

Full Length Research Paper

Cloning, over-expression, and characterization of a new carboxypeptidase A gene of *Bacillus pumilus* ML413 in *Bacillus subtilis* 168

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Carboxypeptidase A (CPAs) are a well-studied group of zinc-containing exopeptidases that facilitate the breakdown of proteins and peptides during metabolism. Carboxypeptidase A is typically produced in mammalian pancreatic, brain and other tissues. A new gene encoding carboxypeptidase A in the prokaryote *Bacillus pumilus* was amplified by polymerase chain reaction (PCR), ligated into the shuttle vector pMA5, and cloned in a GRAS bacteria-*Bacillus subtilis* 168 host. This gene sequence contained a 1621 bp open reading frame that encodes a protein of 540 amino acids. The optimum pH and temperature for enzyme activity were 7.5 and 50°C, respectively. The enzyme was quite stable at neutral pH and maintained about 65% activity following a 24 h incubation at 40°C. The K_m of this CPA was 0.1 mM, much higher than in mammalian species. Glycerol, ammonium sulfate, and sodium citrate improved enzyme activity under optimal culture condition. The carboxypeptidase activity in recombinant *B. subtilis* 168 reached a maximum of 179 U ml⁻¹ in a 5 L fermentator when cultured on improved medium. The over expression of carboxypeptidase A in *Bacillus subtilis* has commercial applications.

Key words: *Bacillus pumilus*, *Bacillus subtilis* 168, over-expression, orthogonal arrays, carboxypeptidase A, metallo-carboxypeptidase.

INTRODUCTION

Carboxypeptidases (CPs) catalyze the release of C-terminal amino acids from proteins and peptides (Sebastian Tanco et al., 2013). CPs serve many important functions in a variety of organisms since originally isolated from bovine pancreatic tissue in 1929

(Suwen et al., 2002). Some non-digestive zinc carboxypeptidases are involved in hormone and neuropeptide processing, bioactive peptide activation or inactivation, or functional modulation of regulatory proteins (Joshi et al., 1999). Thus, CPs are widely used

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using *Bam*HI and *Mlu*I restriction digests. The digested fragment was purified and then ligated with pMA5, which had already been digested using *Bam*HI and *Mlu*I restriction enzyme. The ligation mixture was used to transform *E.coli* JM109 competent cells. Restriction digestion analysis was carried out to verify the presence of the recombinant plasmid pMA5-*cpa* gene. Nucleotide sequence of *cpa* insert was analyzed by Sangon Biotech. Protein and nucleotide sequence comparisons were performed using the BLAST server available from the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and then this recombinant plasmid was transformed into chemically competent *B. subtilis* 168 cells for Carboxypeptidase A production using the procedure described by Spizizen (Jia et al., 2013).

Expression of *cpa* in *B. subtilis*

Recombinant *B. subtilis* 168 was cultivated overnight (12 h) on selective LB medium supplemented with 50 µg/ml kanamycin. The cells were then harvested by centrifugation at 10,000 *g* for 10 min at 4°C, and the supernatant was used for a CPA enzyme activity assay. The precipitated pellets were washed twice with 5 ml of 50 mM Tris/HCl buffer (pH 7.5), then suspended in 5 ml of the same buffer supplemented with 50 µl of lysozyme solution and kept on ice for 2 h. The mixture was then sonicated 10 times for 2 s with 5 s cooling intervals. Cell extracts were centrifuged for 30 min at 10,000 *g* at 4°C in a SIGAMA 4K-15 centrifuge (Sigma-Aldrich Co., Ltd., Shanghai, China) to remove cell debris. The supernatant was used for crude enzyme activity assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12% acrylamide), or stored at -20°C for further analysis.

Enzyme activity assay

Enzyme activities of the intracellular and extracellular portions of the protein were measured spectrophotometrically by recording the increase in absorbance at 254 nm that accompanies the hydrolysis of the peptide substrate hippuryl-L-phenylalanine as described by (Tardioli et al., 2003) with slight modifications. Assays were performed using a UV-1200 spectrophotometer (Pharmacia Biotech), with thermostatically controlled at 25°C. A 100 µl aliquot of enzyme solution was added to the assay solution (1.45 ml of 1 mM hippuryl-L-phenylalanine prepared in 50 mM Tris-HCl buffer, pH 7.5) supplemented with 40 µmol ZnSO₄·7H₂O, and the increase in absorbance was measured for 5 min. The amount of phenylalanine released by CPA action was calculated from the millimolar extinction coefficient at 254 nm (0.36 l mmol⁻¹·cm⁻¹), and enzymatic rate was expressed as concentration of product formed per min (µmol L⁻¹ min⁻¹). One unit (U) was defined as the amount of enzyme that hydrolyses 1.0 µmol of hippuryl-L-phenylalanine per min at 25°C. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard (David et al., 2004).

Enzyme purification

All purifications were performed using an ÄKTA Prime Plus (GE Healthcare Bio-sciences procedures). The CPA-containing supernatant was filtered through a 0.2 µm cellulose acetate membrane (Corning Incorporated, NY, USA) and applied to a 2.5 ml HisTrap column (AmershamBioSciences) equilibrated in buffer A with 50 mM Tris-HCl (pH 7.4). Then the enzyme was eluted in a linear gradient with a change of imidazole concentration (500 mM) using a flow rate of 1.0 mL min⁻¹. The active fractions were

collected (Figure 7) and dialyzed for CPA enzyme assay or storing at -20°C.

Enzyme characterization

Optimum pH was determined by measuring the activity of purified CPA at pH 3.0 to 10.0 (pH 3.0 to 6.5, 50 mM citrate-sodium citrate buffer; pH 6.5 to 8.0, 50 mM Tris-HCl buffer; pH 8.0 to 10.0, 50 mM glycine-NaOH buffer), while the optimum temperature was examined in 50 mM Tris-HCl buffer (pH 7.5) using the standard reaction mixture. The pH and thermal stability of CPA was determined by incubation at different pH levels at 4°C for 24 h and at various temperatures for 24 h, respectively. Effects of various compounds on enzyme activity were examined in the standard reaction mixture supplemented with various cations and EDTA at concentrations of 1 mM. Enzyme in the absence of metal ions or EDTA served as a control. Kinetic parameters were determined in 50 mM Tris-HCl buffer (pH 7.5) at 25°C by changing the concentration of the substrate. Eadie-Hofstee plots were used to calculate kinetic parameters K_m and V_{max} according to the enzyme reactions.

Single-factor-at-a-time experiments for medium optimization

To determine the effect of carbon, nitrogen sources and inorganic salt on enzyme activity, the growth medium was supplemented with sucrose, maltose, lactose, glucose, galactose, glycerol and soluble starch as carbon sources, ammonium chloride, ammonium sulfate, ammonium acetate and urea as nitrogen sources and sodium chloride, potassium chloride, sodium nitrate, potassium nitrate and sodium citrate as inorganic salts. Each source was used at a concentration of 0.5% (w/v). Enzyme activity was determined after 12 h of incubation at 37°C and 200 rpm (revolutions per minute).

Orthogonal array methodology for medium optimization

To examine the interactions among nutritional components of fermentation medium and to optimize their concentrations for CPA production, orthogonal arrays were used as reported by Vijayalakshmi et al. (2011). An L9 orthogonal array in three levels was used consisting of 9 different experimental trials for the medium optimization to increase CPA activity (Tables 3 and 4). The design for the L9 orthogonal arrays was developed and analyzed using “zhengjiaozhushou” Chinese software. Zhengjiaozhushou design is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial design. In this design the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require 3 levels of each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs.

5 L fermentation for CPA production

The recombinant *B. subtilis* 168 harboring plasmid pMA5-*cpa* was scaled up to 2 L in optimized medium in a 5 L bioreactor (Shanghai Baoxing Bioengineering Equipment Co., Ltd). Inoculum (100 ml) was added to the bioreactor. The agitation rate was set to 300 rpm at 37°C. For controlled pH cultivations, the pH was maintained at 7.2 by addition of 0.5 M NaOH and 1 M HCl solution. After 8 h incubation, the parameters like optical density of cell (OD₆₀₀), enzyme activity, and protein concentration were determined at

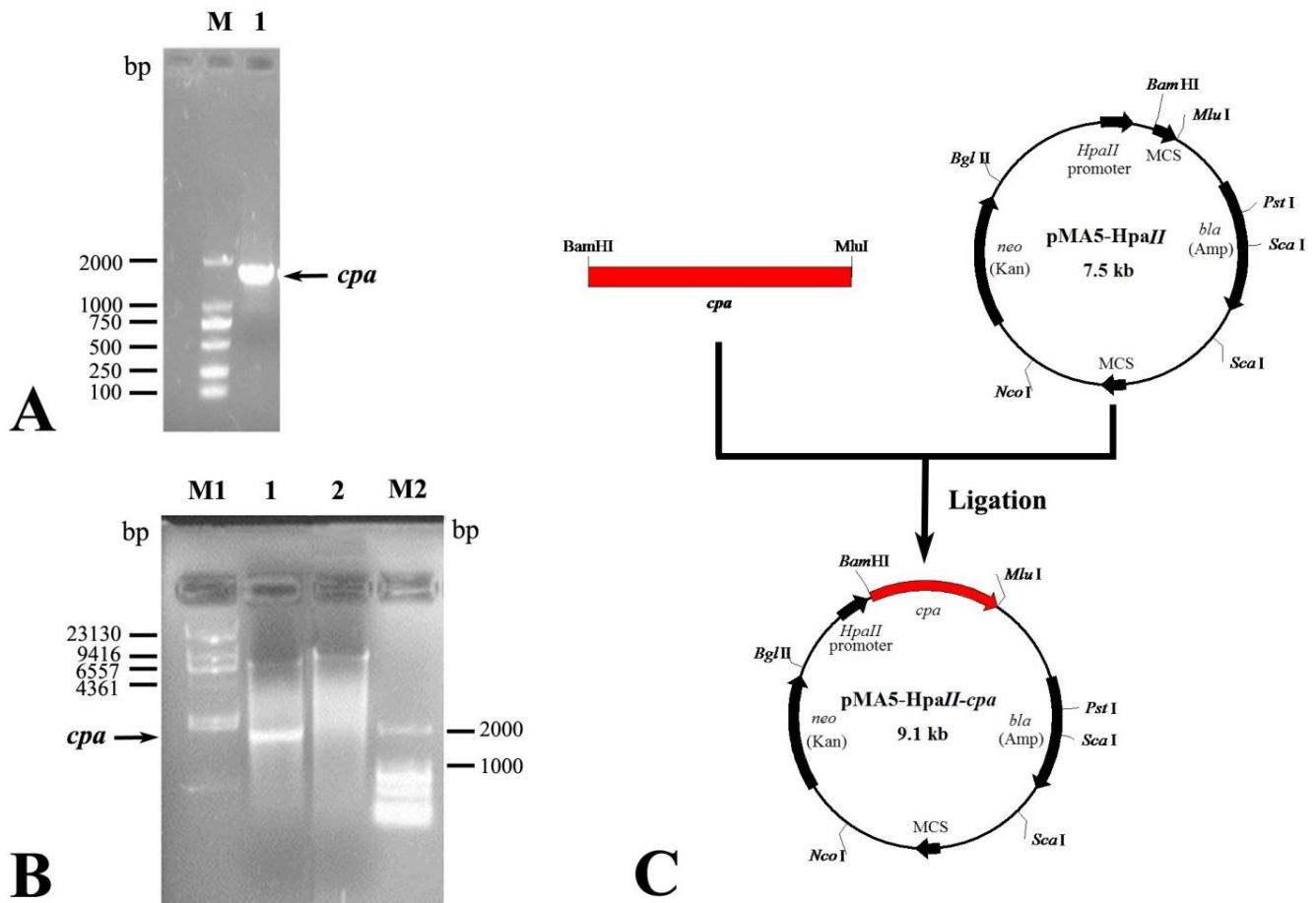


Figure 1. PCR and construction of recombinant vector pMA5-*cpa*. (A) Lane M, DL2000 DNA marker; lane 1, *cpa* PCR product. (B) Lane M1, λ Hind III DNA marker; lane 1, recombinant vector digested with *Bam*HI and *Mlu*I; lane 2, recombinant vector digested with *Bam*HI; lane M2, DL2000 DNA marker. (C) Ligation of the *cpa* gene with shuttle vector pMA5. The *cpa* gene predigested with *Bam*HI and *Mlu*I was ligated into the *Bam*HI and *Mlu*I sites of the pMA5 vector.

regular intervals.

RESULTS AND DISCUSSION

Cloning, construction of recombinant plasmid pMA5-*cpa*

The carboxypeptidase A (CPA) gene was successfully amplified from the genomic DNA of *Bacillus pumilus* ML413 strain by PCR. Electrophoresis revealed that the amplicon consisted of a 1621 bp fragment (Figure 1A). BLAST analysis showed that the gene sequence had a high level of similarity with that of several Peptidase M14 containing strains published in the NCBI database including *B. pumilus* strain W3 (CP011150.1) and *B. pumilus* strain MTCC B6033 (CP007436.1) with similarities of 99 and 98%, respectively. The PCR product

was ligated with pMD18-T to create pMD18-*cpa*, and then digested with *Bam*HI and *Mlu*I. The digestion product was cloned into the expression vector pMA5 under the control of the *Hpa*II promoter, resulting in recombinant vector pMA5-*cpa* (Figure 1C). The recombinant plasmid was digested with *Bam*HI and *Mlu*I to produce two major fragments with sizes of 1621 and 7500 bp (Figure 1B). This result confirmed the successful construction of the recombinant plasmid.

Expression of the recombinant vector in *B. subtilis* 168

The resultant pMA5-*cpa* was used to transform the expression host *B. subtilis* 168 to construct a recombinant *B. subtilis* strain 168/pMA5-*cpa*. The recombinant *B. subtilis* could express CPA under the control of the *Hpa*II promoter. For production of CPA, the

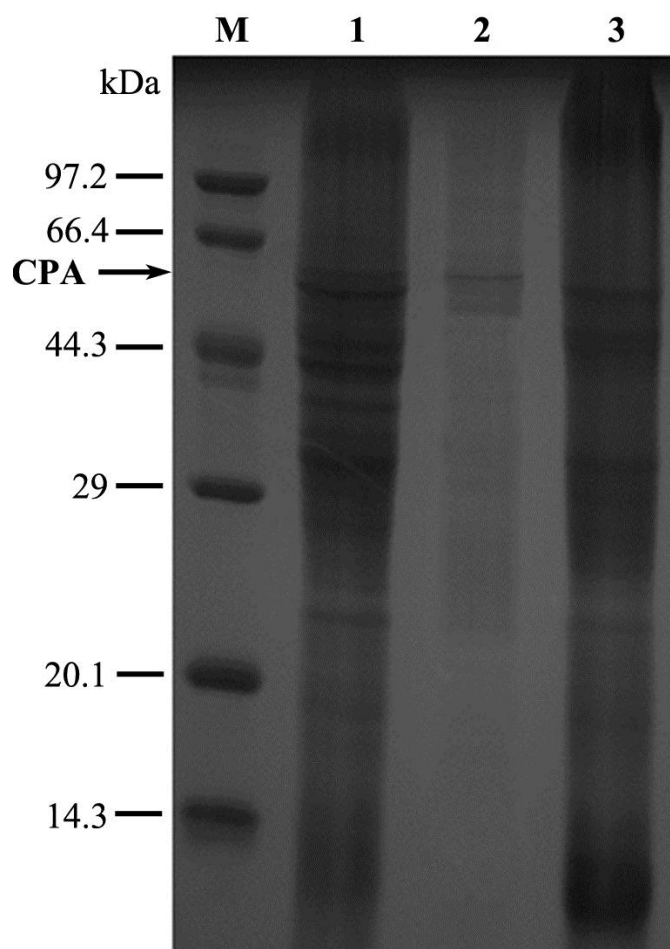


Figure 2. SDS-PAGE analysis of recombinant *B. subtilis* 168/pMA5-*cpa*. Lane M, protein marker; lane 1, *B. subtilis* 168/pMA5-*cap* cell extract; lane 2, Recombinant pure CPA; lane 3, *B. subtilis* 168 cell extract as control.

recombinant *B. subtilis* was used to inoculate 50 ml of LB medium (pH 7.2) supplemented with kanamycin ($50 \mu\text{g ml}^{-1}$) in a 250 ml flask, and incubated at 37°C in a rotary shaker at 200 rpm for 12 h. The recombinant *B. subtilis* showed intracellular and extracellular enzyme activities of 29.49 and 0 U ml^{-1} , respectively. The total enzyme activity was substantially higher than the 7 U ml^{-1} of the host *B. subtilis* 168. A band of 59 kDa corresponding to CPA was produced by the recombinant *B. subtilis* (Figure 2). Unlike other mammal digestive carboxypeptidases which are activated through limited proteolysis in the duodenum (Pedro José et al., 2002), our active recombinant CPA was expressed directly. However, the recombinant CPA could not be expressed outside the host *B. subtilis*, while it performed as an extracellular enzyme in *B. pumilus* (data not shown). Gao et al. (2013) reported that TB medium was suitable for the production of enzymes by recombinant engineered strains. Hence, we cultured the recombinant *B. subtilis* into 50 ml TB

Table 1. Enzyme activity of purified CPA.

Step	Protein (mg ml^{-1})	Activity (U ml^{-1})	Specific activity (Umg^{-1})
Affinity chromatography	4.01	179	45

medium using the other conditions as described for the LB medium. Intracellular and extracellular enzyme activities of CPA in TB were 45 and 0 U ml^{-1} , respectively.

Purification of the enzyme

For affinity purification of recombinant proteins expressed in *B. subtilis* 168, a His tag (Tgg, Tgg, Tgg, Tgg, Tgg, Tgg) was inserted into the sequence of the reverse primer. Affinity tags are useful tools for the production and purification of their fusion partners. Affinity tags may also have a beneficial impact on the yield of recombinant proteins, increase the recombinant protein's solubility, and promote their proper folding (Bown et al., 2004). This facilitates the further study of these proteins. Recombinant CPA was purified by metal affinity chromatography, making use of a His tag at the C terminus of CPA. The SDS-PAGE of lane 2 in Figure 2 indicated that this recombinant CPA was successfully purified with a band of 59 kDa. As a result, the purified enzyme showed a specific activity of 45 U mg^{-1} (Table 1). The specific activity of the recombinant CPA approached the activity of commercial CPA derived from bovine pancreas (52 U mg^{-1}) (Vertesi et al., 1999). This suggests a potential commercial use of our recombinant CPA. SDS-PAGE analysis indicated that the subunit of the enzyme had a molecular mass of about 59 kDa (Figure 2), when the gel was stained with CBB R-250.

Characterization of the Enzyme

The optimum pH values of CPA for hydrolyzing hippuryl-L-phenylalanine was pH 7.5 (Figure 3a). The optimum pH value reported here was different from values reported for carboxypeptidase from Archaeon *Thermococcus sp.* NA1 (pH 6) (Hyun Sook Lee et al., 2006), crayfish carboxypeptidase from *Astacus fluviatilis* (pH 6.5) and carboxypeptidase III from germinating triticale grains (pH 4.6) (Drzymala et al., 2009); but similar to immobilized carboxypeptidase A and carboxypeptidase A from the pancreas of the catfish *Parasilurus asotus* (Yoshinaka et al., 1985).

In the present study, the recombinant CPA showed maximum activity at 50°C , and then abruptly decreased at higher temperatures (Figure 3b). This was in agreement

