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Fibrinolytic Serine Protease Isolation from *Bacillus amyloliquefaciens* An6 Grown on *Mirabilis jalapa* Tuber Powders

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Abstract In this study, *Mirabilis jalapa* tuber powder (MJTP) was used as a new complex organic substrate for the growth and production of fibrinolytic enzymes by a newly isolated *Bacillus amyloliquefaciens* An6. Maximum protease activity (1,057 U/ml) with casein as a substrate was obtained when the strain was grown in medium containing (grams per liter) MJTP 30, yeast extract 6, $CaCl_2$ 1, K_2HPO_4 0.1, and K_2HPO_4 0.1. The strain was also found to grow and produce extracellular proteases in a medium containing only MJTP, indicating that it can obtain its carbon, nitrogen, and salts requirements directly from MJTP. The *B. amyloliquefaciens* An6 fibrinase (BAF1) was partially purified, and fibrinolytic activity was assayed in a test tube with an artificial fibrin clot. The molecular weight of the partially purified BAF1 fibrinolytic protease was estimated to be 30 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. The optimum temperature and pH for the caseinolytic activity were 60 °C and 9.0, respectively. The enzyme was highly stable from pH6.0 to 11.0 and retained 62% of its initial activity after 1 h incubation at 50 °C. However, the enzyme was inactivated at higher temperatures. The activity of the enzyme was totally lost in the presence of phenylmethylsulfonyl fluoride, suggesting that BAF1 is a serine protease.

Keywords *Bacillus amyloliquefaciens* · *Mirabilis jalapa* · Protease · Fibrinase

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Introduction

When fibrin clots are not lysed, they accumulate in blood vessels and cause thrombosis leading to cardiovascular diseases [1, 2]. These diseases, including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure, and stroke, are the leading causes of death throughout the world [3].

For the treatment of thrombotic diseases, at present, fibrinolytic therapy has been often employed mainly with use of the plasminogen activators such as streptokinase, urokinase, and the genetically engineered tissue plasminogen activator (t-PA). Intravenous administration of urokinase and streptokinase has been widely used but these enzymes have low specificity to fibrin and are expensive. However, t-PA has been developed for this therapy because of its efficacy and stronger affinity to fibrin [4]. The other thrombolytic agents used are the plasmin-like protein, e.g., nattokinase [5] and lumbrokinase [6], which can directly degrade the fibrin of blood clots. Nattokinase is the first fibrinolytic enzyme identified from fermented food. It was purified from *Bacillus subtilis* var. natto screened from *natto*, a traditional Japanese soybean fermented food [7, 8]. The oral administration of this enzyme can enhance fibrinolysis in dogs with experimentally induced thrombosis. Moreover, fibrinolytic activity, the amounts of t-PA, and fibrin degradation by-product in the plasma are increased when nattokinase is given to human subjects by oral administration [9]. Other fibrinolytic enzymes that were produced by *Bacillus* strains screened from traditional fermented foods were reported, such as subtilisin CK 11-4 from *Chungkook-Jang* [1], subtilisin DJ-4 from *Doen-Jang* [2] in Korea, and subtilisin DFE from *Douchi* in China [10]. These enzymes have significant potential for food fortification and nutraceutical applications, since their use could effectively prevent cardiovascular diseases [3].

Protease production depends on many factors. For instance, the growth rate of the culture and the composition of the medium play important roles [11, 12]. Indeed, carbon and nitrogen sources were considered determinant factors [13, 14]. Several studies have reported that proteins and peptides are necessary for effective protease production, while glucose repressed protease formation [15, 16]. However, some works reported better protease synthesis in the presence of glucose as a carbon source [17, 18]. Other medium compounds, such as metal ions and phosphorus source, may also affect the amount of enzyme formation. Ghorbel-Frikha et al. [19] reported the production of a calcium-dependent metalloprotease from *B. cereus* BG1. The enzyme was detected only when the strain was cultivated in the presence of calcium. Protease production from species of *Bacillus* using various agricultural residues (such as soybean meal, rice bran, and wheat flour) or marine by-products has been widely described in literature [20–23].

