J Ind Microbiol Biotechnol (2011) 38:845–853
DOI 10.1007/s10295-010-0855-x

ORIGINAL PAPER

A novel nonionic surfactant- and solvent-stable alkaline serine protease from *Serratia* sp. SYBC H with duckweed as nitrogen source: production, purification, characteristics and application

G. Y. Li · Y. J. Cai · X. R. Liao · J. Yin

Received: 1 July 2010 / Accepted: 18 August 2010 / Published online: 12 November 2010

Abstract A novel nonionic surfactant- and hydrophilic solvent-stable alkaline serine protease was purified from the culture supernatant of *Serratia* sp. SYBC H with duckweed as nitrogen source. The molecular mass of the purified protease is about 59 kDa as assayed via SDS-PAGE. The protease is highly active over the pH range between 5.0 and 11.0, with the maximum activity at pH 8.0. It is also fairly active over the temperature range between 30 and 80°C, with the maximum activity at 40°C. The protease activity was substantially stimulated by Mn^{2+} and Na^{+} (5 mM), up to 837.9 and 134.5% at 40°C, respectively. In addition, Mn^{2+} enhanced the thermostability of the protease significantly at 60°C. Over 90% of its initial activity remained even after incubating for 60 min at 40°C in 50% (v/v) hydrophilic organic solvents such as DMF, DMSO, acetone and MeOH. The protease retained 81.7, 83.6 and 76.2% of its initial activity in the presence of nonionic surfactants 20% (v/v) Tween 80, 25% (v/v) glycerol and Triton X-100, respectively. The protease is strongly inhibited by PMSF, suggesting that it is a serine protease. Washing experiments revealed that the protease has an excellent ability to remove blood stains.

Keywords Alkaline serine protease · Solvent-stable protease · Blood stain washing · Duckweed · *Serratia* sp. SYBC H

G. Y. Li · Y. J. Cai (✉) · X. R. Liao (✉) · J. Yin
The Key Laboratory of Industrial Biotechnology, Department of Education, Ministry of Education, School of Biotechnology, Jiangnan University, Lihu Road 1800, 214122 Wuxi, Jiangsu Province, China
e-mail: yu_jie_cai@yahoo.com.cn

X. R. Liao
e-mail: liaoxiangru@163.com

Introduction

Alkaline proteases are an important category of enzymes that hydrolyze protein peptide bonds under alkaline conditions. They are widely used in detergents, leather processing, silk processing, medicine, food, animal feed, environment protection, chemical processing, and (as an alternative for protease K) in DNA isolation [1–5]. Non-aqueous catalytic reactions, e.g., oligo-peptide synthesis, require that proteases, as catalysts in the presence of organic solvents, must be stable. However, most enzymes are either inactivated or very low in activity in non-aqueous media [6, 7]. Various methods (physical and chemical) have been developed for stabilizing enzymes in the presence of organic solvents [8–10]. For synthetic reactions, it is more efficient to possess a protease that is naturally stable and exhibits high activity in organic solvents, thus bypassing all protease-stabilizing procedures.

Several solvent-stable proteases have been reported from solvent-tolerant *Pseudomonas* sp. [6, 11, 12] and *Bacillus* sp. [13]. Their production has conventionally been induced using glucose or starch coupled with expensive nitrogen sources such as yeast extract, peptone or casamino acids [14, 15]. Recent efforts have been directed towards using cost-effective feedstock or agricultural by-products as substrates for protease production [16, 17]. The current study introduced cost-free duckweed as nitrogen source for *Serratia* sp. SYBC H to produce an organic solvent-stable alkaline protease. Duckweed is a floating water plant rich in crude proteins and minerals, and is often used as feed for fish, plankton and poultry. Because of its over 90% water content and anti-nutritional ingredients (e.g., tannin and phytic acid), fresh duckweed is neither easily utilized nor fully absorbed [18, 19]. It is of great significance to explore an alternative and more efficient way to use duckweed. Research has been conducted in this laboratory on using

wind-dried duckweed as nitrogen source for *Serratia* sp. SYBC H fermentation. The protease, a high-value end product, was found to be highly productive, tolerant of alkaline and various organic solvents and surfactants.

In this report we present, for the first time, the production, purification, characteristics and washing application of a water-miscible solvent- and nonionic surfactant-stable alkaline serine protease by *Serratia* sp. SYBC H fermentation with duckweed as nitrogen source.

Materials and methods

Bacterial strain

The strain *Serratia* sp. SYBC H was obtained from decayed *Cyanobacteria* in Taihu Lake, China. The stock culture was maintained on LB slants at 4°C and as a glycerol stock at −20°C.

Duckweed

The duckweed used in this study was collected from Taihu Lake (China), wind dried and foreign objects were removed; it was crushed into powder and filtrated through a 200-mesh sieve.

Assay of protease activity

The protease activity was determined using a slightly modified method reported by Bhosale et al. [20]; 100 µl of the protease sample was mixed with 500 µl of pre-incubated 1% (w/v) azocasein (Sigma) dissolved in Tris-HCl buffer (50 mM, pH 7.8). After incubating at 37°C for 10 min, the reaction was terminated by adding 1.9 ml of 10% trichloroacetic acid (TCA). The supernatant was then obtained by centrifugation at 8,000*g* for 15 min and colored by adding 2 ml of 1 M NaOH. The absorption ($A_{440\,nm}$) was then determined. A unit (U) of protease activity was defined as the amount of enzymes required to increase 0.01 of $A_{440\,nm}$ per minute.