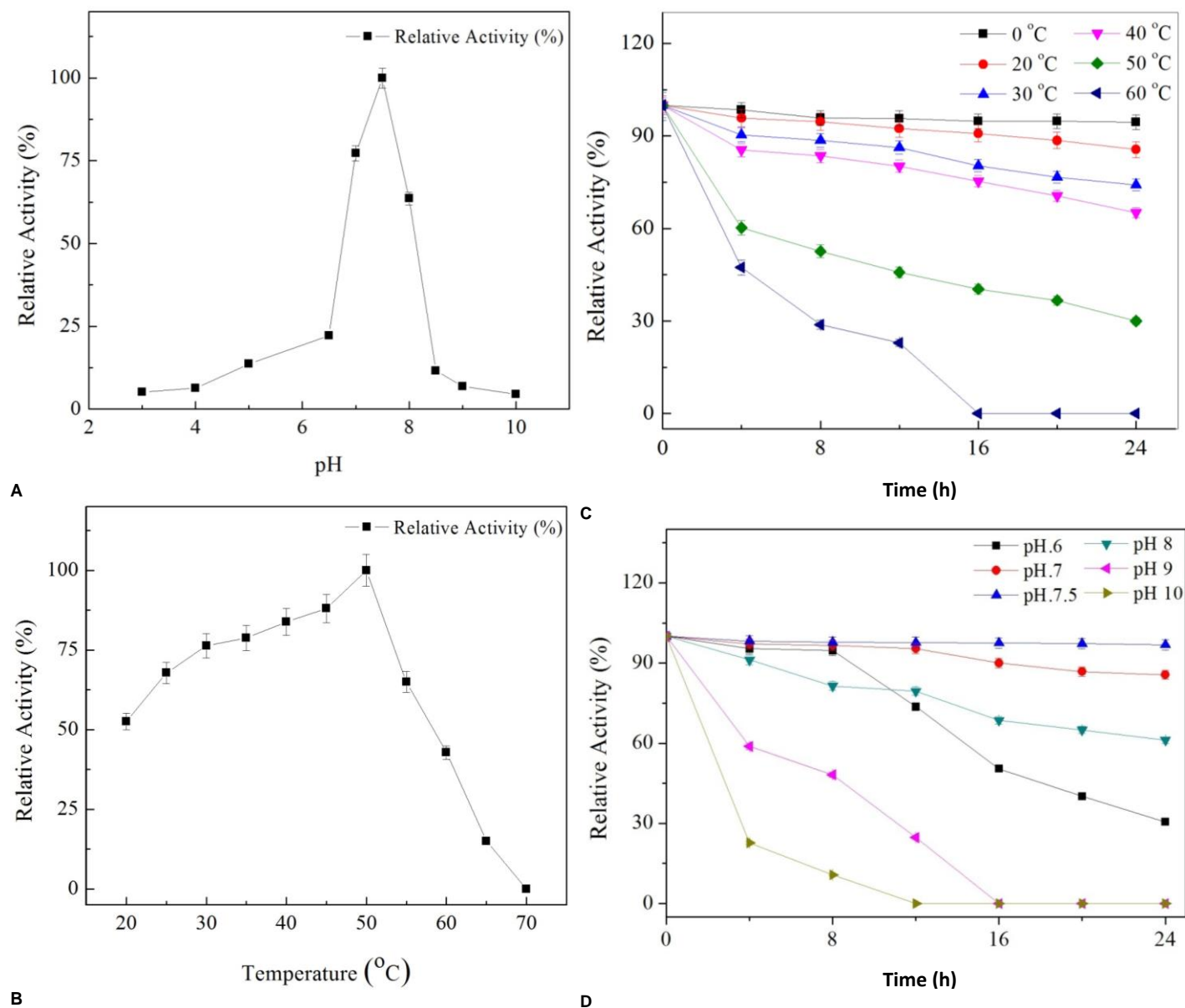


Figure 3. Effects of pH and temperature on recombinant CPA enzyme. (a) Optimum pH was carried out in 50 mM citrate sodium/citrate buffer (pH 3 to 6.5), 50 mM Tris/HCl buffer (pH 6.5 to 8), and 50 mM glycine/NaOH buffer (pH 8 to 10), at a temperature of 37°C. (b) Optimum temperature was carried out in 25 mM Tris/HCl buffer (pH 7.5) with the temperature range of 20 to 70°C. (c) Effect of temperature on stability of the CPA was determined between 0°C and 60°C by incubating the enzyme for a certain time in 25 mM Tris/HCl buffer (pH 7.5). (d) Effect of pH on the stability of the CPA was determined by incubating the enzyme at different pH at 4 °C for 24 h.

with earlier reports of the optimum temperature of carboxypeptidase A from chicken pancreas (Kazuhisa et al., 2000). The thermal stability of our recombinant CPA is shown in Figure 3c. The recombinant CPA was stable when the temperature was less than 40°C. The activity remained at 86 and 65% at 20 and 40°C after 24 h storage, respectively. The effectiveness of pH on the stability of the CPA was examined in the range of pH 6 to 10 (Figure 3d). The recombinant CPA was found to be stable only at pH 7.5 and the activity was 98% after 24 h incubation. The influences of metal ions and EDTA on the

CPA activity are as shown in Figure 4. These results indicated that the CPA enzyme was activated by Li^+ (117%), Cu^{2+} (115%), Co^{2+} (110%) and Ca^{2+} (107%). Mg^{2+} had no effect on CPA activity. Fe^{2+} (74%) and EDTA (57%) inhibited CPA activity. Some metal ions, notably cobalt, slightly enhanced the enzyme activity of the carboxypeptidase involved in the proteolytic cleavage of the influenza haemagglutinin as reported by Garten et al. (1983).

The K_m and K_{cat} values for recombinant CPA using hippuryl-L-phenylalanine as substrate were 0.1 mM and

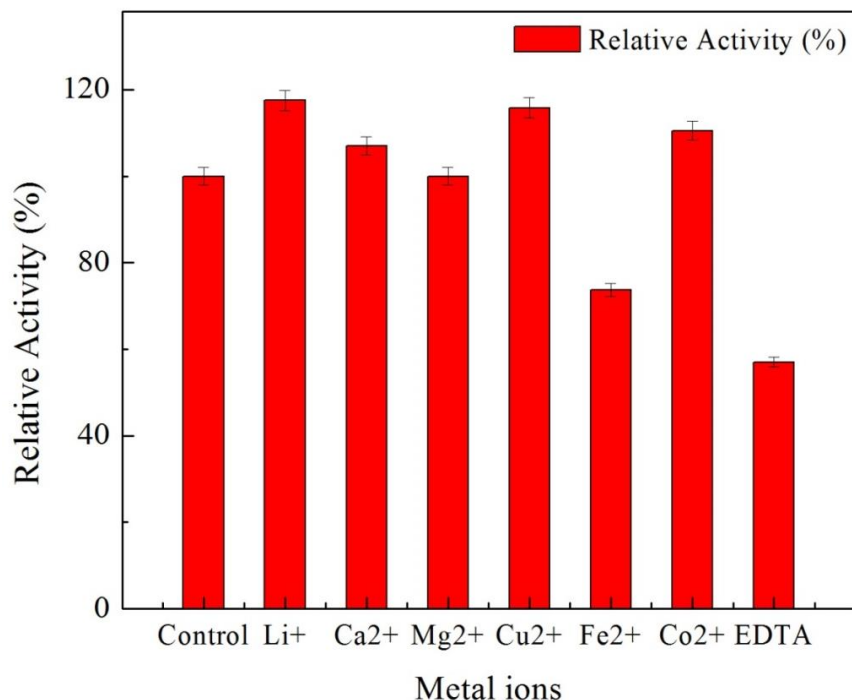


Figure 4. Effects of different metal ions and EDTA at the same concentration (1 mM) on CPA activity (enzyme with absence of metal ions or EDTA served as the control).

Table 2. Kinetic parameters of recombinant CPA with hippuryl-L-phenylalanine as substrate.

Substrate (1mM)	K_m (mM)	V_m ($\mu\text{M s}^{-1}$)	K_{cat} (s^{-1})	$K_{cat}/k_m(\text{mM}^{-1} \text{s}^{-1})$
Hippuryl-L-phenylalanine	0.1	6.36	63.6	636

63.6 s^{-1} , respectively (as shown in Table 2). These values are similar to those of CPA from chicken pancreas, but lower than the values reported for ostrich carboxypeptidase (0.41 mM), bovine carboxypeptidase (1.75 mM), and the value reported by Cheng et al. (1999) for carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (0.9 mM). These results indicate that substrate specificity of our recombinant CPA shows a higher affinity for hippuryl-L-phenylalanine when compared to that of mammals and other avian species. This suggests a potential commercial application.

