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FULL PAPER

Purification and characterization of a fibrinolytic protease from *Aspergillus oryzae* KSK-3

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Abstract An enzyme from *Aspergillus oryzae* KSK-3, isolated from commercial rice-koji for miso brewing, showed fibrinolytic activity in liquefied rice culture and was analyzed. A culture filtrate of *A. oryzae* KSK-3 was concentrated by ultrafiltration and subsequently purified to electrophoretic homogeneity by ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration. The molecular weight of the purified enzyme was estimated to be approximately 30 kDa by SDS-PAGE and high-performance liquid chromatography–size exclusion chromatography. Its maximum fibrinolytic activity was observed at pH 6 and 50°C. The purified protease was stable between pH 4 and 9, at temperatures of up to 50°C. The activity of the enzyme was highest with S-2238 and was considerably inhibited by phenylmethylsufonyl fluoride and pefabloc SC. These results indicate that the enzyme is a serine protease. Moreover, the enzyme is edible and exhibited very high productivity (2,960 U urokinase per milliliter of culture broth). Taken together, the findings of this study indicate that the *A. oryzae* KSK-3 enzyme may be used as a natural agent for oral fibrinolytic therapy and nutraceutical applications.

Keywords Edible fungi · Fibrin · Koji · Serine protease

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Introduction

Cardiovascular diseases, such as high blood pressure, acute myocardial infarction, ischemic heart diseases, valvular heart disease, peripheral vascular disease, arrhythmias, and stroke, are the primary causes of death worldwide (Mine et al. 2005). According to the data of the World Health Organization (WHO), cardiovascular diseases account for 29% of the mortality rate worldwide (Simkhada et al. 2010). Similarly, another report of WHO shows that about 23 million people die of cardiovascular diseases each day. Ischemic heart disease, apoplexy, chronic obstructive pulmonary disease, and respiratory tract infection are predicted to be the four major causes of death by 2030 (World Health Organization 2008).

The principal pathophysiological mechanism underlying cardiovascular diseases is the formation of fibrin (blood clots), which adheres to the unbroken wall of blood vessels. Fibrin is formed from fibrinogen by the action of thrombin. Fibrin is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator. Hemostasis is a complex process that maintains the balance between fibrin formation and fibrinolysis. However, when fibrin hydrolysis is not complete because of a balance disorder, thrombosis, such as a myocardial infarction, can occur. The thrombolytic agents urokinase-type plasminogen activator, tissue plasminogen activator, and streptokinase are used in conventional therapy for thrombosis (Chang et al. 2005). However, these thrombolytic agents are expensive, have low specificities against fibrin, and have undesirable side effects such as gastrointestinal bleeding, resistance to repercussion, and allergic reactions (Blann et al. 2002; Turpie et al. 2002; Hwang et al. 2007).Therefore, continuous efforts have been focused on the search for safer and less expensive thrombolytic agents from various sources.

Until recently, fibrinolytic enzymes with potential thrombolytic applications have been purified from various sources such as plants, animals, mushrooms, snake venom, and microbial sources (Cartwright 1974; Mihara et al. 1991; Fujita et al. 1993; Zhang et al. 1995; Chizhov et al. 1999; Chitte and Dey 2000; Joeng et al. 2001; Maurer 2001; Kim et al. 2006). During the past few decades, thrombolytic agents from microorganisms have increasingly attracted medical interest. These agents include nattokinase produced by *Bacillus natto*, subtilisin DJ-4 secreted by *B. amyloliquefaciens*, subtilisin DFE purified from *B. amyloliquefaciens*, subtilisin CK from *Bacillus* sp. strain CK-11-4, and streptokinase produced by *Streptococcus hemolyticus* (Sumi et al. 1987; Collen and Lijnen 1994; Kim et al. 1996; Kim and Chio 2000; Peng et al. 2003). High fibrinolytic activities have also been found in some fungi, for example, such as *Aspergillus ochraceus* 513 (Batomunkueva and Egorov 2001), *Fusarium oxysporum* (Tao et al. 1998), *Fusarium* sp. BLB (Ueda et al. 2007), *Fusarium* sp. CPCC 480097 (Wu et al. 2009), *Rhizopus chinensis* 12 (Liu et al. 2005), and *Penicillium chrysogenum* H9 (EI-Aassar et al. 1990). Furthermore, fibrinolytic enzymes from the marine algae *Codium latum* (Matsubara et al. 1998), *C. divaricatum* (Matsubara et al. 1999), and *C. intricatum* (Matsubara et al. 2000) were reported.

Koji molds are important fungal strains for Japanese fermentation industries. For instance, *A. oryzae* is used for brewing Japanese sake and miso, *A. sojae* is necessary for soy source brewing, and *A. kawachi* and *A. awamori* are used for shochu-moromi brewing because of their strong production of fermentable sugars. However, research has mainly focused only on diastatic enzymes, especially fungal amylases, and little attention has been given to other hydrolytic enzymes.

In this study, we characterized the fungus *A. oryzae* KSK-3 isolated from kome-koji, which produced a stable fibrinolytic protease under actual fermentation conditions. The purification and characterization of this protease are also described. This is the first report, to our knowledge, on a fibrinolytic protease produced by the edible *Aspergillus oryzae* strain KSK-3.