For this purpose, powder prepared from *Mirabilis jalapa* tubers was recognized as a potentially useful and cost-effective medium ingredient. *M. jalapa* (Nyctaginaceae), commonly known as “four-o’clocks” or “Marvel of Peru”, is an ornamental flowering plant. *M. jalapa* is cheaply cultivated in Tunisia. A number of active compounds may be extracted from different organs of *M. jalapa*, for example, ribosome inactivating protein associated with antiviral activity [24], antifungal phenolic compound [25], antimicrobial peptides [26], and rotenoids showing inhibition of HIV-1 reverse transcriptase [27]. Furthermore, chemical analysis showed that tubers are rich in organic and inorganic compounds, suggesting it as a good candidate for culture media. This paper describes the preparation and the use of powder from *M. jalapa* tubers as a new microbial growth substrate for the production of proteases by *Bacillus amyloliquefaciens* An6 and the partial purification and biochemical characterization of the fibrinolytic BAF1 enzyme.

Materials and Methods

Materials

Fibrin bovine blood was from MP Biomedicals Co. (St. Louis, MO, USA). Casein sodium salt from bovine milk and CM-Sephadex were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Casein peptone and yeast extract were from BioRad (France). Trichloroacetic acid (TCA) was from Carlo Erba Reactifs. Other chemicals were of analytical grade.

Isolation of Fibrinolytic Enzyme-Producing Strains

B. amyloliquefaciens An6 which was used in this study was isolated from the soil of detergent industry in Tunisia. Samples collected were plated onto skim-milk agar plates containing (grams per liter) peptone 5, yeast extract 3, bacteriological agar 12, and skim-milk 250 ml. Plates were incubated 24–48 h at 37°C. A clear zone of skim-milk hydrolysis gave an indication of protease-producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Purified colonies were finally transferred to fibrin agar plates composed of (grams per liter) fibrin 5, ammonium sulfate 2, $CaCl_2$ 1, K_2HPO_4 0.1, KH_2PO_4 0.1, $MgSO_4$ (7 H_2O) 0.2, and agar 18. Plates were then incubated at 37°C for 24 h. Isolates, which formed a clearing zone on fibrin agar medium, were isolated. Depending upon the zone of clearance, strain An6 was selected for further experimental studies. It was identified, according to the methods described in *Bergey's Manual of Determinative Bacteriology* and on the basis of the 16S rDNA sequence analysis, as *B. amyloliquefaciens*.

Cultivation and Media

The strain was routinely grown in Luria–Bertani broth medium composed of (grams per liter) peptone 10, yeast extract 5, and NaCl 5 [28]. The initial medium used for protease production was composed of (grams per liter) hulled grain of wheat (HGW) 10, yeast extract 2, K_2HPO_4 0.1, KH_2PO_4 0.1, and $CaCl_2$ 2 (pH 8). The media were autoclaved at 121°C for 20 min. Cultivations were conducted in 250 ml Erlenmeyer flasks containing 25 ml of culture medium maintained at 37°C. Incubations were carried out with agitation at 200 rpm for 24 h. The culture medium was centrifuged at 10,000×*g* for 5 min at 4°C, and the cell-free supernatants were collected.

Hulled grain of wheat, a by-product of semolina factories, constitutes a good source of starch which is utilized as a food material. The HGW contains 50–60% starch, 8–12% proteins, 15% cellulose, and 5% carbohydrates.

The growth of the microorganisms was estimated by the determination of colony-forming units (CFU per milliliter). All experiments were carried out in duplicate and repeated at least twice.

Preparation and Chemical Composition of *M. jalapa* Tuber Powder

To obtain *M. jalapa* tuber powder (MJTP), raw material was peeled, grinded, and then dried at 80°C for at least 5 h. The dried preparation was minced again to obtain a fine powder and then stored in glass bottles at room temperature. The MJTP contained 32.6±2% starches, 17.3±3% proteins, 20±2% ashes, and low content of lipids [29].

Shrimp waste powder (SWP) and chicken feather powder (CFP), tested as complex organic substrates, were also prepared in our laboratory. To obtain SWP, shrimp waste, collected from the marine food processing industry, was washed thoroughly with tap water and then cooked 20 min at 100°C. The solid material obtained was dried, minced to obtain a fine powder, and then stored in glass bottles at room temperature. The SWP contained high protein content (46–49%), relatively high ash and chitin contents (27–35% and 17–20%, respectively), and low lipid content (5–6.5%) [30]. To obtain CFP, chicken feather was washed thoroughly with tap water, dried at 105°C, and then grinded. The CFP contained 71.48% protein, 3% lipids, and 0.73% ashes.