Single-factor-at-a-time optimization strategy

The production of enzymes from microorganisms is strongly influenced by the composition of the medium, with carbon and nitrogen sources being the most important factors. Of the seven carbon (Figure 5a) and five nitrogen sources (Figure 5b) tested, glycerol and ammonium sulfate resulted in the highest relative activities of 197.21 and 175.85%, respectively. Lower

relative activities were observed with lactose (77%) and ammonium chloride (94%). The carbon and nitrogen sources used to increase production of recombinant CPA in this study are different than those reported by (Imtiaz et al., 2013) for the production of alkaline protease by *B. subtilis*, or other recombinant enzymes using *B. subtilis* as a host (Jia et al., 2013). The highest relative activity of carboxypeptidase (162.28%) was observed when sodium citrate was added to the culture medium as an inorganic salt source (Figure 6c). Sodium citrate was added in its simple form, so it was readily available for protein synthesis. A lower level of activity was observed when medium contained sodium nitrate or potassium nitrate. The inhibitory effects of nitrate were reported by Bhutto et al. (2011).

Optimization of enzyme activity using orthogonal array (OA) methodology

The orthogonal array methodology was used to investigate the influence of glycerol, ammonium sulfate

Table 3. Factors and levels listed for compatibility testing.

Factor	Level 1	Level 2	Level 3
Glycerol	G1	G2	G3
Ammonium sulfate	As1	As2	As3
Sodium citrate	Sc1	Sc2	Sc3

Table 4. Orthogonal array constructed for the compatibility testing.

Test number	Glycerol	Ammonium sulfate	Sodium citrate
Test 1	G1	As1	Sc1
Test 2	G1	As2	Sc2
Test 3	G1	As3	Sc3
Test 4	G2	As1	Sc2
Test 5	G2	As2	Sc3
Test 6	G2	As3	Sc1
Test 7	G3	As1	Sc3
Test 8	G3	As2	Sc1
Test 9	G3	As3	Sc2

and sodium citrate concentrations on CPA production. There are three independent variables (Glycerol, G1= 0.25%; G2 = 0.5%; G3 = 0.75%), (ammonium sulfate, As1 = 0.25%; As2 = 0.5%; As3 = 0.75%), and (sodium citrate, Sc1 = 0.25%, Sc2 = 0.5%; Sc3 = 0.75%). The order of factor effects on CPA production was found to be glycerol > sodium citrate > ammonium sulfate as shown in Table 5. The results of the optimization medium on CPA activity in 5 L fermenter are shown in Figure 6. The optimized medium contained yeast extract 24 g L⁻¹, tryptone 12 g L⁻¹, K₂HPO₄ 9.4 g L⁻¹, KH₂PO₄ 2.2 g L⁻¹, glycerol 5 g L⁻¹, ammonium sulfate 5 g L⁻¹ and sodium citrate 7.5 g L⁻¹. The predicted level of CPA activity was 45 U ml⁻¹ in a 50 ml flask culture. This increased to 179 U ml⁻¹ of recombinant CPA after scaling up to 2 L in a lab scale bioreactor (12 h after inoculation). The optimized medium resulted in a 4-fold higher level of activity when compared to TB basal medium and 6-fold higher activity when compared to LB medium. The activity curve of the recombinant CPA in a bioreactor was similar to the curve of the OD₆₀₀ (optical density, of sample measured at a wavelength of 600 nm), this could be due to the fact that the decline growth phase of the recombinant *B. subtilis* began after 16 h in such an abundant nitrogen source medium. Enzyme activity is generally directly related to the incubation period and metabolite concentrations as reported by Imtiaz et al. (2013).

Conclusion

A new carboxypeptidase A gene isolated from *B. pumilus*

was successfully cloned into a Generally Recognized As Safe (GRAS) bacterium-*B. subtilis* 168. This enzyme was characterized after purification. The recombinant CPA was expressed in a mature form with a molecular weight of 59 kDa. This was in contrast to other mammalian digestive CPAs which need be activated through limited proteolysis with a scission of a ~100 residue N-terminal prosegment. Culture conditions, including incubation period, had a profound effect on the production of enzyme. Glycerol and ammonium sulfate provided the carbon and inorganic nitrogen sources, respectively. The recombinant carboxypeptidase A showed some characteristics in common with CPA isolated from other sources. The CPA has a broad specificity for C-terminal amino acid residues with aromatic or branched aliphatic. With a high activity of 179 U ml⁻¹ in 5 L bioreactor, the recombinant CPA expressed in the GRAS bacterium-*B. subtilis* proves promising as a processing aid for protein hydrolysis in order to improve flavor and reduce bitterness in food protein hydrolysates.

