



Original Research Article

Cloning, expression, and characterization of a porcine pancreatic α -amylase in *Pichia pastoris*Lv-hui Sun ^{a, b}, Tao Qin ^{b, c}, Yan Liu ^b, Hua Zhao ^{b, *}, Xinjie Xia ^{b, d, *}, Xingen Lei ^{b, e, *}^a Department of Animal Nutrition and Feed Science, Huazhong Agricultural University, Wuhan 430070, China^b International Center of Future Agriculture for Human Health, Sichuan Agricultural University, Chengdu 611134, China^c Wuhan Chopper Biology Co., LTD, Wuhan 430070, China^d Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China^e Department of Animal Science, Cornell University, Ithaca, NY 14853, USA

ARTICLE INFO

Article history:

Received 28 September 2017

Received in revised form

24 November 2017

Accepted 29 November 2017

Available online 2 January 2018

Keywords:

Porcine

 α -amylase

Pancreatic

Gene expression

Pichia pastoris

ABSTRACT

Pancreatic α -amylase (α -1, 4-glucan-4-glucanohydrolase, EC.3.2.1.1) plays a primary role in the intestinal digestion of feed starch and is often deficient in weanling pigs. The objective of this study was to clone, express, and characterize porcine pancreatic α -amylase (PPA). The full-length cDNA encoding the PPA was isolated from pig pancreas by RT-PCR and cloned into the pPICZ α A vector. After the resultant pPICZ α A-PPA plasmid was transferred into *Pichia pastoris*, Ni Sepharose affinity column was used to purify the over-expressed extracellular recombinant PPA protein (rePPA) that contains a His-tag to the C terminus and was characterized against the natural enzyme (α -amylase from porcine pancreas). The rePPA exhibited a molecular mass of approximately 58 kDa and showed optimal temperature (50 °C), optimal pH (7.5), K_m (47.8 mg/mL), and V_{max} (2,783 U/mg) similar to those of the natural enzyme. The recombinant enzyme was stable at 40 °C but lost 60% to 90% ($P < 0.05$) after exposure to heating at ≥ 50 °C for 30 min. The enzyme activity was little affected by Cu^{2+} or Fe^{3+} , but might be inhibited (40% to 50%) by Zn^{2+} at concentrations in pig digesta. However, Ca^{2+} exhibited a dose-dependent stimulation of the enzyme activity. In conclusion, the present study successfully cloned the porcine pancreatic α -amylase gene and over-expressed the gene in *P. pastoris* as an extracellular, functional enzyme. The biochemical characterization of the over-produced enzyme depicts its potential and future improvement as an animal feed additive.

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1. Introduction

As a family member of retaining carbohydrases, α -amylase catalyzes the hydrolysis of α -(1, 4) glycosidic linkages in starch and related malto-oligosaccharides (Janecek, 1994). With this unique

function and a broad distribution in microbes, plants, and animals (Muralikrishna and Nirmala, 2005), α -amylase has many applications in food, textile, paper, and feed industries (Eliasson, 1996; Gupta et al., 2003). It represents about 25% to 33% of the world enzyme market and is second to only proteases (Nguyen et al., 2002).

Porcine pancreatic α -amylase (PPA) is a secreted 55.4 kDa glycoprotein. It is an endo-amylase and has a high efficiency in catalyzing the hydrolysis of α -(1, 4)-glucosidic bonds in both amylose and amylopectin through multiple attacks toward the non-reducing end (Darnis et al., 1999; Prodanov et al., 1984; Robyt and French, 1970). Because it plays a crucial role in the intestinal starch digestion (Andersson et al., 2002), insufficient production of PPA in the early life of weaning pigs can be a significant stress that causes sudden pause or reduction of growth rate, hence leads

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.



to major economic loss (Hedemann and Jensen, 2004). Thus, supplementing weanling piglets with amylolytic cultures of *Lactobacillus acidophilus* improved daily gain and feed use efficiency (Rincker et al., 2000). Likewise, supplementing amylase, along with xylanase, to a raw pea diet (Owusu-Asiedu et al., 2002) and supplementing amylase, along with glucanase and glucoamylase, to a barley-based diet (Inbarr and Ogle, 1988) improved feed conversion ratio and (or) reduced incidence of diarrhea in newly-weaned pigs. These results indicate that amylase is limiting in the young pig and therefore, there may be an application for exogenous enzyme. However, current commercial PPA products are mainly isolated from animal pancreatic tissues. The high cost associated with the extraction of PPA and its purification, the limitation of securing pig's pancreatic tissues, and the possibility of microbial contamination have prevented the application of PPA in a large scale in animal feed industry. Because of these factors, there is a need to develop an efficient heterologous expression system for economical, convenient, and safe production of large amount of PPA as an affordable feed additive. Previous attempts have been conducted to produce amylases in heterologous systems; however, the yield results were unsatisfactory (Kato et al., 2001; Li et al., 2011). Because the methylotrophic yeast *Pichia pastoris* has recently been used to manufacture feed enzymes such as phytase (Han and Lei, 1999), the objective of the present study was to determine if PPA could be effectively expressed in this system and how the overly-produced recombinant enzyme was compared with the endogenous enzyme isolated from the pancreas of pigs. After the PPA gene was successfully cloned and expressed as a recombinant PPA (rePPA) in the *Pichia pastoris* system, we found that the enzymatic properties and responses to the intestinal metals of rePPA were similar to those of natural PPA. Our findings suggest a feasible approach to produce PPA for the animal feed industry.

