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Efficient expression and characterization of an acid protease in *Aspergillus niger* for bread making and soy protein modification

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ABSTRACT: Acid proteases are important enzymes in the food industry. In this study, an acid protease (*Ao*pepA) from *Aspergillus oryzae* was high-level expressed extracellularly in *Aspergillus niger* using different expression strategies. The highest protease activity of 407.6 U/mL was yielded in a shake flask by the engineered *A. niger* strain, which was constructed using a mixed expression strategy of constitutive and induction types. High protease activity of 1153.6 U/mL with a protein concentration of 3.2 mg/mL was produced by fed-batch fermentation in a 5 L fermenter. *Ao*pepA displayed optimal activity at pH 2.5 and 50 ℃, respectively. It was stable within pH 2.0-7.0 and up to 45 ℃. *Ao*pepA had a broad substrate specificity and the highest specific activity of 920.8 U/mg towards hemoglobin. The addition of *Ao*pepA (1000 U/kg) during the bread making improved the specific volume by 16.6% and decreased the hardness by 26.9%. Moreover, *Ao*pepA was applied to modify soy protein, resulting in a 2.2-fold increase in foaming capacity and a 1.2-fold increase in foaming stability. The emulsifying activity and emulsion stability indexes were enhanced by 1.3-fold and 2.8-fold, respectively. This study provides a valuable way for the efficient expression of proteases in *A. niger* and broadens the application of acid proteases in the food industry.

Keywords: Acid protease, *Aspergillus niger*, Fed-batch fermentation, Bread making, Soy protein modification

1. Introduction

Aspartic proteases (3.4.23.X), also known as acid proteases, are endopeptidases that catalyze the hydrolysis of peptide bonds in peptides and proteins under acid conditions[1]. They play a crucial role in various fields of the food industry, such as tenderization of pork[2], clarification of juice[3], coagulation of milk in cheese processing[4], preparation of food-derived bioactive peptides[5], particularly in baking foods[6] and protein modification[7]. In bread making, the addition of acid proteases to wheat flour contributes to improving the quality of bread[6]. They can break down the wheat gluten proteins in the dough, facilitating the formation of a good gluten network[8,9]. Moreover, acid proteases are important in the modification of soy protein. Through suitable enzymatic hydrolysis of soy protein, acid proteases can enhance its solubility, surface hydrophobicity, and functional properties[7,10], making it more suitable for application in various food fields.

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Many proteases have been used to improve bread quality[11,12] and modify proteins[13,14]. However, there have been few reports on the use of acid proteases to improve bread quality and modify proteins.

Acid proteases are mainly produced by microorganisms due to their convenience for genetic manipulation and large-scale production[1]. Microbial acid proteases can be classified into two groups: pepsin-like proteases and chymosin-like proteases[15]. These enzymes contain two typical Asp-Thr/Ser-Gly motifs, and the two aspartic acid (Asp) residues in the motifs are located in the active sites to play a catalytic role. Their molecular weights range from 30 to 50 kDa[15]. Typically, they exhibit optimal activity within pH 2.0–6.0 and a temperature range of 40–60 ℃, and they remain stable under acidic conditions[16,17]. Moreover, they show broad hydrolysis activity towards various protein substrates, and their activities are inhibited by pepstatin A[18].

Recently, the development of acid proteases by genetic engineering technology has become an efficient way. The main host strains for heterologous expression of acid proteases are *Escherichia coli* and *Komagataella phaffii* (syn. *Pichia pastoris*). Acid proteases expressed in *E. coli* are generally intracellular, making them inconvenient for application[19]. In *K. phaffii*, methanol is typically used as an inducer for the production of acid proteases[20]. In contrast, *Aspergillus niger* is regarded as a generally recognized as safe (GRAS) strain by the US Food and Drug Administration (FDA) for the production of food enzymes due to its food safety, strong post-translational modification ability, and excellent protein secretion systems[21]. Importantly, acid proteases produced by *Aspergillus niger* are considered to be safe and meet safety requirements for applications in the food industry[22]. Moreover, many biotechnology companies (e.g., AB Enzymes, BASF, Chr. Hansen, Bayer, DSM, Novozymes, DuPont, Puratos, and Roal Oy) have used *A. niger* as a cell factory for enzyme production[21]. Although previous studies have successfully expressed several acid proteases in *A. niger*[5,23], few studies have focused on improving their expression level in this host. Additionally, the expression levels of foreign proteases in *A. niger* are usually low, which may be attributed to the limitations of transcription, translation, and post-translational processing modifications[24]. Therefore, the exploration of various expression strategies for the high-level production of proteases in *A. niger* has attracted widespread attention.

In this study, an acid protease (*Ao*pepA) from *Aspergillus oryzae* was efficiently expressed extracellularly in *A. niger* FBL-B using different expression strategies. Fed-batch fermentation was carried out using the recombinant *A. niger* FBL-B for the high-level production of *Ao*pepA in a 5 L fermenter. *Ao*pepA was further purified and biochemically characterized. Subsequently, the potential application of *Ao*pepA was investigated in bread making and soy protein modification. This study offers a valuable reference for the efficient expression of proteases in *A. niger* and broadens the application of acid proteases in the food industry.

2. Materials and methods

2.1. Strains, plasmids, and reagents

Aspergillus oryzae 3.042 from China General Microbiological Culture Collection Center was used to

clone the acid protease *AopepA* gene (GenBank accession number: D13894.1). *Aspergillus niger* FBL-B (Δ*gla*A, Δ*amy*A, Δ*aamy*, Δ*pyr*G, *hygB*$^{+}$, *Ble*$^{+}$) was used as a host for *Ao*pepA expression. *Escherichia coli* DH5α (TransGen, Beijing, China) was utilized for replication and construction of plasmids. The Bunt-pyrG-PglaA (glaS) and Blunt-amdS plasmids were used as the templates for the construction of *Ao*pepA expression plasmids. All plasmids were constructed using the in-fusion cloning method. The plasmids were extracted by the Plasmid Mini-Preps Kit (TianGen, Beijing, China). FastPfu DNA polymerase and restriction endonuclease were purchased from New England Biolabs (NEB, Frankfurt, Germany). Casein from bovine milk (Sigma-Aldrich, St. Louis, MO, USA) was used as a substrate to determine protease activity. Wheat gluten with a protein content of 68.9% (wet basis) was kindly donated by Longlive Biotechnology Co., Ltd. (Dezhou, China). Soy protein isolate (SPI) was obtained from Yuanye Biotech Co., Ltd (Shanghai, China). All other analytical-grade reagents were commercially available. **Table 1** lists all used plasmids and strains. All primers are given in **Table S1**.

Table 1 Strains and plasmids used in this study

Strains and plasmids	**Source or reference**
Strains	
E. coli DH5α	Transgene
A. oryzae 3.042	Lab stock
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)	This study
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)-pepA-pepS	This study
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)-pepA-glaS	This study
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)-K-pepA-pepS	This study
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)-K-pepA-glaS	This study
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)-SES-pepA-glaS	This study
A. niger FBL-B (Δ*glaA*, Δ*amyA*, Δ*aamy*, Δ*pyr*G, *hyg*B^{+}, *Ble*$^{+}$)-double-pepA	This study
Plasmids	
Blunt-amdS	Lab stock
Blunt-pyrG-PglaA (glaS)	Lab stock
Blunt-pyrG-PglaA-pepA-pepS	This study
Blunt-pyrG-PglaA-pepA-glaS	This study
Blunt-pyrG-PglaA-Kozak-pepA-pepS	This study
Blunt-pyrG-PglaA-Kozak-pepA-glaS	This study
Blunt-amdS-SES-pepA-glaS	This study

2.2. Construction of plasmids for AopepA expression

The Blunt-pyrG-PglaA (glaS) plasmid was used as a template, and the plasmid skeleton (PS) fragment was amplified using two primers PS-F and PS-R **(Table S1)**. SignalP 5.0 server was used to predict the signal peptide of *Ao*pepA (http://www.cbs.dtu.dk/services/SignalP/). *AopepA* fragments containing the native signal peptide sequence and without the native signal peptide sequence were fused to PS fragments to generate the plasmids Blunt-pyrG-PglaA-pepA-pepS and Blunt-pyrG-PglaA-pepA-glaS, respectively. Then, a Kozak sequence (GCCACCATGG) was added at the front of the signal peptide sequence to construct the plasmids Blunt-pyrG-PglaA-Kozak-pepA-pepS and Blunt-pyrG-PglaA-Kozak-pepA-glaS, respectively. The construction of a constitutive *Ao*pepA expression plasmid using a synthetic expression system (SES) consisted of two parts: an artificial transcription factor expression module and a protease *Ao*pepA expression module.

The CP1 core promoter (008CP), synthetic transcription factor (sTF), and *TEF1t* terminator constitute the artificial transcription factor expression module. The eight tandem transcription factor binding site sequences (8BS), CP2 core promoter (201CP), *Ao*pepA (containing the glaS signal peptide) sequence, and *ADH1t* terminator constitute the *Ao*pepA expression module. Then, the two module sequences were cloned into the Blunt-amdS vector to construct the plasmid Blunt-amdS-SES-pepA-glaS. The linearized Blunt-pyrG-PglaA-pepA-pepS, Blunt-pyrG-PglaA-pepA-glaS, Blunt-pyrG-PglaA-Kozak-pepA-pepS, Blunt-pyrG-PglaA-Kozak-pepA-glaS, and Blunt-amdS-SES-pepA-glaS plasmids by *Xho*I were individually transformed into the *A. niger* FBL-B chassis host using the PEG-$CaCl_2$-mediated protoplast transformation method (PTM) for *Ao*pepA expression, respectively. For the mixed expression system of constitutive and induction types, the linearized Blunt-pyrG-PglaA-pepA-glaS and Blunt-amdS-SES-pepA-glaS plasmids were co-transformed into *A. niger* FBL-B chassis host using the same method for *Ao*pepA expression. All recombinant strains were inoculated into 50 mL of fermentation medium and incubated at 30 ℃ for 5 days to evaluate the expression level of *Ao*pepA in *A. niger* FBL-B in shake flasks following the previous method[25].

2.3. High-level production, purification, and zymogram analysis of AopepA

Fed-batch fermentation was carried out using the recombinant *A. niger* FBL-B for the high-level production of *Ao*pepA in a 5 L fermenter (Bxbio, Shanghai, China) following a previously described method[5,25]. In brief, the recombinant *A. niger* with the highest protease activity was inoculated into 150 mL of DPY medium, and incubated at 34 ℃ and 200 r/min for 36–48 h to obtain the seed broth. Then, 300 mL of the seed broth was inoculated into a 5 L fermenter containing 2.7 L of initial fermentation medium [5% (w/v) corn starch hydrolysate, 3% (w/v) corn steep liquor, 3% (w/v) soybean powder, 0.5% (w/v) KH_2PO_4, and 0.05% $CaCl_2$] for fed-batch fermentation. The medium was adjusted to pH 5.5 using 28.0% aqueous ammonia and 10% phosphoric acid. The agitation speed and temperature were controlled at 300 r/min and 34 ℃, respectively. When the starch hydrolysate in the initial fermentation medium was completely consumed (about 60–72 h), the feed medium [20% (w/v) corn starch hydrolysate, 1% (w/v) corn steep liquor, 0.5% (w/v) KH_2PO_4, and 0.05% $CaCl_2$] was fed into the initial fermentation medium at a flow rate of 5.0 mL/h/L. The dissolved oxygen (DO) concentration was maintained at > 5% by controlling the agitation rate and air flux until the end of fermentation. The wet cell weight, protein concentration, and protease activity were measured at 24 h intervals during the fed-batch fermentation. The fermentation supernatant (crude enzyme) was obtained by centrifugation at 12000 × *g* for 10 min at 4 ℃. It was dialyzed with buffer A (50 mmol/L phosphate buffer pH 6.0) and then loaded onto a Q-Sepharose Fast Flow (QSFF) column pre-equilibrated by buffer A. Elution was performed using a gradient NaCl concentration in buffer B (50 mmol/L phosphate buffer pH 6.0, 500 mmol/L NaCl) at a flow rate of 1.0 mL/min. The purified *Ao*pepA was concentrated by ultrafiltration and analyzed using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). The zymogram analysis of the purified *Ao*pepA was carried out following a previously described method[15].

2.4. Protease activity and protein concentration

The Folin-phenol reagent method with minor modifications was used to determine protease activity[26]. In brief, 100 μL of casein solution (1%, w/v) was mixed with 100 μL of diluted enzyme solution in 50 mmol/L lactic acid buffer pH 2.5 and incubated at 50 ℃ for 10 min. Then, 200 μL of 0.4 M trichloroacetic acid solution was added to stop the reaction. The reaction mixture was centrifuged at 12,000×*g* for 3 min. The supernatant (100 μL) was mixed with 500 μL of 0.4 M Na_2CO_3 solution and 100 μL of Folin-phenol reagent, and incubated at 50 ℃ for 20 min. The absorbance values of samples were measured at 680 nm. One unit (U) of enzyme activity was defined as the amount of protease that hydrolyzed casein to produce 1 μg of tyrosine every minute. The Lowry method was employed to determine the protein concentration and bovine serum albumin (BSA) was utilized as a standard protein[27]. Specific activity (U/mg) was the protease activity per milligram of protein.

2.5. Biochemical characterization of AopepA

The optimal pH of *Ao*pepA was determined at 40 ℃ in 50 mmol/L buffers with various pH values (KCl–HCl, pH 1.0–2.0; glycine–HCl, pH 2.0–3.0; lactic acid, pH 2.5–4.0; citrate, pH 3.5–6.0; and phosphate, pH 6.0–8.0). *Ao*pepA was pre-incubated with the above buffers at 40 ℃ for 30 min to assess its pH stability. The optimal temperature of *Ao*pepA was measured in 50 mmol/L lactic acid buffer pH 2.5 at 20–65 ℃. *Ao*pepA was pre-incubated at the above different temperatures for 30 min to evaluate its thermostability.

*Ao*pepA was incubated with 1 mmol/L different metal ions (Fe^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Zn^{2+}, Mg^{2+}, K^+, Na^+, Ca^{2+}, Ag^+, Cu^{2+}, Fe^{2+}, and Cr^{2+}), chemical reagents (β-mercaptoethanol, SDS), and protease inhibitors (EDTA, PMSF, and iodoacetamide at 1 mmol/L, and pepstatin A at 0.05 mmol/L) at 45 ℃ for 30 min. The residual protease activities were determined using the standard assay. The untreated *Ao*pepA was used as a control (100%).

Casein, whey protein, skimmed milk, bovine serum albumin, hemoglobin, soy protein isolate, β-lactoglobulin, gelatin, protamine sulfate, egg albumin, myoglobin, and collagen were used as substrates (1%, w/v) to measure the substrate specificity of *Ao*pepA, and casein was used as the control (100%). The protease activities of *Ao*pepA towards various protein substrates were determined using the standard assay.

2.6. Effect of AopepA on bread quality

The wheat gluten proteins (5%, w/v) were hydrolyzed by *Ao*pepA (E/S: 500 U/g) in 20 mmol/L lactic acid buffer pH 2.5 at 37 ℃ for 15, 30, and 60 min. The hydrolysis pattern of wheat gluten proteins was analyzed using 12.5% SDS–PAGE. The sample hydrolyzed for 60 min was freeze-dried, and its appearance was analyzed using scanning electron microscopy (SEM) (SU3500, Hitachi, Japan) at a magnification of ×1000. The unhydrolyzed sample was used as the control.

The bread making procedure was performed according to the previously described method with slight modifications[28]. In brief, the materials [wheat flour, 700 g; yeast, 7 g; salt, 7 g; sugar, 56 g; water, 378 g; *Ao*pepA, (0–1500 U/kg, based on the weight of flour)] were mixed and stirred at a low speed for 3 min, and then at a high speed for 3 min in a flour mixing apparatus (Sinmag Co., Wuxi, China). Then, 28 g butter was added to the dough, which was stirred at a low speed for 2 min and then at a high speed for 3 min. The dough

was divided into 150 g per piece, shaped at 25 ℃ for 15 min, and fermented at 38 ℃ with 80% humidity for 60 min. Finally, the bread was baked for 20 min (surface temperature 180 ℃, bottom temperature 200 ℃). After baking, the bread was cooled and stored at 4 ℃ for 48 h and 96 h. The specific volume (mL/g) and hardness (N) were determined to evaluate the bread quality according to the previous method[28]. The air-oven method was used to determine the moisture content of bread during low-temperature storage[29].

2.7. Modification of soy protein isolate by AopepA

The soy protein isolate (SPI) solution (5%, w/v) was hydrolyzed by *Ao*pepA (E/S: 1000 U/g) in 20 mmol/L lactic acid buffer pH 2.5 at 40 ℃ for 30, 60, 90, 120, and 180 min. The mixture was placed in boiling water for 10 min to deactivate *Ao*pepA, and the samples were freeze-dried to obtain the modified SPI. The hydrolysis pattern of SPI was analyzed using 12.5% SDS–PAGE. The degree of hydrolysis (DH) was measured by the *O*-phthaldialdehyde (OPA) assay with the following formula:

$$\mathrm{DH}\ (\%) = h/h_{\mathrm{tot}} \times 100\%$$ [30]

Where h is the number of hydrolyzed bonds in the hydrolysates, and h_{tot} is the total number of peptide bonds per protein equivalent.

The foaming and emulsifying properties of the modified SPI were analyzed according to the previously described method[31,32]. The foaming capacity (FC) and foaming stability (FS) were calculated using the following formulas:

$$\mathrm{FC}\ (\%) = \frac{V_0 - 30}{30} \times 100\%$$

$$\mathrm{FS}\ (\%) = \frac{V_{30} - 30}{V_0 - 30} \times 100\%$$

Where 30 is the volume of protein dispersion before homogenization, V_0 is the volume of homogenized modified SPI solution, and V_{30} is the volume of homogenized modified SPI solution after standing for 30 min.

The emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated using the following formulas:

$$\mathrm{EAI}\ \left(\mathrm{m^2/g}\right) = \frac{2 \times 2.303 \times \mathrm{N} \times A_0}{\mathrm{C} \times \Phi \times 10^4}$$

$$\mathrm{ESI}\ (\mathrm{min}) = \frac{A_0 \times 10}{A_0 - A_{10}}$$

Where N is the dilution factor, C is the protein concentration (g/mL) before the formation of the emulsion, and is the volume fraction of the oil phase in the emulsion. A_0 and A_{10} are the absorbances at 0 and 10 min, respectively.

2.8. Statistical analysis

Statistical analysis was performed using one-way ANOVA analysis of IBM SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) with Duncan's multiple range test. All experiments were performed with three replications. All data were shown as mean ± standard deviation (SD). Different lowercase letters were considered as statistically significant differences ($P < 0.05$).

3. Results and discussion

3.1. Effects of different strategies on the expression of AopepA in A. niger FBL-B

The schematic diagram of plasmid construction using different strategies for *Ao*pepA expression in *A. niger* FBL-B is shown in **Fig. 1A**. The protease activities of fermentation supernatants of different recombinant *A. niger* FBL-B strains in shake flasks were determined **(Fig. 1B)**. The strain with the native signal peptide produced a protease activity of 326.8 U/mL, which was lower than that of the strain with the glucoamylase signal peptide (344.7 U/mL) ($P < 0.05$). However, the strain using the native signal peptide combined with the Kozak sequence only produced a protease activity of 265.3 U/mL. The strain containing the glucoamylase signal peptide combined with the Kozak sequence produced a protease activity of 258.1 U/mL.There was no significant difference between the protease activities of the two strains ($P > 0.05$). The strain constructed using an SES-constitutive type expression strategy with the glucoamylase signal peptide produced a protease activity of 229.6 U/mL. In contrast, the strain constructed using a mixed expression strategy of constitutive and induction types with the glucoamylase signal peptide produced the highest protease activity of 407.6 U/mL **(Fig. 1B)**. This was much higher than that of the induction-type strain with the glucoamylase signal peptide (344.7 U/mL) and the constitutive-type strain with the glucoamylase signal peptide (229.6 U/mL) ($P < 0.05$).

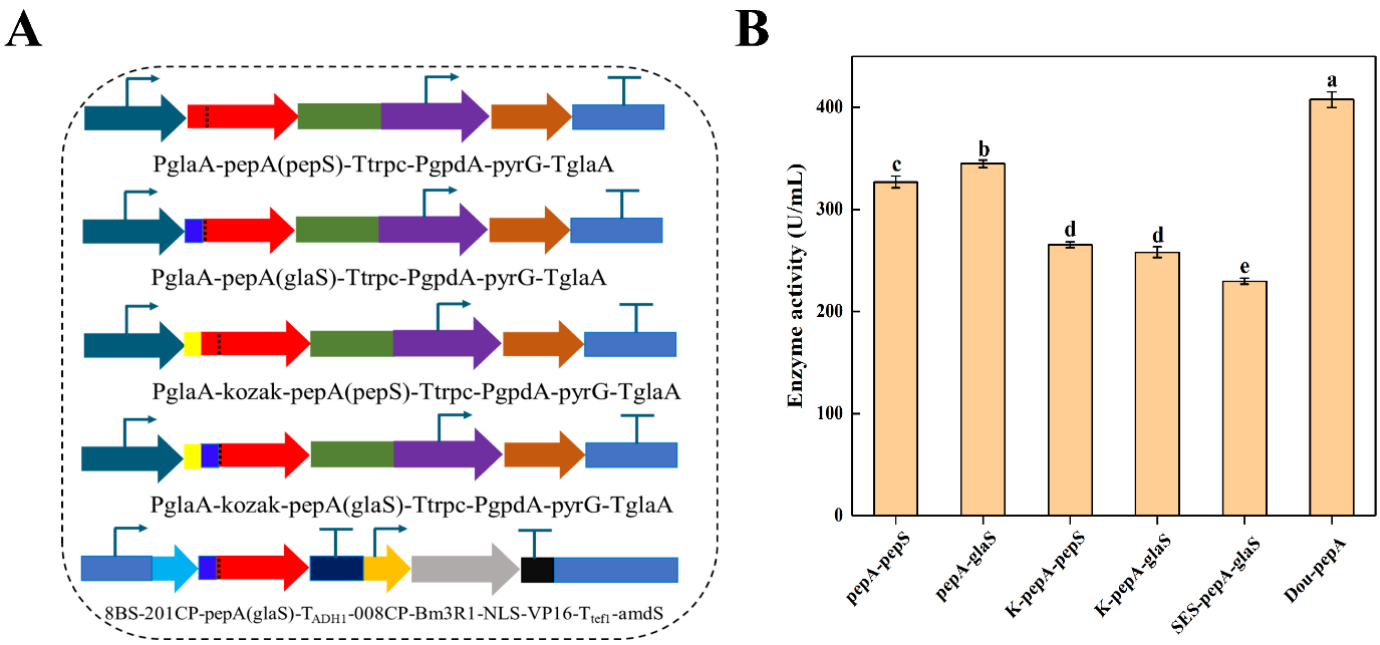

Fig. 1. Schematic diagram of plasmid construction using various strategies for *Ao*pepA expression in *A. niger* FBL-B (A) and the protease activities of fermentation supernatants of different recombinant *A. niger* strains in shake flasks (B). These plasmids included Blunt-pyrG-PglaA-pepA-pepS, Blunt-pyrG-PglaA-pepA-glaS, Blunt-pyrG-PglaA-Kozak-pepA-pepS, Blunt-pyrG-PglaA-Kozak-pepA-glaS, and Blunt-amdS-SES-pepA-glaS. The protease activity was determined using casein (1%, w/v) as a substrate in 50 mmol/L lactic acid buffer pH 2.5 at 50 °C. Different lowercase letters represent significant differences ($P < 0.05$).

The signal peptides play a crucial role in the secretory expression of proteins in *A. niger*[33]. The glucoamylase signal peptide improved the secretory expression level of α-galactosidase in *A. niger*[34]. In this study, the expression level of *Ao*pepA with the glucoamylase signal peptide was slightly higher than that of the native signal peptide **(Fig. 1B)**. However, the native signal peptide showed the secretory expression level of lipase in *A. niger* was higher than that of the glucoamylase signal peptide[33]. The Kozak sequence

(GCCACCATGG) enhances translation efficiency by optimizing the ATG environment to avoid leaky ribosomal scanning. It has been applied to improve the expression levels of foreign genes in *A. niger*[33]. However, the addition of a Kozak sequence after the promoter significantly reduced the expression level of *Ao*pepA **(Fig. 1B)**. This may be because the commonly used Kozak sequence may not be optimal for *Ao*pepA expression[35]. Recently, a universal synthetic expression system (SES) has been developed to increase the expression level of genes in fungi[36]. When using the SES-constitutive expression system, the expression level of *Ao*pepA was lower than that of the glucoamylase-induction type **(Fig. 1B)**. Interestingly, the expression level of *Ao*pepA was significantly improved when using a mixed expression system of constitutive and induction types. This may be due to the simultaneous transcription of the target gene using the two expression modes inside the cells, which increased the mRNA content of *Ao*pepA, thereby improving the expression level of *Ao*pepA in *A. niger* FBL-B[36].

3.2. High-level production, purification, and zymogram analysis of AopepA

The recombinant *A. niger* FBL-B strain produced a high protease activity of 1153.6 U/mL with a protein concentration of 3.2 mg/mL in a 5 L fermenter after fermentation for 168 h. The maximum wet cell weight of 548.5 g/L was obtained after fermentation for 96 h **(Fig. 2A)**. *Ao*pepA was secreted into the fermentation supernatant and a distinct protein band of approximately 40 kDa was observed on SDS–PAGE **(Fig. 2B)**. *Ao*pepA was purified to homogeneity **(Fig. 3A)** with a recovery yield of 59.2% and a specific activity of 563.8 U/mg **(Table 2)**. The zymogram analysis revealed that a clear band of proteolytic activity was observed in the gel **(Fig. 3B).**

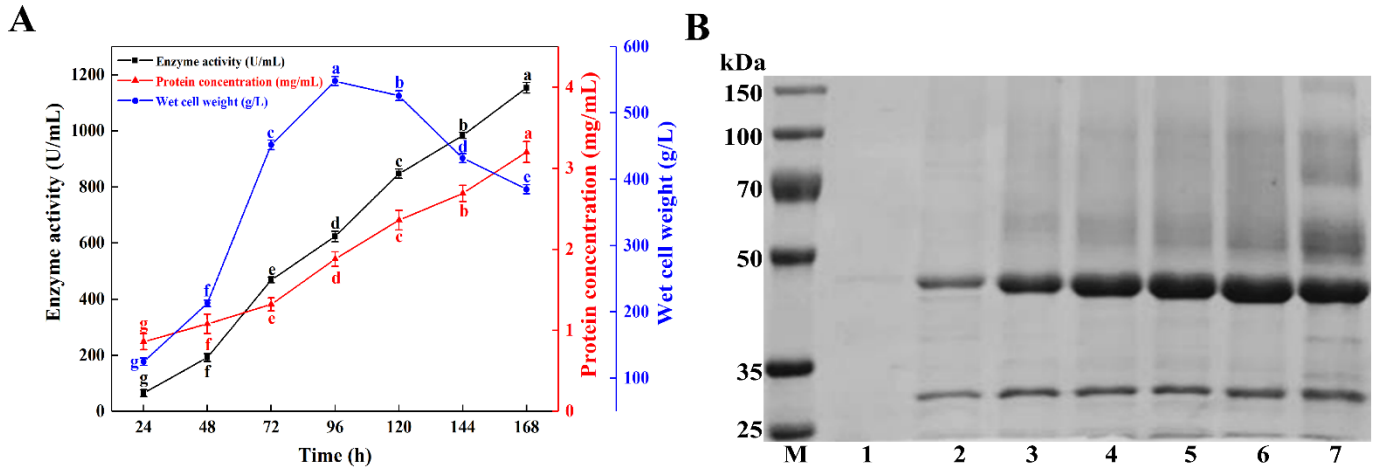

Fig. 2. Time-course of *Ao*pepA produced by fed-batch fermentation in a 5 L fermenter (A) and SDS–PAGE analysis of extracellular proteins (B). Enzyme activity (■), protein concentration (▲), and wet cell weight (●) were measured every 24 h during the fed-batch fermentation**.** The protease activity was determined using casein (1%, w/v) as a substrate in 50 mmol/L lactic acid buffer pH 2.5 at 50 °C. In Fig. 2B, Lane M, standard protein marker; lanes 1-7, the fermentation supernatant samples were withdrawn at 24, 48, 72, 96, 120, 144, and 168 h, respectively. Different lowercase letters represent significant differences ($P < 0.05$).

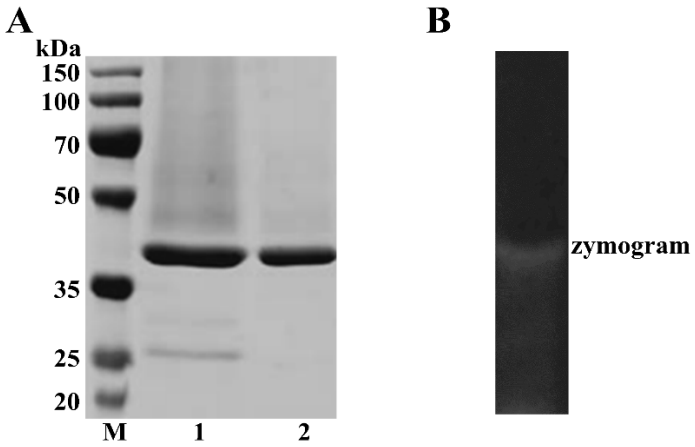

Fig. 3. SDS–PAGE (A) and zymogram (B) analysis of the purified *Ao*pepA. Lane M, standard protein marker; lane 1, crude enzyme; lane 2, the purified *Ao*pepA.

Table 2 Purification summary of *Ao*pepA

Purification step	Total activity [a] / U	Total protein [b] /mg	Specific activity [c]/ (U·mg^{-1})	Purification factor [d]/(-fold)	Recovery [e]/%
Crude enzyme	30192.5	96.4	313.2	1.0	100
QSFF	17872.5	31.7	563.8	1.8	59.2

a: Enzyme activity was determined in 50 mmol/L lactic acid buffer pH 2.5 at 50 ℃ using casein (1%, w/v) as a substrate.
b: The protein concentration was determined using the Lowry method with BSA as the standard.
c: The specific activity was defined as the enzyme activity per milligram of protein (U/mg).
d: The purification factor (-fold) was defined as the ratio of specific activity before and after purification.
e: The recovery (%) was calculated as the ratio of total activity before and after purification.

So far, the main host strains for heterologous expression of acid proteases are *K. phaffii* and *E. coli*, with few reports in *A. niger*. **Table S2** listed the expression levels and activities of various acid proteases in different hosts. In this study, the expression level of *Ao*pepA (1153.6 U/mL) in *A. niger* is close to that of *Rm*proB (1242.2 U/mL) from *Rhizomucor miehei* in *A. niger*[5], and significantly higher than that of pepsinogen A (542.3 U/mL) from *Homo sapiens* in *A. niger*[23]. It is much higher than those of most acid proteases expressed in *K. phaffii*, such as rP6218 (328.1 U/mL) from *Trichoderma harzianum*[37], *Ps*APA (89.3 U/mL) from *Penicillium* sp.[38], *Tl*AP (67.8 U/mL) from *Talaromyces leycettanus*[3]. However, the expression level of *Ao*pepA (1153.6 U/mL) in *A. niger* is much lower than that in *K. phaffii* (17,392.0 U/mL)[39], and also lower than those of several acid proteases expressed in *K. phaffii*, such as *Ta*proA1 (4092.0 U/mL) from *Trichoderma asperellum*[16], *Rm*proA (3480.3 U/mL) from *Rhizomucor miehei*[2], and Apa1 (1500.0 U/mL) from *A. niger*[17].

3.3. Biochemical characterization of AopepA

*Ao*pepA had the optimal activity of pH 2.5 in lactic acid buffer **(Fig. 4A)**. More than 80% of relative activity was retained within pH 2.0–7.0 **(Fig. 4B)**. The optimal temperature of *Ao*pepA was 50 ℃ **(Fig. 4C)**. It retained more than 80% of relative activity up to 45 ℃ **(Fig. 4D)**. Cu^{2+} (125.1%), Mn^{2+} (118.5%), Co^{2+} (111.3%), and Ni^{2+} (110.1%) significantly promoted the activity of *Ao*pepA. SDS (8.1%) strongly inhibited the activity of *Ao*pepA **(Table 3)**. Protease inhibitors PMSF, iodoacetamide, and EDTA did not affect the activity of *Ao*pepA, while pepstatin A completely inhibited its activity **(Table 3)**. As shown in **Table 4**,

*Ao*pepA displayed the highest specific activity towards hemoglobin (163.3%), followed by bovine serum albumin (143%), myoglobin (121.6%), casein (100%), β-lactoglobulin (89.4%), egg albumin (84.5%), skimmed milk (70.8%), whey protein (64.3%), and soy protein isolate (53.0%). The protease activity was not detected in gelatin, collagen, and protamine sulfate.

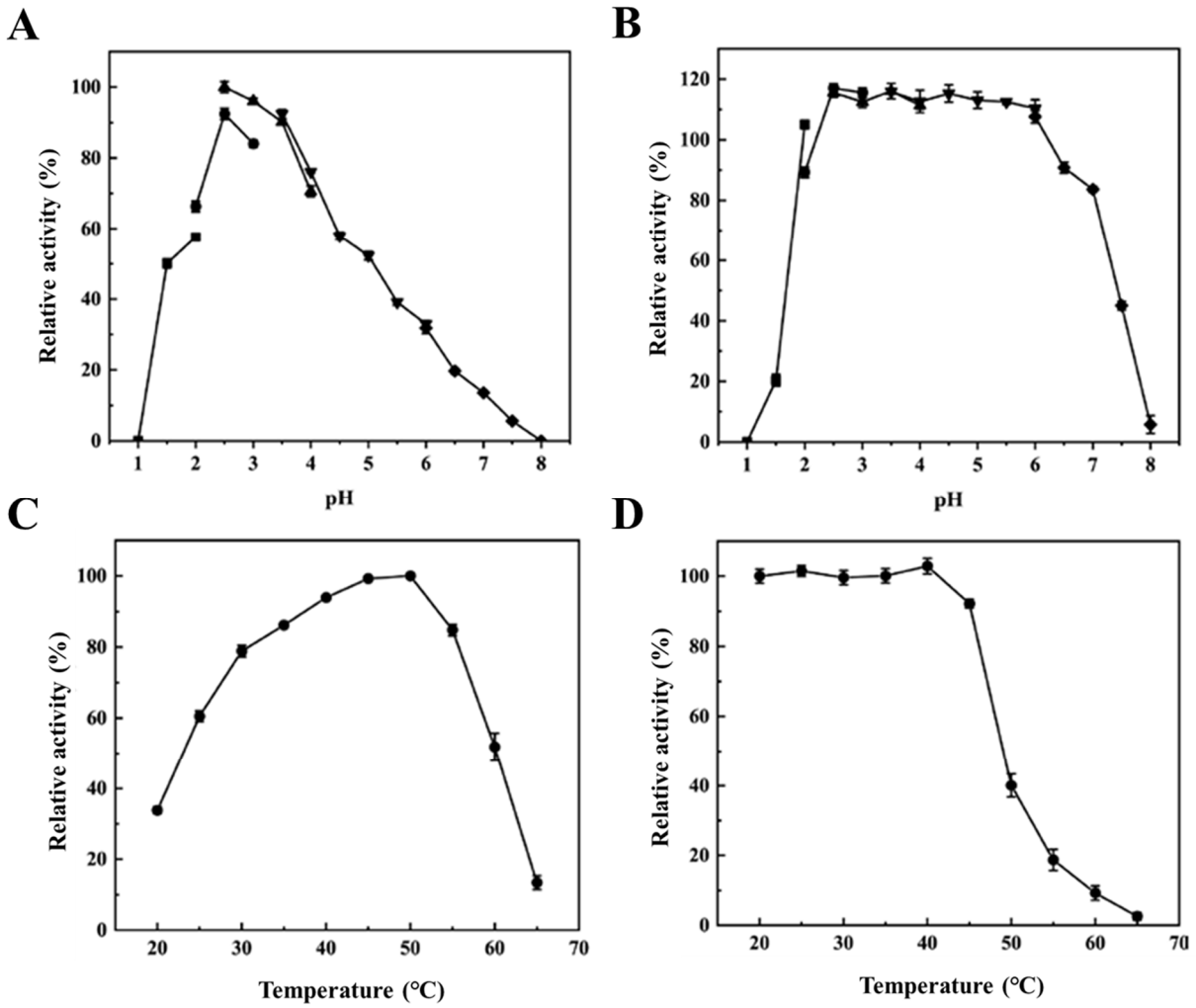

Fig. 4. Optimal pH (A), pH stability (B), optimal temperature (C), and thermostability (D) of the purified *Ao*pepA. The optimal pH of *Ao*pepA was determined in 50 mmol/L various buffers at 40 °C. The buffers included KCl–HCl (■), pH 1.0–2.0; glycine–HCl (●), pH 2.0–3.0; lactic acid (▲), pH 2.5–4.0; citrate (▼), pH 3.5–6.0; and phosphate (◆), 6.0–8.0. The pH stability of *Ao*pepA was assessed by measuring the residual protease activity after the enzyme was pre-incubated at 40 °C for 30 min in the above various buffers. The optimal temperature of *Ao*pepA was determined at different temperatures (20–65 °C) in 50 mmol/L lactic acid buffer pH 2.5. The thermostability of *Ao*pepA was evaluated by measuring the residual protease activity after the enzyme was pre-incubated at the above various temperatures for 30 min in 50 mmol/L lactic acid buffer pH 2.5.

Table 3 Effects of metals ions, chemical reagents, and protease inhibitors on the activity of *Ao*pepA

Metal ions, chemical reagents, and protease inhibitors (1 mmol/L)	Specific activity [a] / ($U \cdot mg^{-1}$)	Relative activity [b] /%
Control	563.8 ± 21.5	100.0 ± 3.8
Cu^{2+}	705.6 ± 13.9	125.1 ± 2.5
Mn^{2+}	667.9 ± 14.0	118.5 ± 2.5
Co^{2+}	627.4 ± 5.1	111.3 ± 0.9
Ni^{2+}	620.9 ± 11.9	110.1 ± 2.1
Ba^{2+}	570.3 ± 15.2	101.1 ± 2.7
Zn^{2+}	563.5 ± 12.5	99.9 ± 2.2
Mg^{2+}	563.5 ± 9.3	99.9 ± 1.6

K^+	559.2 ± 9.2	99.2 ± 1.6
Na^+	557.4 ± 16.8	98.9 ± 3.0
Ca^{2+}	557.4 ± 11.6	98.9 ± 2.1
Ag^+	556.3 ± 17.3	98.7 ± 3.1
Fe^{3+}	554.5 ± 7.1	98.3 ± 1.3
Fe^{2+}	552.4 ± 2.8	98.0 ± 0.5
Cr^{2+}	545.9 ± 7.1	96.8 ± 1.3
β-mercaptoethanol	554.5 ± 8.5	98.3 ± 1.5
SDS	45.6 ± 11.4	8.1 ± 2.0
EDTA	562.1 ± 3.9	99.7 ± 0.7
PMSF	558.8 ± 17.9	99.1 ± 3.2
Iodoacetamide	555.2 ± 4.9	98.5 ± 0.9
Pepstatin A (0.05 mmol/L)	0.0 ± 0.0	0.0 ± 0.0

All samples were determined in triplicate. Specific activity and relative activity were shown as the mean ± SD (n=3).
a: The protease activity was determined under the optimal conditions (pH 2.5, 50 ℃) using casein (1%, w/v) as substrate.
b: The specific activity without mental ions, chemical reagents, and protease inhibitors was defined as 100%.

Table 4 Substrate specificity of *Ao*pepA

Substrates	Specific activity [a] / ($U \cdot mg^{-1}$)	Relative activity [b] / %
Casein	563.8 ± 21.5	100.0 ± 3.8
Hemoglobin	920.8 ± 5.3	163.3 ± 0.9
Bovine serum albumin	806.1 ± 10.2	143.0 ± 1.8
Myoglobin	685.6 ±11.9	121.6 ± 2.1
β-lactoglobulin	504.2 ± 9.2	89.4 ± 1.6
Egg albumin	476.2 ± 19.7	84.5 ± 3.5
Skimmed milk	399.1 ± 9.7	70.8 ± 1.7
Whey protein	362.7 ± 14.6	64.3 ± 2.6
Soy protein isolate	298.8 ± 8.9	53.0 ± 1.5
Protamine sulfate	ND [c]	ND [c]
Gelatin	ND [c]	ND [c]
Collagen	ND [c]	ND [c]

All samples were determined in triplicate. Specific activity and relative activity were shown as the mean ± SD (n=3).
a: The protease activity was determined under the optimal conditions (pH 2.5, 50 ℃).
b: The protease activity towards casein was defined as 100%.
c: No activity detected.

Generally, acid proteases have optimal activity within pH 2.0–6.0 and remain stable under acidic conditions[2,16]. The optimal pH (pH 2.5) of *Ao*pepA is consistent with that of rP6218 from *T. harzianum*[37] and *Rm*proB from *R. miehei*[5]. *Ao*pepA exhibited a wider pH stability range (pH 2.0–7.0) than rP6218 (pH 2.5–6.0) from *T. harzianum*[37], *Ta*proA1 (pH 3.0–6.0) from *T. asperellum*[16], and Apa1 (pH 2.0–5.0) from *A. niger*[17]. The optimal temperature (50 ℃) of *Ao*pepA is higher than that of *Rm*proB (40 ℃) from *R. miehei*[5] and *Ps*APA (30 ℃) from *Penicillium* sp.[38]. *Ao*pepA displayed good thermostability, which is higher than those of rP6218 from *T. harzianum*[37] and *Ps*APA from *Penicillium* sp.[38]. Cu^{2+} and Mn^{2+} activated the activity of *Ao*pepA, which is similar to that of rP6218 from *T. harzianum*[37], *Ps*APA from *Penicillium* sp.[38], and *Tl*AP from *T. leycettanus*[3]. SDS strongly inhibited the protease activity, which may be due to the denaturation of *Ao*pepA[2]. Pepstatin A completely inhibited the protease activity, indicating that *Ao*pepA belongs to the aspartic protease family[19]. Usually, acid proteases exhibit a broad substrate specificity and the highest specific activity towards casein and no activity towards collagen, such as *Rm*proA and *Rm*proB from *R. miehei*[2,5] and *Ps*APA from *Penicillium* sp.[38]. *Ao*pepA hydrolyzed β-lactoglobulin and egg albumin **(Table 3)**. However, *Ta*proA1 from *T. asperellum* and *Ps*APA from *Penicillium* sp. did not have activity

towards β-lactoglobulin and egg albumin, respectively[16,38].

3.4. Effect of AopepA on bread quality

The SDS-PAGE profile of wheat gluten proteins hydrolyzed by *Ao*pepA is shown in **Fig. S1A**. As the hydrolysis time increased, the wheat gluten proteins were gradually degraded. Meanwhile, the network structure of wheat gluten proteins (**Fig. S1B**) was significantly destroyed by *Ao*pepA, as evidenced by the microstructure (**Fig. S1C**). **Fig. 5A** shows the central cross-section of the bread. As the *Ao*pepA dosage increased, the specific volume of the bread was first increased and then decreased. The specific volume and hardness of bread without *Ao*pepA (control) were 4.28 mL/g and 3.34 N, respectively. The addition of *Ao*pepA (250–1500 U/kg) increased the specific volume and decreased the hardness. When the dosage of *Ao*pepA was 1000 U/kg, the specific volume of the bread was 4.99 mL/g, which was 16.6% higher than that of the control. Meanwhile, the hardness of the bread was 2.44 N, which was 26.9% lower than that of the control **(Fig. 5B)**. Further, the bread hardness was monitored during storage at 4 ℃. The hardness of bread without *Ao*pepA (control) was 13.26 N and 15.55 N at 2 and 4 days, respectively. It was significantly higher than those of the addition of 1000 U/kg *Ao*pepA (8.64 N and 9.66 N at 2 and 4 days, respectively). Compared with the control, the hardness of bread was decreased by 35.0% and 37.8% at 2 and 4 days, respectively **(Fig. 5C)**. Additionally, the addition of *Ao*pepA (1000 U/kg) reduced the loss of moisture content in bread compared to the control during low-temperature storage (**Fig. S2**).

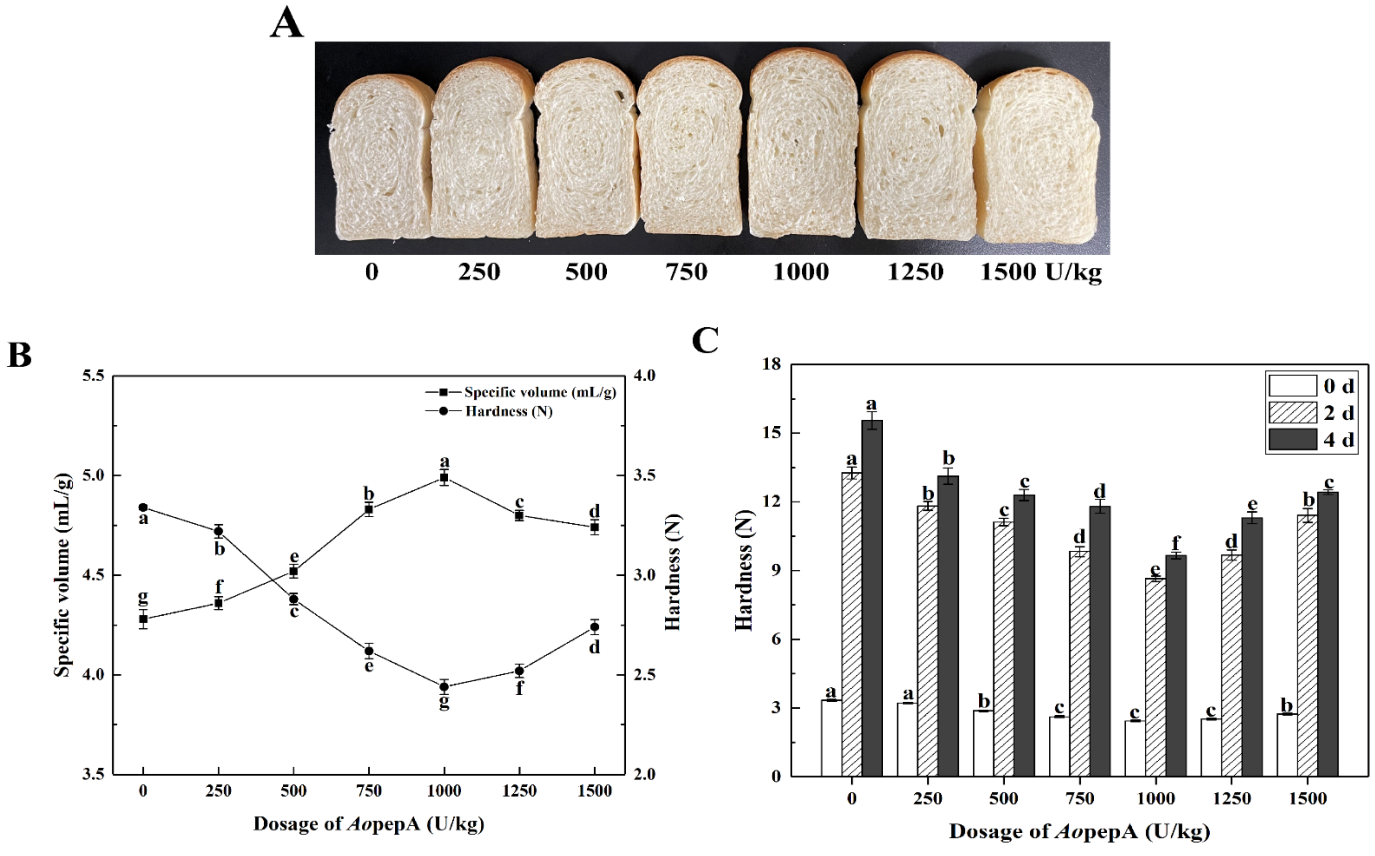

Fig. 5. The central cross-sections of bread (A). Effect of *Ao*pepA on bread quality (B) and bread storage at 4 ℃ for 4 days (C). The specific volume (mL/g) (■) was calculated using the rapeseed replacement method. The hardness (N) (●) was measured with a TMS-Pro Texture Analyzer. Different lowercase letters represent significant differences ($P < 0.05$).

In the baking industry, proteases play a crucial role in improving bread quality by hydrolyzing the proteins into peptides or amino acids[40]. Several proteases have been reported to improve bread quality, such as Flavourzyme[11], bacillolysin (metalloprotease), subtilisin (serine protease), papain (cysteine protease)[12], and thermoase (a commercial protease from *Bacillus stearothermophilus*)[41]. Acid proteases, important enzymes in the food industry, are rarely reported to improve bread quality. In this study, the addition of *Ao*pepA (250–1500 U/kg) significantly increased the specific volume of bread ($P < 0.05$) **(Fig. 5A)**. However, when the *Ao*pepA dosage exceeded 1000 U/kg, the specific volume of bread began to decrease **(Fig. 5B)**. Possibly, an appropriate dosage of *Ao*pepA can promote dough expansion and thereby increase the specific volume of bread[42,43]. However, excessive addition of *Ao*pepA may cause over-hydrolysis of wheat gluten proteins, resulting in a decrease of dough viscoelasticity and gas retention, thereby decreasing the specific volume of bread[11]. Generally, low-temperature storage causes a decrease in bread quality due to an increase in bread hardness[44]. The addition of *Ao*pepA (250–1500 U/kg) to bread significantly decreased its hardness during low-temperature storage ($P < 0.05$) (**Fig. 5C**), suggesting that *Ao*pepA can delay the aging of bread and help to maintain a better texture during low-temperature storage. In bread making, some enzymes such as α-amylases and xylanases are commonly used to reduce the hardness of wheat bread during low-temperature storage[45,46]. Generally, α-amylases improve the softness of wheat bread by decreasing the rigidity of the starch gel network, improving starch structure, and retarding starch retrogradation[45,47]. Xylanases reduce the hardness of wheat bread by degrading wheat arabinoxylan, strengthening the gluten network, and improving moisture distribution[46,48]. Compared with these enzymes, *Ao*pepA shows a different mechanism in reducing the hardness of wheat bread during low-temperature storage. In this study, *Ao*pepA can hydrolyze wheat gluten proteins to destroy its network structure **(Fig. S1)**, which may weaken the starch-protein interactions[49]. Meanwhile, the addition of *Ao*pepA reduced the loss of moisture content in bread **(Fig. S2)** and the hardness of bread **(Fig. 5C)** during low-temperature storage, thereby delaying the staling of bread. These findings indicated that the acid protease *Ao*pepA from *A. oryzae* can be used as a novel food additive to improve bread quality.

3.5. Modification of soy protein isolate by AopepA

Compared with the SPI (control), the β-conglycinin and glycinin subunits of SPI were effectively hydrolyzed by *Ao*pepA, resulting in a protein band with a molecular weight of approximately 50 kDa **(Fig. 6A)**. Additionally, the DH reached a maximum value of 37.8% at 120 min with the extension of hydrolysis time **(Fig. S3)**. Compared with the SPI (control), the FC and FS of the modified SPI were significantly improved. When the hydrolysis time was 180 min, FC and FS of the modified SPI reached maximum values of 42.8% **(Fig. 6B)** and 95.3% **(Fig. 6C)**, which were 2.2-fold and 1.2-fold higher than those of the control, respectively. Moreover, the EAI of the modified SPI reached a maximum value of 23.8 m^2/g at 60 min, which was 1.3-fold higher than that of the control. With the extension of the hydrolysis time, the EAI of the modified SPI did not change significantly ($P > 0.05$) **(Fig. 6D)**. At 60 min, the ESI of the modified SPI reached a maximum value of 129 min, which was 2.8-fold higher than that of the control **(Fig. 6E)**. However, the ESI of

the modified SPI gradually decreased with the extension of the hydrolysis time **(Fig. 6E)**.

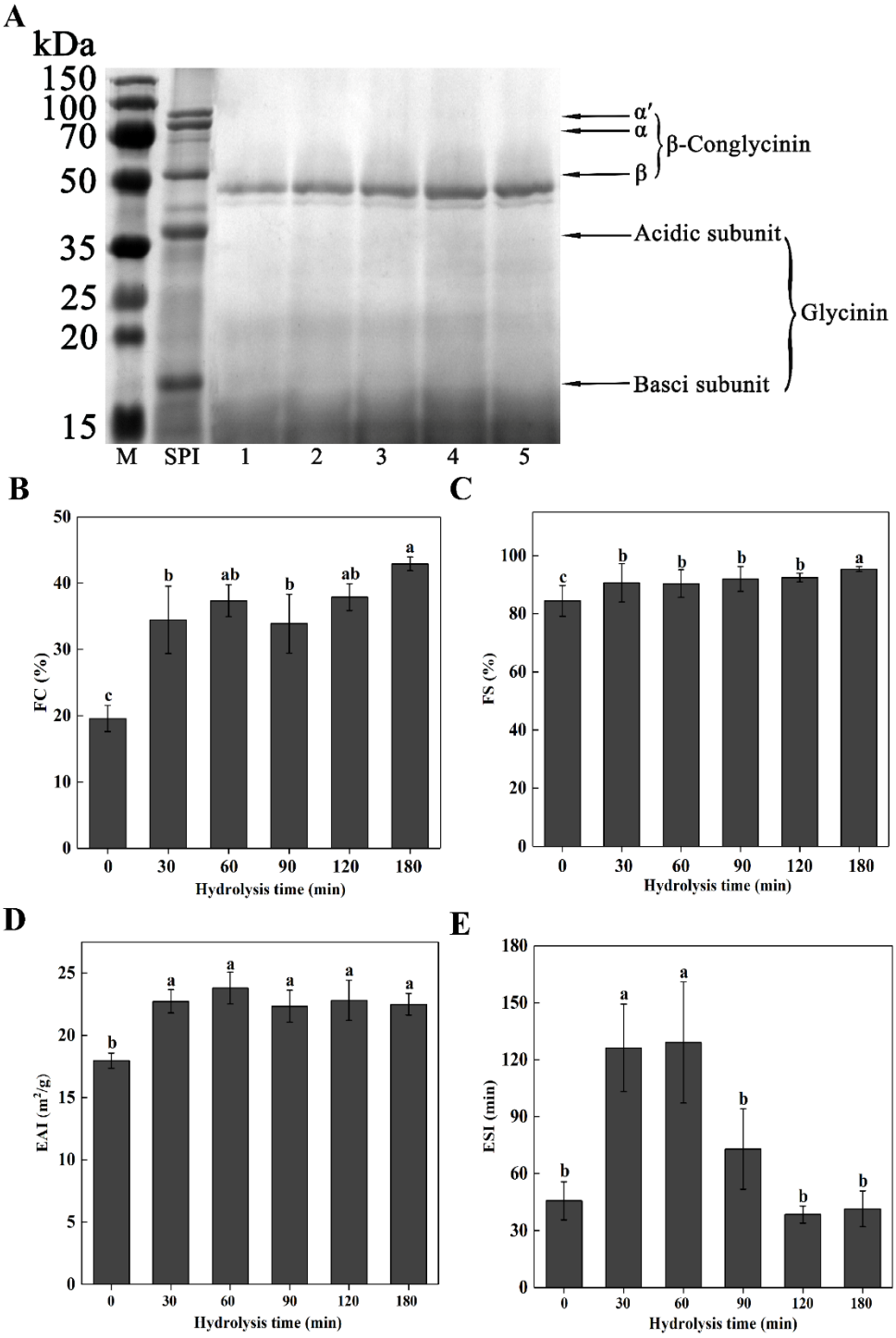

Fig. 6. SDS–PAGE analysis of the soy protein isolate (SPI) hydrolyzed by *Ao*pepA (A). In Fig. 5A, Lane M, standard protein marker; lane SPI, control; lanes 1-5, the SPI hydrolyzed by *Ao*pepA at 30, 60, 90, 120, and 180 min, respectively. The foaming capacity (B), foaming stability (C), emulsifying activity index (D), and emulsion stability index (E) of the modified SPI at different hydrolysis times. Different lowercase letters represent significant differences ($P < 0.05$).

Proteases, such as trypsin (porcine pancreas)[50] and alcalase 2.4 L[13], have been widely applied to improve the functional properties of proteins, but the use of acid proteases is rarely reported. Moreover, the DH and pH of the environment have considerable effects on the foaming and emulsifying properties of proteins[13,51]. In this study, the SPI hydrolyzed by *Ao*pepA at pH 2.5 with a DH of 34.2% exhibited maximum values of FC **(Fig. 6B)** and FS **(Fig. 6C)** compared to the SPI (control). This may be attributed to the modified

SPI exposing more hydrophobic groups, having a looser spherical conformation, and a higher proportion of small molecule peptides, which is conducive to the formation of a liquid film at the interface between air and water[13]. However, as the hydrolysis time increased, the EAI of the modified SPI was initially increased and then decreased **(Fig. 6D)**. This may be due to the destruction of the protein spherical structure, which exposes more hydrophobic residues, thereby increasing the EAI of SPI[13]. Moreover, a higher DH may lead to a decrease in the flexibility of SPI and an increase in interactions between protein molecules, consequently reducing its EAI[13,52]. The decrease in the ESI of the modified SPI (**Fig. 6E**) is likely caused by a decrease in the molecular flexibility of the proteins[53]. Therefore, these results suggested that the acid protease *Ao*pepA from *A. oryzae* can be employed to modify SPI.

4. Conclusion

An acid protease (*Ao*pepA) from *A. oryzae* was efficiently expressed extracellularly in *A. niger* using various expression strategies. The recombinant *A. niger*, constructed using a mixed expression strategy of constitutive and induction types, produced a high protease activity of 1153.6 U/mL in a 5 L fermenter. *Ao*pepA displayed optimal activity at pH 2.5 and 50 ℃, as well as high specific activity towards hemoglobin (920.8 U/mg). The addition of *Ao*pepA to wheat flour during the bread making improved bread quality. Additionally, *Ao*pepA improved the functional properties of soy protein. This study provides a valuable reference for the efficient expression of other enzymes in *A. niger* and highlights the application potential of acid proteases in the food industry.

Declaration of competing interest

The authors state that they have no known competing financial interests or personal relationships that could have appeared to influence the work described in this study.

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References

[1] J. Mamo, F. Assefa, The role of microbial aspartic protease enzyme in food and beverage industries, J. Food Quality 1 (2018) 7957269. https://doi.org/10.1155/2018/7957269.

[2] Q. Sun, F. Chen, F. Geng, et al., A novel aspartic protease from *Rhizomucor miehei* expressed in *Pichia pastoris* and its application on meat tenderization and preparation of turtle peptides, Food Chem. 245 (2018) 570-577. https://doi.org/10.1016/j.foodchem.2017.10.113.

[3] Y. Guo, T. Tu, P. Yuan, et al., High-level expression and characterization of a novel aspartic protease from *Talaromyces leycettanus* JCM12802 and its potential application in juice clarification, Food Chem. 281 (2019) 197-203. https://doi.org/10.1016/j.foodchem.2018.12.096.

[4] L. Zhang, G. Wu, D. Li, et al., Isolation and identification of milk-clotting proteases from *Prinsepia utilis Royle* and its application in cheese processing, Food Res. Int. 183 (2024) 114225. https://doi.org/10.1016/j.foodres.2024.114225.

[5] S. Wang, P. Zhang, Y. Xue, et al., Characterization of a novel aspartic protease from *Rhizomucor miehei* expressed in *Aspergillus niger* and its application in the production of ACE-inhibitory peptides, Foods 10 (2021) 2949. https://doi.org/10.3390/foods10122949.

[6] C.G. Rizzello, J.A. Curiel, L. Nionelli, et al., Use of fungal proteases and selected sourdough lactic acid bacteria for making wheat bread with an intermediate content of gluten, Food Microbiol. 37 (2014) 59-68. https://doi.org/10.1016/j.fm.2013.06.017.

[7] G. Liang, W. Chen, X. Qie, et al., Modification of soy protein isolates using combined pre-heat treatment and controlled enzymatic hydrolysis for improving foaming properties, Food Hydrocolloids 105 (2020) 105764. https://doi.org/10.1016/j.foodhyd.2020.105764.

[8] L. Deng, Z. Wang, S. Yang, et al., Improvement of functional properties of wheat gluten using acid protease from *Aspergillus usamii*, PLoS One 11 (2016) e0160101. https://doi.org/10.1371/journal.pone.0160101.

[9] M.G. Gänzle, J. Loponen, M. Gobbetti. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality, Trends Food Sci. Tech. 19 (2008) 513-521. https://doi.org/10.1016/j.tifs.2008.04.002.

[10] B. Yuan, J. Ren, M. Zhao, et al., Effects of limited enzymatic hydrolysis with pepsin and high-pressure homogenization on the functional properties of soybean protein isolate, LWT-Food Sci. Technol. 46 (2012) 453-459. https://doi.org/10.1016/j.lwt.2011.12.001.

[11] M. Gu, T. Hong, Y. Ma, et al., Effects of a commercial peptidase on rheology, microstructure, gluten properties of wheat dough and bread quality, LWT-Food Sci. Technol. 160 (2022) 113266. https://doi.org/10.1016/j.lwt.2022.113266.

[12] Y. Honda, N. Inoue, R. Sugimoto, et al., Dynamic viscoelasticity of protease-treated rice batters for gluten-free rice bread making, Biosci. Biotech. Bioch. 82 (2018) 484-488. https://doi.org/10.1080/09168451.2018.1427549.

[13] J. Hao, Z. Zhang, M. Yang, et al., Micronization using combined alkaline protease hydrolysis and high-speed shearing homogenization for improving the functional properties of soy protein isolates, Bioresour. Bioprocess. 9 (2022) 77. https://doi.org/10.1186/s40643-022-00565-9.

[14] Q. Wang, Y. Wang, M. Huang, et al., Ultrasound-assisted alkaline proteinase extraction enhances the yield of pecan protein and modifies its functional properties, Ultrason. Sonochem. 80 (2021) 105789. https://doi.org/10.1016/j.ultsonch.2021.105789.

[15] P.M. Souza, G. Werneck, B. Aliakbarian, et al., Production, purification and characterization of an aspartic protease from *Aspergillus foetidus*. Food Chem. Toxicol. 109 (2017) 1103-1110. https://doi.org/10.1016/j.fct.2017.03.055.

[16] Y. Xue, Q. Yan, X. Li, et al., Characterization of a novel aspartic protease from *Trichoderma asperellum* for the preparation of duck blood peptides, Appl. Microbiol. Biotechnol. 108 (2024) 131. https://doi.org/10.1007/s00253-023-12848-y.

[17] M. Wei, P. Chen, P. Zheng, et al., Purification and characterization of aspartic protease from *Aspergillus niger* and its efficient hydrolysis applications in soy protein degradation, Microb. Cell Fact. 22 (2023) 42. https://doi.org/10.1186/s12934-023-02047-9.

[18] K. Purushothaman, S. Bhat, S. Singh, et al., Aspartic protease from *Aspergillus niger*: Molecular characterization and interaction with pepstatin A, Int. J. Biol. