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Application of cysteine protease of *Euphorbia nivulia* Buch.-Ham. latex in laundry detergent formulation

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ABSTRACT

A new cysteine protease named nivulian was partially characterized from the latex of *Euphorbia nivulia* Buch.-Ham., a member of Euphorbiaceae family. It had good stability in the presence of local detergents viz., Tide®, Ujalla®, Nirma® and also Triton X-100 and Tween-80 at 37^{0}C. The protein stains (blood and egg yolk) were removed within 10 – 15 min from the test fabric (cotton) cloth by the treatment of this enzyme. Thus this application of plant origin 'nivulian' demonstrates feasibility for inclusion in laundry detergent formulation.

KEYWORDS

Cysteine protease;
Euphorbia nivulia;
Euphorbiaceae;
Latex;
Nivulian.

INTRODUCTION

Proteases are enzymes which potentially hydrolyze anything containing peptide bond, from a dipeptide up to a large protein containing thousands of amino acids[1]. It is beyond question that the results of research on proteolytic enzymes, or peptidases, are already benefiting mankind in many ways, and there is no doubt that research in this area has the potential to contribute still more in future[2]. These enzymes are used in industry owing to their high stability at various conditions[3]. Since last 50 years, proteases are frequently used in laundry detergents for removing proteinaceous materials (in stain form) from fabrics and account for approximately 59% of total worldwide sales of enzyme[4]. The uses of enzyme in detergent formulations depend on its compatibility with the detergent[5]. An ideal detergent enzyme must be stable and active in the detergent solutions and have adequate thermal stability to be effective in a wide range of washing temperatures[6]. In 1914, the Röhm Company in Germany isolated the first enzyme preparation for industrial use i.e. the protease trypsin, which was marketed as a powerful detergent[7]. Several detergents like Tide, Dynamo and Era Plus (Procter and Gamble Company, USA) contains proteases[8]. Proteases, used as cleaning agents, stable in the presence of chlorine as water supplies are chlorinated in many parts of world including India and some detergent compositions may include bleaching agents[9]. Protease performance in laundry detergents is evaluated by using soiled test fabrics and the efficiency is measured either visually or by measuring the reflectance of light under standard conditions[10]. Present paper reports the stability of plant origin cysteine protease called nivulian of *E. nivulia* latex in the presence of detergents and its washing performance for the first time. Hence its incorporation in laundry detergent formulation is warranted.

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MATERIALS AND METHODS

Chemicals

All chemicals were of the highest purity, analytical HPLC grade purchased from Sigma Chemicals, USA; Himedia Laboratories, Mumbai; SRL Chemicals, Bangalore; Qualigen Fine Chemicals, Mumbai; Merck Chemicals, India; Bangalore Genie, India.

Plant material, collection of latex, crude enzyme preparation and purification of cysteine protease (nivulian)

The detail information about identification, collection, preservation, preparation of crude enzyme and its proteolytic activity of *Euphorbia nivulia* Buch.-Ham latex is described in our previous communication[11]. Method of purification of protease was done using acetone precipitation, DEAE cellulose chromatography, dialysis and followed by rechromatography on DEAE cellulose column as described in our earlier communications[11]. The protease activity was expressed as amount of enzyme required to produce peptide equivalent to μg of tyrosine/min/mg protein at 37^{0}C and protein content was measured according to Lowry's method using Bovine serum albumin as the standard protein[11].

Compatibility of nivulian with laundry detergents

The compatibility of nivulian in the presence of commercial solid laundry detergents was examined by incubating enzyme for 1 h at 37^{0}C with various common detergent preparations, and the residual enzyme activity was determined as per the discussed method[11]. The enzyme activity of a control sample (without detergent), incubated under the similar conditions, was taken as 100%. The solid detergents used were Ariel Oxy Blue, Tide (Procter and Gamble Company, USA), Fena Ultra, New Impact (Fena (P) Ltd., New Delhi, India), Nirma (Nirma Ltd., Ahmedabad, India), Wheel Active, Rin, Surf Excel (Hindustan Unilever Ltd., India), Ghari (Ghari Group of Companies, New Delhi, India), Sasa (Shri Mahila Griha Udyog Lijjat Papad, Mumbai, India) and Ujala (Jyothy Laboratories, Mumbai, India). The detergents were diluted in tap water to give a final concentration of 7 mg/ml to simulate effective washing conditions. The endogenous proteases contained in these detergents were inactivated by incubating the diluted detergents at 65^{0}C for 1 h prior to the addition of enzyme.