Assay of Proteolytic Activity

Protease activity was measured by the method of Kembhavi et al. [31] using casein as a substrate. A 0.5-ml aliquot of the culture supernatant or the purified enzyme, suitably diluted, was mixed with 0.5 ml of 100 mM glycine-NaOH (pH9.0) containing 1% casein and incubated for 15 min at 60°C. The reaction was stopped by the addition of 0.5 ml TCA (20%, *w*/*v*). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000×*g* for 15 min to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0–50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

Fibrinolytic Assays

Fibrinolytic activity was assayed by incubating crude enzyme preparation (7.5 U/mg of fibrin clots) appropriately diluted in 1 ml potassium phosphate buffer (pH7.4) with fibrin clots (*m*=0.04 g) at 37°C for 3 h. The fibrin clot incubated with phosphate buffer (pH7.4) without enzyme is considered as a control.

Partial Purification of Subtilisin BAF1 from *B. amyloliquefaciens* An6

The culture supernatant (75.0 ml), containing the extracellular enzymes, was first subjected to acetone precipitation. Acetone fraction of 0–80% (*v*/*v*) was collected by centrifugation at 10,000×*g*, and the obtained pellet was solubilized in a minimal volume (4.0 ml) of 100 mM sodium acetate buffer, pH6.5, containing 2 mM $CaCl_2$. The acetone fraction was subjected to CM-Sephadex column (2×25 cm) equilibrated with 25 mM sodium acetate buffer (pH6.5) containing 2 mM $CaCl_2$. After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–0.4 M in the equilibrating buffer. Fractions of 4.85 ml were collected at a flow rate of 82 ml/h and analyzed for protease activity and protein concentration. All the purification steps were conducted at temperatures not exceeding 4°C.

Protein Determination

Protein concentration was determined by the method of Bradford [32] using bovine serum albumin as standard. During the course of the purification, the protein concentration was determined by measuring the absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the purity and molecular weight of the purified enzyme, as described by Laemmli [33], using a 5% (*w*/*v*) stacking and a 15% (*w*/*v*) separating gels. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit (Sigma) as markers. The molecular mass markers used were bovine serum albumin (66,000 Da), egg white ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), bovine carbonic anhydrase (29,000 Da), bovine trypsinogen (24,000 Da), and bovine α-lactoalbumin (14,200 Da).

Detection of Protease Activity by Zymography

Zymography is a sensitive and rapid assay method for analyzing protease activity on SDS-PAGE impregnated with a protein substrate. Casein zymography was performed on SDS-PAGE according to the method of Garcia-Carreno et al. [34] with slight modification. The sample was not heated before loading in the gel. After electrophoresis, the gel was submerged in 100 mM glycine–NaOH buffer (pH9.0) containing 2.5% Triton X-100, with shaking for 45 min to remove SDS. Triton X-100 was then removed by washing the gel three times with 100 mM glycine–NaOH buffer (pH9.0). The gel was then incubated with 1% (*w*/*v*) casein in 100 mM glycine–NaOH buffer (pH9.0) for 30 min at 50°C. Finally, the gel was stained with Coomassie brilliant blue R-250 for zymography analysis. The development of clear zones on the blue background of the gel indicated the presence of protease activity.

Effect of pH on Protease Activity and Stability

The optimum pH of the partially purified enzyme was studied over a pH range of 6.0–11.0 at 60°C using casein as a substrate. For the measurement of pH stability, the enzyme was incubated for 1 h at 30°C in different buffers, and then the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM sodium acetate buffer, pH6.0; 100 mM phosphate buffer, pH7.0; 100 mM Tris-HCl buffer, pH8.0; and 100 mM glycine–NaOH buffer, pH9.0–11.0.

Effect of Temperature on Protease Activity and Stability

To investigate the effect of temperature, protease activity was tested using casein as a substrate at different temperatures ranging from 40°C to 75°C in 100 mM glycine–NaOH buffer, pH9.0. Thermal stability was examined by incubating subtilisin BSF1 for 1 h at 50, 60, and 70°C. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH9.0 and 60°C. The nonheated enzyme was considered to be control (100% activity).

Influence of Enzyme Inhibitors on Protease Activity

Proteases can be classified by their sensitivity to various inhibitors [35]. In order to determine the nature of the proteolytic enzyme from *B. amyloliquefaciens* An6, the effects of a variety of enzyme inhibitors on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and β-mercaptoethanol. The enzyme was preincubated with inhibitors for 30 min at 30°C, and the remaining enzyme activity was determined using casein (1%, *w*/*v*) as a substrate

at pH9.0 and 60 °C. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

Result and Discussion

Screening of Fibrinolytic Activity Producing Strains

To obtain microorganisms which produced fibrinolytic enzymes, protease producing strains selected by growth on skim milk agar were plated on fibrin plates. Among more than ten strains isolated in the laboratory and screened for fibrinolytic activity, An6 strain was selected. This strain exhibited prominent clear zone around the colony on fibrin plate indicating that is secretes fibrinolytic enzymes (Fig. 1). The strain was identified as *B. amyloliquefaciens* based on 16S rDNA sequence analysis.

