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# Enzymatic Characteristics of a Recombinant Neutral Protease I (rNpI) from *Aspergillus oryzae* Expressed in *Pichia pastoris*

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Supporting Information

**ABSTRACT:** A truncated neutral protease I (NpI) from *Aspergillus oryzae* 3.042 was expressed in *Pichia pastoris* with a high enzyme yield of 43101 U/mL. Its optimum pH was about 8.0, and it was stable in the pH range of 5.0−9.0. Its optimum temperature was about 55 °C and retained >90% activity at 50 °C for 120 min. Recombinant NpI (rNpI) was inhibited by $Cu^{2+}$ and EDTA. Eight cleavage sites of rNpI in oxidized insulin B-chain were determined by mass spectrometry, and five of them had high hydrophobic amino acid affinity, which makes it efficient in producing antihypertensive peptide IPP from $\beta$-casein and a potential debittering agent. The high degree of hydrolysis (DH) of rNpI to soybean protein (8.8%) and peanut protein (11.1%) compared to papain and alcalase makes it a good candidate in the processing of oil industry byproducts. The mutagenesis of $H^{429}$, $H^{433}$, and $E^{453}$ in the deduced zinc-binding motif confirmed rNpI as a gluzincin. All of these results show the great potential of rNpI to be used in the protein hydrolysis industry.

**KEYWORDS:** *Aspergillus oryzae, recombinant neutral protease I, Pichia pastoris, expression, enzymatic characteristics*

## INTRODUCTION

*Aspergillus oryzae*, a fungus widely used in the production of traditional Asian foods such as rice wine, soy sauce, and soybean paste, secretes abundant hydrolysis enzymes such as amylases and protease. *A. oryzae* is considered to be a "safe" organism to produce proteases used in the food industry.[1] *A. oryzae* proteases show high efficiency in the hydrolysis of plant protein, such as soybeans.[2] It was recently reported that a crude protease extract from *A. oryzae* produced a higher degree of peanut meal protein hydrolysis (43.4%), compared to commercial proteases Alcalase, Protamex, and papain.[3] In addition, its hydrolysate had better sensory taste than other hydrolysates.

The whole genome of *A. oryzae* was sequenced in 2007.[4] About 134 peptidase genes consisting of 69 exopeptidases and 65 endopeptidases were predicted and occupy roughly 1% of total genes in its genome.[5] Much work has been carried out to purify and characterize the peptidase from *A. oryzae*,[1,6] but only a few peptidase amino acid and/or nucleotide sequences (about 20) have been reported.[5] To acquire higher production of *A. oryzae* proteases for characteristic identification and utility, recombination technology has been used to overexpress some of these proteases, such as the aspartyl aminopeptidase,[7] prolyl aminopeptidase,[8] leucine aminopeptidase,[9] alkaline protease (Alp),[10] and serine carboxypeptidase.[11] *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Pichia pastoris*, and *A. oryzae* itself are usually used as hosts to express *A. oryzae* proteases.[9,10] For the expression of Alp, the *P. pastoris* expression system seems to be most efficient.[10]

Among the *A. oryzae* proteases, two neutral metalloproteases, neutral protease I (NpI) and neutral portease II (NpII), have been purified since 1973.[12] NpII shows high activities on peptide bonds formed by basic amino acids, such as His and Arg, and its cDNA has been cloned and expressed in *S. cerevisiae*.[12] The complete cDNA of NpI has also been cloned and sequenced with a putative protein molecule weight of 69 kDa (NCBI accession no. AF099904). However, the enzyme characteristics of NpI were not well described, and its heterogeneous expression has not been reported. In 2009, when the proteolytic enzymes from *A. oryzae* capable of producing antihypertensive peptide Ile-Pro-Pro (IPP) were identified, NpI was purified as the key protease in the release of IPP, and it is efficient in cleaving an -X-Pro-Pro sequence from casein's C-terminus.[6]