Protease production

Activated plate strain *Serratia* sp. SYBC H was scratched in one or two loops into 35 ml of LB medium followed by incubation at 30°C and 200 rpm for 10 h. The grown culture was then used as a 5% inoculum and transferred into 50 ml of the sterilized fermentation medium in a 250-ml Erlenmeyer flask containing (g/l): wheat flour 10, duckweed powder 20, Tween80 6.0 and NaCl 5.0, and incubated at 30°C and 200 rpm for 18 h. The initial pH of the fermentation medium was adjusted to 7–7.2 with 50 mM NaOH and HCl. After cultivation, the culture was centrifuged at 8,000*g* and 4°C for 15 min. The supernatant obtained was used for protease purification.

Purification of the protease

The crude protease was purified in three steps involving $(NH_4)_2SO_4$ salt precipitation, DEAE-cellulose anion-exchange chromatography and Sephadex G-75 gel filtration. At first, the culture supernatant was concentrated by adding ammonium sulfate to 20% saturation. After 24 h incubation at 4°C, the solution was centrifuged at 8,000*g* for 20 min to remove the precipitated hybrid proteins. Ammonium sulfate was then added to the supernatant up to 60% saturation, and the solution was incubated at 4°C for 24 h. Precipitate was obtained by centrifugation and dissolved in a small volume of Tris-HCl buffer (50 mM, pH 7.8). The sample was dialyzed against the same buffer for 24 h at 4°C, filtrated through a microporous membrane (pore size 0.22 µm), loaded onto a pre-equilibrated DEAE-cellulose anion exchange column and eluted with Tris-HCl buffer (50 mM, pH 7.8) at a flow rate of 1 ml/min until the A_{280} reading was less than 0.02. The column was then eluted with a linear salt gradient (0–1 M NaCl) at a flow rate of 1 ml/min. The fractions with proteolytic activity were collected, dialyzed against Tris-HCl buffer (50 mM, pH 7.8), concentrated with polyethylene glycol 20,000 and loaded onto a pre-equilibrated Sephadex G-75 gel column for further purification. The gel column was eluted with Tris-HCl buffer (50 mM, pH 7.8) at a flow rate of 0.5 ml/min. The active fraction as a single peak from the column was collected, concentrated by lyophilization and further checked on native and non-natural PAGE, and used for enzymatic characterization study.

SDS-PAGE and zymography with azocasein

Zymography activity staining was performed with azocasein on native PAGE according to the method of Davis [21] with some slight modifications. A solution of azocasein (1%) was incorporated into 12% separating gel before polymerization. After electrophoresis, the gel was incubated in Tris-HCl buffer (50 mM, pH 7.8) at 40°C for 60 min. Finally, the gel was stained with Coomassie brilliant blue R-250 in methanol-acetic acid-water solution (3:1:6, v/v/v), and then destained with methanol-acetic acid-water solution (1:1:8, v/v/v).

The homogeneity and molecular mass of the purified protease were determined by SDS-PAGE using 5% stacking gel and 12% separating gel (mass fractions) as described by Laemmli [22]. The molecular weight was estimated with the following proteins as molecular markers: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14 kDa).

Effect of temperature on protease activity

The effect of temperature on the protease activity was examined by incubating the enzyme reaction mixtures at different temperatures ranging from 20 to 80°C, using 1% (w/v) azocasein in 50 mM buffer (pH 7.8) as substrate. The protease activity was measured according to the method described above. The maximum activity was used as 100% of the relative activity. All data were the mean of three parallel experiments, with each experiment repeated two times.

Effect of $MnCl_2$ and $CaCl_2$ on thermostability of the protease at high temperature

To study the effect of $MnCl_2$ and $CaCl_2$ on the thermostability of the protease at high temperatures, the purified protease was incubated with 5 mM $MnCl_2$ and $CaCl_2$ at 60°C and in 50 mM buffer (pH 7.8), respectively. Aliquots were withdrawn at desired time intervals to test the residual protease activity under standard conditions as described above (37°C). The purified protease in the absence of an additive was considered to be the control. All the results presented were the mean of three parallel experiments, with each experiment repeated two times.

Effect of pH on protease activity and stability

The purified protease was adjusted to different pH values (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0), and azocasein solution 1% (w/v) was prepared with an equivalent pH. The pH was adjusted using one of the following buffers (0.05 M): glycine-HCl (pH 3.0–5.0), phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0–11.0). The protease activity was measured under normal assay conditions described above (37°C), and the maximum activity was regarded as control (100% of the relative activity). All the results presented were the mean of three parallel experiments, with each experiment repeated two times.

The pH stability was determined by incubating the purified protease dissolved in buffers of pH 4.0, pH 8.0 or pH 11.0 for 7 days at 4°C, then incubating at 40°C for 30 min. The residual activity was then assayed according to the normal assay procedure described above. All the results presented were the mean of three parallel experiments, with each experiment repeated two times.

Effect of organic solvents on protease activity

The effect of various hydrophilic organic solvents (50%; v/v) on the protease activity was tested. An aliquot of 50 µl protease solution and 50 µl organic solvent was shaking incubated at 40°C for 60 min, after which the residual protease activity was measured according to the normal assay procedure described above. The results were compared with the control tests in which no organic solvent was added. All the results presented were the mean of three parallel experiments, with each experiment repeated two times.

Effect of various metal ions on protease activity

The effect of metal ions on the protease activity was investigated by individually adding Ca^{2+}, Mg^{2+}, Fe^{2+}, Na^{+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} or Cu^{2+} into the purified protease to a 5-mM final concentration and incubating at 40°C for 30 min. The residual protease activity was then measured according to the normal assay procedure described above. The results were compared with control tests in which no metal was added. All the results presented were the mean of three parallel experiments, with each experiment repeated two times.