2. Materials and methods

2.1. Strains, plasmids, and reagents

Escherichia coli TOP10 (Invitrogen, Beijing, China) was used for plasmid amplification. The plasmid pPICZ α A (Invitrogen, Beijing, China) was used for the production of His-tagged PPA proteins, and the *P. pastoris* X-33 strain (Invitrogen, Beijing, China) was used as the protein expression host (Zhao et al., 2014). The *E. coli* TOP10 strain was grown in LB medium at 37 °C and *P. pastoris* X-33 strain was grown in yeast extract-peptone-dextrose medium at 28 to 30 °C (Zhao et al., 2014). The AMV reverse transcriptase, T4 DNA ligase, Taq DNA polymerase, pMD18-T vector, restriction enzymes (*Xba*I, *Kpn*I, *Sac*I), DL2000 DNA marker, protein marker were purchased from TaKaRa (Dalian, China). Plasmid Mini-prep Kit, Gel Extraction Kit, and Cycle-pure Kit were purchased from OMEGA (Chengdu, China). Ni-NTA His Binding Resin (GE Healthcare, Piscataway, NJ, USA) was used for the purification of recombinant PPA (Zhao et al., 2014). Other chemicals used in this experiment were of analytical grade and are commercially available.

2.2. Cloning of the PPA gene and construction of the expression plasmid

Total RNA was isolated from the porcine pancreas (Sus scrofa, Duroc \times Large White \times Landrace) using TRIzol reagent (Invitrogen, Beijing, China). The cDNA was generated by RT-PCR using the AMV Reverse transcriptase. The forward primer was

(5'-ATGAAGTGTTCCTGCTGCTTC-3') and the reverse primer was (5'-CAATTTGGATTACGATGAATTGCA-3'). After the amplified DNA fragment was purified using the Gel Extraction Kit, it was ligated into the pMD18-T vector, and transformed into the *E. coli* TOP10 strain by calcium chloride activation (Dagert and Ehrlich, 1979). The positive colonies were identified by DNA sequencing (Invitrogen, Shanghai, China). After that, the verified pMD18-T-PPA was used as a template to amplify the cDNA fragment encoding the mature PPA protein (without the signal peptide) by PCR. The forward primer was (5'-GATCGGTACCCAGT-ATGCCCCACAAACC-3', *Xba*I site underlined), and the reverse primer was (5'-TTTGTTCTA-GACTTAATTTGGA TTCAGCATG-3', *Kpn*I site underlined). The PCR product was purified, digested with *Xba*I and *Kpn*I, and ligated into the expression vector pPICZ α A. The pPICZ α A-PPA plasmid was transformed into *E. coli* TOP10 (Dagert and Ehrlich, 1979), and positive transformants were selected by using zeocin (25 μ g/mL) resistance and restriction mapping (Invitrogen, USA), along with a final verification of sequencing.

2.3. Transformation and expression of PPA in *P. pastoris*

The recombinant plasmid pPICZ α A-PPA was transformed into *P. pastoris* X-33 by electroporation (Kim et al., 2006). Single colonies of the transformants were selected for expression according to a protocol of EasySelect *Pichia* Expression Kit (Invitrogen, Beijing, China). After 3 days of methanol induction, total RNA was extracted from the cultured cells to screen for high-level expression transformants using real-time quantitative PCR analysis (Zhao et al., 2017). The expressed extracellular PPA protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Benicia, CA, USA). The *P. pastoris* transformants containing the expression vector pPICZ α A without the PPA gene insert were used as the negative control.

2.4. Purification of recombinant porcine pancreatic α -amylase

After 72 h of induction with methanol, cells were removed by centrifuge the fermentation broth at 14,000 \times g at 4 °C for 10 min (Zhao et al., 2014). After that, the supernatant was added to 0.5 mol/L NaCl and adjusted to pH 7.4, followed by filtration through a 0.45 μ m filter. The supernatant was then applied to a Ni Sepharose (GE Healthcare, Piscataway, NJ, USA) affinity column (Bio-Rad, Richmond, CA, USA) pre-equilibrated with a binding buffer (20 mol/L NaH₂PO₄, pH 7.4, 500 mol/L NaCl, 20 mol/L imidazole). After the column was washed with binding buffer to remove the unbinding proteins, the PPA was eluted with elution buffer (20 mol/L NaH₂PO₄, pH 7.4, 500 mol/L NaCl, 500 mol/L imidazole). The harvested protein was stored at -20 °C for subsequent analysis. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.5. Characterization of rePPA and comparison with the native enzyme

Activities of rePPA were measured as described by Bogdanov (2002), using 100 μ L 2.0% soluble starch (Kelong, Chengdu, China) as a substrate (Anitha Gopala and Muralikrishnaa, 2009). One unit of α -amylase activity was defined as the amount of enzyme needed for hydrolyzing 1.0 mg starch per minute at pH 7.5 at 37 °C. The pH-activity profile of rePPA was assayed at 37 °C using acetate buffer (pH 3.0 to 5.0), phosphate buffer (pH 5.5 to 8.0), and Tris-HCl buffer (pH 8.5 to 9.5). The optimal temperature

of rePPA was determined using the phosphate buffer (pH 7.5) from 20 to 80 °C. The thermal stability of rePPA was determined by the residual activity after the enzyme was incubated at 40, 50 and 55 °C for 30 min.