Both NpI and NpII belong to the zinc-dependent class of metalloendopeptidases.[12] According to the MEROPS classification system (http://merops.sanger.ac.uk), they belong to the zincins tribe of the metallopeptidases (MPs) class, characterized by a catalytic zinc ion that cleaves peptide bonds with the participation of a solvent molecule and a polarizing general base/acid, usually a glutamate.[13] Gomis-Ruth suggested a "standard orientation" for MPs, which entails a frontal view of the horizontally aligned active site cleft.[14] The back of the cleft is framed by a helix comprising a zinc-binding

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consensus sequence, HEXXH or HXXEH, which provides two histidines as the first and second protein ligands to bind catalytic metal and glutamate required for catalysis. In the zincins clan, the gluzincin family is also characterized as having glutamate as the third zinc ligand, and it is separated from the second ligand by a long amino acid spacer.[14,15] In the cDNA of NpI, a consensus motif, HExxH-19aa-E, of gluzincins has been found.

In this paper, to identify NpI's enzyme characteristics, the cDNA of NpI from *A. oryzae* 3.042 was cloned and expressed in *P. pastoris*. The recombinant NpI was purified, and its optimum pH, optimum temperature, thermal stability, pH stability, and inhibitors were tested. The substrate specificity of rNpI was assessed using the oxidized insulin B-chain. Site-directed mutagenesis of deduced zinc ligand anmino acids was carried out to verify if NpI belongs to gluzincins, and the structure associated with hydrolysis was discussed. Hydrolysis efficiency on vegetable proteins was also evaluated with soy and peanut proteins.

## MATERIALS AND METHODS

**Strains, Plasmids, Enzymes, Reagents.** *A. oryzae* 3.042 was preserved in our microbiology laboratory. *P. pastoris* KM71 strain ($his^-$,$mut^s$), pPIC9K plasmid vector, and Trizol reagent were from Invitrogen (Carlsbad, CA, USA). *E. coli* DH5$\alpha$, pSIMPLE-18 *Eco*R V/BAP vector, and enzymes for manipulating DNA and RNA were purchased from Takara (Dalian, China). Primers used in this study were obtained from Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). All of the protocols and culture media for *P. pastoris* KM71 strain were performed according to the manual of the *Pichia* Expression Kit from Invitrogen.

**NpI cDNA Cloning from *A. oryzae*.** Ten grams wheat bran medium (96.41% wheat bran, 1.23% maltose, 1.56% peptone, 0.74% $KH_2PO_4$, 0.06% $FeSO_4{\cdot}7H_2O$, and 1 mL water per gram dry wheat bran) was sterilized at 115 °C for 15 min, cooled to 25 °C, and inoculated with a piece of mycelia of *A. oryzae* 3.042 (0.5 cm × 0.5 cm). The mixture was cultured for 48 h at 24.1 °C. Mycelia of *A. oryzae* were collected and quickly frozen in liquid nitrogen. The total RNA of mycelia was extracted according to the guideline of Trizol reagent. Reverse transcription was performed using an M-MLV RTase cDNA Synthesis Kit (Dalian, China). Primer 1, 5′-ATGCGGGGT-CTTCTACTAGC-3′, and primer 2, 5′-CTAGCAAGCATCAGA-GGGCACCT-3′, were designed on the basis of the sequence reported in Genbank (accession no. AF099904.1). PCR conditions were as follows: 1 cycle of 94 °C for 2 min, 29 cycles at 98 °C for 10 s, 55 °C for 15 s, 72 °C for 2 min, followed by 1 cycle of 72 °C for 10 min. The purified PCR product was cloned to pSIMPLE-18 *Eco*R V/BAP vector and then transformed into *Escherichia coli* DH5$\alpha$ competent cells. The transformants (pSIMPLE-18 *Eco*R V/BAP/Np I) were screened on an LB plate. The pSIMPLE-18 *Eco*R V/BAP/Np I vector was sequenced and identified.

**Construction of Recombinant NpI (rNpI) Expression Vector.** The identified pSIMPLE-18 *Eco*R V/BAP/Np I vector was used as a template. The truncated NpI gene (without signal peptide encoding sequence) was amplified by PCR using primer 3 (5′-CCGGAATTC-CATCCTACCCATCATGCAC-3′) and primer 4 (5′-CAGTT-TAGCGGCCGCTTAGTGGTGGTGGTGGTGGTGGCAAGCAT-CAGAGGGCACCT-3′). The underline indicates the *Eco*RI, *Not*I restriction site and a 6× His tag. The PCR product was purified and cut with *Eco*RI and *Not*I and then ligated to a pPIC9k vector. The pPIC9K/Np I vector was transformed to *E. coli* DH5$\alpha$. Finally, the pPIC9K/Np I vector was identified by PCR, restriction digestion, and sequencing.