Effect of protease inhibitors and surfactants on protease activity

The effect of various protease inhibitors and surfactants, such as serine inhibitor [phenylmethyl sulphonylfluoride (PMSF)] and metalloproteinase inhibitor [ethylene diamine tetra acetic acid (EDTA)], SDS, glycerol, Tween 80 and Triton X-100, was studied by adding them individually into the purified protease solution and incubating for 30 min at 40°C. The residual activity was then measured according to the method described above and compared with the control tests with neither inhibitors nor surfactants. All data represented the mean of three parallel experiments, with each experiment repeated two times.

Blood-stain washing experiments

The primary applications of alkaline proteases are as cleaning additives for the detergent industry. In order to investigate the ability of this *Serratia* sp. SYBC H alkaline protease to remove blood stains, washing experiments were conducted. A gauze was stained with blood drops obtained from a slaughterhouse and baked dry at 65°C. The blood-stained gauze was then treated with several drops of purified protease solution (with protease activity greater than 500 U/ml) and incubated at 40°C for half an hour, then rinsed and bake dried.

Results

SDS-PAGE and zymogram with azocasein

The protease from culture supernatant was purified by the three-step procedure described above. The purified protease and crude protease sample from culture supernatant were evaluated using zymogram activity staining with the

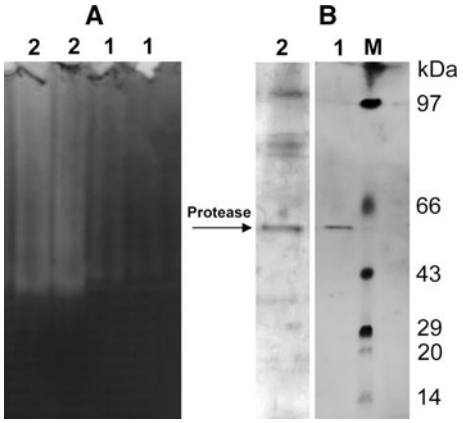

Fig. 1 **a** Zymography activity staining of *Serratia* sp. SYBC H protease with native PAGE. Samples were electrophoresed under non-denaturing conditions in a 12% separating gel containing 1% (w/v) azocasein. *Parallel lane 1*: purified protease; *parallel lane 2*: crude protease from fermentation supernatant. **b** SDS-PAGE analysis of *Serratia* sp. SYBC H protease. *Lane M*: standard protein markers of different molecular weights. *Lane 1*: purified protease. *Lane 2*: crude protease from culture supernatant

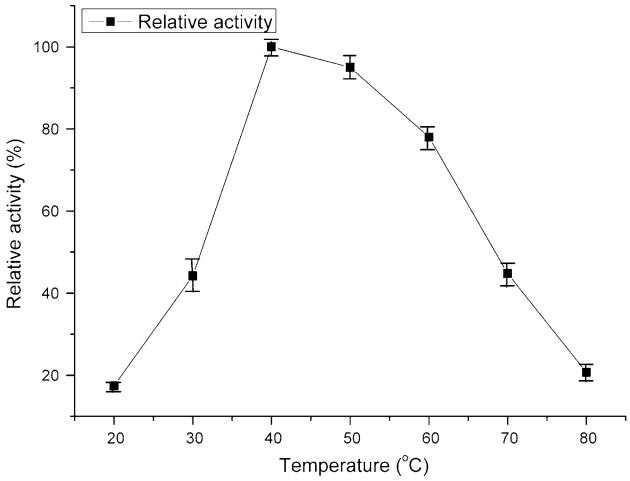

Fig. 2 Effect of temperature on the activity of purified serine protease from *Serratia* sp. SYBC H. The activity at 40°C is taken as 100%

modified native PAGE. As shown in Fig. 1a, a clear zone of proteolytic activity against the blue background for the purified sample, essentially similar to that for the crude protease sample obtained from the fermentation supernatant, indicated the homogeneity of the purified protease.

SDS-PAGE of the crude and purified protease preparations is shown in Fig. 1b. The purified protease was homogenous and showed a single band on the SDS-PAGE, with an estimated molecular mass of 59 kDa.

Effect of temperature on protease activity

The effect of temperature on the protease activity is shown in Fig. 2. The protease was active over a fairly wide range of temperature from 30 to 80°C. The maximum activity was observed at 40°C. The protease exhibited low activity below 20°C, with the activity less than 20% of the maximum activity (at 40°C). At 30°C, the protease activity was 44.2% of the maximum value at 40°C. Above 30°C, the protease activity increased rapidly with increasing temperature and reached a peak value at 40°C, then decreased slowly with further increase in temperature. At 50, 60, 70 and 80°C, the protease retained 95.3, 79.0, 44.8 and 20.7% of its maximum activity (at 40°C), respectively, which indicated that the protease was fairly active at high temperatures.

Effect of $MnCl_2$ and $CaCl_2$ on thermostability of the protease at high temperature

The effect of $CaCl_2$ and $MnCl_2$ on the thermostability of the purified protease was examined by incubating the protease in the presence of 5 mM $CaCl_2$ or $MnCl_2$ at 60°C before measuring the residual activity at 37°C from 0 to 90 min at 15-min intervals (Fig. 3). The purified protease appeared to be highly stable and retained its full activity even after incubation for 90 min at 60°C in the presence of 5 mM $MnCl_2$. However, in the case of $CaCl_2$, the protease retained only 38.2% of its initial activity after incubation for 90 min at 60°C, which was less than the control test with no metal added (55.3%).

