Cloning, Expression and Biochemical Characterization of a new Carboxypeptidase from Aspergillus Niger

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Research Article

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Abstract

A new serine carboxypeptidase gene, *capA*, was identified in *Aspergillus niger* CBS 513.88 by reading genomic information and performing sequence alignment, and then, the gene was cloned and expressed in *Pichia pastoris*. In a shake-flask, the enzyme activity of the recombinant strain GS115 (pPIC9K-*capA*) reached 109.7 U/mL. The optimum temperature and pH for enzyme activity were determined to be 45°C and 6.0, respectively. After incubation at 40°C-50°C or at pH 4.0–8.0 for 1 h, the enzyme retained more than 80% or 60% of its initial activity. Mg^{2+} enhanced the activity of CapA, whereas Cu^{2+}, Fe^{2+}, Co^{2+} and PMSF inhibited its activity. Among the six substrates tested, five could be hydrolyzed by CapA. CapA had a broad substrate specificity and preferred the hydrophobic amino acids Leu and Lys in the C-terminus of proteins, and CBZ-Phe-Leu was the preferred substrate for CapA. The good thermostability, pH stability and hydrolysis characteristics provide a solid foundation for the application of CapA in the food and biotechnology fields.

Introduction

Carboxypeptidases (CPSs) are exoproteases that hydrolyze the C-terminal peptide bonds of proteins or polypeptides and release free amino acids individually. Carboxypeptidases are divided into serine carboxypeptidases (EC 3.4.16.), metal carboxypeptidases (EC 3.4.17.), and cysteine carboxypeptidases (EC 3.4.18.) based on the differences in their catalytic mechanisms (Jing et al. 2012). Carboxypeptidases are commonly used in the food industry to produce amino acids (Pinto et al. 2008), prepare oligopeptides (Morita et al. 2009), debitter protein hydrolysates and enhance flavor (Fu and Yang 2011). Some special carboxypeptidases are also used to cleave specific polypeptides (Pozzuolo et al. 2008) and amino acid sequences of polypeptides (Gao and Wang 2007) in the field of biotechnology.

Carboxypeptidases are found in animals, plants, fungi and bacteria (Breddam 1986), but the content of carboxypeptidase in animals and plants is low, the composition is complex, and the extraction cost is high. Microbial fermentation is the main method of obtaining carboxypeptidases. At present, carboxypeptidases from various sources, mainly microbial carboxypeptidases and especially fungal carboxypeptidases, have been cloned and expressed (Chen 2014). Among these identified carboxypeptidases, two kinds of carboxypeptidases originating from *A. niger* have been cloned and expressed: CPD-I (PepF) and CPD-II (PepG) (Dal et al. 1992).

The genomic sequence of *A. niger* CBS 513.88 was analyzed and published in 2007, but the *A. niger* protein database shows that there are many proteins with undetermined functions (Pel et al. 2007). In this paper, a new gene encoding a serine carboxypeptidase was found by analyzing the genome of *A. niger* CBS 513.88. The gene was cloned and expressed in *Pichia pastoris*, and the enzymatic properties of the recombinant enzyme were systematically analyzed. The research has laid a good foundation for further exploring the application value of this enzyme.

1 Materials And Methods

1.1 Materials and reagents

A.niger CGMCC 3.7193 was stored in the China Common Microbe Culture Collection Management Center (CGMCC); *E. coli* JM109, *P. pastoris* GS115 and the pPIC9K plasmid were obtained and stored in our laboratory. *A. niger* and *E. coli* were cultured on PDA medium and LB medium, respectively. *P. pastoris* was cultured in YPD, MD, BMGY and BMMY media. The preparation and culture methods were performed according to the Pichia Expression Kit (Version M) provided by Invitrogen. The PCR primers for CapA were F: 5'- GTAGTCCTCCAGCCAGAGGAACCATC-3', and R: 5'-TGCTCTAGATCACTCAGTAAAC GATGCCCCG-3'; the restriction enzyme sites are underlined, and these primers were generated by Sangon Biotech Co., Ltd. (Shanghai). Restriction endonuclease, *Pyrobest* DNA polymerase and T4 DNA ligase were purchased from the TaKaRa company; the Plasmid Extraction Kit and DNA Purification and Recovery Kit were from Beijing Zoman Biotechnology Co., Ltd; the RNAqueous™-Micro Total RNA Isolation Kit and SuperScript III First-Strand Synthesis System were from Invitrogen; and the following chemical substrates were synthesized by Ontores Biotech Co: CBZ-ala-Arg, CBZ-Pro-Gly, CBZ-Ala-Lys, CBZ-Gly-Ala, CBZ-Ala-Glu and CBZ-Phe-Leu.