**Transformation and Screening of the Recombinant *P. pastoris*.** The pPIC9K/Np I vector was linearized with *Sac*I and transformed into *P. pastoris* KM71 with a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA). The strain transformed by vacant pPIC9K vector was used as a control. The conditions of electrotransfomation were as follows: 2 mm electroporation cuvette, 1.5 kV, 20 $\mu$F, 200 Ω, 5−10 ms. After the pulse, the electroporation cuvette was quickly put into 0.8 mL of ice-cold 1.0 M sorbitol and incubated in ice water for 5 min. Then the transfomants were spread on the MD plates and grown at 28 °C until single colonies appeared (2−4 days). To verify the NpI gene's integration into the genome of *P. pastoris* KM71 strains, the transformants were identified by genomic PCR test using two pairs of primers, primer 3, primer 4, and AOX primers (5′AOX, 5′-GACTGGTTCCAATTGACAAGC-3′; and 3′AOX, 5′-GGCAAATGGCATTCTGACATCC-3′).

**Expression of rNpI in Triangular Flask and Fermenter.** The recombinant *P. pastoris* KM71 was inoculated to 25 mL of BMGY medium in a 250 mL triangular flask and shaken (200 rpm) for about 24 h at 30 °C to the log phase growth ($OD_{600}$ = 5−6). The yeast cells were harvested by centrifuging at 6000*g* for 10 min at 4 °C and then resuspended with 50 mL of BMMY medium in 500 mL triangular flasks. The initiated OD of the BMMY media was 2.0−4.5. The flask was shaken (250 rpm) at 28 °C for 6 days. The expression of rNpI was induced by the addition of 500 $\mu$L of methanol every 24 h. The supernatant of the fermentation broth at different time points (every 24 h) was collected by centrifugation at 10000*g* for 5 min at 4 °C. The wet weight of yeast cells was recorded. SDS-PAGE was performed, and the gel was stained by 0.2% CBB R-250. The rNpI with 6× His tag was confirmed using InVision His-tag In-gel stain from Invitrogen.

For expression of the rNpI in the fermenter, the recombinant *P. pastoris* KM71 strain was inoculated into a triangular flask containing YPD medium and grown at 30 °C and 250−300 rpm until $OD_{600}$ reached 8.0−10.0. The culture was inoculated in an FUS-10L full automatic fermenter (Guo-qiang, China) for fermentation, which contained 6.3 L of fermentation basal salts medium (pH of the medium was adjusted to 5.0 with 28.0% ammonium hydroxide). The fermentation process included three stages. The first stage was the glycerol batch phase. In this stage, dissolved oxygen (DO) was kept above 40% by controlling the air flow and stirring speed. When the glycerol in the mediumwas completely consumed, the DO quickly increased to 100% (about 18−24 h), which indicated the end of the first stage. The second stage was the glycerol-fed batch phase. Glycerol mixture (50% w/v glycerol/water), containing 12 mL of PTM trace salts per liter was fed to initial fermentation volume from stage 1. The feed rate was 12 mL/h/L and with the DO kept above 20%. This stage ended when the wet weight of yeast cells was >180.0 g/L fermentation broth. The third stage was the methanol-fed batch induction phase. At this phase, the temperature was adjusted to 28.3 °C, the pH adjusted to 6.0 with 28.0% ammonium hydroxide, and DO kept between 20 and 30% by controlling the stirring speed. rNpI was induced to express by the addition of methanol, which contained 12 mL PTM trace salts per liter of methanol. The fermentation volume from stage2 was fed methanol at an initiate rate of 1 mL/h/L. After 2 h, the feed rate was increased by 20% increments every 1 h. The final feed rate of 5 mL/h/L was maintained until the end of fermentation. Throughout these three stages, the wet weight of yeast cells was monitored and the protease activity was tested.