All the results showed that the thermostability of the protease from *Serratia* sp. SYBC H against thermal inactivation was significantly enhanced in the presence of Mn^{2+}, whereas $CaCl_2$ decreased the thermostability of the protease.

Effect of pH on protease activity and stability

The protease activity at various pH levels is shown in Fig. 4. The maximum activity was assayed at pH 8.0. At pH 9.0, 10.0 and 11.0, the protease activity was 92.3, 80.4 and

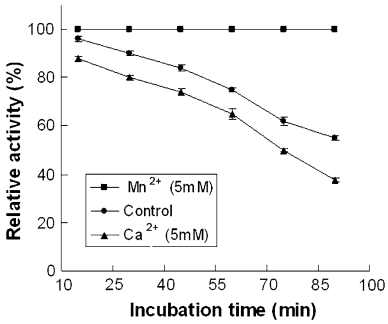

Fig. 3 Effect of Ca^{2+} and Mn^{2+} on the thermostability of purified serine protease from *Serratia* sp. SYBC H. The protease was incubated at 60°C in 50 mM buffer, pH 7.8, in the absence of additive (control) (*filled circle*), in the presence of Mn^{2+} (5 m M) (*filled square*) and in the presence of Ca^{2+} (5 mM) (*filled triangle*)

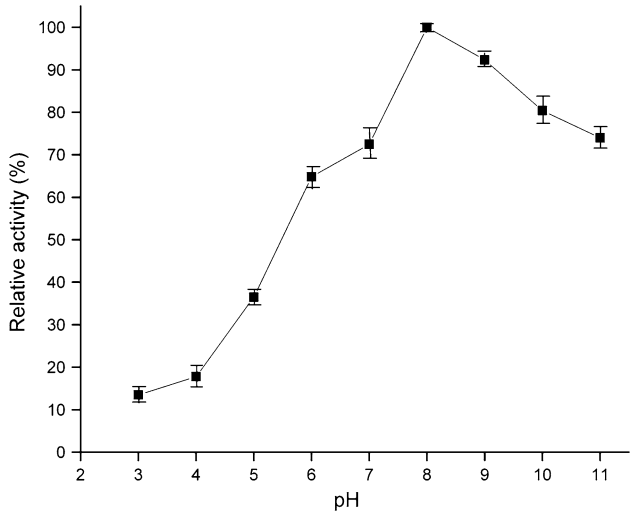

Fig. 4 Effect of pH on the activity of purified serine protease from *Serratia* sp. SYBC H. The activity of the protease at pH 8.0 is taken as 100%

74% of the maximum value (at pH 8.0), respectively. At a pH level of 4.0, 5.0, 6.0 and 7.0, the protease activity was 20, 38.5, 64.8 and 72.5% of the maximum activity value (at pH 8.0), respectively. Based on these results, it is clear that the protease was fairly active over a wide pH range between 5.0 and 11.0 at 37°C with the optimal pH of 8.0 for hydrolysis of azocasein.

For the pH stability study, the purified protease in buffers of pH 4.0, 8.0 or 11.0 was incubated at 4°C for 7 days, followed by incubation at 40°C for 30 min; the residual protease activity was then determined. The results showed that at pH 11.0, 8.0 and 4.0, the residual protease activity was 76.0, 94.6 and 23.5% of its initial activity value, respectively. This indicates that the protease was stable under alkaline conditions as high as pH 11.0 and exhibited appreciable activity at acid conditions of pH 4.0.

Effect of organic solvents on protease activity

Enzymes are usually inactivated or show low activity in the presence of organic solvents, especially in water-miscible solvents. The protease in this study was not only stable and active in the presence of hydrophobic solvents such as isooctane, decane, dodecane and hexadecane (unpublished results), but also stable and active in the presence of various hydrophilic solvents. In order to study the protease activity in the presence of hydrophilic solvents, the protease was incubated with 50% (v/v) hydrophilic solvents for 60 min at 40°C before the residual activity was determined. The results are shown in Table 1. The protease retained more than 80% activity in the presence of the water-miscible solvents ethanol, ethyl acetate and acetonitrile. More remarkably, the protease retained more than 90% activity after exposure to water-miscible solvents DMF, DMSO, Acetone and MeOH, except that relatively great loss in the protease activity was observed in the case of isopropanol and THF, in which the protease activity was reduced by 27.6 and 30.7%, respectively.

Table 1 Effect of organic solvents on purified protease activity

Organic solvent (50% v/v)	Relative protease activity[a] (%)
Control	100
DMF	90.8 ± 1.82
DMSO	92.1 ± 1.38
Ethanol	82.6 ± 1.72
Acetone	97.2 ± 1.26
Methanol	94.1 ± 1.51
Ethyl acetate	81.3 ± 2.03
Acetonitrile	83.9 ± 0.92
Isopropanol	72.4 ± 1.16
THF	69.3 ± 0.62

[a] Mean ± SD ($n = 6$)

Effect of metal ions on protease activity

The effect of various metal ions at 5 mM concentration on the protease activity has been studied at 40°C. It was observed that Na^+ and Mn^{2+} increased the protease activity to 134.5 and 837.9% of the control value, respectively. However, Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} significantly decreased the activity to 0, 15.3, 20.3, 22 and 45.8% of the control value, respectively (Table 2). Unlike most reported alkaline serine proteases, which were activated by Ca^{2+} and Mg^{2+}, the protease in this study was inhibited by Ca^{2+} and Mg^{2+}, and reduced to 87 and 89.8% of the control activity value, respectively. These results indicated that Mn^{2+} is the best activator for this *Serratia* sp. SYBC H protease.