Materials and methods

Strains

Five hundred and fifty-four strains isolated from 44 commercial koji cultures were screened for fibrinolytic protease production. Thirteen strains, shown in Fig. 1, exhibited high fibrinolytic activities (more than 20 U urokinase per milliliter of culture broth) after a 7-day culture (28°C) in GYP liquid medium (1% glucose, 0.3% yeast extract powder, 0.5% polypeptone, 0.3% extract malt). Among these strains, KSK-3, isolated from *kome-koji* (Yamataka Miso, Nagano, Japan), was the most potent producer of fibrinolytic protease and was used in this study. Sequences of the 28S rDNA D1/D2 region from strain KSK-3 showed 100% homology with that of *A. oryzae* RIB40 (data not shown), thus confirming KSK-3 to be a strain of *A. oryzae*. *Aspergillus oryzae* KSK-3 was maintained in a Czapek agar slant (0.2% sodium nitrate, 0.1% dipotassium hydrogen phosphate, 0.02% magnesium sulfate, 0.05% potassium chloride, 0.0005% ferrous sulfate, 3% sucrose, 1.8% agar, pH 5.6), and the spore suspension was inoculated into GYP liquid medium for enzyme production.

Mixed culture of koji and yeast for screening

Mixed culture of koji and yeast were prepared with isolated koji molds and *Saccharomyces cerevisiae* kyoukai No.7 (Brewing Society of Japan, Tokyo, Japan).

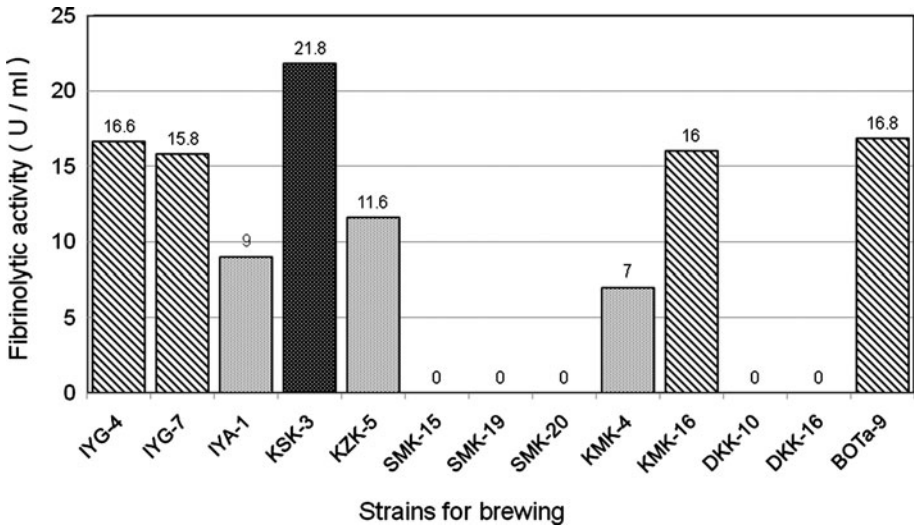

Fig. 1 Residual fibrinolytic activity of various strains in brewed mash (moto). Fibrinolytic activity was determined by the fibrin plate method (using the standard curve of urokinase). The 13 strains with the highest levels of fibrinolytic activity (≥20 U/ml) were incubated in yeast moto. KSK-3 maintained its fibrinolytic activity, whereas the other strains showed a decrease or inactivation of fibrinolytic activity

Fifty grams of the rice was gelatinized in 100 ml boiled distilled water and subsequently liquefied by with 50 µl Termamyl 120L (α-amylase; Novozyme Japan, Chiba, Japan). A spore suspension (prepared from the Czapek slant with 10 ml 0.1%Tween 80 solution) of isolated koji mold was inoculated into liquefied rice liquid, and the culture was incubated at 28°C on a rotary shaker (170 rpm) for 2–4 days.

Alcohol fermentation yeast was precultured in 7 ml GYP liquid medium at 28°C on a rotary shaker (170 rpm) for 24 h. The preculture was inoculated into 100 ml of GYP liquid medium in a shaking flask, and the culture was incubated at 28°C on a rotary shaker (170 rpm) for 2–4 days.

Both yeast and koji cultures were prepared as earlier described, and lactic acid (5 ml) was added to 200 ml liquefied rice to make mash; the mash was then incubated for fermentation at 15°C for 14 days. The mash was mixed gently with a glass stick once a day to release the gas. The mash filtrate was used for the experiment.

Culture conditions for enzyme production

The culture conditions of *A. oryzae* KSK-3 for enzyme production were preliminarily optimized (28°C, 170 rpm). A 2-day culture of *A. oryzae* KSK-3 in 7 ml GYP liquid medium was used as a preculture. The preculture was inoculated into 250 ml GYP liquid medium in a shaking flask (500 ml), which was incubated at 28°C on a rotary shaker (170 rpm) for 7 days. The culture broth was obtained by suction filtration and used for subsequent enzyme purification.