**Protease Activity Assay.** The protease activity of rNpI was determined by using the Folin−phenol reagent method. Briefly, 1 mL of diluted fermentation broth was added into 1 mL of 2% casein solution (dissolved in 0.02 M, pH 7.5, phosphate buffer) and kept at 40 °C for 10 min. The reaction was terminated by adding 2 mL of 0.4 M trichloroacetic acid. After incubation for 20 min at 40 °C, the mixture was centrifuged at 13000*g* for 10 min. One milliliter of supernatant was added into 5.0 mL of 0.4 M $Na_2CO_3$ and 1.0 mL of Folin−phenol reagent and incubated for 20 min at 40 °C. The OD value was then measured at 660 nm. One unit of protease activity was defined as the amount of enzyme that hydrolyzed the casein and produced 1 $\mu$g of tyrosine per minute at 40 °C.

**Purification of rNpI.** The supernatant of the fermentation broth was collected by centrifuging at 10000*g* for 10 min at 4 °C. After dialysis in 0.02 M phosphate buffer (pH 7.4) at 4 °C overnight, the supernatant was filtered through a 0.22 $\mu$m filter and collected as the

crude protease solution. The crude protease solution was then loaded onto a nickel-affinity chromatography column (HisTrap FF 5 mL, GE Healthcare). The column was washed with buffer A (20 mM phosphate buffer, 20 mM imidazole, 0.5 M NaCl, pH 7.4) until the eluant's OD value was <0.0001 at 280 nm. The column was then eluted by a gradient mixture of buffer A and buffer B (20 mM phosphate buffer, 500 mM imidazole, 0.5 M NaCl, pH 7.4). The eluant containing the rNpI was collected and concentrated by an ultrafilter system. The concentrated eluant was then loaded onto a Sephadex G-75 column (GE Healthcare), which was prebalanced with 500 mM phosphate buffer (pH 8.0) at a flow rate of 35.0 mL/h. The eluant with rNpI was collected and concentrated as purified rNpI sample for further study. SDS-PAGE was performed to verify its purity.

**Zymography of rNpI.** Zymography was carried out to visualize the activity and homogeneity of the purified rNpI. The sample of purified rNpI was mixed with loading buffer without $\beta$-mercaptoethanol and was not heated before electrophoresis. Twelve percent polyacrylamide separating gel contained 0.3% casein as the substrate was used. After a run at 4 °C, the gel was immersed in phosphate buffer (pH 7.5) at 40 °C for 30 min and then stained with 0.2% CBB R-250 overnight. The gel was destained until a clear transparent band was visible against a blue background. The transparent band in the gel indicated the hydrolysis activation of rNpI.

**Biochemical Characterization of rNpI.** Optimum temperature, optimum pH, thermal stability, pH stability, metal ion test, and inhibitor tests of the recombinant protease were measured as described before.[9] The metal cations and protease inhibitors that were tested are listed in Table 1.

**Table 1. Effects of Metal Cations and Protease Inhibitors on rNpI Activity**

| reagent | concentration (mM) | enzyme activity[a] (%) |
|---|---|---|
| none | | 100 |
| $Cu^{2+}$ | 1 | 10.7 ± 2.1 |
| $Fe^{2+}$ | 1 | 45.4 ± 2.5 |
| $Co^{2+}$ | 1 | 55.5 ± 3.2 |
| $Mn^{2+}$ | 1 | 95.1 ± 2.4 |
| $Mg^{2+}$ | 1 | 93.8 ± 4.6 |
| $Ca^{2+}$ | 1 | 92.0 ± 3.4 |
| $Zn^{2+}$ | 1 | 47.9 ± 2.2 |
| pepstatin | 1 | 101 ± 3.1 |
| EDTA | 1 | 10.1 ± 3.8 |
| PMSF | 1 | 98.2 ± 2.9 |

[a]The enzyme activity of recombinant rNpI without the addition of metal cations and protease inhibitors was defined as 100%. The effects of metal cations and protease inhibitors on rNpI activity were calculated as the mean and standard error of three trials.