Conflict of interest

The authors have not declared any conflict of interest

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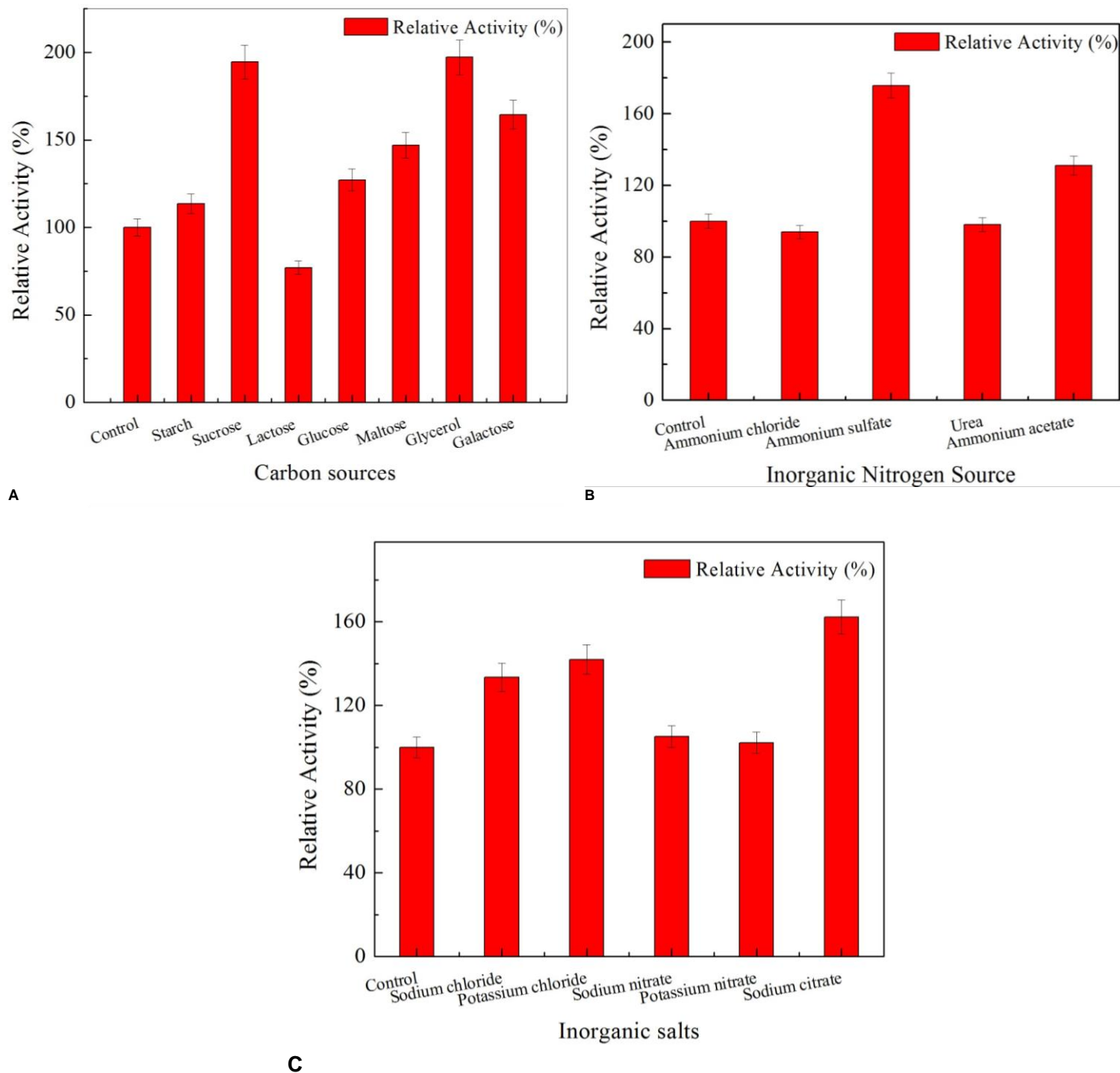


Figure 5. Effects of carbon, inorganic nitrogen sources, and inorganic salt for the medium on enzyme activity. a. Effect of carbon sources. b. Effect of inorganic nitrogen sources. c. Effect of inorganic salt.

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Table 5. Result of compatibility testing.

Test number	1	2	3	Result
	G	As	Sc	
Test 1	1	1	1	16.35
Test 2	1	2	2	15.14
Test 3	1	3	3	17.01
Test 4	2	1	2	25.11
Test 5	2	2	3	34.23
Test 6	2	3	1	17.95
Test 7	3	1	3	22.51
Test 8	3	2	1	18.25
Test 9	3	3	2	31.01
K 1	16.167	21.323	17.517	
K 2	25.763	22.540	23.753	
K 3	23.923	21.990	24.583	
Factors effects	9.596	1.217	7.066	

G: glycerol; As: Ammonium sulfate; Sc: Sodium citrate.