1.2 Methods

1.2.1 Gene cloning and recombinant *P. patoris* construction

Total RNA was extracted from *A. niger*, and the first-strand cDNA was synthesized by reverse transcription. The first-strand cDNA was used as a template for PCR amplification with the synthesized primer, and the subsequent PCR product purification, enzyme digestion, ligation, *E. coli* JM109 transformation, and the positive clone screening was performed according to routine laboratory methods (Zhu and Wang 1994). The recombination plasmid linearization and electric transformation of *P. pichia* GS115 were conducted, and the recombinant *P. pastoris* GS115 (pPIC-*capA*) were screened, according to the methods provided by the Pichia Expression Kit.

1.2.2 Induction of expression and preliminary purification of recombinant enzyme

Recombinant *P. pastoris* GS115 (pPIC-CapA) was purified on YPD plates. Single colonies were selected and inoculated into YPD liquid medium (25 mL) and cultured at 30°C and 200 r/min for 18 h to 20 h. Then, 1% of the inoculation was transferred into BMGY medium (25 mL). The bacteria were cultured at 30°C and 200 r/min for 16 h-18 h until the cells reached the logarithmic stage of growth (OD600=2.0-6.0). Then, the bacteria were centrifuged at 8,000 r/min for 5 min for collection, and the bacteria were suspended in the proper volume of BMMY medium and cultured until the OD600 value reached 1.0. For the induction, the bacteria were cultured at 30°C and 200 r/min. Methanol was added every 24 h until the final concentration reached 0.5%. The fermentation was maintained for 120 h and then ended. The

supernatant of the fermentation broth was centrifuged at 4°C and 8,000 r/min to collect the crude enzyme broth. The crude enzyme solution was precipitated by 30%-70% ammonium sulfate and dialyzed by a dialysis bag with a molecular weight cut-off of 50 kDa to achieve a preliminary purification.

1.2.3 Determination of recombinant carboxypeptidase activity

Carboxypeptidase activity was determined by referring to the method described by Morita et al (2009), and a simple optimization was performed. Using CBZ-Phe-Leu as the substrate, a 1 mmol/L substrate solution was prepared with a pH 6.0 disodium hydrogen phosphate and citric acid buffer solution (0.1 mol/L). A total of 450 μl substrate and 50 μl of the suitably diluted enzyme solution were mixed and reacted at 37°C for 60 min. A 500-μl aliquot of a 0.5% indanone solution was immediately added; the mixture was heated in a water bath at 100°C for 15 min, and then it was cooled by tap water for 5 min. The absorbance value of A570 was determined by spectrophotometry (SP-2012UV spectrophotometer: Shanghai Spectral Instrument Co., Ltd.). Standard tyrosine solutions of different concentrations were prepared and reacted with ninhydrin under the same conditions. The standard curve was generated.

The definition of the enzyme activity is as follows: the unit of enzyme activity (U) is the amount of enzyme that hydrolyzes the substrate to generate 1 μg tyrosine at 37°C for 1 min.

1.2.4 Analysis of the enzymatic properties of recombinant carboxypeptidase

1.2.4.1 Determination of optimum temperature and temperature stability

The activity values of carboxypeptidase were determined at 30°C-70°C and pH 6.0, and the optimal reaction temperature of the enzyme was determined.

