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Characterization of Acidic Protease from *Aspergillus niger* BCRC 32720

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ABSTRACT: An acid protease from the broth of a 24 h cultivated *Aspergillus niger* BCRC 32720 was purified to electrophoretical homogeneity by CM Sepharose FF and Sephacryl S-100 HR chromatographs. The specific activity, purification fold, and yield were 23.29 kU/mg, 2.5, and 24.2%, respectively. Molecular mass (M) and N-terminal amino acid sequence were 47.5 kDa and SKGSAVTT, whereas the pH and temperature optima were at 2.5 and 50 °C, respectively. It was stable at pH 2.0–4.0 or ≤40 °C and activated by Fe^{2+} and cysteine, but partially inhibited by phenylmethanesulfonyl fluoride and tosyllysine chloromethyl ketone and highly inhibited by Ag^+, Sn^{2+}, Fe^{3+}, Sb^{3+}, and pepstatin A. It was considered to be an aspartic protease.

KEYWORDS: acid protease, *Aspergillus niger*, purification, characterization

■ INTRODUCTION

Microbial enzymes could be easily isolated they have, thus, been extensively studied and long been directly or indirectly used in food fermentation, baking, and alcohol-making industries.[1] Among these microbial enzymes, proteases amount to up to 65% of the global market and are frequently used in detergent, leather, pharmaceuticals, and food industries.[2,3] Acid proteases are mostly used in the production of seasonings, protein hydrolysate, and soy sauce or as digestive aids.[2] They are also widely applied in improving the texture of flour paste and fibril muscle and the clarification of beer and fruit juice.[4]

Filamentous fungi are exploited for the production of industrial enzymes due to their ability to grow on solid substrate or broths and produce a wide range of extracellular enzymes. Among the advantages of enzyme production by fungi, its low cost, high productivity, fast production, and ease with which the enzymes can be modified are most concerned. Several studies have recently been performed on the isolation of acid proteases from different fungi such as *Aspergillus saitoi*,[5] *Aspergillus oryzae*,[6−8] *Aspergillus niger*,[9,10] *Mucor pusillus*,[11] *Rhizopus hangchow*,[12] *Monascus pilosus*,[13−15] and *Penicillium duponti*.[16] However, many reported activity levels were still poor. Therefore, new species for the production of acid proteases are still being sought by several scientists.[17−20] The present work reports the characteristics of an acid protease from *A. niger* BCRC 32720.

■ MATERIALS AND METHODS

Materials. CM-Sepharose and Sephacryl S-100 High Resolution were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, bis(acrylamide), and Coomassie Brilliant Blue G-250 were obtained from Merck (Darmstadt, Germany), whereas the malt extract, peptone, and PDA were the products of Difco. Hemoglobin, bovine serum albumin (BSA), glucose, sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylethylenediamine (TEMED), 1-(L-trans-epoxy-succinylleucylamido-4-guanidinobutane) (E-64), phenylmethanesul-fonyl fluoride (PMSF), iodoacetic acid (IAA), N-ethylmaleimide (NEM), leupeptin, pepstatin A, tosyllysine chloromethyl ketone (TLCK), and tosylphenylalanine chloromethyl ketone (TPCK) were obtained from Sigma (St. Louis, MO, USA). Protein−dye binding reagent was obtained from Bio-Rad (Hercules, CA, USA). $FeCl_3$, $FeCl_2$, $MgCl_2$, $MnCl_2$, NH_4Cl, urea, ethylenediaminetetraacetic acid (EDTA), and trichloroacetic acid (TCA) were the products of Hayashi Pure Chemical Industries (Osaka, Japan).

Purification of Acidic Protease. *Preparation of the Crude Enzyme.* *A. niger* BCRC 32720, obtained from the Bioresources Collection and Research Center (BCRC) (Food Industry Research and Development Institute, Hsinchu, Taiwan), was activated in malt extract broth (containing 20 g/L malt extract, 20 g/L glucose, 1.0 g/L peptone) at 30 °C for 4 days. *A. niger* broth (0.1 mL) was inoculated to potato dextrose agar (PDA) and incubated at 30 °C for another 4 days. The spores, washed from PDA using 5 mL of 0.05% Tween 20, were used as starter. They were inoculated into malt extract broth and incubated at 30 °C with 150 rpm shaking for 24 h and then centrifuged at 10000g for 20 min. The supernatant was passed through a 0.22 μm membrane to remove the cells. Finally, it was dialyzed against 20 mM citrate buffer, pH 3.5, to exclude the salt and used as crude enzyme.

Ion Exchange Chromatograph. The CM-Sephacel Fast Flow column (2.6 × 13 cm) was equilibrated with 20 mM citrate buffer, pH 3.5, and washed with the same buffer after the crude enzyme was loaded. The acidic protease was then eluted with 0−400 mM NaCl in 20 mM citrate buffer (pH 3.5) at a flow rate of 0.5 mL/min (3.0 mL/tube). Fractions with acidic protease activity were collected and concentrated to a minimal volume by Amicon Ultrafiltration (cutoff 10000).

Gel Filtration. The Sephacryl S-100 High Resolution column (1.6 × 90 cm) was equilibrated with 20 mM citrate buffer, pH 3.5, containing 50 mM NaCl. Acidic protease collected on the CM-Sephacel column was concentrated to a minimal volume. The resulting sample was

loaded on Sephacryl S-100, which was equilibrated with 20 mM citrate buffer containing 50 mM NaCl, pH 3.5, and then eluted with the same buffer at a flow rate of 0.5 mL/min (3.0 mL/tube). Fractions with acidic protease activity were collected and subjected to the following assays. All purification procedures were performed at 4 °C.

Determination of Enzyme Activity. The activity of acidic protease was measured mainly as described by Narahara et al.[21] To 1.0 mL of 2% hemoglobin in 100 mM citrate buffer, pH 3.5, was added 1.0 mL of the appropriate concentration of enzyme. After 30 min of reaction at 37 °C in a water bath with shaking, 1.0 mL of 20% TCA was added to stop the reaction. The resulting samples were centrifuged at 10000g for 20 min to remove the precipitate. Absorbance at 280 nm (A_{280}) of the supernatant was determined by using a spectrophotometer (Hitachi U-2001, Hitachi, Japan). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze hemoglobin and release equivalent to 1.0 μg of tyrosine within 1 min of reaction at 37 °C.

Protein Concentration. Protein concentration was determined according to the protein−dye binding method[22] using BSA (Sigma) as standard.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Purified enzyme in 62.5 mM Tris-HCl buffer containing 2% SDS, 5% β-mercaptoethanol (β-Me), and 0.002% bromophenol blue was boiled at 100 °C for 5 min and then subjected to SDS-PAGE analysis (12.5% acrylamide gel) according to the method of Laemmli.[23] Gels were stained with Coomassie Brilliant Blue R-250 according to the method of Neuhoff et al.[24] Phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20 kDa) were used as marks (Life Technologies, Gaithersburg, MD, USA).

Optimal pH and pH Stability. The activity of acidic protease in 100 mM buffer with various pH values (citrate buffer, pH 2−6; sodium phosphate, pH 6−8; Tris-HCl, pH 8−9) was measured as described by Narahara et al.[21] To determine the pH stability, the acidic protease in 100 mM buffer with various pH values was incubated at 37 °C for 30 min and then the residual activity determined.[21]

Optimal Temperature and Thermal Stability. The activity of acidic protease in 100 mM citrate buffer, pH 3.5, was measured at various temperatures (4−80 °C).[21] To measure the thermal stability, the acidic protease in 100 mM citrate buffer, pH 3.5, was incubated at various temperatures (4−90 °C) for 30 min. At definite time intervals, the enzyme solution was cooled immediately in ice water for 5 min. The activity was measured.[21]

Effect of Metal Ions. Various metals (1.0−10 mM) suspended in 100 mM citrate buffer, pH 3.5, were added to the purified acidic protease. The resulting samples were incubated at 37 °C for 30 min. The residual activity was measured.[21]

Effect of Inhibitors. Various inhibitors (0.5−2.0 mM) suspended in 100 mM citrate buffer, pH 3.5, were added to the purified acidic protease. The resulting samples were incubated at 37 °C for 30 min. The residual activity was measured.[21]

N-Terminal Amino Acid Sequence Analysis. Purified acid protease was subjected to SDS-PAGE analysis[23] and then electrotransferred onto polyvinylidene difluoride membrane (PVDF). Proteins were stained with Coomassie Blue R-250 after electrophoresis. N-Terminal amino acid sequences were analyzed by Edman degradation[25] after being electrotransferred onto PVDF. Comparison of the sequence with homologous strains in GenBank was performed using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was inferred from BLAST at the National Center for Biotechnology Information (NCBI).

Statistical Analysis. One-way analysis of variance (ANOVA) was run using the Statistical Analysis System (SAS/STAT, release 8.0; Cary, NC, USA). Duncan's multiple-range test was used to determine the significance of differences within treatments. For each treatment, three replicates were measured and the mean values were calculated. Values were considered to be significantly different when $P < 0.01$.

■ RESULTS AND DISCUSSION

Growth, pH, and Protease Activity of *A. niger* BCRC 32720 during Incubation. One percent of starter was
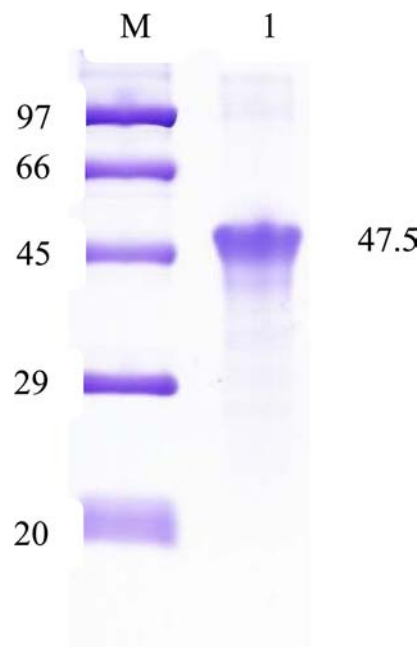

Figure 1. SDS-PAGE profile of purified acid protease from *A. niger* BCRC 32720. The enzyme was analyzed by SDS-PAGE with 12.5% acrylamide and stained with Coomassie Brilliant Blue R-250. Lanes: 1, purified acid protease; M, markers (phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa)).

Table 1. Summary of the Purification of Acid Protease from *A. niger* BCRC 32720

procedure	total activity (kU)	total protein (mg)	specific activity (kU/mg)	purification (fold)	yield (%)
crude enzyme	20.22	2.17	9.32	1.00	100
CM Sepharose FF	13.42	0.77	17.43	1.87	66.2
Sephacryl S-100 HR	4.89	0.21	23.29	2.50	24.2

inoculated into 100 mL of malt extract broth using a 250 mL flask and incubated at 30 °C with 150 rpm shaking for 2 days. At 12 h intervals, the pH, mycelial dry weight, and protease activity were determined. According to Fukamoto et al.,[26] during the early stage of *A. oryzae* fermentation, proteases were produced inside the mycelia and then secreted gradually to the broth. The highest production of proteases occurred at the stage during spore formation. The mycelial dry weight and protease activity increased with the incubation time and reached a plateau after 24 h of incubation, whereas the acidic protease activity was about 370 units/mL (data not shown). According to Fumiyosi et al.,[27] the types and production of proteases produced by Aspergilli were highly related to the ratio of carbon to nitrogen source (C/N ratio). At low C/N ratio, the dominant proteases would be neutral and alkaline proteases. However, the dominant proteases were acidic protease type at high C/N ratio.[27] In our preliminary
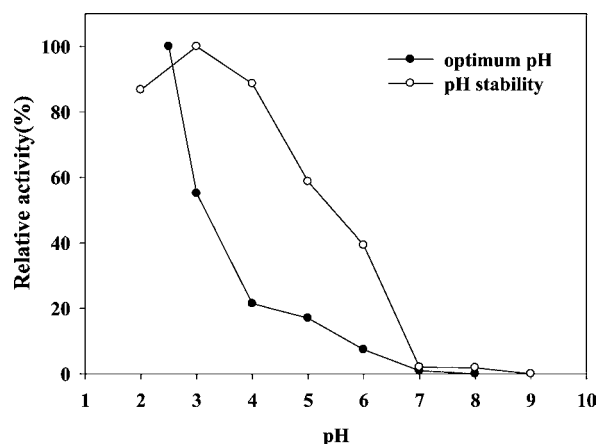
Figure 2. pH optimum and pH stability of the purified acid protease from *A. niger* BCRC 32720. The relative activity was expressed as percentage relative to the highest activity (highest activity = 100%).

Figure 3. Optimum temperature and thermal stability of the purified acid protease from *A. niger* BCRC 32720. The relative activity was expressed as percentage relative to the highest activity (highest activity = 100%).
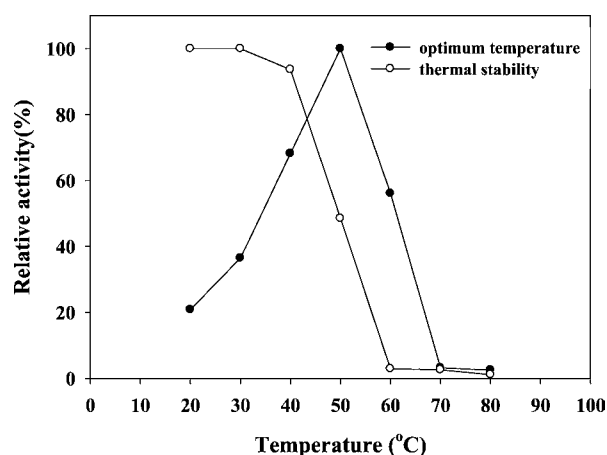
experiments, during 7 days of cultivation in malt extract broth at 30 °C with 150 rpm shaking, the pH decreased from 5.23 to 3.13, whereas acidic protease activity increased to 370 units/mL after 24 h of incubation. No further decline in pH or increase in activity was observed during the prolonged incubation. However, the mycelial dry weight increased with the duration up to 5 days of cultivation (data not shown).

Purification. The acidic protease was purified to electrophoretical homogeneity after DEAE-Sephacel Fast Flow and Sephacryl S-100HR chromatographs (Figure 1). About 2.5-fold of purification and 24.2% recovery were achieved after Sephacryl S-100 HR chromatography (Table 1). According to SDS-PAGE analysis, the purified acidic protease was a monomer with M of 47.5 kDa (Figure 1).

Effects of pH and Temperature. The purified protease was very stable at pH 2.0−4.0 with an optimum pH at 2.5 (hemoglobin as substrate) (Figure 2). There was ≥80% activity left even after 30 min of incubation at ≤40 °C. This result was similar to those reported by Nunokawa,[28] Li,[29] Sawada,[30] Vicente et al.,[31] Wu and Hang,[32] and Eneyslaya et al.[33] According to Tsujita and Endo's study,[34] the optimal pH values of acidic proteases A₁ and A₂ purified from commercial *A. oryzae* (Takadiastase) were 3.0 (casein as substrate) and 5.8

Table 2. Effect of Metal Ions on Acid Protease Activity from *A. niger* BCRC 32720

metal ion	relative activity[a] (%)		
	1 mM	5 mM	10 mM
none	100	100	100
LiCl	100.2	99.6	98.5
NaCl	99.5	98.4	97.6*
KCl	101.2	99.3	98.8
AgNO₃	18.9*	4.6*	2.5*
NH₄Cl	89.7*	99.3	100.2
MgCl₂	98.6	99.0	97.5*
CaCl₂	90.1*	91.6*	94.4*
BaCl₂	97.8	101.7	98.8
FeCl₂	165.4*	211.0*	224.6*
CoCl₂	101.1	103.9*	106.5*
NiCl₂	98.8	97.1*	97.6*
CuCl₂	98.2	95.2*	91.6*
ZnCl₂	101.2	98.8	97.2*
HgCl₂	93.3*	88.7*	78.4*
MnCl₂	104.7*	96.1*	92.7*
CdCl₂	102.1	98.3	99.3
PbCl₂	66.8*	68.3*	70.2*
SnCl₂	69.1*	59.2*	32.2*
SrCl₂	65.8*	76.2*	75.5*
FeCl₃	64.1*	54.8*	46.4*
CrCl₃	63.9*	67.8*	68.8*
SbCl₃	54.3*	40.1*	14.3*

[a]Values with an asterisk differ significantly ($p < 0.01$).

Table 3. Effect of Various Chemicals on Acid Protease Activity from *A. niger* BCRC 32720

chemical[b]	relative activity[a] (%)			
	0.1 mM	0.5 mM	1 mM	2 mM
none		100.0	100.0	100.0
EDTA		108.5*	105.9*	102.3*
NEM		98.2	93.6*	88.5*
IAA		102.2*	94.8*	86.1*
NaN₃		100.4	99.6	98.1*
SDS		72.7*	62.4*	10.9*
urea		101.6	100.1	99.5
PMSF	27.6*	28.5*	29.2*	

chemical[b]	relative activity[a] (%)			
	0.1 μM	1 μM	10 μM	100 μM
E-64	99.8	99.4	99.1	
pepstatin A	0.0*	0.0*	0.0*	
leupeptin		84.5*	77.0*	62.6*
TLCK		77.0*	64.2*	11.1*
TPCK		84.6*	79.1*	74.2*

[a]Values with an asterisk differ significantly ($p < 0.01$). [b]EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; NaN₃, sodium azide; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; E-64, 1-(ʟ-*trans*-epoxysuccinyl-leucylamido-4-guanidinobutane); TLCK, tosyllysine chloromethyl ketone; TPCK, tosyl phenylalanine chloromethyl ketone.

(hemoglobin as substrate), respectively. The optimal pH values of all acidic proteases, E₁, E₁ₐ, E₁ᵦ, and E₂, extracted from the broth of *A. oryzae* were 4.2 (hemoglobin as substrate).[35] From the data obtained in this study, the optimal pH for the hemoglobin was much lower than those from *Aspergillus* cited above. The optimal temperature of purified acidic protease was

Table 4. Effect of Various Reductants on Acid Protease Activity from *A. niger* BCRC 32720

reductant[b]	relative activity[a] (%)		
	1 mM	5 mM	10 mM
none	100.0	100.0	100.0
cysteine	126.8*	136.1*	140.3*
DTT	108.9*	106.7*	106.1*
GSH	100.3	105.9*	111.1*
β-ME	104.6*	105.2*	106.7*

[a]Values with an asterisk differ significantly ($p < 0.01$). [b]DTT, dithiothreitol; GSH, glutathione; β-ME, β-mercaptoethanol.

50 °C; however, its activity was quickly disappeared at 60 °C (Figure 3). This phenomenon was similar to the aspartic protease purified from *Phycomyces blakesleeanus*.[31] In this study, the purified acidic protease was very stable at temperature ≤40 °C, but unstable at temperature ≥50 °C (Figure 3). *A. oryzae* can produce various proteases with optimal pH at 2.5−5.0 and optimal temperatures at 50−70 °C.[28,35−37] They were very stable at pH 2.5−6.5.[10,26] However, their thermostability highly varied with the species such as *Rhizopus oryzae* (30−45 °C), *A. oryzae* MTCC 5341 (40−57 °C),[36,38] *A. niger* I1 (30−40 °C),[10] and *M. pilosus* (25−55 °C).[37] The acidic proteases A$_1$ and A$_2$ extracted from commercial *A. oryzae* (Takadiastase) were very stable even after 10 min of incubation at 55 and 40 °C, respectively, using hemoglobin as substrate.[34] According to Fukumoto et al.,[26] about 90% activity of the acidic protease from *Rhizopus chinensis* was left after 15 min of heating at 60 °C. These phenomena were quite similar to that obtained in this study, which was very stable at temperature ≤40 °C, but unstable at temperature ≥50 °C, with 50% of activity lost after 30 min of incubation at 50 °C and completely lost after 30 min of incubation at 60 °C (Figure 3). According to the previous studies, the acidic proteases seemed to be heat-unstable.

Effect of Metal Ion. Fe^{2+} highly activated, whereas Ag^+, Hg^{2+}, Pb^{2+}, Sn^{2+}, Sr^{2+}, Fe^{3+}, Cr^{3+}, and Sb^{3+} strongly inhibited, the purified acid protease ($p < 0.01$) (Table 2). Sawada[30] reported that Hg^{2+} highly inhibited, whereas Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Sr^{2+}, and Zn^{2+} activated, the acid protease from *Paecilomyces varioti*.[28] Hg^{2+} highly inhibited that from *A. oryzae*,[28] whereas Fe^{2+} strongly inhibited that from *R. chinesis*.[26] However, according to Siala et al.,[10] most of the metals, except Cu^{2+}, used in their study did not affect that from *A. niger* I1. Cu^{2+} and Mn^{2+} activated, but Mo^{3+} strongly inhibited, the MpiAP1 and MpiAP2 from *M. pilosus*.[37]

Effects of Inhibitors and Chemicals. Pepstatin A completely inhibited, whereas leupeptin, PMSF, TLCK, and TPCK highly inhibited, the purified acid protease ($p < 0.01$) (Table 3). However, E-64, EDTA, IAA, NaN_3, NEM, and urea did not affect the purified acid protease (Table 3). According to Siala et al.,[10] and Vishwanatha et al.,[38] pepstatin A highly inhibited the acid proteases from *A. niger* I1 and *A. oryzae* MTCC 5341, but EDTA, IAA, PMSF, and antipain hydrochloride did not affect their activity. This phenomenon was similar to that from *P. blakesleeanus*.[31] Pepstatin is a potent inhibitor of aspartyl proteases and nearly all acid proteases,[39] whereas leupeptin inhibits serine, cysteine, and threonine proteases, but does not inhibit α-chymotrypsin or thrombin.[40] TPCK is the irreversible inhibitor of chymotrypsin. It also inhibits some cysteine proteases.[41] According to the data obtained, it might be a aspartic protease, with cysteine groups near the active site.

Effect of Reductants. β-ME, cysteine, DTT, and GSH could significantly activate the purified acid protease, except 1 mM GSH (Table 4). However, 5 mM β-ME had no effect on the acid protease from *A. niger* I1.[10] These data further confirmed that the purified acid protease contained cysteine near the active site.

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence was SKGSAVTT (Table 5). After the comparison with those on the NCBI (www.ncbi.nlm.nih.gov) BLAST system, this N-terminal amino acid sequence is the same as that of CBS 513.88 aspergillopepsin A-like aspartic endopeptidase from *A. niger*. This enzyme was composed of 394 amino acids with 1−69 of leader sequence (prosequence) and 70−394 of mature protein. Inhibitor binding sites were at 101, 103, 143−145, 283, and 285−287, whereas enzyme catalyzing sites were at 101−104 and 283−286. Among these amino acids, 101 and 283 were aspartic acid. This could further confirm the purified acid protease is aspartic protease.

In conclusion, the pure acid protease could have almost 25% recovery, with an optimal pH at 2.5 and thermal stability at ≤40 °C, which would be good for use as a digestive aid.

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Notes

The authors declare no competing financial interest.

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Table 5. Comparison of N-Terminal Amino Acid Sequences of Acid Protease from *A. niger* BCRC 32720 with the Amino Acid Sequences of *A. niger* CBS 513.88 Aspergillopepsin A-like Aspartic Endopeptidase[a]

origin	
1	MVVFSKTAAL VLGLSSAVSA APAPTRKGFT INQIARPANK TRTINLPGMY ARSLAKFGGT
61	VPQSVKEAA*S KGSAVTT*PQN NDEEYLTPVT VGKSTLHLDF DTGSADLWVF SDELPSSEQT
121	GHDLYTPSSS ATKLSGYTWD ISYGDGSSAS GDVYRDTVTV GGVTTNKQAV EAASKISSEF
181	VQNTANDGLL GLAFSSINTV QPKAQTTFFD TVKSQLDSPL FAVQLKHDAP GVYDFGYIDD
241	SKYTGSITYT DADSSQGYWG FSTDGYSIGD GSSSSSGFSA IADTGTTLIL LDDEIVSAYY
301	EQVSGAQESE EAGGYVFSCS TNPPDFTVVI GDYKAVVPGK YINYAPISTG SSTCFGGIQS
361	NSGLGLSILG DVFLKSQYVV FNSEGPKLGF AAQA

[a]The N-terminal amino acid sequences of acid protease from *A. niger* BCRC 32720 are the same as those in *italics* and underlined from *A. niger* CBS 513.88 aspergillopepsin A-like aspartic endopeptidase.

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