**Hydrolysis of the rNpI to Oxidized Insulin B-Chain.** The cleavage sites of oxidized insulin B-chain were identified as described by Shahrzad Bakhtiar with some modification.[16] One hundred micrograms of oxidized insulin B-chain (Sigma) was dissolved in 1 mL of ammonia−water (pH 8.0), and 30 U of purified rNpI was added. After incubation at 40 °C for 1 h, the mixture was immersed in boiling water for 5 min to inactivate the enzyme. The cleavage products of oxidized insulin B-chain were identified by a mass spectrometer (API4000 Q-Trap, AB SCIEX, USA).

**Hydrolysis of Soy Protein and Peanut Protein by rNpI.** Hydrolysis of the recombinant protease to soy protein and peanut protein was performed according to the method of Zhao with some modification.[3] One hundred milliliters of 5.0% soy protein and peanut protein were respectively prepared with phosphate buffer (pH 8.0), followed by incubation in boiling water for 15 min, and then cooled to 50 °C. The protein solution was added to 10000 U of rNpI and was incubated for 4 h, followed by centrifugation at 8000*g* for 10 min. The degree of hydrolysis (DH) was determined by formol titration method as described before.[3] Hydrolysis of commercial proteases, papain and Acalse, was allso carried out according to the same method described for their optimum pH and temperature.

**Site-Directed Mutagenesis of Zinc Ligands of rNpI.** To verify if NpI belongs to gluzincins, mutagenesis of three deduced zinc ligands $H^{429}$, $H^{433}$, and $E^{453}$ (including mutants H429G, H433G, E453G, H433E, H433D, E453H, E453C, E453D, and H433E-E453H) was performed using a TaKaRa MutanBEST Kit according to the manufacturer's instructions. The plasmid, pSIMPLE-18 *Eco*RV/BAP/Np I, was used as the template for PCR of site-directed mutagenesis. The mutants were confirmed by sequencing. The mutational genes were transformed into the *P. pastoris* KM71 strain and induced for expression as described above. The expression of mutational recombinant proteases was detected by SDS-PAGE, and their protease activities were tested as described above.

## RESULTS AND DISCUSSION

**cDNA Cloning of NpI from *A. oryzae* 3.042 and Expression Vector Construction.** The cDNA of NpI from *A. oryzae* 3.042 was cloned into the pSIMPLE-18 *Eco*R V/BAP vector. Its sequence has some differences from the sequence reported in Genbank (accession no. AF099904.1). The reported base sequence T, A, T, C, and G at sites 828, 891, 1066, 1067, and 1512, respectively, was substituted by G, G, C, T, and A in the sequence we cloned (see Figure 1 in the Supporting Information). Only one missense mutation of $S^{356}$ was substituted by $L^{356}$; the other four mutations were synonymous mutations. A truncated NpI gene without signal peptide obtained from pSIMPLE-18 *Eco*R V/BAP/Np I plasmid was cloned into pPIC9K vector at the site downstream of the vector's $\alpha$-factor signal site. After verification by sequencing, this expression vector pPIC 9K/Np I (see Figure 2 in the Supporting Information) was transformed to *P. pastoris* KM71. The genomic PCR tests of the transformants showed the NpI gene had been integrated into the genome of *P. pastoris* KM71. Transformants containing multiple NpI copies were screened by YPD-Geneticin plates.

**Expression and the rNpI in *P. pastoris*.** The recombinant *P. pastoris* was induced to express the neutral protease gene by adding methanol. The SDS-PAGE of the supernatant of fermentation broth collected at 120 h from 500 mL triangular flasks is shown in Figure 1A. In this gel, a band of rNpI was seen and confirmed using InVision His-tag In-gel Stain from Invitrogen (Figure 1B). After separation by nickel-affinity chromatography and Sephadex G-75 chromatography, a clear purified rNpI bank on the gel was seen (Figure 1C), and its activity was confirmed by the zymography (Figure 1D). The molecular mass of purified rNpI was calculated as 57 kDa according to the plot of relative mobility versus molecular mass. The mature wild-type NpI was isolated by Gotou from *A. oryzae*, and its molecular weight is about 46 kDa.[6] The putative open reading frame of NpI gene was reported as 69014 Da (NCBI accession no. AAF04628). The putative molecular weight of this recombinant truncated NpI without its own singnal peptide, but with a His tag, is 43225. However, the molecular weight of rNpI in this study was about 57 kDa. This may be due to the glycosylation when it is expressed in *P. pastoris* KM71.