Protease assay

Protease activity was determined by the Folin–Ciocalteu method (Kunitz 1974). Five hundred microliters of the enzyme solution was added to 1.5 ml 1.14% Hammarsten casein solution in a 0.1 M Mcllvaine buffer (0.2 M disodium dihydrogen phosphate, 0.1 M citric acid, pH 8.0). After incubation for 30 min, the reaction was stopped by the addition of 2.0 ml 0.44 M TCA, followed by centrifugation at 10,000 rpm for 10 min. The supernatant (0.5 ml) was mixed with 2.5 ml 0.44 M sodium carbonate and 0.5 ml Folin–Ciocalteu reagent solution. The optical density of the color that developed at 37°C after 20 min of incubation was measured at 660 nm using a microplate reader (Model 550; Bio-Rad Japan, Tokyo, Japan). One unit (U) of protease activity was defined as the amount of enzyme required to release 1 µg tyrosine from Hammarsten casein per minute under the assay conditions described earlier.

Fibrinolytic activity

Fibrinolytic activity was determined using the method of Astrup and Mullertz (1952) with slight modification. The fibrin plate was prepared as follows: 5 ml bovine plasma fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) solution (0.6% in 50 mM Tris-HCl buffer, pH 7.55) was mixed with 200 µl thrombin (Sigma-Aldrich) solution (10 U/ml) and 5 ml 0.7% calcium chloride solution in Petri dishes. After clot formation (usually after 2 h of incubation at room temperature), 20 µl of sample solution was carefully placed on a plate. The plate was incubated at 37°C for 18 h. The activity of the fibrinolytic enzyme was estimated by measuring the area of the clear zone on the fibrin plate. The area of the clear zone was measured with the Image J software (National Institutes of Health, Bethesda, MD, USA) (Abramoff et al. 2004), and the number of units was determined using the standard curve of urokinase (Wakamoto, Tokyo, Japan).

Purification of fibrinolytic protease

The culture filtrate (500 ml) was concentrated by ultrafiltration [Advantec P0200 (molecular weight cutoff, 20 kDa); Toyo Roshi Kaisha, Tokyo, Japan] and subsequently treated using a salting-out procedure at 4°C with ammonium sulfate at 60% saturation. The resulting precipitate was collected by centrifugation at 15,000 *g* at 4°C for 30 min. The precipitated pellet was dissolved in a small volume of phosphate buffer (20 mM, pH 7.0), and the solution was dialyzed against the same buffer overnight at 4°C. The dialysate was concentrated by ultrafiltration [Advantec P0200 (molecular weight cutoff, 20 kDa); Toyo Roshi Kaisha] and subsequently loaded onto a DEAE-Toyopearl 650C column (Tosoh, Tokyo, Japan: 1.5 × 15 cm) equilibrated with 20 mM phosphate buffer and then washed with the same buffer. The DEAE nonabsorbed eluent was concentrated with ultrafiltration and then applied to a Sephacryl S-100 (GE Healthcare Japan, Tokyo, Japan) column (1.5 × 100 cm). The column was previously equilibrated with phosphate buffer and eluted with the same buffer at a flow rate of 0.1 ml/min. The protease-containing fraction was pooled, and the enzyme solution was concentrated. The fraction with fibrinolytic activity was subjected to a Superdex 75 10/300 GL (GE Healthcare Japan) column (1.0 × 30 cm) previously equilibrated with phosphate buffer, and eluted with the same buffer at a flow rate of 0.2 ml/min. The protease-containing fraction was pooled and the enzyme solution was concentrated for further analysis.

Protein determination

Protein concentration was determined by the Bradford method, using the standard curve of bovine serum albumin (Bradford 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 10% polyacrylamide running gel according to the method of Laemmli (1970). The molecular mass was calibrated using a molecular mass marker (Precision plus Protein standards; Bio-Rad Japan, Chiba, Japan) as a standard. Protein bands were detected by staining with Coomassie brilliant blue R-250.

Fibrin zymography

Fibrinolytic activity was analyzed using a fibrin zymogram gel. Fibrin zymography was carried out as described by Kim et al. (1998). Fibrinogen and thrombin were mixed with 10% polyacrylamide gel solution, and the mixture was electrophoresed on a fibrin gel. The molecular mass was calibrated with a molecular mass marker as a standard. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h, rinsed three times with distilled water, and incubated in reaction buffer (0.1 M glycine, pH 8.4) at 37°C for 18 h. The staining and destaining procedures were similar to those of SDS-PAGE.

Zymogram gel analysis

Zymogram gel (Invitrogen, Carlsbad, CA, USA) was also used for the molecular mass analysis of protease activity (Heussen 1980). After electrophoresis, the gel was renatured in zymogram renaturing buffer for 30 min. The buffer was decanted, and a zymogram developing buffer was added for 30 min; the gel was incubated in the same buffer at 37°C overnight. The gel was stained with colloidal blue staining kit, and then destained with distilled water at room temperature for 7 h.