Effect of surfactant and oxidizing agent on nivulian

The effect of 1% (v/v) final concentration of different surfactant (sodium dodecyl sulphate, Triton X-100 and Tween 80) and oxidizing agent (H_2O_2) on enzyme activity was studied by preincubating enzyme for 1 h at 37^{0}C in the above chemical surfactants and oxidizing agent before analysis. A parallel control was kept with enzyme and buffer with substrate and the value of the control activity was considered as 100%.

Animal material - blood sample

Fresh blood samples of domestic animals viz., goat (*Capra hircus*), buffalo (*Bubalus bubalis*) and ox (*Ovibos moschatus*) of either sexes were collected under the supervision of Dr. N. M. Pawar, Veterinary Practitioner of Paldhi Unit, Jalgaon District, Maharashtra, India. Blood sample of healthy hen was collected from local chicken shops of Jalgaon city.

Evaluation of washing performance of nivulian

Application of Nivulian (5 U mg protein^{-1}) in 0.02 mol L^{-1} phosphate buffer pH 7.4 as a detergent additive was studied on white cotton cloth pieces (1.5" x 1.5") stained with blood samples of different animals (ox, buffalo, goat, hen and human being). The stained cloth pieces were taken in separate trays. The following groups were setup: (A) Tray with 50 ml of 0.02 mol L^{-1} phosphate buffer pH 7.4 and blood stained cloth. (B) Tray with 50 ml Nivulian protease (5 U mg protein^{-1}) in 0.02 mol L^{-1} phosphate buffer pH 7.4 and blood stained cloth. (C) Tray with 50 ml detergent (7 mg/ml) and blood stained cloth. (D) Tray with 50 ml mixture of detergent (7 mg/ml) and Nivulian protease (5 U mg protein^{-1}) and blood stained cloth[12]. These trays were incubated at 30^{0}C for 25 min. The cloth pieces were taken out from each set at regular intervals of 5 min, rinsed with water, dried and visually examined. Untreated cloth pieces stained with blood were taken as control. Additionally, after washing performance, dried cotton pieces were subjected for cutting. The resulting little pieces of individual destained cotton cloth piece were suspended in saline at 30^{0}C and centrifuged at 10000 rpm for 20 min. The progress of destaining of

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blood stain was monitored by measuring the absorbance of resulting supernatant at 420 nm. The test cotton fabric pieces stained with egg yolk were also treated under similar conditions at 30^{0}C. Stain removal was checked qualitatively by visualization.

RESULTS AND DISCUSSION

Euphorbia nivulia Buch.-Ham. belongs to the Euphorbiaceae family, whose members usually develop secretory tissues (laticifers) which frequently include proteolytic and milk clotting enzymes. The young stem latex of *E. nivulia* possesses proteolytic and milk clotting cysteine protease enzyme with optimum pH and temperature is pH 6.6 and 45^{0}C respectively[13].

The effect of surfactant and oxidizing agent at 1 % concentration on the proteolytic activity at 37^{0}C is summarized in TABLE 1. In presence of SDS and H_2O_2 protease activity was inhibited upto 48.49 % and 31.82 % respectively and activity was unaffected by exposure to Triton X-100 and Tween-80, indicating that the purified protease could not be lipoprotein. Our results are in accordance with the earlier observations reported by Patil and Chaudhari, 2009[14] in the purification of metalloprotease of *Pseudomonas aeruginosa*. As seen from TABLE 2 nivulian was considerably stable with all commercial detergents tested. After one hour, 80 to 90% activity was retained with Surf Excel, Fena, Rin and Ghari detergent, more than 92% with Wheel and Ariel, 78% with Sasa and 67% with Impact. Surprisingly protease activity of nivulian was increased by 1 to 34% with Tide, Ujala and Nirma. A similar stability profile was observed in protease of *Bacillus circulans* BM15[15].

TABLE 1 : Stability of protease in surfactants (Sur) / oxidizing agent (OA) at 1% concentration.

Sr. No.	Sur / OA	Residual activity (%)
1	SDS^{++}	51.51 ± 0.18
2	$H_2O_2^{++}$	68.18 ± 0.35
3	Tween 80^{++}	243.03 ± 0.69
4	Triton X-100^{++}	190.39 ± 1.04

Data represented in average values ± SD of n=6 experiment;

The results (Figure 1) of evaluation of enzyme for washing performance pointed out that the blood stains on the cloth pieces remained as they were even after 15 min of rinsing in the case of controls and commercial detergents. Blood stain was completely removed from the cloths after rinsing them with a combination of detergent and Nivulian for a period of 15 min, whereas it was removed after 25 min when rinsed with Nivulian individually. These results clearly indicate that the enzyme is fairly stable as an ingredient in the presence of detergents. Our results of washing performance of Nivulian are good in accordance with the earlier observations reported in washing performance of protease of *Pseudomonas aeruginosa*[16] and *Streptomyces gulbergensis*[12].

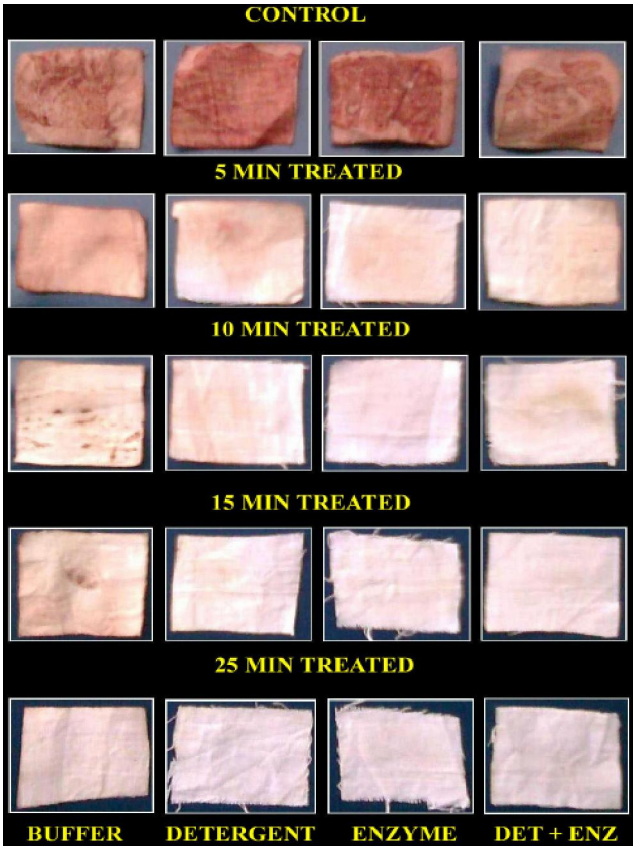

Figure 1 : Evaluation of nivulian for washing of goat's blood stains from cloth.

In order to evaluate the performance of Nivulian with respect to its capability of removing stains, different blood samples were used viz., human being, ox, buffalo and hen and also egg yolk stain. On incubating several pieces of stained cloth at 30^{0}C for 25 min results of this findings are interesting, the use of enzyme (Nivulian) alone showed more effective removal of blood and egg yolk stains (Figure 2). In fact, Nivulian facilitate the release of proteinaceous materials in a much

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Figure 2 : Removing blood and egg yolk stains from cloth by the application of nivulian and detergent after 25 min; First column: Untreated blood stained cloth. (Control); Second column: Blood stained cloth washed (BSCW) by buffer; Third column: BSCW by enzyme (nivulian); Fourth column BSCW by detergent; Fifth column: BSCW by detergent with nivulian. First, second, third and fourth row: Cloth stained by blood sample of human being, ox, buffalo and hen respectively. Fifth row: Cloth stained by egg yolk.

TABLE 2 : Stability of protease enzyme preparation of *E. nivulia* latex in various local detergents.

Sr. No.	Detergent	Residual activity (%)	
		7 mg/ml	10 mg/ml
1	Control	100	100
2	Tide	101.4 ± 0.20	90.38 ± 0.57
3	Ujalla	104.2 ± 0.16	107.8 ± 1.38
4	Wheel	98.85 ± 0.22	98.65 ± 0.52
5	Impact	67.25 ± 0.69	65.08 ± 2.56
6	Rin	87.87 ± 0.04	85.03 ± 0.17
7	Nirma	101.2 ± 0.44	103.1 ± 0.72
8	Surf Excel	89.36 ± 0.31	83.66 ± 1.47
9	Ariel	92.49 ± 0.78	83.24 ± 0.57
10	Fena	82.54 ± 0.65	74.55 ± 0.83
11	Ghari	80.33 ± 0.11	72.48 ± 0.48
12	Sasa	78.43 ± 0.07	61.21 ± 1.26

Data represented in average values ± SD of n=3 experiment

easier way than the commercially available detergent. Furthermore, the combination of Nivulian with detergent resulted in complete stain removal (Figure 1 and 2). A similar study reported on the usefulness of alkaline proteases from *Bacillus brevis* and *Bacillus pumilus*[17].

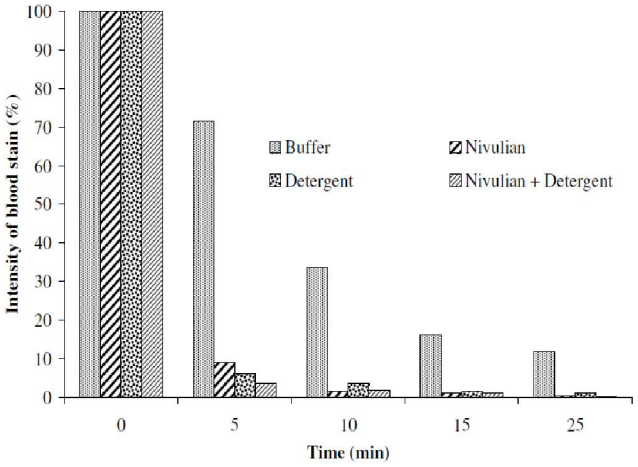

Figure 3 : Destaining profile of human blood stain by nivulian.

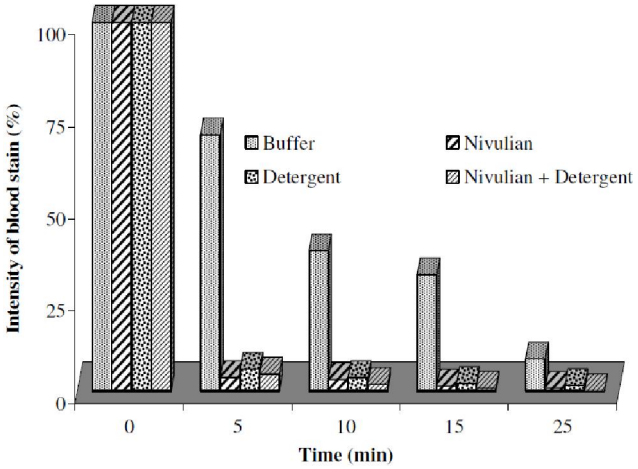

Figure 4 : Destaining profile of ox blood stain by nivulian.

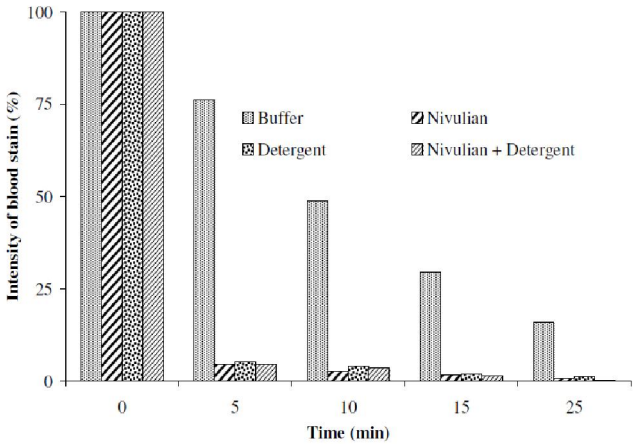

Figure 5 : Destaining profile of buffalo blood stain by nivulian.