Kinetic constants of K_m and V_{max} were determined at pH 7.5 and 37 °C using the Lineweaver–Burk method (Lineweaver and Burk, 1934). To test the function mechanism of rePPA under the intestinal conditions, the purified enzyme was incubated with different concentrations of chloride metal ions (Zn^{2+} , Cu^{2+} , Fe^{3+} , Ca^{2+}) in the phosphate buffer at 37 °C for 10 min. The changes in the action against the untreated control were detected.

2.6. Data analysis

Data were analyzed by SAS 8.2 (SAS Institute, Cary, NC, USA), and simple t-test was used to compare mean differences. Significance was set at $P < 0.05$ ($n = 3$).

3. Results

3.1. Cloning, expression, and purification of the PPA gene

A 1,533 bp cDNA fragment of the coding sequence was isolated from porcine pancreases and cloned into pMD18-T by RT-PCR (Fig. 1A). The cloned cDNA showed 99.3% DNA and 99.8% amino acids sequence homology to that of porcine pancreatic α -amylase listed by NCBI (GenBank: AF064742.1, Appendix Fig. 1). After the cloned expression vector *pPICZ α A-PPA* was digested with *KpnI* and *XbaI*, a 1,500 bp target gene band and a 3,600 bp expression vector band were shown on the 1% agarose gel (Fig. 1B). After 0.5% methanol had induced the *P. pastoris* X-33 transformant for 72 h, the targeted protein was purified by Ni Sepharose affinity chromatography. The purified rePPA showed a single band on 12% SDS-PAGE gel with a molecular size of approximately 58 kDa (Fig. 2, lane 4). The yield of the recombination protein in the medium supernatant was 65 mg/L after 72 h fermentation.

3.2. Characterization of the rePPA related to the native enzyme

As shown in Fig. 3, rePPA shared similar pH-activity and temperature-activity profiles with the natural form of the PPA enzyme. The optimal pH of the rePPA was 7.5. However, more than

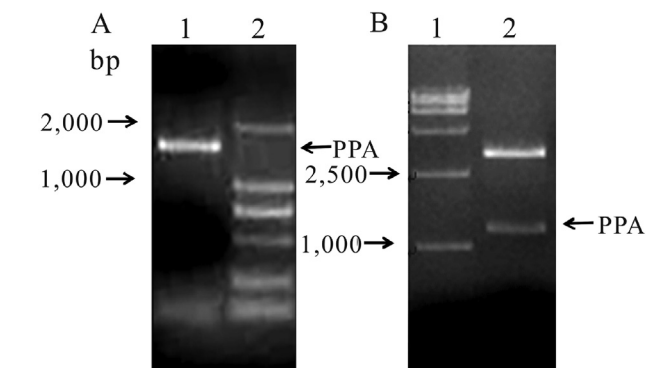


Fig. 1. (A) Cloning of the porcine pancreatic α -amylase (PPA) cDNA. Lane 1: PCR products of the PPA cDNA. Lane 2: molecular size markers. (B) Construction of the PPA expression plasmid. Lane 1: molecular size markers. Lane 2: double-digest restriction mapping.

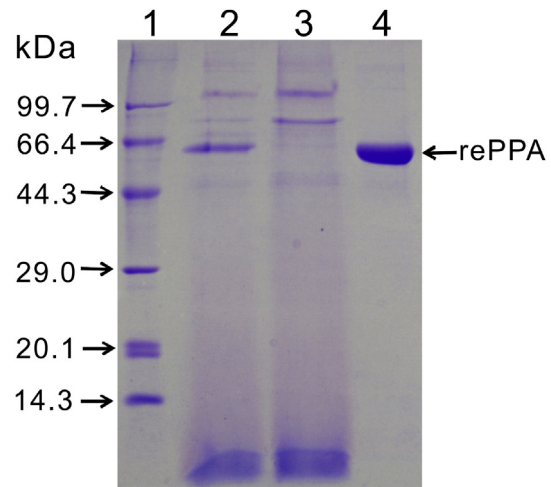


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant porcine pancreatic α -amylase (rePPA) expressed in *Pichia pastoris*. Lane 1: molecular size markers; lane 2: supernatant protein of *P. pastoris* containing pPICZ α A-PPA after methanol-induced; lane 3: supernatant protein of *P. pastoris* containing pPICZ α A after methanol-induced; lane 4: purified rePPA expressed in *P. pastoris*.

50% of the enzymatic activity maintained between pH 5.5 and 9.5, with a reduction ($P < 0.05$) to less than 30% at a pH lower than 5 (Fig. 3A). The optimal temperature of the rePPA was 50 °C, with 60% to 94% of activity at 30 to 55 °C. The activity decreased sharply ($P < 0.05$) at temperatures over 55 °C (Fig. 3B). Incubating the purified rePPA at 40 °C for 30 min had a little impact on its activity, but treating the enzyme at 50 or 55 °C resulted in 60% or 90% activity loss ($P < 0.05$) (Fig. 3C). The purified rePPA showed a K_m for soluble starch as 47.82 mg/mL and V_{max} as 2,783 U/mg protein, whereas the native form of PPA had a K_m of 40.45 mg/mL and V_{max} 2.3 U/mg protein (non-purified crude enzyme) (Fig. 4). As shown in Fig. 5, the activity of rePPA demonstrated a significant dose-dependent increase by the incubation with Ca^{2+} (Fig. 5). In contrast, the enzymatic activity showed dose-dependent decrease ($P < 0.05$) by incubating with Zn^{2+} , Cu^{2+} , or Fe^{3+} .

4. Discussion

In the current study, we successfully cloned porcine pancreatic α -amylase gene and expressed it in *P. pastoris*. Although the cloned PPA cDNA from the current study displayed 99.3% DNA sequence homology to the one reported by Darnis et al. (1999), with only one amino acid difference between the proteins that were coded by them. The difference in the sequences between the 2 clones might be due to the spices variation (Duroc \times Large White \times Landrace vs. Large White). Interestingly, our method was effective as the a-factor signal peptide in the yeast expression vector guided the secretion of the recombined rePPA into the culture broth. This approach can overcome the complicated purification procedures and can be applied for a direct industrial application for amylase (Romanos, 1995). The enzyme yield (65 mg/L) by the methanol-inducible *Pichia* expression system was higher than that of *Rhizopus oryzae* α -amylase (20 mg/L) produced in *Kluyveromyces lactis* (Li et al., 2011), but lower than that of mouse salivary α -amylase (240 mg/L) expressed in *P. pastoris* (Kato et al., 2001). This can be explained as the production of the heterologous proteins within the intracellularly and extracellularly through the expression system of *P. pastoris* were up to 3 and

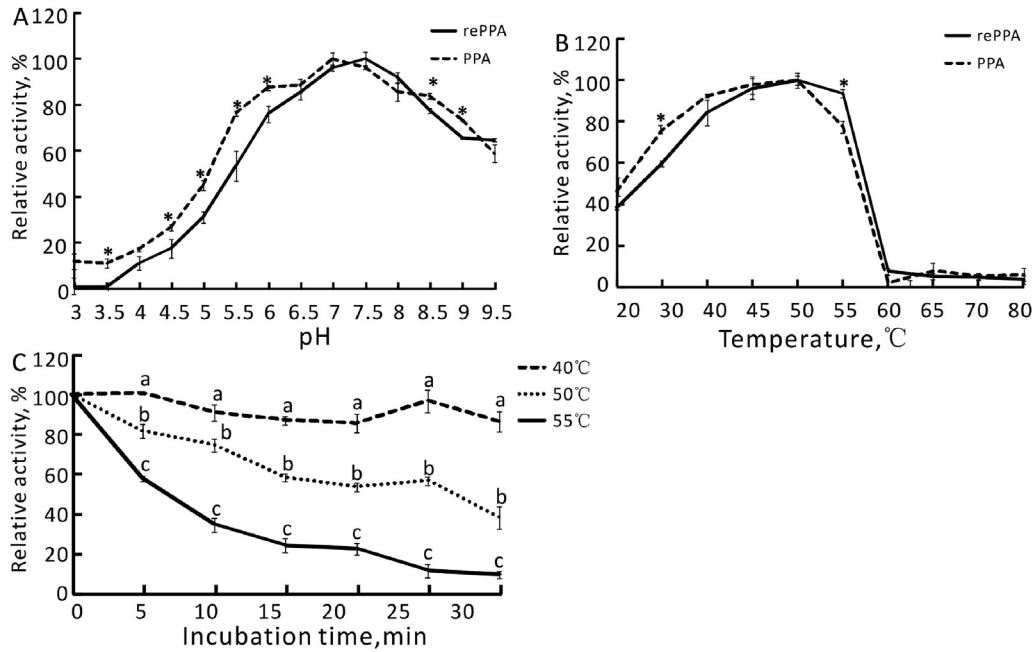


Fig. 3. Effect of pH (A) and temperature (B) on rePPA activity. The thermostability of rePPA at different temperatures was determined by preincubating the enzyme at these temperatures in the absence of substrate for 5, 10, 15, 20, 25, and 30 min before measuring its activity (C). The rePPA activity prior to the preincubations at different temperature was taken as 100%. An asterisk indicate a significant difference ($P < 0.05$) between rePPA and PPA at each point of pH or temperature ($n = 3$). Different letters indicate a significant difference ($P < 0.05$) between different temperatures preincubating the enzyme at each time point ($n = 3$). PPA = porcine pancreatic α -amylase; rePPA = recombinant porcine pancreatic α -amylase.