The enzyme solution was incubated at 30°C-70°C for 0.5, 1, 1.5 and 2 h. The enzyme activity was measured according to the method described in section 1.2.3. The enzyme solution without heat treatment was used as a control (the enzyme activity was 100%) to calculate the relative enzyme activity and investigate the thermal stability of carboxypeptidase.

1.2.4.2 Determination of the optimum pH and pH stability

The activity values of carboxypeptidase at pH 4.0-8.0 and 45°C were measured to determine the optimal pH of the enzyme.

The enzyme solution was incubated in a pH 4.0-8.0 buffer solution for 1 h (45°C), and the enzyme activity was determined according to the method described in section 1.2.3. The maximum value of the enzyme

activity was 100%, and the pH stability of carboxypeptidase was investigated. The buffer used was the 0.1 mol/L disodium hydrogen phosphate-citric acid buffer (pH 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0).

1.2.4.3 Influence of metal ions and chemical reagents on enzyme activity

Metal ions and chemical reagents at final concentrations of 1 mmol/L were added to the carboxypeptidase and substrate reaction systems. The enzyme activity was determined according to the method described in section 1.2.3. The enzyme activity in the system without metal ions and chemical reagents was 100%, and the relative enzyme activity in the presence of the metal ions and chemical reagents was calculated.

1.2.4.4 Substrate specificity analysis

Carboxypeptidase was reacted with 1 mmol/L CBZ-Ala-Arg, CBZ-Pro-Gly, CBZ-Ala-Lys, CBZ-Gly-Ala, CBZ-Ala-Glu or CBZ-Phe-Leu, and the enzyme activity was determined according to the method described in section 1.2.3. When CBZ-Phe-Leu was used as the substrate, the enzyme activity was 100% in the assay. The relative enzyme activity value of other substrates reacting with carboxypeptidase was calculated.

1.2.5 Bioinformatics analysis

NCBI (https://www.ncbi.nlm.nih.gov/genbank/) was used to identify the serine carboxypeptidase amino acid sequences from *A. niger* and other sources; Clustal X2 and Biodit were used for sequence alignment analysis. The phylogenetic relationship was analyzed with Mega 5.0 by the neighbor-joining method (Tamura et al. 2011).

2 Results And Discussion

2.1 Cloning and sequence analysis of the carboxypeptidase gene

The *A. niger* CBS 513.88 genome (EMBL AM270980-AM270998) has been sequenced (Pel et al. 2007), and a novel suspected carboxypeptidase (CapA) gene has been identified by analyzing its genome information and BLAST. The cDNA of *A. niger* F0510 was extracted as a template, and the gene was successfully amplified using CapA-F and CapA-R as primers. The recombinant plasmid pPIC-CapA was constructed. Sequencing confirmed that CapA had an intact open reading frame (ORF), and the nucleotide sequence was identical to the published sequence of the *A. niger* CBS 513.88 genome. The serine carboxypeptidase gene contains 1479 bp, and it encodes 492 amino acids.

Clustal X2 was used to analyze and compare the amino acid sequences of the serine carboxypeptidases from different sources, which are listed and summarized in Fig. 1. There are four conserved domains involved in the substrate binding and catalysis of CapA, among which domain 1 is the conserved substrate binding domain and domains 2-4 are the conserved catalytic domains, which includes the conserved triplet of serine (S), aspartic acid (D) and histidine (H) (Morita et al. 2009; Breddam 1986). A conserved G-X-S-X sequence (located in domain 1) spatially includes the active serine residues (Jung et al. 1998). There is a conserved glutamate (Glu) in front of the catalytic serine residue (located in domain 2), which is thought to be the reason why serine carboxypeptidase performs the best catalysis under acidic conditions (Jung et al. 1998). Similar to other carboxypeptidases, CapA belongs to the S10 family of the SC carboxypeptidases (Mahoney 2001).