The yeast cell's wet weight of fermentation broth from fermentation in triangular flasks and fermentor was tested. The enzyme activity of broth supernatant is shown in Figure 2. It was shown that protease in triangle flash fermentation reached its highest activity (8469 U/mL fermentation broth) at 120 h. The wet weight increased until 72 h and plateaued at about

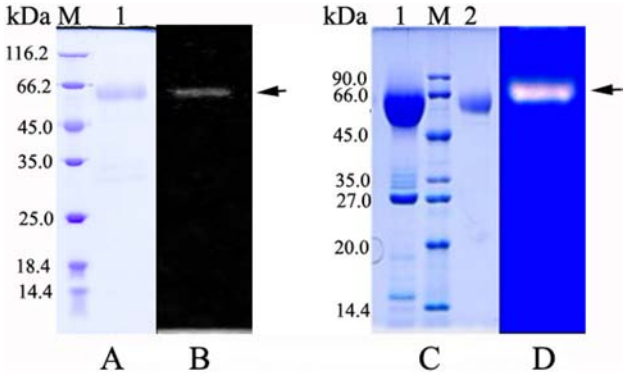


**Figure 1.** Expression and purification of rNpI. (A) SDS-PAGE analysis of the supernatant of fermentation broth in 500 mL triangular flask. Lanes: M, protein molecular weight markers; 1, supernatant of fermentation broth. (B) Gel stained with InVision His-tag In-gel stain. The arrow shows rNpI with a C-termial His-tag. (C) SDS-PAGE analysis of the purified rNpI. Lanes: M, protein molecular weight markers; 1, enzyme through nickel-affinity chromatography; 2, purified rNpI through Sephadex G-75 chromatography. (D) Zymography of the purified rNpI, showing proteolytic activity of the rNpI.

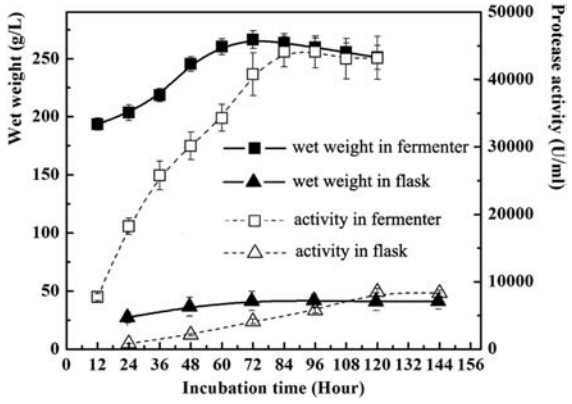


**Figure 2.** rNpI expression of reconstructed *P. pastoris* in 500 mL triangular flask and 10 L fermentor: (■, ▲) yeast wet weight of fermentation broth in 10 L fermentor and 500 mL triangular flask, respectively; (□, △) protease activity of fermentation broth in 10 L fermentor and 500 mL triangular flask, respectively.

41.05 g/L fermentation broth for the remaining time, whereas in the 10 L fermentor, the peak protease activity of about 43101 U/mL fermentation broth was reached at 84 h and the peak wet weight of 266.4 g/L fermentation broth was reached at 72 h. With the aerating and agitation system, fermentation in the 10 L fermentor yielded a higher density of yeast cells and produced much more enzyme compared with fermentation in triangular flasks.

*A. oryzae* has a long industrial history for protease production by solid-state or submerged formation. However, numerous problems exist in both processes such as incomplete nutrient utilization and frequent bacterial contamination.[17] The purification of *A. oryzae* proteases is also complicated due to its high diversity of protease production, which limits their utility. The rNpI obtained from our constructed recombinant *P. pastoris* is highly expressed and easily purified from fermentation broth, which holds great potential to be used in the protein hydrolysis industry.