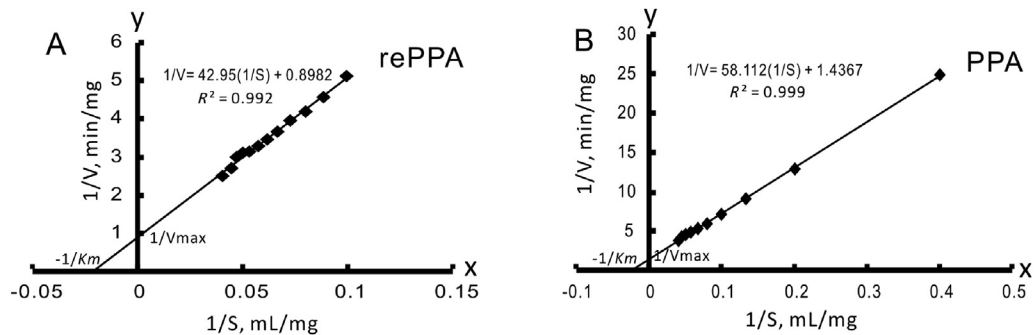


Fig. 4. The K_m value of rePPA (A) and native PPA (B) was determined by the Lineweaver–Burk method. R^2 means the correlation coefficient between $1/V$ and $1/S$. The intercept of the function with x-axis represents $-1/K_m$, while the intercept with y-axis gives $1/V_{max}$. PPA = porcine pancreatic α -amylase; rePPA = recombinant porcine pancreatic α -amylase; K_m = Michaelis constant, the substrate concentration at which the reaction velocity is 50% of the V_{max} ; V = reaction velocity; V_{max} = maximal reaction velocity; S = substrate concentration.

12 g/L, respectively (Barr et al., 1992; Clare et al., 1991), the rePPA yield obtained in the present study was relatively low. Therefore, further research will be required to maximize the production of the rePPA. The reason of that could be there is a limited or rare usage of several codons within the PPA gene in *P. pastoris* (Qiao et al., 2010; Teng et al., 2007), optimizing these codon usages may improve the protein production of the rePPA by the yeast host. As well, increasing the expression number of the plasmid copies could increase the expression of the recombinant protein (Romanos, 1995). Furthermore, optimizing the fermentation conditions such as temperature, pH, and methanol concentration can effectively lead to a better protein production (Muralikrishna and Nirmala, 2005).

In the present study, similar enzymatic properties were detected between the over-expressed rePPA in *P. pastoris* and those of the natural form, which were similar to the findings in previous studies. Precisely, the rePPA and the natural form of PPA (Sigma) had K_m for soluble starch: 47.8 and 40.5 mg/mL, respectively. In the current study, the estimated V_{max} (2,783 U/mg), optimal pH (7.5), and optimal temperature (50 °C) of the rePPA were similar to those identified for the natural enzyme by Anitha Gopala and Muralikrishna (2009) and Wakim et al. (1969). These similarities illustrate that the enzymatic properties were not altered by the heterologous expression of the rePPA in the *Pichia* yeast. Practically, the recombinant amylase can be supplemented into the diet of young pigs as a