Table 2 Effect of metal ions on purified protease activity

Metal ion (5 mM)	Relative protease activity[a] (%)
Control	100
Fe^{2+}	22.0 ± 0.42
Na^+	134.5 ± 2.02
K^+	90.4 ± 1.81
Mg^{2+}	89.8 ± 1.44
Mn^{2+}	837.9 ± 9.01
Ca^{2+}	87.0 ± 1.48
Cu^{2+}	20.3 ± 0.18
Zn^{2+}	45.8 ± 0.60
Co^{2+}	0.0
Ni^{2+}	15.3 ± 0.24

[a] Mean ± SD ($n = 6$)

Effect of protease inhibitors and surfactants on protease activity

The effect of inhibitors and surfactants on the protease activity was measured, as shown in Table 3. The protease was strongly inhibited by serine protease inhibitor (PMSF) at 5 mM concentration, indicating that the *Serratia* sp. SYBC H protease in this study is a serine protease. Thiol reagent (DTT) had a weak influence on the activity of the purified protease, with 13.1% of protease activity lost, indicating that the disulfide bond of the protease plays a role in stabilizing the protease structure or activity. Metal-chelating agents such as EDTA at 5 mM concentration did not inactivate the protease, with the proteolytic activity up to 116.4% of the control value, confirming that this protease is not a metalloprotease.

As shown in Table 3, the protease was highly stable and active in the presence of non-ionic surfactants such as Tween 80, glycerol and Triton X-100. The protease retained 81.7, 83.6 and 76.2% activity after incubating for 30 min at 40°C in the presence of 20% (v/v) Tween 80, 25% (v/v) glycerol and Triton X-100, respectively (no previous report can be found that used such a large concentration of non-ionic surfactants). There was a 50.9% reduction in protease activity in the presence of the anionic surfactant SDS at 1% (w/v) concentration.

Table 3 Effect of inhibitors and surfactants on purified protease activity

Inhibitor/ surfactant	Concentration	Relative protease activity[a] (%)
Control	–	100
PMSF	5 mM	38.3 ± 0.34
DTT	5 mM	86.9 ± 1.09
EDTA	5 mM	116.4 ± 1.10
Glycerol	25% v/v	83.6 ± 1.34
Tween 80	20% v/v	81.7 ± 0.65
Triton X-100	25% v/v	76.2 ± 1.05
SDS	1% w/v	49.1 ± 0.54

[a] Mean ± SD ($n = 6$)

Blood-stain washing performance

Alkaline proteases added to laundry detergents enable the release of proteinaceous materials such as blood, milk, whey and sweat from stains. A comparison of the blood-stained gauze before and after treatment by this protease is shown in Fig. 5. The results demonstrated effective blood-stain removal ability of the alkaline protease produced by *Serratia* sp. SYBC H with duckweed, indicating its promising potential application in the detergent industry.

Discussion

This report has provided the first study on a nonionic surfactant- and solvent-stable alkaline serine protease by *Serratia* sp. SYBC H fermentation with duckweed as nitrogen source. The biotechnological applications of the microbial fermentation of duckweed to produce proteases have not been explored prior to this study.

Several studies have been reported previously by other researchers on investigating proteases from different strains of *Serratia*; among them, *Serratia marcescens* strains have been the most studied [23–27]. When *Serratia* sp. SYBC H was grown in a simple optimized medium with duckweed as nitrogen source, it secreted a single protease into the medium, as observed from SDS-PAGE and native PAGE. This is distinctively different from the most studied *Serratia marcescens* strains, for which two types of proteases are produced, a major metalloprotease and a minor serine protease [23, 26, 27]. These differences are attributed to differences in the proteinaceous substrates and strains.

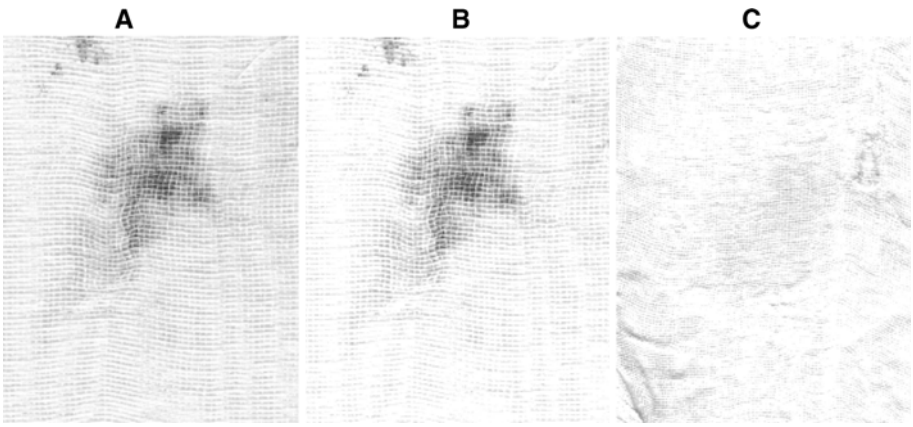

Fig. 5 Washing performance of protease from *Serratia* sp. SYBC H. **a** Blood-stained gauze. **b** Blood-stained gauze washed with distilled water. **c** Blood-stained gauze washed with *Serratia* sp. SYBC H protease

Most *Serratia* proteases normally have high molecular mass. The molecular mass of the protease from *Serratia* sp. SYBC H is about 59 KDa, similar to that of *Serratia marcescens* AP3801 metalloprotease (58 kDa) [28] and *Serratia marcescens* HR-3 metalloprotease (61 kDa) [25], and lower than that of *Serratia marcescens* ATCC 25419 serine protease (66.5 kDa) [23] and *Serratia rubidaea* metalloprotease CP-1 (97 kDa) [29], and higher than that of *Serratia marcescens* metalloprotease (50 kDa) [30], *Serratia marcescens* NRRLB-23112 metalloprotease (50.9 kDa) [24], and *Serratia marcescens* CCEB 415 proteases I and II (both 37 kDa) [31]. Thus, in terms of molecular mass, the *Serratia* sp. SYBC H protease is within the range of reported *Serratia* proteases.

The optimum temperature of this protease is around 40°C. *Serratia* proteases that had the same optimum temperature as this protease included *Serratia rubidaea* protease [29], *Serratia ureilytica* proteases P1 and P2 [32], and *Serratia marcescens* AP3801 metalloprotease [28], but were lower than that of *Serratia marcescens* ATCC 25419 serine protease (48°C) [23], and S*erratia marcescens* CCEB 415 proteases I and II (both 50°C) [31]. With an approximate 20% activity at 80°C, this protease is more active at high temperatures than most of the reported *Serratia* proteases such as *Serratia rubidaea* protease metalloprotease [29], *Serratia marcescens* NRRLB-23112 metalloprotease [24], *Serratia marcescens* ATCC25419 serine protease [23], *Serratia ureilytica* metalloproteases P1 and P2 [32], *Serratia marcescens* AP3801 metalloprotease [28], S*erratia marcescens* CCEB 415 proteases I and II [31], all of which were completely inactivated at temperatures higher than 60 or 65°C.

The optimal pH of this protease is 8.0, similar to that of *Serratia rubidaea* protease CP-2 [29], S*erratia marcescens* AP 3801 metalloprotease [28] and S*erratia marcescens* ATCC 25419 metalloprotease [23], but lower than that of *Serratia marcescens* ATCC 25419 serine protease (pH 9.5) [23]. The protease exhibits considerable activity and stability at a pH level of 11.0, similar to *Serratia rubidaea* protease CP-1 [29], *Serratia ureilytica* metalloproteases P1 and P2 [32], and *Serratia marcescens* CCEB 415 serine protease [31], and better than *Serratia rubidaea* serine protease CP-2 [29] and *Serratia marcescens* NRRLB-23112 metalloprotease [24], which are dramatically inactivated at pH values over 10.0.

When a protease is used for kinetic- and equilibrium-controlled synthesis, it is generally preferred that a small to moderate amount of highly solvating organic media such as DMF, DMSO, acetonitrile or MeOH be used with homogeneous aqueous-organic mixtures [33]. Most of the reported solvent-stable proteases are produced by *Pseudomonas* sp. [6, 11, 12] and *Bacillus* sp. [13]. In this study, a novel *Serratia* alkaline serine protease with strong hydrophilic solvent-stable properties was introduced, which was produced by *Serratia* sp. SYBC H with duckweed as nitrogen source. To date, very limited reports can be found on *Serratia* proteases that are stable and active in the presence of 50% (v/v) hydrophilic solvents. Recently, Wan et al. [34] reported *Serratia marcescens* MH6 metalloprotease retained 50, 24 and 10% activity in 50% (v/v) DMSO, DMF and isopropanol, respectively. In comparison, the protease from *Serratia* sp. SYBC H in the current study retained 92.1, 90.8 and 72.4% activity in 50% (v/v) corresponding solvents, namely, DMSO, DMF and isopropanol, respectively. In addition, it should be pointed out that no similar experiment has been done on *Serratia marcescens* MH6 metalloprotease in 50% (v/v) hydrophilic solvents of acetonitrile, methanol, acetone, ethanol and THF. Thus, this is the first report on a *Serratia* alkaline serine protease with strong hydrophilic solvent-stable properties. The *Serratia* sp. SYBC H protease in this study retained more than 90% activity after incubation with 50% (v/v) water-miscible solvents DMF, DMSO, acetone and MeOH for 60 min at 40°C. With these distinctive properties, this protease has successfully catalyzed sucrose ester synthesis in hydrophilic organic solvents DMF, DMSO and pyridine as media (results not published).

Considering nonionic surfactants stability of *Serratia* proteases, to the best of our knowledge, the nonionic surfactant concentrations reported in previous studies were negligible when compared with that of 20 or 25% (v/v) used in the current study. These low nonionic surfactant concentrations reported in previous studies are listed as follows: the maximum concentration of 0.5% (v/v) Triton X-100 and Tween 80 to study *Serratia rubidaea* proteases CP-1 and CP-2 [29], 1% (w/v) Tween 20, 1% (w/v) Triton X-100, 5% (v/v) ethanol and 0.5% (v/v) 2-mercaptoethanol to study *Serratia marcescens* NRRLB-23112 metalloprotease [24], 1% (v/v) Tween 80 and Triton X-100 to study *Serratia marcescens* MH6 metalloprotease [34], and 2% nonionic surfactants of Tween 20, Tween 40 and Triton X-100 to study *Serratia* sp. TKU016 protease [35]. The *Serratia* sp. SYBC H protease in this study retained 81.7, 83.6 and 76.2% activity in the presence of 20% (v/v) Tween 80, 25% (v/v) glycerol and Triton X-100, respectively, indicating its strong nonionic surfactant stability, which is distinct from other *Serratia* proteases.

The protease activity is reduced by 50.9% in the presence of anionic surfactant SDS (1.0%, w/v). Compared with the reported *Serratia* proteases, the stability of *Serratia* sp. SYBC H protease against SDS is higher than that of some *Serratia* proteases such as *Serratia ureilytica* TKU013 proteases P1 and P2 (95 and 93% activity loss with 2 mM SDS, respectively) [32]; *Serratia rubidaea* protease CP-1 (67% activity loss with 0.5% (w/v) SDS) [29]; *Serratia marcescens* MH6 protease (100% activity loss

with 1% (w/v) SDS) [34]. However, the stability of *Serratia* sp. SYBC H protease against SDS is lower than *Serratia marcescens* NRRLB-23112 protease [only 5% activity loss with 1% (w/v) SDS] [24]. The results indicate that the protease from *Serratia* sp. SYBC H is relatively sensitive to anionic surfactant SDS.

Serine proteases are normally activated by Mn^{2+}, Ca^{2+} and Mg^{2+}. In particular, most reported serine proteases, such as subtilisin, are activated by Ca^{2+} [36, 37]. Both the activity and the thermostability of this *Serratia* sp. SYBC H protease with 5 mM Mn^{2+} were increased by more than one fold compared to the control, suggesting it to be a Mn^{2+}-dependent serine protease. By contrast, Ca^{2+} not only reduced *Serratia* sp. SYBC H serine protease activity, but also significantly decreased its thermostability. This is significantly different from most other serine proteases, for which the protease activity and stability were reported to increase dramatically with Ca^{2+}, and/or their thermal stability significantly reduced when Ca^{2+} was removed from the strong binding site [36–38]; except for Mn^{2+}, which stimulated *Serratia* sp. SYBC H serine protease activity, all other tested divalent heavy metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}) strongly inhibited the protease activity, causing more than 50% inactivation. In particular, Co^{2+} completely inhibited the protease activity. This phenomenon suggests that these mental ions might correlate with the active center of the protease and/or with the toxic effects on the protease directly. The mechanism remains to be clarified. The property of strong inhibition by heavy metal ions except Mn^{2+} is also different from some other serine proteases, such as alkaline serine protease from *Bacillus mojavensis* A21, which retained 84.4% activity in the presence of 5 mM Cu^{2+} [36]; alkaline serine protease from *Bacillus sp*. SSR1, which retained 83.4 and 113.4% activity in the presence of 10 mM Zn^{2+} and Cu^{2+}, respectively [37]; alkaline serine protease from *Aspergillus parasiticus,* which retained 100% activity in the presence of 5 mM Co^{2+} and Cu^{2+} [38]. Therefore, the protease of *Serratia* sp. SYBC H in the current study might be considered as a distinctive serine protease with respect to the metal ion requirement.

In summary, this study has introduced a novel solvent- and nonionic surfactant-stable serine protease of *Serratia* sp. SYBC H, unlike most other reported solvent-stable proteases that are produced from *Pseudomonas* sp. and *Bacillus* sp. The *Serratia* sp. SYBC H alkaline serine protease shares some similar characteristics with some other *Serratia* proteases such as molecular weight, optimum temperature (40°C), optimum pH (8.0), stability and activity at pH levels over 10.0. It also possesses unique characteristics such as its appreciable activity at high temperature of 80°C, and more distinctively, its tolerance and stability in the presence of non-ionic surfactants such as Tween 80, glycerol and Triton X-100, its activity and stability in the presence of various hydrophilic organic solvents, and its high activity and thermostability in the presence of Mn^{2+}. With these special properties, this protease could find its potential industrial applications in laundry detergents, textile processing and synthetic reactions in organic solvent media.

Acknowledgments This work was financially supported by the National High Technology and Development Program of China (863 Program, grant no., 2010AA101501).

References

1. Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme application. Curr Opin Biotechnol 13:345–351. doi:10.1016/S0958-1669(02)00328-2
2. Mukherjee AK, Adhikarl H, Rai SK (2008) Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using imperata cylindrica grass and potato peel as low-cost medium: characterization and application of enzyme in detergent formulation. Biochem Eng J 39:353–361. doi:10.1016/j.bej.2007.09.017
3. Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561–594. doi:10.1016/S0734-9750(99)00027-0
4. Kwon YT, Kim JO, Moon SY, Lee HH, Rho HM (1994) Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. Biotechnol Lett 16:413–418
5. Mei C, Jiang X (2005) A novel surfactant-and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL33–51. Process Biochem 40:2167–2172. doi:10.1016/j.procbio.2004.08.007
6. Gupta A, Roy I, Khare SK, Gupta MN (2005) Purification and characterization of solvent stable protease from *Pseudomonas aeruginosa* PseA. J Chromatogr A 1069:155–161. doi:10.1016/j.chroma.2005.01.080
7. Ogino H, Watanabe F, Yamada M, Nakagawa S, Hirose T, Noguchi A (1999) Purification and characterization of organic solvent-stable protease from organic solvent-tolerant *Pseudomonas aeruginosa* PST-01. J Biosci Bioeng 87:61–68. doi:10.1016/S1389-1723(99)80009-7
8. Persson M, Wehtje E, Adlercreutz P (2002) Factors governing the activity of lyophilised and immobilized lipase preparations in organic solvents. Chem Biochem 3:566–571. doi:10.1002/1439-7633(20020603)3:6<566:AID-CBIC566>3.0.CO;2-7
9. Gupta MN, Roy I (2004) Enzymes in organic media. Eur J Biochem 271:2575–2583. doi:10.1111/j.1432-1033.2004.04163.x
10. Chen KQ, Robinson AC, Van Dam ME, Martinez P, Economou C, Arnold FH (1991) Enzyme engineering for nonaqueous solvents, II. Additive effects of mutations on the stability and activity of subtilisin E in polar organic media. Biotechnol Prog 7:125–129. doi:10.1021/bp00008a007
11. Tang XY, Pan Y, Li S, He BF (2008) Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. Bioresour Technol 99:7388–7392. doi:10.1016/j.biortech.2008.01.030
12. Geok LP, Razak CNA, Rahman RNZA, Basri M, Salleh AB (2003) Isolation and screening of an extracellular organic solvent-tolerant protease producer. Biochem Eng J 13:73–77. doi:10.1016/S1369-703X(02)00137-7
13. Ghorbel B, Kamoun AS, Nasri M (2003) Stability studies of protease from *Bacillus cereus* BG1. Enzyme Microb Technol 32:513–518. doi:10.1016/S0141-0229(03)00004-8
14. Dey G, Mitra A, Banerjee R, Maiti BR (2001) Enhanced production of amylase by optimization of nutritional constituents using

response surface methodology. Biochem Eng J 7:227–231. doi:10.1016/S1369-703X(00)00139-X
15. Chauhan B, Gupta R (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. Process Biochem 39:2115–2122. doi:10.1016/j.procbio.2003.11.002
16. Sandhya C, Sumantha A, Szakacs G, Pandey A (2005) Comparative evaluation of neutral protease production by *Aspergillus oryzaein* submerged and solid-state fermentation. Process Biochem 40:2689–2694. doi:10.1016/j.procbio.2004.12.001
17. Prakasham RS, Subba Rao CH, Sarma PN (2006) Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. Bioresour Technol 97:1449–1454. doi:10.1016/j.biortech.2005.07.015
18. Tacon AG (1990) Standard methods for the nutrition and feeding of farmed fish and shrimp, vol.2. Nutrient sources and composition. Argent Laboratories Press, Redmond, pp 84–89
19. Ray AK, Das I (1994) Apparent digestibility of some aquatic macrophytes in rohu. *labeo rohita* (Ham.), fingerlings. J Aquacult Trop 9:335–342
20. Bhosale SH, Rao MB, Deshpande VV, Srinivasan MC (1995) Thermostability of high-activity alkaline protease from *conidiobolus coronatus* (NCL 86.8.20). Enzyme Microb Technol 17:136–139
21. Davis BJ (1964) Disc electrophoresis, II. Methods and application to human serum protein. Ann NY Acad Sci 121:404–427
22. Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature 227:680–685
23. Romero FJ, Garcia LA, Salas JA, Diaz M, Quiros LM (2001) Production, purification and partial characterization of two extracellular proteases from *Serratia marcescens* grown in whey. Process Biochem 36:507–515
24. Salamone PR, Wodzinski RJ (1997) Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens*. Appl Microbiol Biotechnol 48:317–324. doi:10.1007/s002530051056
25. Tao K, Long Z, Liu K, Tao Y, Liu S (2006) Purification and properties of a novel insecticidal protein from the locust pathogen *Serratia marcescens* HR-3. Curr Microbiol 52:45–49. doi:10.1007/s00284-005-0089-8
26. Romero F, Garcia LA, Diaz M (1998) Protease production from whey at high concentrations by *Serratia marcescens*. Res Environ Biotechnol 2:93–115
27. Miyazaki H, Yanagida N, Horinouchi S, Beppu T (1990) Specific excretion into the medium of a serine protease from *Serratia marcescens*. Agric Biol Chem 54:2763–2765
28. Morita Y, Kondoh K, Hasan Q, Sakaguchi T, Murakami Y, Yokoyama K, Tamiya E (1997) Purification and characterization of a cold-active protease from psychrotrophic *Serratia marcescens* AP3801. JAOCS 74:1377–1383. doi:10.1007/s11746-997-0240-8
29. Doddapaneni KK, Tatineni R, Vellanki RN, Gandu B, Panyala NR, Chakali B, Mangamoori NL (2007) Purification and characterization of two novel extra cellular proteases from *Serratia rubidaea*. Process Biochem 42:1229–1236. doi:10.1016/j.procbio.2007.05.019
30. Ulrich B (1994) Crystal structure of the 50 kDa metallo protease from *Serratia marcescens*. J Mol Biol 242:244–251. doi:10.1006/jmbi.1994.1576
31. Kaska M, Lysenko O, Chaloupka A (1976) Exocellular proteases of *Serratia marcescens* and their toxicity to larvae of *Galleria mellonella*. Folia Microbiol 21:465–473. doi:10.1007/BF02876938
32. Wang SL, Lin CL, Liang TW, Liu KC, Kuo YH (2009) Conversion of squid pen by *Serratia ureilytica* for the production of enzymes and antioxidants. Bioresour Technol 100:316–323. doi:10.1016/j.biortech.2008.06.026
33. Bordusa F (2002) Proteases in organic synthesis. Chem Rev 102:4817–4867. doi:10.1021/cr010164d
34. Wan MH, Wu B, Ren W, He BF (2010) Screening, characterization, and cloning of a solvent-tolerant protease from *Serratia marcescens* MH6. J Microbiol Biotechnol 20:881–888. doi:10.4014/jmb.0910.10038
35. Wang SL, Chang TJ, Liang TW (2010) Conversion and degradation of shellfish wastes by *Serratia* sp. TKU016 fermentation for the production of enzymes and bioactive materials. Biodegradation 21:321–333. doi:10.100/s10532-009-9303-x
36. Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M (2009) A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. Process Biochem 44:29–35. doi:10.1016/j.procbio.2008.09.003
37. Singh J, Batra N, Sobti RC (2001) Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. Process Biochem 36:781–785. doi:10.1016/S0032-9592(00)00275-2
38. Tunga R, Shrivastava B, Banerjee R (2003) Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus*. Process Biochem 38:1553–1558. doi:10.1016/S0032-9592(03)00048-7