Blue native polyacrylamide gel electrophoresis

The molecular mass of the native protein was analyzed using Native-PAGE Bis-Tris gel (Invitrogen) (Schägger and von Jagow 1991). The molecular mass was calibrated with a molecular mass marker as a standard. After electrophoresis, destaining was done with wash solution at room temperature for 30 min.

High-performance liquid chromatography–size exclusion chromatography

Molecular mass was analyzed using by high-performance liquid chromatography (HPLC) (LC-10A system; Shimadzu, Kyoto, Japan) using the calibration curve of molecular weight marker proteins (Oriental Yeast, Tokyo, Japan). The analytical conditions were as follows: column, Tosoh TSKgel G3000SW$_{XL}$ (7.8-mm I.D. × 30 cm); eluent, 0.05 M phosphate buffer (pH 7.0) + 0.3 M sodium chloride; flow rate, 1.0 ml/min; temperature, 25°C; UV detection at 280 nm.

Effects of pH and temperature on activity

The activities of the purified enzyme were measured using the synthetic substrates at 37°C in glycine-HCl (pH 3.0), acetic acid (pH 4.0–5.0), phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 10.0). All buffer concentrations were 20 mM. Proteinase activity was analyzed at temperature ranging from 20° to 80°C.

Effects of pH and temperature on enzyme stability

Analysis of the effect of pH level on enzyme stability was carried out by incubating the enzyme for 4 h at 37°C in the following buffers: glycine-HCl, sodium acetate, phosphate, and glycine-NaOH. All buffer concentrations were 20 mM. After incubation, the activity remaining was determined using the casein Folin–Ciocalteu method. For measuring thermal stability analysis, the purified protease was incubated in 20 mM phosphate buffer at pH 7.0 for 30 min at 20°–70°C. After incubation, remaining activity was determined using the casein Folin–Ciocalteu method.

Effects of protease inhibitors on enzyme activity

Inhibition of the protease was determined by measuring its activity at pH 7.0 in the presence of protease inhibitors. Enzyme samples were separately incubated at 37°C for 10 min with the following inhibitors: antipain dihydrochloride, aprotinin, bestatin, chymostatin, E-64, pefabloc SC, pepstatin, and phosphoramidon (all; Roche Diagnostics, Basel Schweiz, Switzerland). Ethylenediaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Wako (Osaka, Japan). Residual activity was determined.

Substrate specificity

The hydrolytic activity of the protease was studied using the following chromogenic substrates (from Sigma):

S-2238 (D-Phe-Pro-Arg-*p*-nitroanilide dihydrochloride), S-2288 (D-Ile-Pro-Arg-*p*-nitroanilide dihydrochloride), and S-2251 (D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride). Glu-Gly-Arg-*p*-nitroanilide dihydrochloride was obtained from Peptide Institute (Osaka, Japan). Each reaction mixture contained 2.5 mM substrate in 20 mM phosphate buffer at pH 7.0.

Results

Stability of the fibrinolytic enzyme under actual brewing conditions

To evaluate the stability of the enzyme, its fibrinolytic activity was determined and compared with those of other enzymes in small-scale sake mash cultures (same as actual fermentation conditions with *S. cerevisiae*). Sake mash cultures were prepared using the 13 strains with the highest levels of fibrinolytic activity in GYP liquid medium. The sake mush with *A. oryzae* KSK-3 had the highest residual activity (21.8 U/ml), whereas most of the other strains decreased or lost their fibrinolytic activity under the same condition (Fig. 1). Therefore, we decided to use strain KSK-3 in the enzyme production and purification experiments.

Purification of the fibrinolytic enzyme

The yield and purity of the fibrinolytic enzyme at each purification step are summarized in Table 1. The enzyme was purified about 182.7 fold with a yield of 0.005%. The Sephacryl S-100 gel-filtration pattern is shown in Fig. 2. Although four peaks of protease activities were observed, only the 55th fraction exhibited fibrinolytic activity. The active fraction was concentrated and further purified using a Superdex 75 10/300 GL gel-filtration column. The purified enzyme was detected as a single band on SDS-PAGE (Fig. 3a) and was calibrated to be 30 kDa with size-exclusion chromatography on HPLC (Fig. 4). Fibrin zymography and zymogram gel analysis also showed the molecular mass of the fibrinolytic enzyme to be 30 kDa (Fig. 3b).

Effects of temperature and pH

The optimum pH for the purified enzyme was 6.0 (Fig. 5a). When the enzyme was incubated at 37°C for 4 h in buffer

Table 1 Purification of fibrinolytic protease from *Aspergillus oryzae* KSK-3

Step	Total activity (U)[a]	Total protein (mg)	Specific activity (U/mg protein)	Fold	Yield (%)
Culture filtrate	2,225	405	5.5	1	100
0–60% $(NH_4)_2SO_4$	380.7	50.5	7.5	1.4	17.1
Ultrafiltration	224	13.6	16.5	3	10.1
DEAE-Toyopearl 650C	108	3.8	27.9	5.1	4.9
Sephacryl S-100HR	24.5	0.1	245	44.5	1.1
Superdex 75 HR 10/30	20.1	0.02	1,005	182.7	0.005

[a] Protease activity was assayed using Folin–Ciocalteu method. Casein was used as substrate