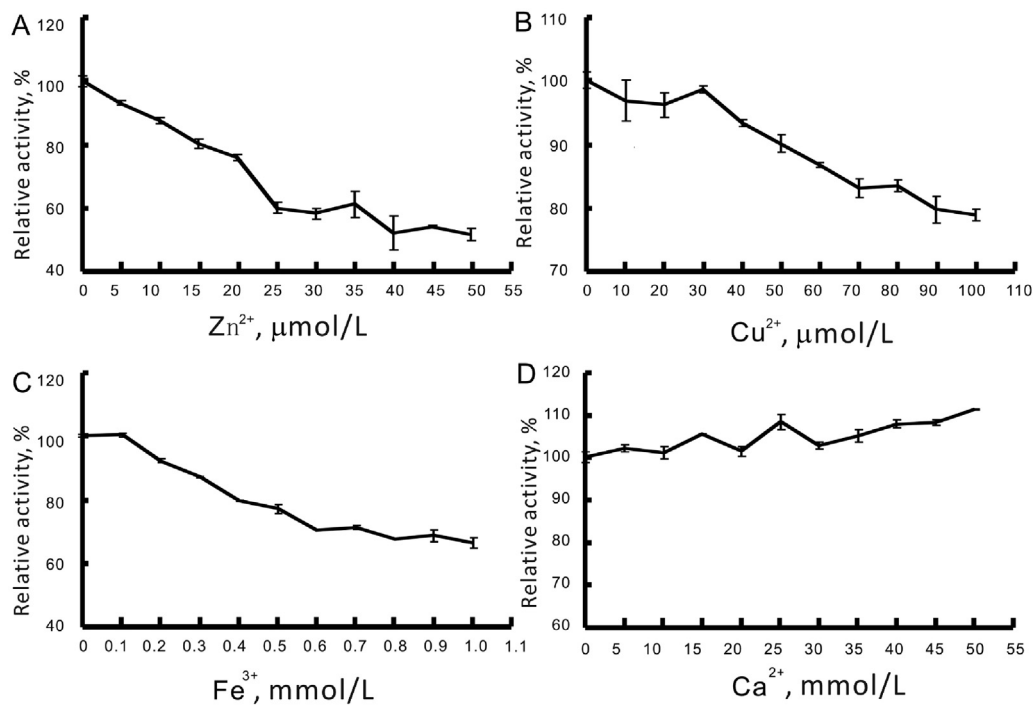


Fig. 5. Effect of metal ions, Zn²⁺ (A), Cu²⁺ (B), Fe³⁺(C), and Ca²⁺(D), on rePPA activity. Y-axis: relative enzyme activity; X-axis: the concentration of the metal ions. rePPA = recombinant porcine pancreatic α -amylase.

replacement or enhancement to the endogenous enzyme within the gastrointestinal tract. It is even more remarkable to notice that the rePPA was actually a fusion protein with 13 additional amino acid residues in the N-terminus and in the C-terminus there are 21 His-tag amino acid residues. Apparently, these additional amino acid residues had little effect on the enzymatic activity or catalytic function. This flexibility may open the door for more genetic or molecular engineering to improve the enzymatic fermentation yield or modify the non-catalysis-related properties.

Furthermore, the recombinant rePPA was tested for its heat tolerance and the response to divalent metals, as the most 2 applicable measurements in animal feeding industries. Due to the usage of a large quantity of feed in a pelleted form for monogastrics (i.e., pigs), exogenous enzymes must have resistance to heat and steam from the pelleting process (Svihus and Zimonja, 2011). Despite that the purified rePPA was reasonably stable at 40 °C and the optimal temperature of rePPA was 50 °C, most of its activity was reduced after exposure to 50 to 55 °C for 30 min. For that reason, if this enzyme is to be used in a large scale in animal feed industries, the thermostability must be improved by different approaches, such as protein engineering (Zhang and Lei, 2008) or chemical coating (Chen et al., 2001). In the digesta of pigs, free ion concentrations (mol/L) were found to be as follows: 5.5 to 31.6 for Cu, 3 to 29 for Fe, 44 to 132 for Zn, 1,100 to 5,400 for Ca (Dintzis et al., 1995). According to the activity response curves of rePPA to different ions (Fig. 5), there is a minor effect of the digesta concentrations of Cu or Fe in inhibiting the rePPA activity, whereas the digesta concentrations of Ca presumably enhanced the enzymatic activity. It has been explained as PPA may bind Ca at the functional site (Buisson et al., 1987; Steer and Levitzki, 1973). However, the available Zn concentration in the digesta was within

the range that may inhibit the activity of the rePPA by 40% to 50%. This inhibition of the enzymatic activity by Zn may be attributed as Zn may bind to the catalytic residues or replace Ca²⁺ from the substrate-binding site of the enzyme (Anitha Gopala and Muralikrishnaa, 2009). Accordingly, it is important to improve the enzymatic resistance to the inhibition caused by Zn and (or) regulate dietary Zn concentration for an efficient supplementation of the rePPA.

5. Conclusion

The present study has successfully cloned the porcine pancreatic α -amylase gene and proved the feasibility to over-express the gene into an extracellular, functional enzyme in *P. pastoris*. Our biochemical characterization of the over-produced enzyme underscores not only potential suitability but also needed improvement for its application in animal feed.

Acknowledgements

We thank Jiaqiang Huang, Krystal Lum and Mahmoud Mohamed Khalil for their technical assistance. This project was supported by the 863 program or State High-Tech Development Plan, funded and administered by the Government of the People's Republic of China (2007AA100602 and 2007AA100601-6), and by the Chang Jiang Scholars Program of the Chinese Ministry of Education (to X.G. Lei).