Preparation of *M. jalapa* Tuber Powder

The MJTP was prepared as described in "Materials and Methods" section. The main chemical composition of MJTP is given in Table 1. These data show that MJTP is relatively rich in both organic and inorganic materials. Notably, it contains the essential substances required in microbial media such as sources of carbon, nitrogen, and minerals. The MJTP has a relatively high starch content 32.6±2%. The protein content is 17.3±3%. The powder is very stable and may be conserved several years. The stability of MJTP could be explained by the weak water content and the low lipid content.

Protease Production by the An6 Strain in Media Containing MJTP

Protease production was first tested in media containing only 10 g/l of different agricultural residues and marine by-products such as HGW, MJTP, CFP, and SWP. Among the substrates tested, MJTP was found to be the best complex organic substrate (71 U/ml) followed by CFP (50 U/ml; Table 2).

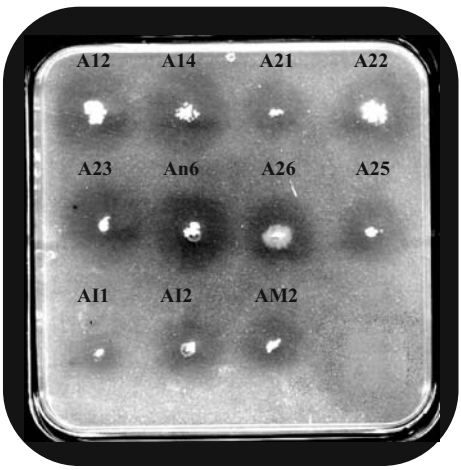

Fig. 1 Screening of fibrinolytic activity producing strains on fibrin agar plates

Table 1 The main chemical composition of MJTP (percent).

Starch	32.6±2
Proteins	17.3±3
Ash	20±2
Lipids	0.5
PSA	20.3±2
Lignin	6±0.5
Waters	2.5±0.5
Ca^{2+}	5.04
K^{+}	0.3
Na^{+}	0.78
PO_4^{2-}	0.56
Cl^{-}	0.24

PSA polysaccharides without starch

As MJTP was found to be the best complex substrate, the effect of its concentration (5–40 g/l) on bacterial growth and protease production was also investigated. As shown in Table 3, protease activity reached a maximum value at 30 g/l (161 U/ml). Further increase of MJTP did not increase enzyme production, although biomass increased continuously. The fact that the strain can grow in media containing only MJTP indicated that it can obtain its carbon and nitrogen requirements directly from the complex organic substrates present in tuber powder.

The growth substrate represents more than 30% of the industrial enzyme production cost [36]. Considering this fact, the use of cost-effective growth medium could significantly reduce the cost of protease production.

The effect of addition of various nitrogen sources (4 g/l) on protease production in a medium containing only 30 g/l of MJTP was also carried out. Of the tested nitrogen source, yeast extract and casein peptone increased protease synthesis (Table 4). Addition of yeast extract or casein peptone to the medium increased the production of the enzyme activity about three and two times, respectively, that of the medium without nitrogen source. Based on these observations, yeast extract was selected and the effect of its concentration on protease production was studied. As shown in Table 5, protease activity increased with the increase of yeast concentration and reached a value of about 522 U/ml with 6 g/l. Beyond 6 g/l, the level of protease activity did not vary significantly, although biomass continued to increase.

The effect of adding $CaCl_2$ (1 g/l) and/or KH_2PO_4 (0.1 g/l)/K_2HPO_4 (0.1 g/l) in medium containing 30 g/l MJTP and 6 g/l yeast extract on protease synthesis was also studied (Table 6). The supplementation of the culture medium with $CaCl_2$ or KH_2PO_4/K_2HPO_4 slightly increased the growth of An6 strain and the enzyme production, thereby indicating the requirements of these components for protease production by this organism. Further, the addition of both $CaCl_2$ and KH_2PO4/K_2HPO_4 enhanced the production up to 1,057 U/ml,

Table 2 Effect of different carbon sources on protease production by *B. amyloliquefaciens* An6.

