, pH-7.0) for 30 min at 37 °C in the presence of 5 mM PMSF. The content was centrifuged after 1 h at 2,000×*g* for 10 min and the production of sulfhydryl group from oxidized glutathione was detected by addition of 50 µl of DTNB (5 mg/ml) to 1.5 ml of the supernatant. The absorbance of the solution was measured at 412 nm after 5 min of stable color development. Enzyme control was prepared in a similar manner except that 1 ml of phosphate buffer (pH-7) was added instead of substrate. Also, a substrate control containing 1 ml glutathione and 1 ml phosphate buffer (pH-7.0) was used. One unit of sulfhydryl reductase was equivalent to the amount of enzyme required to release 1 micro mole (µmole) of sulfhydryl per ml per min at 37 °C.

Enzyme inhibition

The effect of protease inhibitors on protease activity was measured. The protease inhibitors PMSF, EDTA, E-64, and

Pepstatin A were used to see the inhibition of the keratinolytic protease.

Amino acid composition

Strain DCUW was grown on whole feather-supplemented broth for 96 h and the culture supernatant was obtained by centrifugation at 6,000×*g* for 10 min. The culture supernatant was acetone (80% v/v) precipitated. The supernatant obtained after precipitation contained peptides and free amino acids. The total amino acids were obtained by peptide hydrolysis with 6 M HCl, at 110 °C for 24 h and the free amino acid content after total protein removal was determined by using HPLC system (LC-10A, Shimadzu). The system employed derivatisation reaction of free amino acids with ophthalaldehyde (OPA). A similar approach was taken for analyzing total amino acid content of feather hydrolysate without separating proteins.

Cell cytotoxicity test [(3-(4,5,-dimethyl-thiazol-2-yl)-2,5,diphenyl tetrazonium bromide) (MTT) assay]

Human epithelial (HeLa) cells were maintained in DMEM media supplemented with 5% (v/v) calf serum, 2 mmole/l glutamine and 1% (v/v) penicillin (streptomycin). Cells (T-25 flask) were cultured at 37 °C in a humid atmosphere containing 5% CO_2, and sub cultured every 2–3 days. HeLa cells were harvested with Trypsin, and a suspension containing 2×10^5 cells per ml was prepared. This assay was performed in 96-well, flat-bottomed, gamma irradiated, microtitre plates with lids. Filter sterilized (0.22 μm), cell-free feather-supplemented culture supernatant fluid (50 μl), 4, 6, and 10 days old were serially diluted 100-fold. Negative controls (50 μl phosphate-buffer saline) and positive controls (50 μl Triton X-100) were included. The HeLa cells were grown in the wells for 24 h up to 70–80% confluence. The wells were then inoculated aseptically with 50 μl of different dilutions of the samples, negative controls and the positive controls, and the plates were incubated at 37 °C in a humid atmosphere containing 5% CO_2 for 24 h. After the incubation period, 50 μl of an aqueous solution of MTT (2 mg/ml) were added to each well and the plates incubated for a further 4 h at 37 °C in a humid atmosphere containing 5% CO_2. After this, the liquid medium in the wells was removed and 200 μl dimethyl sulphoxide (DMSO) was added to each well. The absorbance was determined at 450 nm in a micro plate reader. The toxic effect of the cell-free bacterial culture supernatant fluids on the HeLa cell line was calculated from the following equation:

$$[1 - (OD_{Negativecontrol} - OD_{Test})] \times 100\%$$

Samples were considered to be toxic if the optical density of the test well was >20% less than that detected in the negative control wells [3].

Preparation of RNA

Total RNA was extracted using Tripure reagent (Roche) according to manufacturer's protocol. Cells for RNA isolation were collected from feather-supplemented medium at 24 h, before the stationary phase was reached in the medium. The feather medium was filtered through cheese cloth to remove feather residues and the cells were collected by centrifugation. Isolated RNA sample was treated with DNase I (Boehringer) (100 Unit/ml) at 37 °C for 1 h. The total RNA was then re-purified by phenol–chloroform–isoamyl alcohol extraction technique. All buffers and distilled water used in RNA isolation were treated with diethyl pyrocarbonate to inhibit RNase activity. The integrity of the isolated total RNA was checked using MOPS gel electrophoresis.

Reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting analysis

Reverse transcription (RT) experiments were performed with 1 μg of total RNA and a reverse primer specific to one of the extracellular protease open reading frame (ORF). Selection of genes was mainly based on observations during purification and characterization of the protease, e.g., high-molecular weight serine protease, large protease complex (as evident from gel filtration chromatography) etc. We screened all the extracellular protease ORFs in the genome of *B. cereus* ATCC 14579 and 10987; and checked expressions of npr (DQ129688, 5′-CGGGCTCCTTAGTTTACGCCAACAGC-3′), vpr((AE016877/protein_id = "NP_834063.1", 5′-CGGGATCCTTATTTCACTTCTAAATTAACAG-3′), Zn-metalloprotease(AE016877/protein_id = "NP_830373.1", 5′-TCACTTTACTGATAATGAG-3′) [17] and FT-protease (AB096094, 5′-CGGGATCCCTATTCAATAGTCAACACATCTG-3′)[28] in FSM grown *B. cereus* DCUW. Reverse primers specific to all the above-mentioned genes were designed for RT-PCR analysis to check their expression in feather supplemented medium grown *B. cereus* DCUW. A similar work was performed with Luria Broth (LB) grown *B. cereus* DCUW as control experiment. The absence of the contaminating DNA was tested by performing PCR reaction on RNA with 10 pico moles (pmole) of each of the reverse primer and the corresponding forward primer. In the reverse transcription reaction total RNA and reverse primer (VprR; 5′-CGGGATCCTTATTTCACTTCTAAATTAACAG-3′) that anneals to the 3′ termini of the corresponding RNA were heated together at 85 °C for 10 min and immediately placed on ice. Subsequently, reverse M-MuLV transcriptase (Roche) was added in cDNA synthesis buffer and the mixture was incubated at 42 °C for 1 h. RT product was used for PCR amplification with Pfu polymerase (Fermentas) and forward primer set (VprF;

5′-GGAATTCCATATGAAAAAAACTACATCTACAC-3′) according to manufacturer's instructions and the products were analyzed in 0.8% agarose gel. PCR products were sequenced after gel extraction in Genetic Analyzer 3130 (Applied Biosystem, Foster City, USA) using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. Blast search was performed with the obtained sequences against public data base at NCBI server.

Small probes were designed using sequenced RT-PCR products and PCR amplification was performed to synthesize radio-labeled (α-P^{33}) probes specific to single expressed ORF. Nothern blotting was performed with total RNA using capillary transfer protocol.

Results

Organism and enzyme production

Keratinolytic protease synthesis by *B. cereus* DCUW was stimulated by the native feather keratin. The enzyme production was found to be associated with increase in pH of the fermentation medium, which is a characteristic property, discussed previously [18]. At regular interval, strain DCUW was sub cultured on feather meal agar plates. Strain DCUW grew at 25–40 °C and produced its highest keratinolytic activity at 30–37 °C. The keratinolytic nature of the protease was confirmed by calculating the activity ratio against chopped feather keratin and casein. The feather keratin: casein ratio was found to be 0.64 (Table 1).

Enzyme purification

The protease was purified approximately 25-fold by using DEAE-Sephadex, α-casein agarose, and Sephacryl S-300 size exclusion chromatography (Table 2). However, this protease was found to form large protease complex in extracellular milieu and a considerable fraction of protease activity was found to be eluted in void volume during gel filtration chromatography. Further purification of the protease using other techniques like ion exchange chromatography and other gel filtration chromatography were attempted, but no further purification was achieved.

Table 1 Comparative proteolytic activity against chopped feather keratin and Hammerstein casein under identical conditions

Substrate(s)	Specific activity (U/mg protein)	Feather keratin: casein ratio
Feather keratin (chopped)	1.4	0.64
Casein (Hammerstein)	0.9	

Table 2 Purification of the keratinolytic protease from the culture medium of strain DCUW

Purification step	Total protein (mg/ml)	Total activity (umole/ml min^{-1})	Specific activity (U/mg)	Yield (%)	Purification
Culture supematant	0.505	0.70	1.38	100	1
Ammonium sulphate	0.386	0.68	1.76	97	1.27
DEAE Sephadex	0.183	0.63	3.44	90	2.5
Casein agarose	0.028	0.50	17.66	71.5	13
Sephacryl S-300	0.010	0.35	34.5	50	25

An 80 kDa purified protein band along with some low molecular weight contaminating bands were resulted on SDS-PAGE (Fig. 1). Moreover, the attempts to purify the protease using different chromatographic techniques resulted in varied molecular weight of purified protease (70 ± 10 kDa). The active void volume fraction was also showing activity bands at 70 ± 10 kDa in zymography gel.

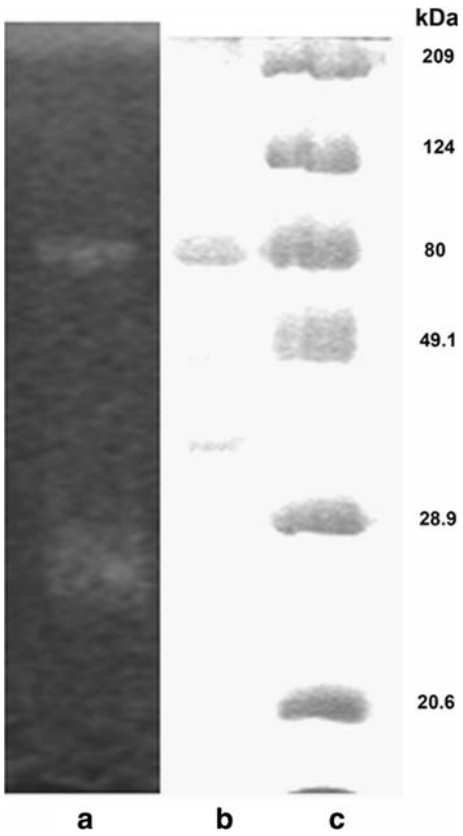

Fig. 1 Zymography and SDS-PAGE analysis of purified protease: gelatin zymography band (**a**), purified enzyme after gel filtration (**b**), molecular weight marker (Broad Range, Bio Rad) (**c**)

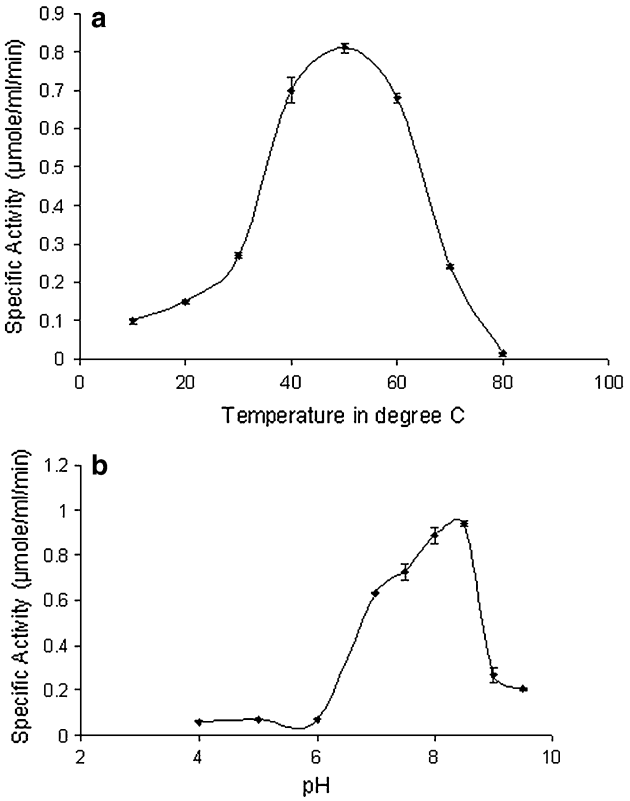

Fig. 2 The temperature and pH optimum. **a** Effects of various incubation temperatures on protease activity. **b** Effects of various pH values on proteolytic activity at 50 °C, using different buffer systems. *Error bars* correspond to standard deviations from triplicate replicas

Several groups [19, 30, 35] have reported similar behavior of extracellular protease, while they were working with minor extracellular protease (Vpr) from *B. subtilis*. Auto processing of Vpr was described as the reason for this unusual behavior during the purification process. Probably, auto-processing of pro-form of the protease resulted in molecular weight of 70 ± 10 kDa during the course of purification.

Effect of temperature and pH on protease activity

The temperature optimum for DCUW keratinolytic protease was found to be 50 °C (Fig. 2a), although the enzyme was found to be active over broad range of temperature. Figure 2b shows the protease activity at different pH. Optimum activity was evident at pH 8.5 (Fig. 2b).

Determination of kinetic parameters

The activity of the purified enzyme was measured in the presence of increasing concentrations of Azo-casein substrate. The V_{max} and K_m were found to be 17.73 Unit/ml and 0.161 mg/ml, respectively (Fig. 3).

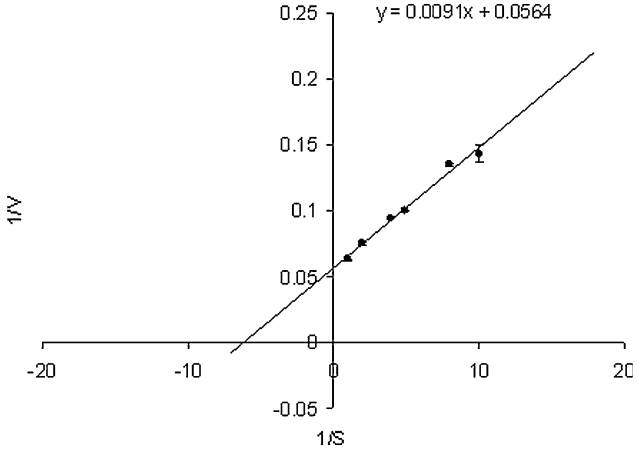

Fig. 3 Lineweaver–Burk plot of the purified enzyme sample ($Y = 0.0091x + 0.0564$, $R_2 = 0.99$). *Error bars* correspond to standard deviations from triplicate replicas

Sulfhydryl group production and Disulfide reductase activity

Keratin contains large numbers of disulfide bonds and that makes this protein different from other proteins. Keratin degradation by keratinases is still a matter of debate as some research groups showed that keratinase alone could bring about complete degradation of raw feather [24], whereas other groups showed that keratin degradation was initiated and assisted by disulfide reductase enzyme [32]. We found that in feather-supplemented medium there was a linear increase in reduced thiol groups as feather degradation progressed (Fig. 4). Reduction of disulfide bonds may occur chemically or enzymatically [4]. Thus, we measured the disulfide reductase activity. The extracellular fraction has showed disulfide reductase activity that was found to increase with time. After 96 h, disulfide activity was found to reach saturation. Based on these results, we conclude that

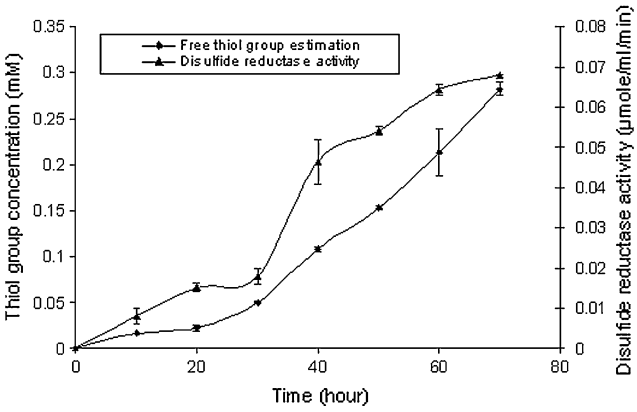

Fig. 4 Time kinetics of extracellular thiol-groups and disulfide reductase activity in feather-supplemented medium. *Error bars* correspond to standard deviations from triplicate replicas

Table 3 Effect of different protease inhibitors on the enzyme activity of protease from *B. cereus* DCUW

Inhibitors	Concentration (mM)	% Activity (residual)
Control without inhibitors		100
PMSF	0.1	25
	0.5	21
	1.0	1
Pepstatin	1	90
E-64	1	96
EDTA	1	100
	5	98
	10	96

there was an enzymatic reduction of disulfide groups in feather keratin by disulfide reductase that actually assisted the feather degradation by keratinolytic protease. Moreover, disulfide reductase activity in extracellular medium was found to be less, compared to intra-cellular pool. The colonization of bacterial cells on feather provides continuous supply of disulfide reductase that ultimately helps keratinolytic protease to complete the feather digestion [4].

Enzyme inhibition

Protease activity (Azocasein and feather meal) was measured in the presence of protease inhibitors and the results are summarized in the Table 3. Strain *B. cereus* DCUW exhibited serine protease activity, but no significant effect of inhibitors against metallo-protease (EDTA), cysteine protease (E-64), aspartic acid protease (pepstatinA) has been observed.

Amino acid composition

The amino acid composition of *B. cereus* DCUW feather-supplemented medium after protein removal is summarized in Table 4. The aim was to separate the feather-degrading extra cellular keratinolytic protease from the degradation products that were generated during the degradation of native feather. The amino acids obtained after Protein separation can be useful in the supplementation of the feed and food products for the poultry and cattle industries. Moreover, the separated enzyme could be useful in different biotechnological industries. The present protease was checked for its usefulness in different industries after separation from small peptides and free amino acids and was found to be highly efficient in leather-bating process (Central Leather Research Institute, Chennai, India), in detergent formulation, and in leather-dehairing process in combination with mild sulfide treatment.

Table 4 Amino acid composition of chicken feather hydrolysate

Amino acid	Amount (μg/ml)
Aspartic acid	2.7
Threonine	4.9
Serine	2.59
Glutamic acid	4.6
Proline	1.44
Glycine	2.36
Alanine	2.27
Valine	2.23
Methionine	0.633
Isoleucine	2.0
Leucine	3.35
Tyrosine	3.59
Phenylalanine	1.67
Histidine	3.4
Lysine	9.5
Tryptophan	1.33
Arginine	2.5

Cell cytotoxicity assay (MTT assay)

Assessment of the metabolic status of tissue culture cells using MTT has been made previously [3]. The tetrazonium ring of MTT is cleaved in the mitochondria of metabolically active cells, resulting in a color change from pale yellow to purple. Only viable cells can produce this formazone reaction product, which makes MTT a sensitive compound for assessment of living cells. *B. cereus* enterotoxin has a cytostatic effect on cultured cells, and this inhibition of cell proliferation can be monitored using MTT cytotoxicity assay. The main objective of the cytotoxicity assay was to determine whether *B. cerus* DCUW has any enterotoxin production during or even after completion of the feather degradation. The entire study was carried out using the supernatants from 4, 6 and 10 days culture, respectively. The time points were selected keeping in our mind that the highest protease activity was achieved after 4 days and the entire feather degradation needs 6 days. The result of the cell cytotoxicity assay has showed that the cell-free supernatant of the feather-supplemented medium had actually very little effect on the cell proliferation. The percent of viability with respect to the negative control is summarized in Fig. 5. Only the 10-day old culture supernatant has showed about 15% decrease in the viability.

RT-PCR and Northern blotting

The *B. cereus* DCUW has produced an extracellular keratinolytic protease, which was inhibited by PMSF,

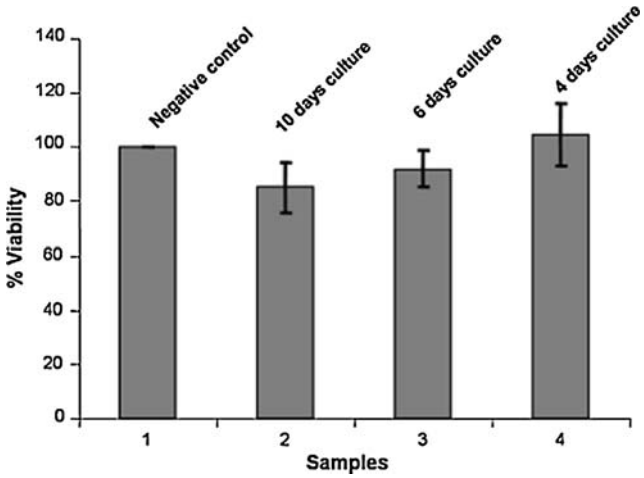

Fig. 5 Cytotoxic effect of strain *B. cereus* DCUW grown in feather-supplemented medium on HeLa cells

indicating the presence of a serine protease. This new protease was found to be secreted as a large complex and upon purification gave an 80-kDa band on 10% SDS-PAGE as well as in zymography (zelatin, casein, collagen, and fibrin).

Keeping the nature of keratinolytic protease in mind, several synthetic oligonucleotides were designed, specific to some of the probable extracellular proteases in already published genome sequence of *B. cereus* (ATCC 14579 and 10987). These oligonucleotides were used to perform RT-PCR after isolating total RNA from feather-supplemented culture cells and we found only in case of oligonucleotides against minor extracellular protease (Vpr) gene, a 2,754 nucleotide PCR product was obtained. Expression of Vpr was also confirmed by northern blot analysis using total RNA isolated from feather-supplemented medium grown *B. cereus* DCUW and Vpr specific probe (designed from the unique C-terminal sequence of Vpr from published genome sequence of *B. cereus* ATCC 14579 and 10987) (Fig. 6).

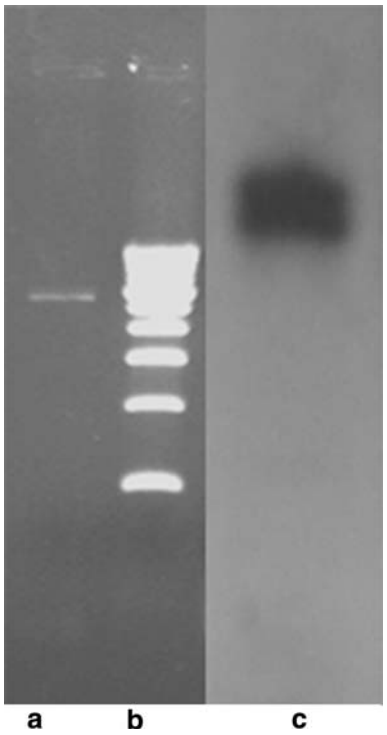

Fig. 6 RT-PCR and Northern blotting: **a** RT-PCR product from primers specific to minor extracellular protease (Vpr), **b** 1 kb DNA molecular weight marker (Fermentas), **c** Northern blotting using total RNA and specific radio-labeled probes against C-terminal domain of Vpr

Discussion

Degradation of feather waste and utilization of the degraded products in the feed and foodstuffs as supplements have attracted attention for the last one decade. People have tried to isolate keratinolytic proteases from different organisms. The socioeconomic need in countries like India demands reutilization of the proteinaceous solid wastes generated from poultry. Keeping this in mind, we have tried to design bio-degradation method where not only we could degrade raw feather but also could reutilize the byproducts for supplementation in chicken feed, fish meal etc.

The major difference between keratin and other proteins lies is the presence of a higher level of disulfide bonds in keratin. Reports on feather degradation propose that keratinases act on disulfide bonds, which are responsible for the mechanical stability of keratin, thus making it easier for proteolytic enzymes to act. Other reports suggest that the reduction of disulfide bonds by disulfide reductase [4] or the production of sulfite and thiosulphate [20] to be involved in keratin degradation. Involvement of call-bound redox system for disulfide bond reduction has also been suggested in prokaryotes [4]. Therefore it has been well documented that keratinolysis is often assisted by disulfide reductase activity in extracellular milieu. In most cases of keratinolytic proteases, it was found that the purified enzyme cannot degrade keratin [16], and only in case of keratinase from *B.licheniformis* PWB-1, it has been shown that the purified enzyme was capable of degrading feather keratin [24]. Therefore, it can be said that for feather degradation, reduction in sulfhydryl groups is the crucial step. Our results further support the sulfhydryl group reduction as we found a linear increase in reduced thiol groups in the extracellular broth as feather degradation progressed. Moreover, disulfide reductase activity in extracellular as well as intracellular enzyme pool further supports the theory of biological reduction of disulfide

bonds in feather by feather-supplemented medium grown strain DCUW.

Microbial keratinases are inducible enzymes, as reported by many authors [25]. The keratinolytic *B. cereus* DCUW produces a high molecular weight inducible protease, which exhibits an extraordinary activity against insoluble native feather. This keratinolytic protease was partially purified and tested for its capacity to degrade other protein substrates like fibrin, collagen, gelatin, albumin etc. The unusual ability of this protease to form large protease complex as evident from gel filtration profile suggest its utility as an external proteolytic translocational processor of different secreted proteins in extracellular medium [8]. We have identified the protease as minor extracellular protease (Vpr) by RT-PCR technique and also by Northern blot analysis. Additionally, we also tried to perform MALDI-TOF experiment with the excised purified band from SDS-PAGE, but unfortunately, the peptide mass finger printing (PMF) of the purified protease (80 kDa) obtained here did not match the PMF for the preproVpr described by Kho et al. [19], or any other PMF pattern found in the database. A similar observation was recently reported by Ageitos et al. [1], where they have suggested Vpr as milk-clotting protease in *B. licheniformis*.

Using purified enzyme, we have determined the optimum pH, optimum temperature, K_m, V_{max} and also determined the effect of different inhibitors. During purification, we failed to purify mature single molecular weight protease and we found that the molecular weight oscillated between 70 ± 10 kDa. Similar behavior was also reported by Sloma et al. [35]; while they were working with Vpr of *B. subtilis*. Protease Vpr from *B. subtilis* is a serine protease that, during processing normally varies from 94 to 65 kDa although forms of even only 28.5 kDa have been reported. We found that along with Vpr, sulfydryl reductase was also playing crucial role in feather-degradation process. Moreover, we have demonstrated the probable biosafe utilization of the end product of feather degradation by measuring amino acids in feather hydrolysate and also by determining the cytotoxic potential of strain DCUW.

This is probably the first report of minor extracellular protease (Vpr) from *B. cereus*. In our isolate we found that Vpr was involved in feather waste degradation although it was found to be capable of degrading other protein substrates like fibrin, collagen, albumin, casein, gelatin etc. Previously, people have reported Vpr as an extracellular protease that could process other secretary proteins (lantibiotic processing by Corvey et al. [8]) and also signaling proteins (CSF) [22]. Also, this protein has been implicated in biofilm formation and quorum sensing [22]. Vpr was also found to be fibrinolytic protease [19] and milk clotting protease [1]. The *B. subtilis* homologue of Vpr was found to auto process its long C-terminal protease-associated domain and thereby produce several smaller proteolytically active fragments. Sequence homology result showed that Vpr from *B. cereus* has 73% homology with its homologue in *B. subtilis* (T-Coffee software). Moreover, a similar long C-terminal protease-associated domain is present in Vpr of *B. cereus*. This is important in the context of auto-processing pattern found in Vpr of *B. cereus* and probably because of that behavior; we found active protease fragments ranges 70 ± 10 kDa in present study. Molecular study involving investigation of the auto-processing in Vpr could be an interesting path to follow. Our next aim is the cloning of the minor extracellular protease (Vpr) from *B. cereus* DCUW and its domain-based analysis to understand the mechanism of auto processing and reason for large protease complex formation.

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