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix

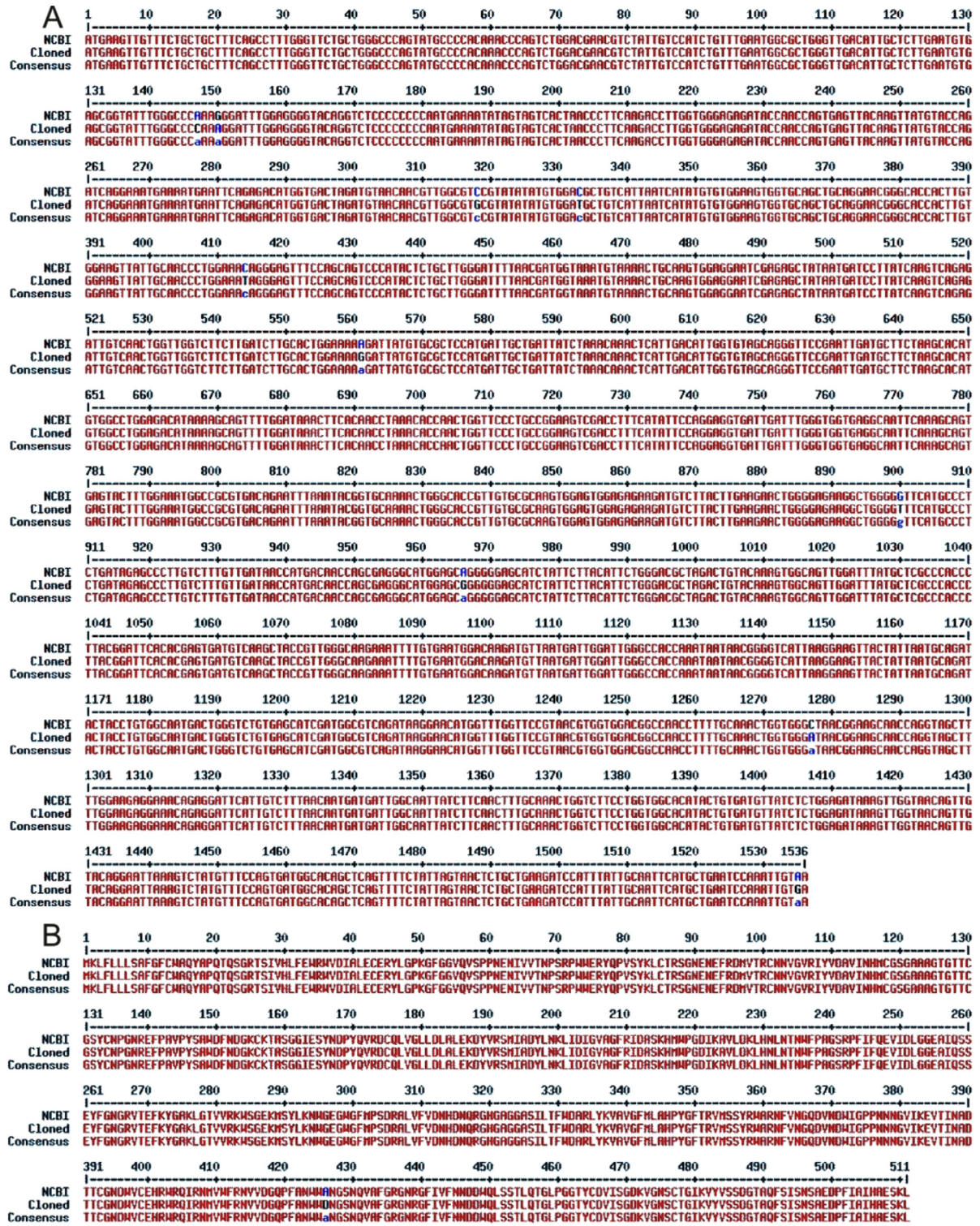


Fig. 1. The nucleic acids (A) and amino acids (B) sequence comparison between the cloned PPA cDNA and the NCBI released PPA cDNA (GenBank: AF064742.1). PPA = porcine pancreatic α-amylase. The nucleic acids or amino acids that labeled in blue or black color means the difference between the cloned PPA cDNA and the NCBI released PPA cDNA. NCBI = national center for biotechnology information.