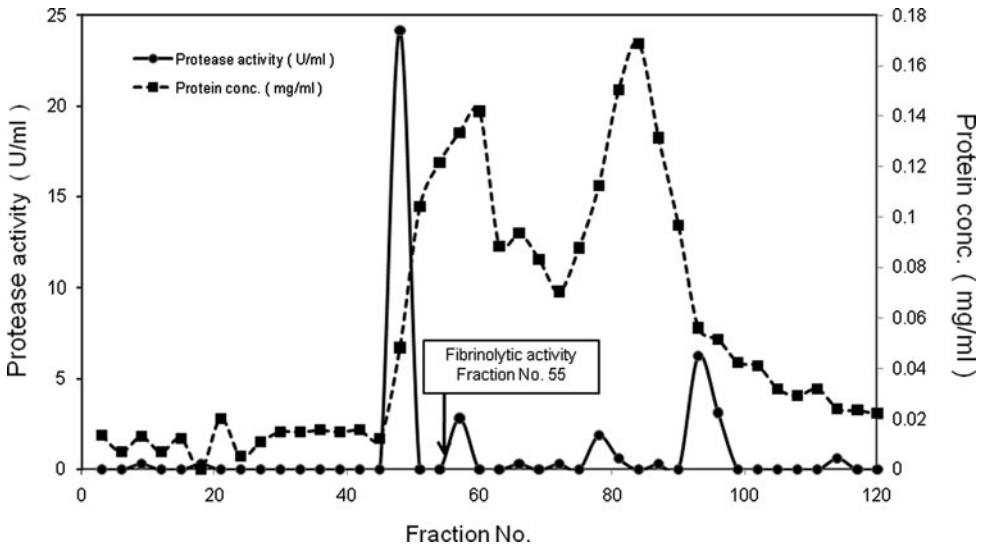

Fig. 2 Elution profile of protease activity on Sephacryl S-100 HR. Protease activity (U/ml) was measured with the Folin–Ciocalteu method (*filled circles*) and protein concentration (mg/ml) was measured using Bradford's method (*filled squares*). Fibrinolytic activity was detected only in the 55th fraction

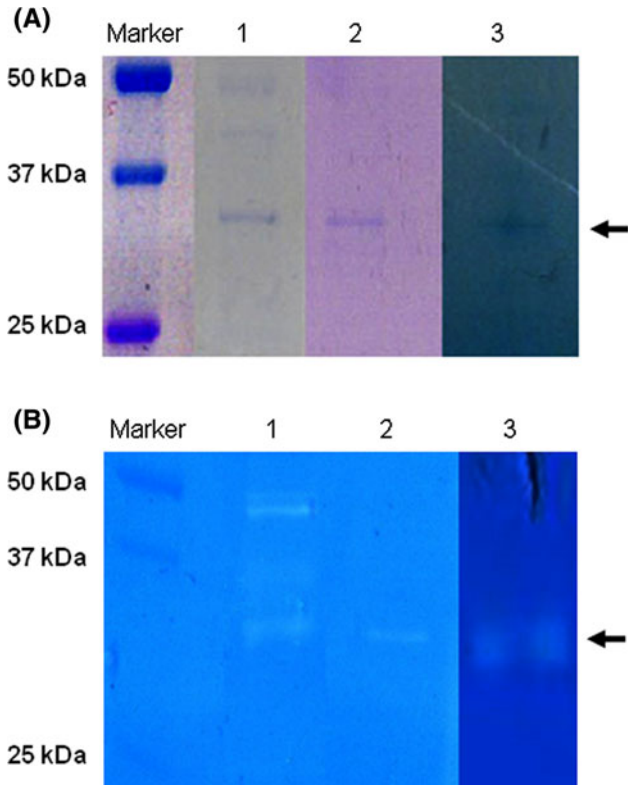

Fig. 3 Electrophoretic analyses of the purified enzyme from strain KSK-3. **a** SDS-PAGE (*lanes 1*, *2*) and Blue Native-PAGE (*lane 3*) analyses. A 10% polyacrylamide gel was used for the analysis. *Lane 1*, partially purified enzyme; *lanes 2* and *3*, purified enzyme. The *arrow* shows the band of fibrinolytic protease of *Aspergillus oryzae* KSK-3. **b** Zymogram gel (*lanes 1*, *2*) and fibrin zymography (*lane 3*) analyses. *Lane 1*, partially purified enzyme; *lanes 2* and *3*, purified enzyme. *Arrow* shows the band of fibrinolytic protease of *Aspergillus oryzae* KSK-3