**Biochemical Characterization of the rNpI.** The pH and temperature effects on the activity of rNpI are shown in Figure 3. Its optimum pH was about 8.0. When the pH was <6.0 or >9.0, the enzyme activity of rNpI rapidly decreased (Figure

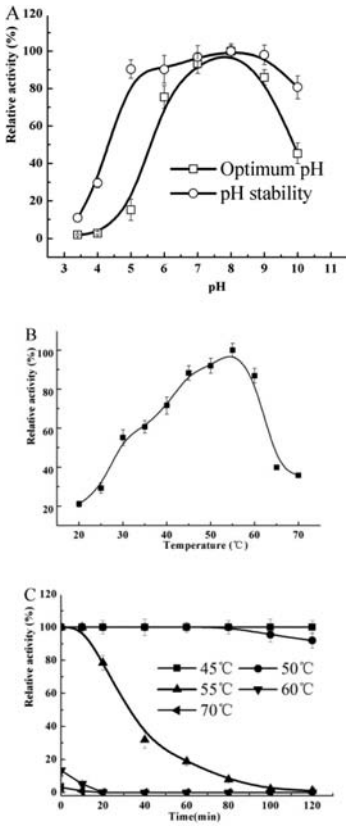


**Figure 3.** Effects of pH and temperature on activity and stability of rNpI: (A) effects of pH on the activity and stability of rNpI; (B) effects of temperature on the activity of rNpI; (C) effects of temperature on the stability of rNpI.

3A). For pH stability, the rNpI showed to be quite stable in the pH range of 5.0−9.0, but quickly lost its activity at pH >9.0 and <5.0 (Figure 3A). The optimal pH and pH stability of rNpI were similar to those of other neutral proteases, such as the neutral protease from *Bacillus stearothermophilus*, *Bacillus halodurans*, and *Thermoactinomyces* species 27a.[18−20] The optimal temperature of rNpI was about 55 °C (Figure 3B). For temperature stability, rNpI retained 100% activity at 45 °C and >90% activity at 50 °C for 120 min, whereas it rapidly lost its activity at 55 and 60 °C (Figure 3C). The temperature stability of rNpI was higher than that of the neutral protease from *Bacillus halodurans* but lower than that of the neutral protease from *B. stearothermophilus*, which had about 80% activity after 1 h at 65 °C.

The effects of metal cations and protease inhibitors on the activity of rNpI are shown in Table 1. $Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ had no significant effect, whereas $Cu^{2+}$ had the strongest inhibitory effect on rNpI's activity. The inhibitory effects of $Cu^{2+}$ on the rNpI might be due to binding of the inhibitory metal cations monohydroxide to the rNpI's catalytic metal ion (zinc).[21] Among protease inhibitors, EDTA strongly inhibited rNpI's activity, but PMSF and pepstatin did not. These results indicate that rNpI is a metallopeptidase.

**Cleavage Sites of the rNpI in the Oxidized Insulin B-Chain.** Cleavage sites of the rNpI in the oxidized insulin B-chain were determined by mass spectrometry (see Figure 3 in

the Supporting Information). Our results show rNpI had eight cleavage sites. Compared to other commercial proteases, such as trypsin,[22] papain,[22] subtilisin,[22] subtilisin BPN,[23] and elastase,[24] it has more cleavage sites (Figure 4). This indicates

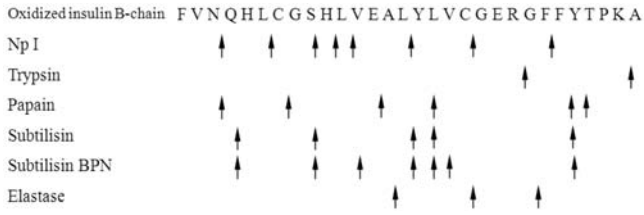


**Figure 4.** Cleavage sites of rNp I in oxidized insulin B-chain determined by mass spectrometry compared with reported commercial proteases. Arrows show the cleavage sites.

rNpI may have a high efficiency in protein hydrolysis and potential to be used in the food industry. It is interesting to find that five of these cleavage sites, including HL|CG, SH|LV, HL|VE, AL|YL, and GF|FY, have a hydrophobic amino acid in either the P1 or P1′ position or two hydrophobic amino acids in both the P1 and P1′ positions. Its high affinity to hydrophobic amino acids may provide the possibility of using rNpI in food protein hydrolysate debittering.[25] This result is coincident with previous reports that proteases from *A. oryzae* have an advantage for their utilization as debittering agents.[26] Zhao recently also reported that the peanut protein hydrolysate produced by a crude protease extract from *A. oryzae* has better taste than other hydrolysates.[3] Gotou reported that NpI purified from *A. oryzae* is important for the release of IPP (an antihypertensive peptide) from $\beta$-casein due to its efficient cleavage at a PP|LT site with hydrophobic amino acid in both the P1 and P1′ positions, which is coincident with our results.

**Hydrolysis of Soy Protein and Peanut Protein by rNpI.** Soy protein and peanut protein were used as the substrate and hydrolyzed by rNpI and compared with two commercial proteases, papain and Alcalase. For soybean protein, the DH of rNpI was 8.8%, higher than that of papain (7.5%) but lower than that of Alcalase (10.5%). For peanut protein, the DH of rNpI was 11.1%, much higher than that of both papain (7.5%) and Alcalase (7.0%). These results show that rNpI is quite efficient in plant protein hydrolysis and can be a good candidate in plant food industrial processing. Zhao et al. have reported that when peanut meal was hydrolyzed by the crude protease extract from *A. oryzae* at pH 7.0, its DH was 43.4%, significantly higher than that of papain, Protamex, and Alcalase.[3] *A. oryzae* has two neutral proteases, NpI and NpII.[12] Because our results show that rNpI has high DH for peanut protein, we believe that NpI may contribute a lot to the hydrolysis of peanut meal by *A. oryzae*'s crude protease.

**Site-Directed Mutagenesis of Zinc Ligands of rNpI.** To verify if NpI belongs to the gluzincins, three residues ($H^{429}$, $H^{433}$, and $E^{453}$) in the deduced zinc-binding motif HExxH-19aa-E of NpI were mutated. When these three residues were mutated to Gly, all three mutants, H429G, H433G, and E453G, showed no expression of rNpI in *P. pastoris*, which indicates these three residues are important for zinc binding. When they were mutated to be Gly, the rNpI may not have folded correctly without the zinc binding and may have been degraded by proteases in *P. pastoris*. It has been reported that in zinc enzymes zinc forms complexes with nitrogen, oxygen, and sulfur of His, Glu, Asp, and Cys.[15] When we mutated $H^{429}$, $H^{433}$, and $E^{453}$ to be these four amino acids, all mutants H433E, H433D, E453H, E453C, and E453D did not express rNpI. When we changed the position of $H^{433}$ and $E^{453}$, the mutant H433E-E453H also did not express rNpI. All of these results indicate that NpI is a gluzincin and that its HExxH-19aa-E motif is highly conserved.

In summary, we have cloned the NpI gene from *A. oryzae* and identify it as a gluzincin with a highly conserved consensus HExxH-19aa-E active motif. rNpI was efficiently expressed in *P. pastoris* with high enzyme yield of 43101 U/mL in a 10 L fermentor. Its extensive cleavage sites in the oxidized insulin B-chain and its high affinity to hydrophobic amino acids make it a good candidate in the protein hydrolysis industry and a potential debittering agent in protein hydrolysates. Soybean and peanut protein hydrolysis show rNpI is quite efficient in plant protein processing, which hold great potential for use in the processing of oil industry byproducts. The applied research of rNpI combined with other exo- or endopeptidases will be carried out using different substrate,s and its utility will be evaluated in our future studies. The purified rNpI is currently being crystallized in our laboratory to identify the three-dimensional structure for elucidation of its hydrolysis mechanisms.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS USED

PCR, polymerase chain reaction; NpI, neutral protease I; rNpI, recombinant neutral protease I; AOX, gene encoding alcohol oxidase; MD, minimal medium containing dextrose; OD, optical density; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; YPD, yeast extract−peptone−dextrose medium; CBB R-250, Coomassie Brilliant Blue R-250; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid

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