References

- Andersson L, Rydberg U, Larsson H, Andersson R, Aman P. Preparation and characterisation of linear dextrans and their use as substrates in vitro studies of starch branching enzymes. *Carbohydr Polym* 2002;47:53–8.
- Anitha Gopala B, Muralikrishna G. Porcine pancreatic α -amylase and its isoforms: purification and kinetic studies. *Int J Food Prop* 2009;12:571–86.
- Barr K, Hopkins S, Sreekrishna K. Protocol for efficient secretion of HSA developed from *Pichia pastoris*. *Pharm Eng* 1992;12:48–51.
- Bogdanov S. Harmonised methods of the International Honey Commission. 2002. p. 34–7.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- Buisson G, Duee E, Haser R, Payan F. Three dimensional structure of porcine pancreatic alpha-amylase at 2.9 Å resolution. Role of calcium in structure and activity. *EMBO J* 1987;6:3909–16.
- Chen CC, Hunag CT, Cheng KJ. Improvement of phytase thermostability by using sorghum liquor wastes supplemented with starch. *Biotechnol Lett* 2001;23:331–3.
- Clare JJ, Rayment FB, Ballantine SP, Sreekrishna K, Romanos MA. High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Biotechnology (NY)* 1991;9:455–60.
- Dagert M, Ehrlich SD. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* 1979;6:23–8.
- Darnis S, Juge N, Guo XJ, Marchis-Mouren G, Puigserver A, Chaix JC. Molecular cloning and primary structure analysis of porcine pancreatic alpha-amylase. *Biochim Biophys Acta* 1999;1430:281–9.
- Dintzis FR, Laszlo JA, Nelsen TC, Baker FL, Calvert CC. Free and total ion concentrations in pig digesta. *J Anim Sci* 1995;73:1138–46.
- Eliasson AC. Carbohydrates in food. New York: Marcel Dekker; 1996. p. 34–553.
- Gupta R, Gigras P, Mohapatra H, Goswami V, Chauha B. Microbial α -amylases: a biotechnological perspective. *Process Biochem* 2003;38:1599–616.
- Han Y, Lei XG. Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (phyA) in *Pichia pastoris*. *Arch Biochem Biophys* 1999;364:83–90.
- Hedemann MS, Jensen BB. Variations in enzyme activity in stomach and pancreatic tissue and digesta in piglets around weaning. *Arch Anim Nutr* 2004;58:47–59.
- Inbarr J, Ogle RB. Effect of enzyme treatment of piglet feeds on performance and post weaning diarrhea. *Swed J Agric Res* 1988;18:129–33.
- Janecek S. Sequence similarities and evolutionary relationships of microbial, plant and animal alpha-amylases. *Eur J Biochem* 1994;224:519–24.
- Kato S, Ishibashi M, Tatsuda D, Tokunaga H, Tokunaga M. Efficient expression, purification and characterization of mouse salivary alpha-amylase secreted from methylotrophic yeast, *Pichia pastoris*. *Yeast* 2001;18:643–55.
- Kim T, Mullaney EJ, Porres JM, Roneker KR, Crowe S, Rice S, et al. Shifting the pH profile of *Aspergillus niger* PhyA phytase to match the stomach pH enhances its effectiveness as an animal feed additive. *Appl Environ Microbiol* 2006;72:4397–403.
- Li S, Shen W, Chen X, Shi G, Wang Z. Secretory expression of *Rhizopus oryzae* α -amylase in *Kluyveromyces lactis*. *Afr J Biotechnol* 2011;10:4190–6.
- Lineweaver H, Burk D. The determination of enzyme dissociation constants. *J Am Chem Soc* 1934;56:658–66.
- Muralikrishna G, Nirmala M. Cereal α -amylases—an overview. *Carbohydr Polym* 2005;20:1–11.
- Nguyen Q, Rezessy-Szabo J, Claeysens M, Stals I, Hoschke A. Purification and characterisation of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626. *Enzym Microb Technol* 2002;31:345–C352.
- Owusu-Asiedu A, Baidoo SK, Nyachoti CM. Effect of heat processing on nutrient digestibility in pea and supplementing amylase and xylanase to raw, extruded or micronized pea-based diets on performance of early-weaned pigs. *Can J Anim Sci* 2002;82:367–74.
- Prodanov E, Seigner C, Marchis-Mouren G. Subsite profile of the active center of porcine pancreatic alpha-amylase kinetic studies using maltooligosaccharides as substrates. *Biochem Biophys Res Commun* 1984;122:75–81.
- Qiao J, Rao Z, Dong B, Cao Y. Expression of *Bacillus subtilis* MA139 beta-mannanase in *Pichia pastoris* and the enzyme characterization. *Appl Biochem Biotechnol* 2010;160:1362–70.
- Rincker MJ, Carter SD, Gilliland SE. Potential of amylolytic cultures of *Lactobacillus acidophilus* to improve dietary starch utilization in weanling pigs. *Okla State Univ Anim Sci Res Rep* 2000;P-980:142–6.
- Robytt JF, French D. Multiple attack and polarity of action of porcine pancreatic alpha-amylase. *Arch Biochem Biophys* 1970;138:662–70.
- Romanos M. Advances in the use of *Pichia pastoris* for high-level gene expression. *Curr Opin Biotechnol* 1995;6:527–33.
- Steer ML, Levitzki A. The metal specificity of mammalian-amylase as revealed by enzyme activity and structural probes. *FEBS Lett* 1973;31:89–92.
- Svihus B, Zimonja O. Chemical alterations with nutritional consequences due to pelleting animal feeds: a review. *Anim Prod Sci* 2011;51:590–6.
- Teng D, Fan Y, Yang YL, Tian ZG, Luo J, Wang JH. Codon optimization of *Bacillus licheniformis* beta-1,3-1,4-glucanase gene and its expression in *Pichia pastoris*. *Appl Microbiol Biotechnol* 2007;74:1074–83.
- Wakim J, Robinson M, Thoma JA. The active site of porcine-pancreatic alpha-amylase: factors contributing to catalysis. *Carbohydr Res* 1969;10:487–503.
- Zhang W, Lei XG. Cumulative improvements of thermostability and pH-activity profile of *Aspergillus niger* PhyA phytase by site-directed mutagenesis. *Appl Microbiol Biotechnol* 2008;77:1033–40.
- Zhao H, Chen D, Tang J, Jia G, Long D, Liu G, et al. Partial optimization of the 5-terminal codon increased a recombination porcine pancreatic lipase (opPPL) expression in *Pichia pastoris*. *PLoS One* 2014;9, e114385.
- Zhao L, Sun LH, Huang JQ, Briens M, Qi DS, Xu SW, et al. A novel organic selenium compound exerts unique regulation of selenium speciation, selenome, and selenoproteins in broiler chicks. *J Nutr* 2017;147:789–97.