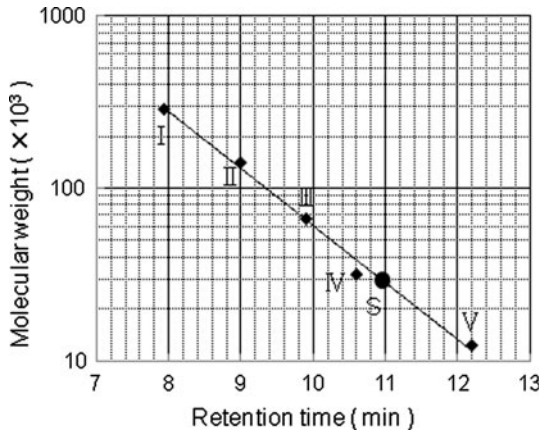

Fig. 4 Molecular mass calibration of the purified enzyme by HPLC–SEC. Molecular mass was estimated using HPLC. Molecular markers: *I*, glutamate dehydrogenase (290,000); *II*, lactate dehydrogenase (142,000); *III*, enolase (67,000); *IV*, myokinase (32,000); *V*, cytochrome *C* (12,400); *S*, purified enzyme from *Aspergillus oryzae* KSK-3

at various pH values, more than 20% of the activity was retained at pH values between 4 and 9 (Fig. 5b). The optimum temperature for the enzyme was 50°C (Fig. 5c), with enzyme stability observed up to 50°C (Fig. 5d).

Effects of metal ions

The effects of metal ions on the protease activity of KSK-3 were also evaluated (Table 2). The reactions were carried out using the Folin–Ciocalteu method under the standard protease assay conditions with 5 mM of the tested compounds. $AlCl_3$ and $PbCl_2$ slightly inhibited the reaction (32.5% and 26.4%, respectively), whereas $MnCl_2$ promoted enzyme activity (154%).

Effects of protease inhibitors on enzyme activity

The effects of various protease inhibitors on protease activity were also examined. The enzyme activity was considerably inhibited by serine protease inhibitors PMSF and Pefabloc SC, but not by chelator agent EDTA (Table 3).

Substrate specificities

The hydrolytic activity of KSK-3 fibrinolytic protease was measured using several chromogenic substrates (Table 4). The highest level of hydrolytic activity was observed with S-2238 (for thrombin), whereas low hydrolytic activity levels were found with S-2251 (for plasmin and streptokinase activated plasminogen) and S-2288 (for serine proteases). No hydrolysis of Glu-Gly-Arg-*p*-nitroanilide dihydrochloride (for urokinase) was observed.

Discussion

This study described the purification and characterization of a fibrinolytic enzyme from *A. oryzae* KSK-3 isolated from commercial koji (molded grain prepared from *A. oryzae*). The enzyme was purified to electrophoretic homogeneity by combination of chromatographic steps on DEAE-Toyopearl 650C column chromatography, Sephacryl S-100HR gel-filtration column chromatography, and Superdex 75 10/300 GL gel-filtration column chromatography. The purified *A. oryzae* KSK-3 enzyme had an estimated molecular mass of 30 kDa, which was similar to the size of fibrinolytic enzymes from *Streptomyces* sp. (SW-1) and *Bacillus subtilis* (Subtilisin FS33) (Wang et al. 1999, 2006) (Table 5). It was stable over a wide range of pH values from 4 to 9, at temperatures below 60°C. This KSK-3 enzyme was pH resistant, as evidenced by its higher stability compared with the protease from *Fusarium* sp. Fu-P

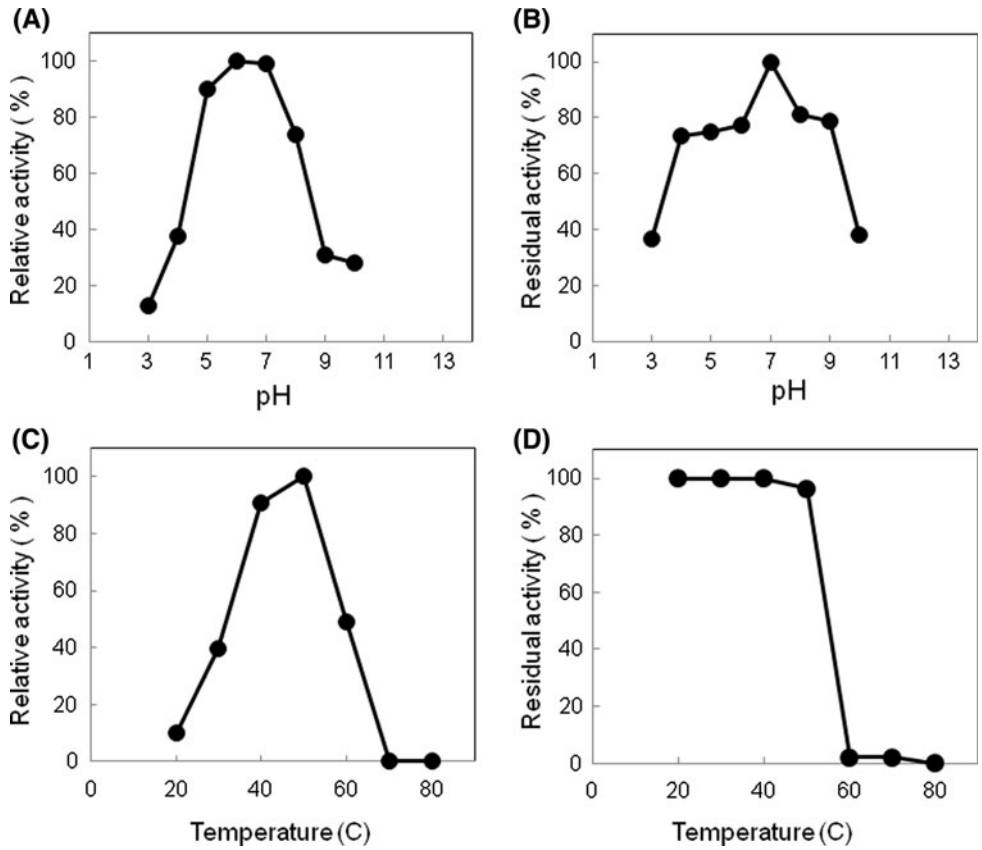

Fig. 5 Effects of pH and temperature on the activity and stability of the KSK-3 enzyme. **a**, **b** Effects of pH on activity and stability of the enzyme. **a** Activities of purified enzymes were measured using the synthetic substrate at 37°C in glycine-HCl (pH 3.0), acetic acid (pH 4.0–5.0), phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 10.0). All buffers were used at 20 mM concentration. **b** Effect of pH on enzyme stability was investigated by incubating the enzyme for 4 h at 37°C in the following buffers: glycine-HCl, sodium acetate, phosphate, Tris-HCl, and glycine-NaOH. Remaining activity was determined using the casein Folin–Ciocalteu method at pH 7.0. **c**, **d** Effects of temperature on activity and stability of the enzyme. **c** Effect of temperature on protease activity was studied at temperatures between 20° and 80°C. **d** To measure the thermal stability of the enzyme, the purified protease was incubated in a 20 mM phosphate buffer at pH 7.0 for 30 min at temperature ranging from 20° to 70°C. The remaining activity was measured using the casein Folin–Ciocalteu method at 37°C

(Wu et al. 2009), *Bacillus amyloliquefaciens* Subtilisin DFE (Peng et al. 2003), *Streptomyces* sp. SW-1 (Wang et al. 1999), *Streptomyces* sp. FP84 (Simkhada et al. 2010), *S. megasporus* SD5 (Chitte and Dey 2000) and *Rhizopus chinensis* 12 (Liu et al. 2005) (Table 5). To identify the type of *A. oryzae* KSK-3 protease, the substrate specificity of the enzyme and the effect of various protease inhibitors on its activity were examined. The *A. oryzae* KSK-3 enzyme exhibited high activity in S-2238, a characteristic similar to the fibrinolytic enzymes from *Fusarium* sp. BLB and earthworms (Mihara et al. 1991; Ueda et al. 2007). KSK-3 enzyme activity was inhibited by PMSF and pefabloc SC, but not by other inhibitors including chelator agent EDTA. This strong inhibition with PMSF and pefabloc SC indicates that the KSK-3 enzyme is a serine protease. *Leishmania* protease is also activated by the manganese cation (Silva-Lopez and Giovanni-De-Simone

Table 2 Effects of metal ions on the activity of *Aspergillus oryzae* KSK-3

Compound	Relative activity (%)
None	100
$AgNO_3$	82.1 ± 3.3
$AlCl_3$	67.5 ± 0.4
$BaCl_2$	92.5 ± 3.5
$CaCl_2$	84.6 ± 2.0
$CoCl_2{}^+$	83.5 ± 5.9
$CuCl_2$	97.0 ± 0.2
$FeCl_2$	83.3
KCl	85.0 ± 4.5
LiCl	86.6 ± 2.0
$MgCl_2$	85.4 ± 8.9
$MnCl_2$	154.1 ± 1.2
NaCl	81.1 ± 0.6
$NiCl_2$	89.4 ± 6.9
$PbCl_2$	73.6 ± 2.8
$ZnCl_2$	85.2 ± 9.6

Reactions were carried out under standard protease assay conditions (Folin–Ciocalteu method) with 5 mM of the tested compounds. Results represent averages of three independent experiments

Table 3 Effect of protease inhibitors on the activity of *Aspergillus oryzae* KSK-3

Inhibitors	Comment	Concentration	Relative activity (%)
Control	–	–	100
EDTA	Metalloproteases	4.5 mM	100
PMSF	Serine proteases	4.5 mM	82.2 ± 0.8
Antipain dihydrochloride	Papain, trypsin	50 μg/ml	100
Bestatin	Amino peptidases, including amino peptidase B, leucine aminopeptidase, tripeptide aminopeptidase	40 μg/ml	94.1 ± 1.4
Chymostatin	α-, β-, γ-, δ-Chymotrypsin	60 μg/ml	100
E-64	Cysteine proteases	10 μg/ml	93.7 ± 4.0
Leupeptin	Serine and cysteine proteases such as plasmin, trypsin, papain, cathepsin B	20 μg/ml	92.2 ± 3.6
Pepstatin	Aspartate proteases such as pepsin, renin, cathepsin D, and chymosin	2.8 μg/ml	88.4 ± 1.4
Phosphoramidon	Metallo-endopeptidases, specifically thermolysine, collagenase, and metallo-endoproteinases	200 μg/ml	91.2 ± 4.7
Pefabloc SC	Serine proteases, such as trypsin, chymotrypsin, plasmin, and thrombin	4 mg/ml	39.5 ± 2.4
Aprotinin	Serine proteases	8 μg/ml	89.2 ± 1.3

Results shown represent the averages of three independent experiments. Protease activity was assayed using the Folin–Ciocalteu method. Casein was used as substrate

EDTA ethylenediaminetetraacetic acid, *PMSF* phenylmethylsulfonyl fluoride

Table 4 Hydrolysis of chromogenic substrate by *Aspergillus oryzae* KSK-3 fibrinolytic enzyme

Substrates	Relative activity (%)
D-Phe-Pro-Arg-*p*-nitroanilide dihydrochloride (for thrombin)	100
D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride (for plasmin)	44.5 ± 4.3
D-Ile-Pro-Arg-*p*-nitroanilide dihydrochloride (for broad spectrum of serine proteases)	30.4 ± 2.0
Glu-Gly Arg-*p*-nitroanilide dihydrochloride (for urokinase)	0

Results shown represent averages of three independent experiments

2004). However, the mechanism is not clear, and further investigation on the effect of metal ions is necessary. The overall findings on the KSK-3 fibrinolytic enzyme with regard to molecular weight, effects of temperature and pH, effects of metal ions, and inhibitors, substrate specificity, and fibrinolytic activity indicate that the KSK-3 enzyme differs from other known fibrinolytic enzymes. A potent fibrinolytic enzyme, nattokinase, was previously isolated from traditional Japanese fermented foods. This enzyme is an extracellular serine protease produced by *B. subtilis*. Furthermore, Sumi et al. (1990) demonstrated that oral ingestion of natto or nattokinase capsules enhanced fibrinolysis in the plasma of an experimental thrombotic model. The calculated fibrinolytic activity of the nattokinase was approximately 1,600 U urokinase/g wet natto. The KSK-3 enzyme showed higher productivity (~3,000 U urokinase/ml culture broth) compared with nattokinase, and maintained its fibrinolytic activity in moto (actual sake-mash culture) for at least 14 days. Moreover, *A. oryzae* KSK-3, a producer strain, used for making fermented food in Japan, is completely edible. These findings indicate that the fibrinolytic enzyme from *A. oryzae* KSK-3 may be used as a natural agent for oral fibrinolytic therapy and nutraceutical applications in the prevention of cardiovascular diseases. Studies are under way to determine the N-terminal amino acid sequence of the enzyme.

Table 5 Comparative characterization of fibrinolytic proteases

Enzyme	MW (kDa)	Opt. pH	Opt. temp. (°C)	pH stability	Temp. stability (°C)	Comments	Reference
Aspergillus oryzae KSK-3	30	6	50	4–9	<60	Serine protease	This work
Aspergillus fumigatus	33	9	37–42	ND	<40	ND	(Larcher 1992)
Aspergillus ochraceus 513	36.5	ND	ND	ND	ND	Serine protease	(Batomunkueva and Egorov 2001)
Fusarium sp. BLB	27	9.5	50	2.5–11.5	<50	Serine protease	(Ueda et al. 2007)
Fusarium sp. Fu-P	28	8.5	45	6–9	<37	Serine metalloprotease	(Wu et al. 2009)
Bacillus natto NK	27.7	ND	ND	7–12	<50	Serine protease	(Fujita et al. 1993)
Bacillus amyloliquefaciens Subtilisin DFE	28	9	48	6–10	<50	Subtilisin-family serine protease	(Peng et al. 2003)
Bacillus subtilis Subtilisin QK-2	28	8.5	55	3–12	40 for 30 min	Subtilisin-family serine protease	(Ko et al. 2004)
Bacillus sp. CK-11-4	28.2	10	70	7–10.5	<50	Serine protease	(Kim et al. 1996)
Bacillus sp. Subtilisin DJ-4	29	ND	40	4–11	48 h at RT	Plasmin-like serine protease	(Kim and Chio 2000)
Bacillus subtilis Subtilisin FS33	30	8	55	ND	ND	Subtilisin-like serine protease	(Wang et al. 2006)
Bacillus sp. KA 38	41	7	40	ND	ND	Metalloprotease	(Kim et al. 1997)
Streptomyces sp. SW-1	30	ND	ND	4–9	4–37	Serine metalloprotease	(Wang et al. 1999)
Streptomyces sp. FP84	35	7.5	45	6–9	<40	Serine metalloprotease	(Simkhada et al. 2010)
Streptomyces megasporus SD5	35	8	55	6–9	37–60	Chymotrypsin-like serine peptidase	(Chitte and Dey 2000)
Armillaria mellea AMMP	21	6	33	ND	ND	Chymotrypsin-like metalloprotease	(Lee et al. 2005)
Oidiodendron flavum	22	8	ND	6–11	<65	ND	(Nagwa and Tharwat 2006)
Rhizopus chinensis 12	18	10.5	45	6.8–8.8	37 for 24 h	Metalloprotease	(Liu et al. 2005)

ND indicates missing information

MW molecular weight, *opt*. optimum, *temp.* temperature, *RT* room temperature

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