	MJTP	HGW	CFP	SWP
Proteolytic activity (U/ml)	71	7.5	50	32
10^8UFC/ml	17	85	7	59

Cultivations were performed for 24 h at 37°C in media containing different carbon sources. Values are means of three independent experiments. Standard deviations±2.5% (based on three replicates)

Table 3 Effect of MJTP concentration on protease production by *B. amyloliquefaciens* An6.

	MJTB (g/l)				
	5	10	20	30	40
Proteolytic activity (U/ml)	44	74	101	161	165
10^8UFC/ml	12	14	19	30	69

Cultivations were performed for 24 h at 37°C in media containing different concentrations of MJTP. Values are means of three independent experiments. Standard deviations±2.5% (based on three replicates)

which was approximately twice that of the medium containing only MJTP and yeast extract.

Partial Purification of Subtilisin BAF1 from *B. amyloliquefaciens* An6 and Description of the Fibrinolytic Activity

The An6 protease from *B. subtilis* was partially purified by the two-step procedure described under "Materials and Methods" section. In the first step, the culture supernatant was precipitated with 80% acetone (*v*/*v*). The precipitate was solubilized in 25 mM sodium acetate buffer (pH6.5) containing 2 mM $CaCl_2$ and applied to cation exchange chromatography column (CM-Sephadex) that had been equilibrated and washed with the same buffer. The elution profiles of the protease activity and proteins from CM-Sephadex yielded two peaks of protease activity: One eluted during washing the column and the second with a specific activity of 22,345.86 U/mg was eluted with a linear gradient of NaCl (0–0.4 M; Fig. 2; Table 7).

The purity of the enzyme in the second peak was evaluated using zymogram activity staining with casein. As shown in Fig. 3b, a unique clear band of caseinolytic activity was observed in the gel for the second peak, indicating the homogeneity of the purified protease. However, peak eluted during washing the column showed at least two clear zones indicating the presence of multiple extracellular proteases (Data not shown). Our results are in line with several works that reported the production of more than one extracellular

Table 4 Effect of different nitrogen sources on protease production by *B. amyloliquefaciens* An6.

Nitrogen source	Proteolytic activity (U/ml)	10^8UFC/ml
None	160	33
Ammonium sulfate	207	28
Urea	0	29
Soya peptone	140	48
Casein peptone	353	390
Gelatin	183	35
Ammonium chloride	175	24
Ammonium nitrate	202	35
Yeast extract	448	526

Cultivations were performed for 24 h at 37°C in media consisting of (grams per liter) MJTP 30 and nitrogen source 4. Values are means of three independent experiments. Standard deviations±2.5% (based on three replicates)

Table 5 Effect of yeast extract concentration on protease production by *B. amyloliquefaciens* An6.

	Yeast extract (g/l)					
	0	2	4	6	8	10
Proteolytic activity (U/ml)	169	296	431	522	575	590
10^8UFC/ml	41	477	473	502	737	1,013

Cultivations were performed for 24 h at 37°C in media consisting of (grams per liter) MJTP 30 and different concentrations of yeast extract. Values are means of three independent experiments. Standard deviations± 2.5% (based on three replicates)

fibrinolytic protease. In fact, Ko et al. [37] have purified from the supernatant of *B. subtilis* QK02 culture two fibrinolytic enzymes QK-1 (a plasmin-like serine protease) and QK-2 (a subtilisin family serine protease). By adding sodium chloride (2.5%) into a *B. amyloliquefaciens* DJ-4 culture broth, Choi and Kim [38] demonstrated on the SDS fibrin zymogram that two serine-type fibrinolytic proteases with a molecular weight of 29 (subtilisin DJ-4) and 38 kDa were stimulated. However, through a fibrin zymography assay, three fibrinolytic enzymes (38, 53, and 80 kDa) were detected when cells were cultivated in the presence of 10% NaCl. In addition, Kho et al. [39] identified two extracellular fibrinolytic enzymes of *B. subtilis* 168, namely Vpr and WprA. On the other hand, many works reported the production of only one fibrinolytic enzyme by *Bacillus* species [1, 5, 8, 10, 40–42].