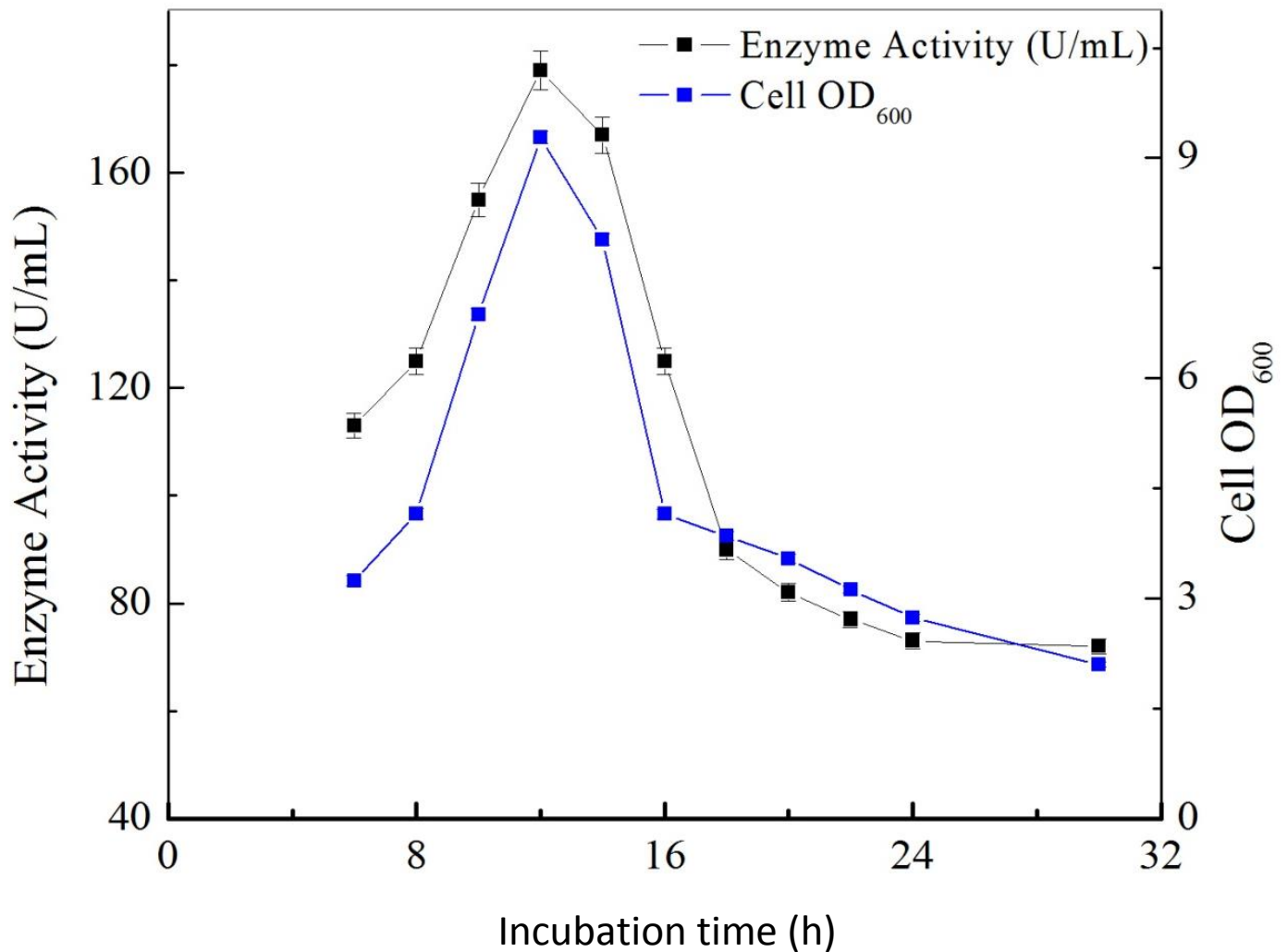


Figure 6. Trend curve of recombinant CPA activity and biomass (OD₆₀₀) using the optimum medium in a 5 L fermentator.

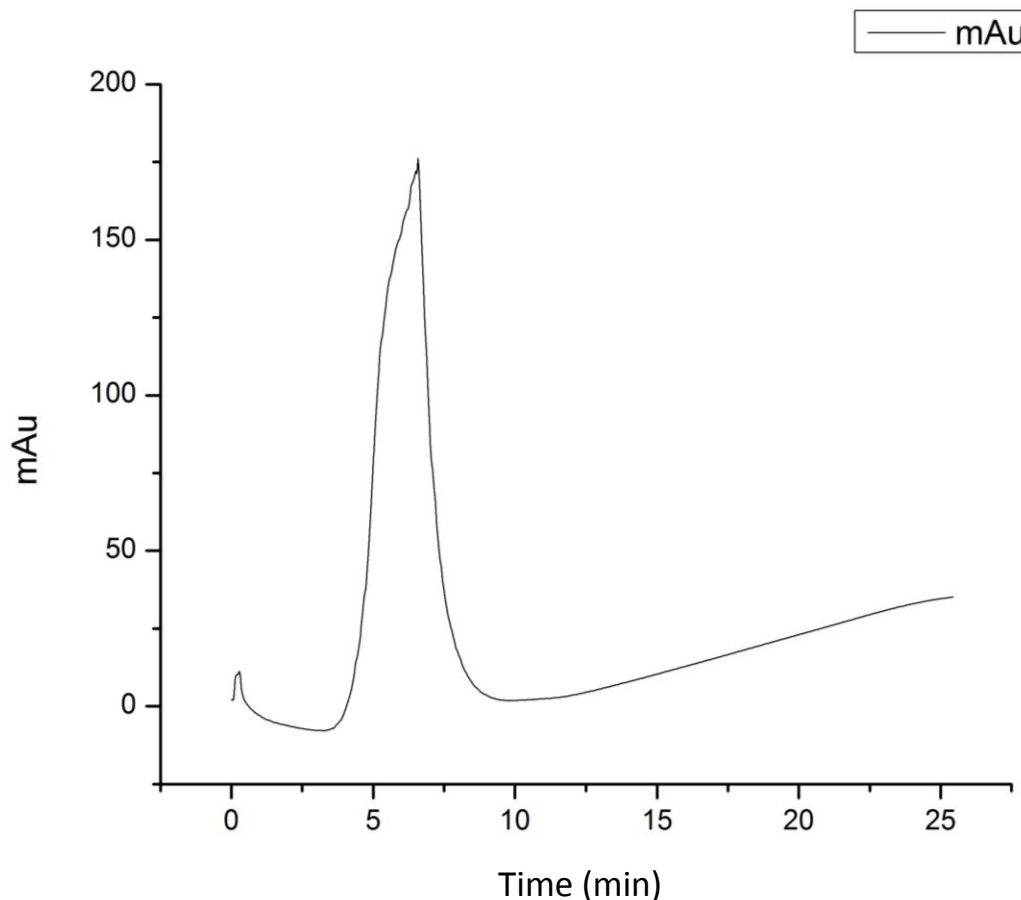


Figure 7. Graph of protein purification.

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