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The Figure 3 to 7 illustrates the destaining profile of human, ox, buffalo, goat and hen blood samples respectively. These destaining profiles clear the idea about removal of blood stains with minimum use of commercial detergent within 25 min. Rapid blood stain removal was noticed with supplementation of commercially available detergents (TABLE 3). Similar results of destaining of blood with combination of protease and detergent was noticed by Rao et al., 2009[18].

TABLE 3 : Destaining profile of different blood stain after 25 min of treatment.

Sr.No.	Treatment	Destaining efficacy (%)*				
		A	B	C	D	E
01	Control	11.89	8.69	15.91	15.66	8.74
02	Nivulian	0.62	0.80	0.74	0.97	0.71
03	Detergent	1.18	1.51	1.09	1.16	0.79
04	Niv. + Det.	0.24	0.12	0.26	0.10	0.24

***The intensity if blood stain at 0 min treatment was taken as 100% for all blood samples; (A): Human; (B): Ox; (C): Buffalo; (D): Goat and (E): Hen blood stained spot.**

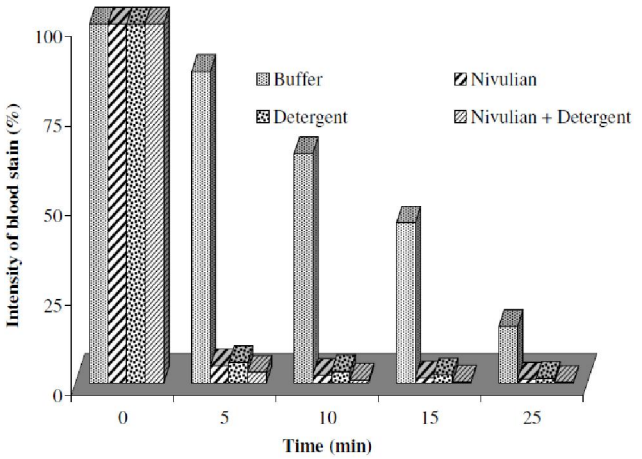

Figure 6 : Destaining profile of goat blood stain by nivulian.

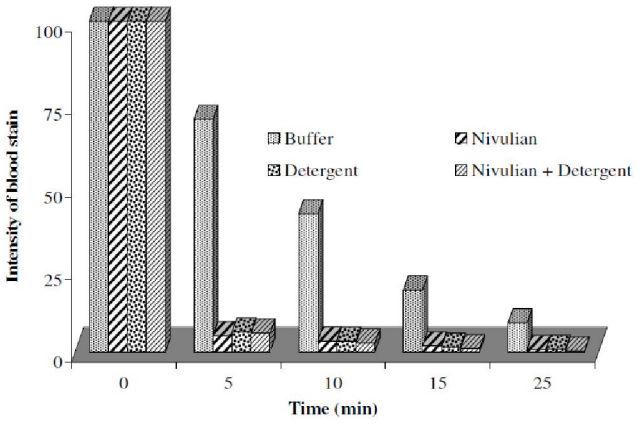

Figure 7 : Destaining profile of hen blood stain by nivulian.

CONCLUSION

A new cysteine protease, named nivulian is partially characterized for the first time from the latex of *E. nivulia*, using simple purification procedure. Easy availability of *E. nivulia* latex, with simple and economic purification process of nivulian, provides great possibility for its large scale preparation. In the light of all these experimental observations it is envisaged that the present protease has properties that it render suitable for (i) as a detergent ingredient and (ii) removal of recalcitrant proteinous stains from fabrics. It could be subjected to many useful applications in biotechnological industries i.e. enzyme based detergent industry.

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