The fibrinolytic activity of the An6 strain was also evaluated with the crude protease preparation and the partially purified enzyme, in a test tube, in the presence of an artificial fibrin clot prepared by coagulation in a glass test tube using fresh human plasma. The clot was completely solubilized after 3 h of incubation at pH7.4 and 37°C in the presence of crude enzyme and the partially purified enzyme BAF1 (peak 2 on the CM-Sephadex) indicating that these proteolytic enzyme(s) are able to degrade fibrin (Fig. 3c lanes 1 and 2). In the presence of the proteases from the peak 1 of CM-Sephadex, the fibrin clot persists, indicating that these enzymes are enable to degrade fibrin.

Biochemical Characterization of Subtilisin BAF1 from *B. amyloliquefaciens* An6

Effect of pH on Enzyme Activity and Stability

The pH activity profile of the partial purified BAF1 fibrinolytic enzyme was determined using different buffers of varying pH values. Interestingly, BAF1 enzyme was highly active in the pH range of 7.0–10.0, with an optimum around pH9.0 (Fig. 4a). The relative activities at pH7.0, 10.0, and 11.0 were about 83%, 92%, and 61.7%, respectively, of that measured at pH9.0. These findings are in accordance with several earlier reports showing that pH optima of the fibrinolytic enzymes belonging to

Table 6 Effect of adding $CaCl_2$ and/or KH_2PO_4/K_2HPO_4 in medium containing 30 g/l MJTP and 6 g/l yeast extract on protease production by *B. amyloliquefaciens* An6.

	None	$CaCl_2$	$K_2HPO_4+KH_2PO_4$	$CaCl_2+K_2HPO_4+KH_2PO_4$
Proteolytic activity (U/ml)	519.5	686	586.4	1,057
10^8UFC/ml	558	949	921	1,330

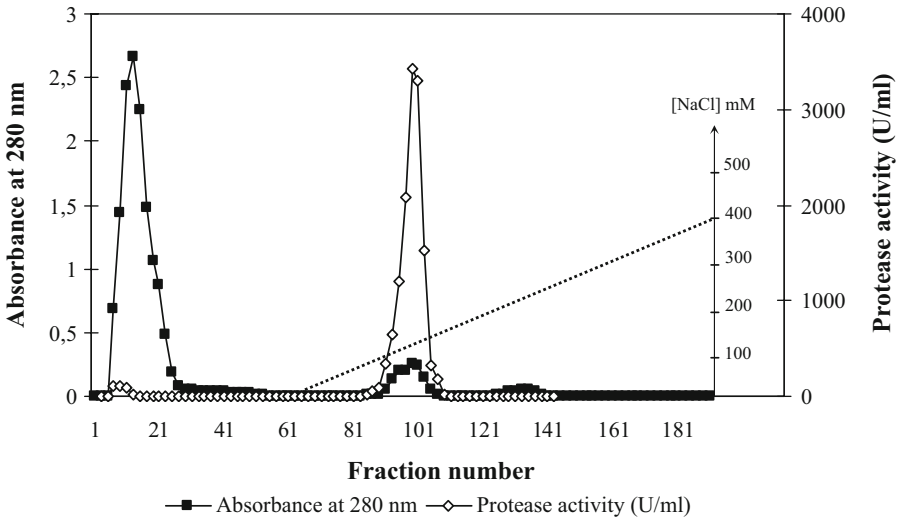

Fig. 2 Elution profile of *B. amyloliquefaciens* An6 proteases from a CM-Sephadex column. The 0–80% acetone precipitate was applied to a 2×25-cm column equilibrated with 25 mM sodium acetate buffer (pH6.5) containing 2 mM $CaCl_2$ and eluted with a linear gradient of NaCl (0–0.4 M) in 25 mM sodium acetate buffer (pH6.5) with $CaCl_2$ (2 mM) at a flow rate of 82 ml/h

serine proteases are generally neutral and alkaline between pH8.0 and 10.0. For example, the subtilisin DFE from *B. amyloliquefaciens* DC-4 exhibited maximum activity at pH 9.0. In the same way, the optimum activity of subtilisin FS33 from *B. subtilis* DC33 [43], subtilisin BSF1 from *B. subtilis* A26 [44], and subtilisin LD8547 from *B. subtilis* LD-8547 [45] is pH 8.0. However, the fibrinolytic enzyme CK from *Bacillus* sp. CK11-4 exhibited an optimum activity between pH10.0 and 12.0 [1].

The pH stability profile showed that the partially purified enzyme is highly stable in a broad pH range (Fig. 4b). The enzyme retained more than 75% of its activity over a wide range of pH from 6.0 to 11.0.

Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on *B. amyloliquefaciens* An6 protease activity was examined at pH9.0 and various temperatures. The result showed that the BAF1 enzyme was active between 40°C and 70°C with an optimum at 60°C (Fig. 5a). The relative activities at 50°C and 70°C were about 62.4% and 21%, respectively, of that at 60°C. The optimum temperature was higher than that of Nattokinase from *B. subtilis* YF38 [46] and serine

Table 7 Summary of the partial purification of BAF1 protease from *B. amyloliquefaciens* An6.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude extract	41.62	178,964	4,299.95	100	1
Acetone precipitation (0–80%)	13.07	161,890.88	12,386.44	90.46	2.88
CM-Sephadex	5.74	128,265.23	22,345.86	71.67	5.19

All operations were carried out at 4°C

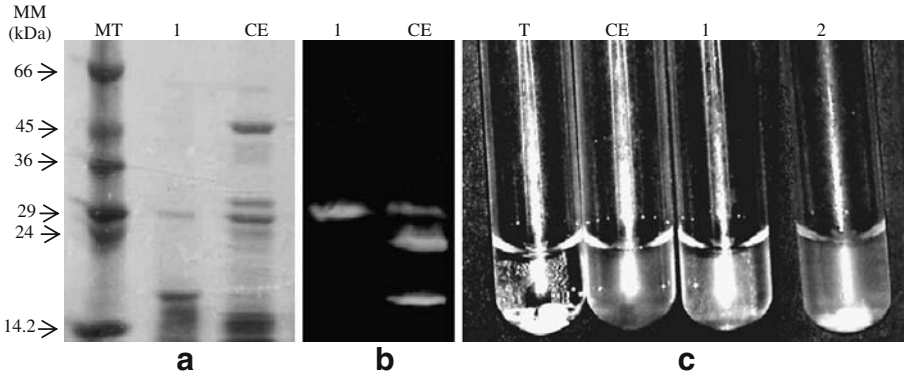

Fig. 3 SDS-PAGE (**a**), casein zymography analysis (**b**) of purification step of *B. amyloliquefaciens* An6 protease, and fibrinolytic activity assay (**c**) on the fibrin clot. *CE* crude enzyme, *2* peak 1, *1* peak 2, *T* fibrin clot incubated without enzyme. For fibrinolytic activity assay, the fibrin clot (0.04 g) was incubated with enzyme (7.5 Umg^{-1} fibrin clot) at 37°C

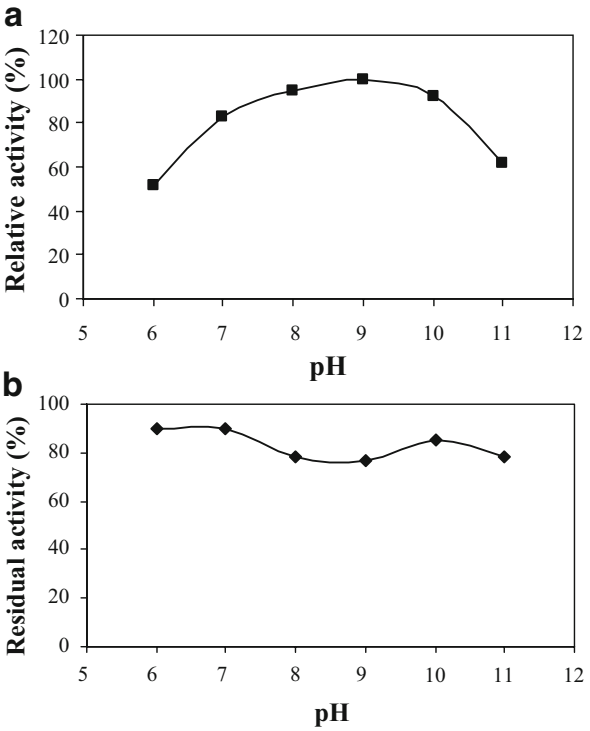

Fig. 4 Effect of pH on activity (**a**) and stability (**b**) of the purified An6 protease. The protease activity was assayed in the pH range 6.0–11.0 at 60°C using casein as a substrate. The maximum activity obtained at pH 9.0 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 1 h at 30°C, and the residual activity was measured at pH9.0 and 60°C. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in "Materials and Methods" section

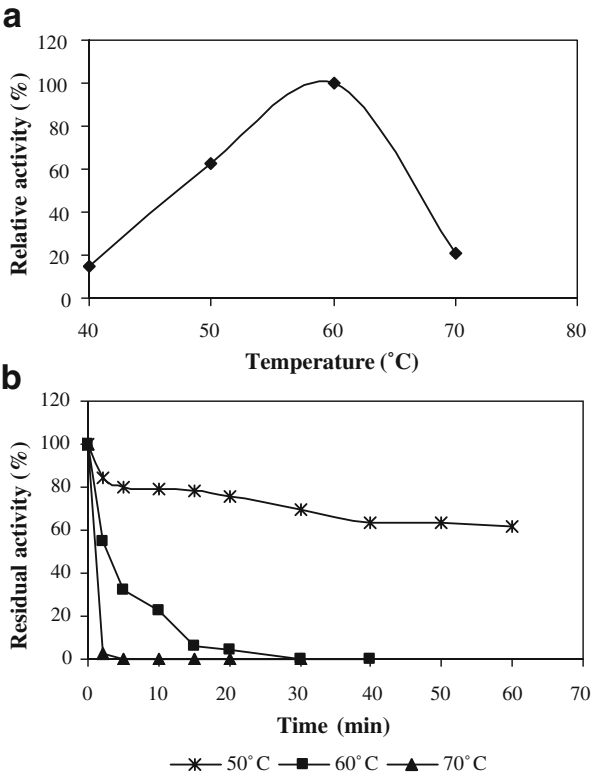

Fig. 5 Effect of temperature on activity (**a**) and stability (**b**) of the purified fibrinolytic protease An6. The temperature profile was determined by assaying protease activity at temperatures between 40°C and 70°C using casein as a substrate. The activity of the enzyme at 60°C was taken as 100%. The temperature stability was determined by incubating the purified enzyme at 50°C, 60°C, and 70°C for 1 h. Aliquots were withdrawn at regular intervals and the residual enzyme activity was measured under the standard conditions assay at different times. The original activity before preincubation was taken as 100%

fibrinolytic protease from *B. subtilis* LD-8547 [45], which exhibited maximum activities at 50°C.

Thermal stability of BAF1 enzyme was also investigated. As shown in Fig. 5b, the enzyme was relatively stable at 50°C, retaining about 62% of its initial activity after 1 h

Table 8 Effect of inhibitors on protease activity.

	Concentration (mM)	Activity (%)
Control	–	100
PMSF	1	0
EDTA	1	18.13
β-Mercaptoethanol	1	100

The remaining protease activity was measured after preincubation of enzyme with each inhibitor at 30°C for 30 min. Enzyme activity measured in the absence of any inhibitor was taken as 100%

PMSF phenylmethylsulfonyl fluoride, *EDTA* ethylenediaminetetraacetic acid

incubation (Fig. 5b). However, the enzyme was found to be unstable at higher temperatures, and it was completely inactivated after 20 min at 60 °C and 2 min at 70 °C. Comparatively, the activity of a fibrinolytic serine protease purified from *B. subtilis* DC33 was completely inactivated after 10 min above 65 °C [43]. The thermal stability of BAF1 enzyme was lower than that of subtilisin LD8547 from *B. subtilis* LD-8547, which showed 82% and 11% residual activities after 30 min incubation at 60 °C and 70 °C, respectively [45].

Effect of Inhibitors on Enzyme Activity

The effects of a variety of enzyme inhibitors, such as chelating agent and a group-specific reagent on the BAF1 enzyme activity, are summarized in Table 8. The BAF1 enzyme was completely inhibited by serine protease inhibitor (PMSF). The proteolytic activity was also affected by EDTA (1 mM) with 81.8% of its original activity being lost, indicating the importance of Ca^{2+} in enzyme stabilization. On the other hand, β-mercaptoethanol did not show any inhibitory effect. Therefore, these results indicated that partially purified enzyme BAF1 is a serine protease.

Conclusion

This work describes the production of protease from a newly isolated *B. amyloliquefaciens* An6 strain cultivated in medium containing a new cheap complex fermentation substrate. The strain was found to produce multiple proteases, and fibrinolytic BAF1 enzyme was partially purified and characterized. The fibrinase was highly active from pH7.0 to 10.0, with an optimum at pH 9.0, and higher stability was observed over a wide range of pH from 6.0 to 11.0. The enzyme was optimally active at 60 °C.

In conclusion, the strain An6 may be of potential interest for biotechnological use because it produces, in cheap fermentation medium, multiple proteases that completely degraded artificial fibrin clot. Further works should be done to purify and characterize the other proteases.

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