CapA was further compared with the amino acid sequences reported for serine carboxypeptidases using MEGA 5.0, and a phylogenetic tree was constructed, as shown in Fig. 2. The phylogenetic tree reflects the genetic distance between 10 different serine carboxypeptidases, and a short genetic distance and clustering indicate a close genetic relationship. The amino acid sequence similarity between CapA and other serine carboxypeptidases ranges from 15.76% (SpCap) to 93.09% (AlCap), with an average value of 30.45%. CapA and AlCap have the highest similarity, which is 93.09%.

2.2 Induction of carboxypeptidase expression

The recombinant plasmid pPIC-CapA was linearized by the restriction enzyme *Sac*Ⅰ and electrically transformed into *P. pastoris* GS115. After a series of screenings, the recombinant strain GS115 (pPIC-CapA) was obtained. The maximum enzyme activity of the crude enzyme reached 109.7 U/mL after 120 h of culture with methanol in a shaking flask. After the preliminary purification of the crude CapA enzyme solution by salting out and dialysis, SDS-PAGE (Fig. 3) analysis showed that the molecular weight of CapA was approximately 60.0 kDa, which was slightly higher than the theoretical molecular weight of 52.7 kDa, and this was determined to be caused by glycosylation, which is generally required in the synthesis process of serine carboxypeptidase (Breddam 1986).

2.3 Enzymatic properties of carboxypeptidase

2.3.1 Optimal temperature and temperature stability

Carboxypeptidase activity was detected in the range of 30°C to 70°C, and the results are shown in Fig. 4a. The optimal temperature of CapA was 45°C, and the relative activity could be maintained at more than 70% at temperatures of 30°C to 55°C. The thermal stability study (Fig. 4b) showed that after incubation at 30°C -50°C for 1 h, the enzyme activity of CapA remained at more than 80%, and after incubation at 60°C and 70°C for 2 h, the enzyme activity remained at more than 30% and 10%, respectively.

The optimal reaction temperature and thermal stability value of CapA were significantly higher than those of the *A. oryzae* carboxypeptidase (which was 30°C; the enzyme activity was less than 10% after 30 min incubation at 60°C) (Morita et al. 2009). The optimal values of CapA were also higher than the optimal temperature (30°C) and thermal stability of *S. cerevisiae*-derived recombinant carboxypeptidase Y (after 60°C incubation for 1 h, the enzyme activity was almost undetectable) (Yu et al. 2015). CAPA has better heat resistance than the other carboxypeptidases, so it has the advantages of simplifying the process, improving the efficiency and reducing the cost in application (Yantao et al. 2014).

2.3.2 Optimal pH and pH stability

Carboxypeptidase activity was detected in the pH range of 4.0-8.0, and the results are shown in Fig. 5a. The optimal pH of CapA was 6.0, and the relative activity could be maintained at more than 60% in the range of pH 5.0-6.5. When the pH was less than 5.0 or greater than 6.0, enzyme activity decreased rapidly. Studies on the pH stability showed (Fig. 5b) that CapA was relatively stable at pH 4.0-8.0, and the enzyme activity remained above 60% after 1 h of incubation. CapA was similar to the carboxypeptidases from other sources, as they all exhibited the best hydrolysis in acidic conditions. However, unlike the optimal pH of the carboxypeptidase from most filamentous fungi and yeasts, which is near 4.0 (Morita et al. 2009; Hayashi et al. 1975; Min et al. 2013), the optimal reaction pH and stable pH range of CapA are more neutral. Similar to the reported carboxypeptidase Y from *S. cerevisiae* (Yu et al. 2015), CapA plays a role in the reaction systems that have a neutral pH, which is more convenient for the subsequent product processing after the enzyme catalysis has occurred.

2.3.3 Influence of metal ions and chemical reagents on enzyme activity

The effects of metal ions or chemical reagents on CapA are shown in Table 1. Mg^{2+} significantly increased the CapA activity, Cu^{2+}, Fe^{2+} and CO^{2+} significantly inhibited the CapA activity, and Ca^{2+}, Zn^{2+} and Mn^{2+} had little effect on the CapA activity. CapA is a serine protease, and its active center does not need the assistance of metal ions (Min et al. 2013), which is also demonstrated by the fact that the enzyme activity is not affected after ion chelation by EDTA. Therefore, metal ions should form coordination bonds with some of the key amino acids of the enzyme and change its conformation, thus affecting the activity of the enzyme (Ewert et al. 2018). PMSF can inhibit the activity of recombinant enzymes by more than 80%. As a specific inhibitor of serine protease, PMSF can inhibit serine carboxypeptidase (OcpC) and *Monascus* carboxypeptidase (Morita et al. 2009).

Table 1

Effect of metal ions or chemicals on the enzymatic activity of CapA

Metal ions or chemical reagents	Relative activity/%
Control	100±2.1
Cu^{2+}	20.7±0.3
Mg^{2+}	119.4±3. 6
Fe^{2+}	42.5±1.6
Ca^{2+}	99.4±2.1
Zn^{2+}	97. 9±1.7
Mn^{2+}	96.5±1.3
Co^{2+}	76.4±1.3
Na^{2+}	106.4±4.2
EDTA	107.4±2.7
PMSF	14.9±0.8

2.3.4 Specific substrate of CAPA hydrolysis

Using 6 CBZ-AA-AA as substrates, the hydrolysis specificity of CapA was measured, and it is summarized in Fig. 6. CBZ-Phe-Leu is the optimal substrate for CapA. The ability of CapA to hydrolyze the 6 substrates is as follows: CBZ-Phe-Leu > CBZ-Gly-Ala > CBZ-Ala-Lys > CBZ- Pro-Gly > CBZ-Ala-Arg. CapA has a wide range of substrate specificities, and it prefers the carboxy-terminal hydrophobic amino acids Leu and Lys, which cause the bitterness of oligopeptides; thus, it has good potential for application in protein C-terminal sequencing and debittering oligopeptide (Gao and Wang 2007; Chen 2014; Cheung et al. 2015), which need to be further studied.

3 Conclusion

In this study, a serine carboxypeptidase CapA from *A niger* was identified for the first time, and its heterologous expression in *P. pastoris* was successfully carried out. The optimal reaction temperature, temperature stability, optimal reaction pH, pH stability, effects of metal ions and chemical reagents on enzyme activity, substrate specificity and other basic enzymatic characteristics of CapA were analyzed. The results show that the optimal reaction temperature and pH of CapA are 45°C and 6.0, respectively. Compared with the reported carboxypeptidase, CapA has better heat resistance (30% of the enzyme activity can be retained after incubation at 60°C for 2 h) and pH stability (60% of the enzyme activity can be maintained after incubation at pH 4.0–8.0 for 1 h). The characteristics of its hydrolyzed substrate

indicate that CapA has potential application in protein C-terminal sequencing and debittering oligopeptides.

Declarations

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Author contributions

Wei Feng contributed to the conception of the study;

Peng Song, Wei Xu, Yang Zhang and Fei Wang performed the experiment;

Peng Song, Xiuling Zhou and Haiying Shi contributed significantly to analysis and manuscript preparation;

Peng Song and Haiying Shi performed the data analyses and wrote the manuscript;

Wei Xu and Wei Feng helped perform the analysis with constructive discussions.

Conflict of Interest

All authors declare that they have no conflict of interest.

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Figures

Fig. 1

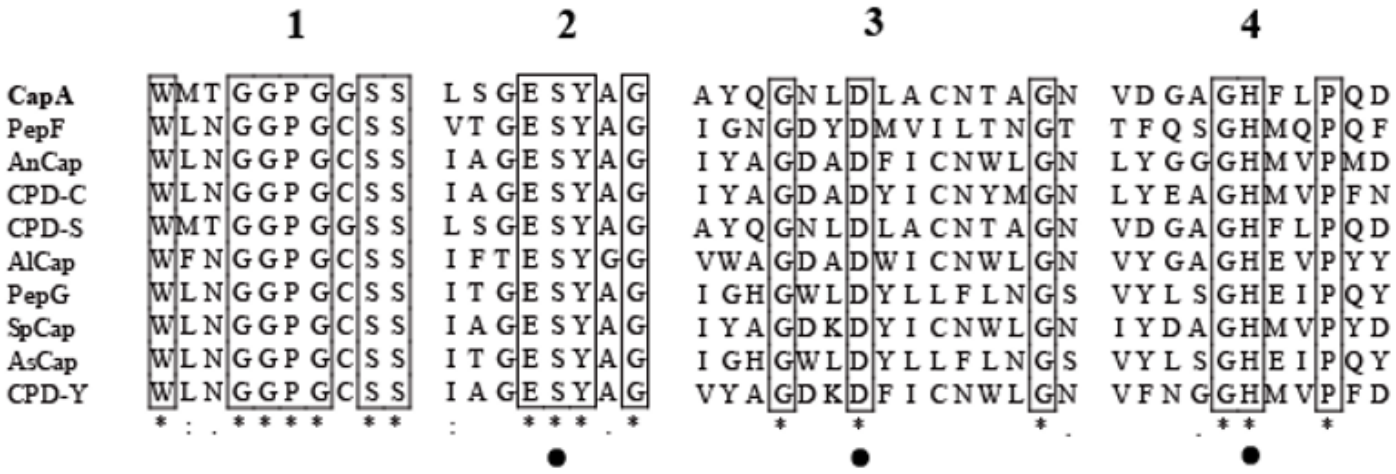

Figure 1

Multiple sequence alignment of serine carboxypeptidases from different microorganisms (*) : conserved amino acid; (:) : conservative replacement; (.) : semi-conservative substitution; and the amino acid residues of the active sites are marked with black dots. The four conservative domains (1, 2, 3, and 4) are indicated above.

Fig. 2

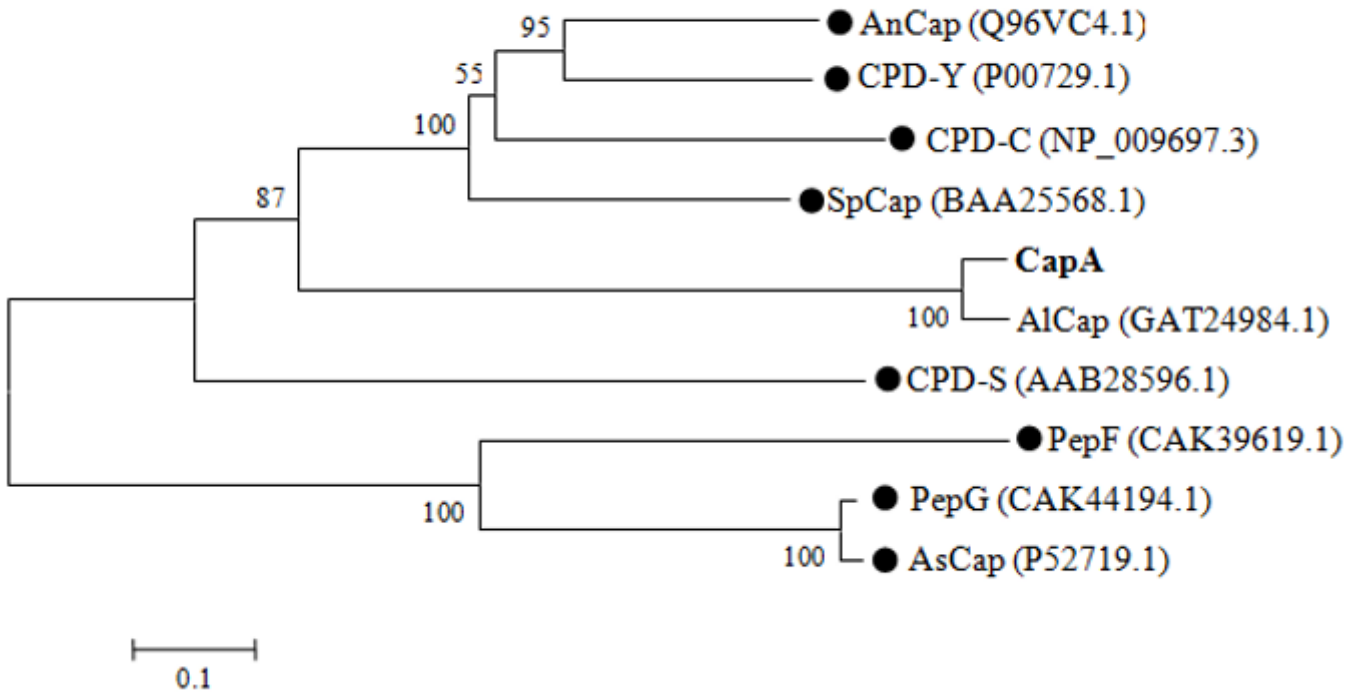

Figure 2

Phylogenetic tree describing the genetic distances among various serine carboxypeptidases from different microorganisms The serine carboxypeptidase in this research is shown in bold. The length of the line segment is the distance calculated by MEGA 5.0. The number on the branch node represents the bootstrap percentage. Values less than 50% are not shown. The black dots indicate that the carboxypeptidase has been reported in the literature. The entry number of the enzyme protein in GenBank is indicated in brackets. AnCap (Ohsumi et al. 2001) is from A. nidulans, CPD-Y (Yu et al. 2015) and CPD-C (Parzych et al. 2018) is from S. cerevisiae, SpCap (Takegawa et al. 2003) is from S. pombe, AlCap is from A. luchuensis. CPD-S (Svendsen et al. 1993) is derived from P. janthinellum, PepF (Dal et al. 1992) and PepG (Dal et al. 1992) from A. niger, and AsCap (Chiba et al. 1995) from A. saitoi.

Fig. 3

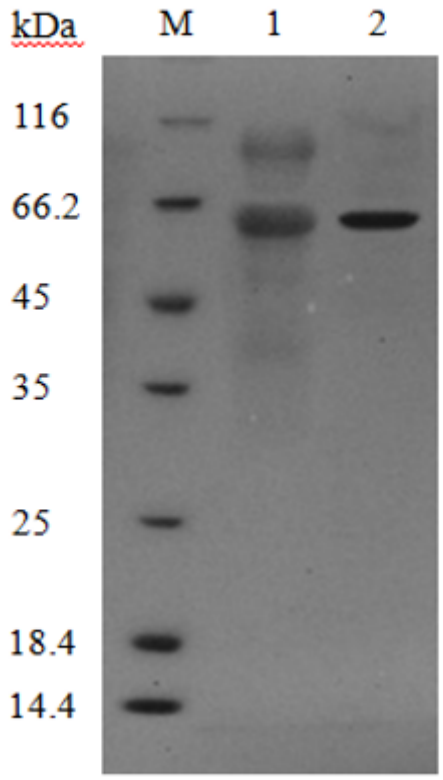

Figure 3

SDS-PAGE analysis of the purification of the recombinant enzyme CapA M: Protein molecular weight standard; 1: Crude enzyme solution; 2: Purified CAPA

Fig. 4 a

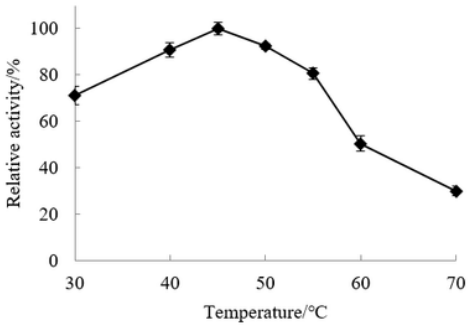

Fig. 4 b

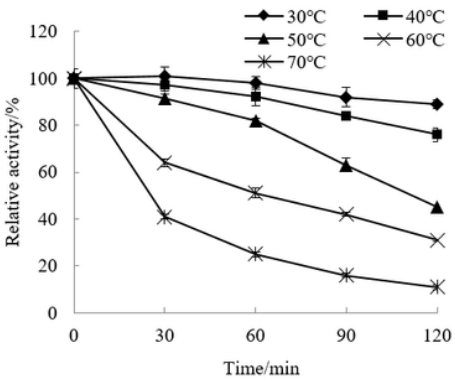

Figure 4

Effect of temperature on the activity (a) and stability (b) of CapA

Fig. 5 a

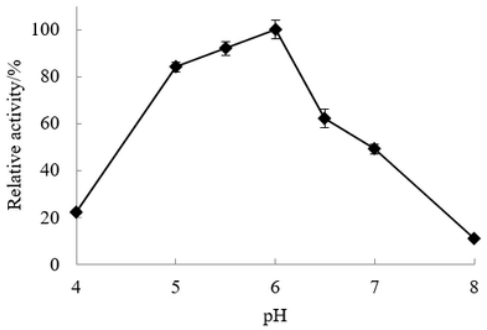

Fig. 5 b

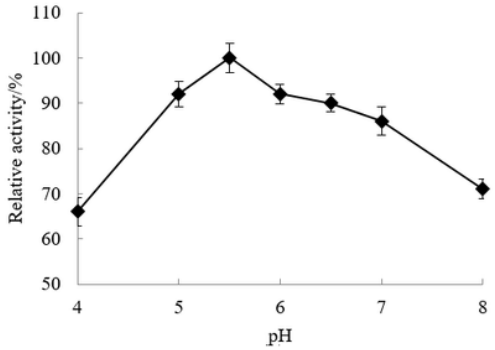

Figure 5

Effect of pH on the activity (a) and stability (b) of CapA

Fig. 6

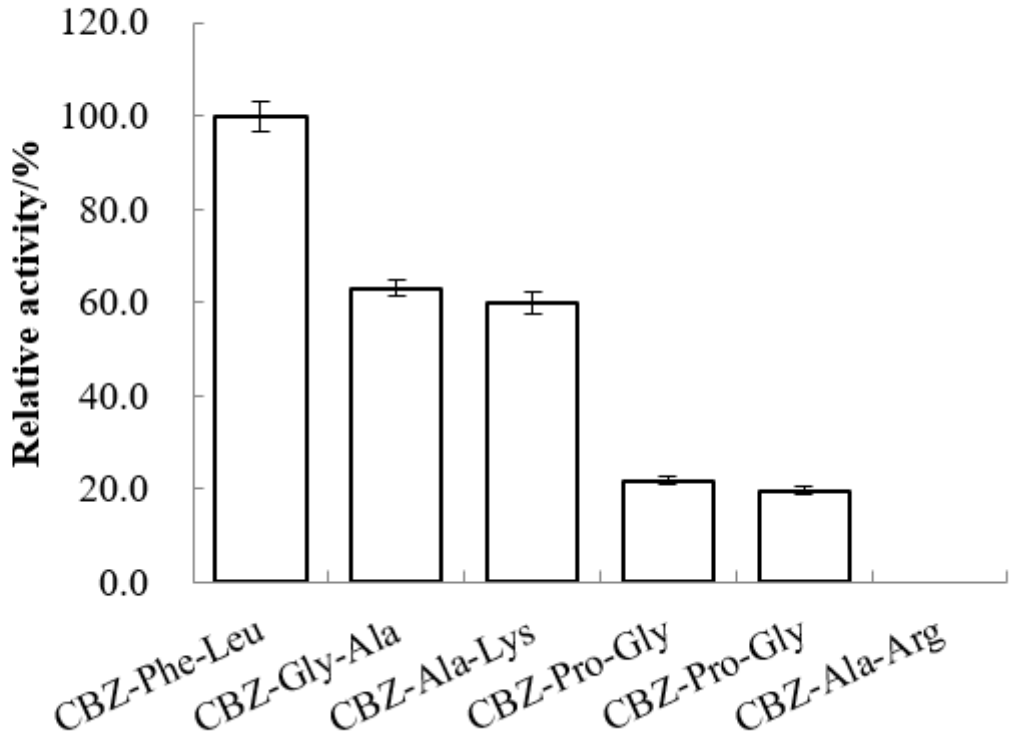

Figure 6

Substrate specificity of recombinant carboxypeptidase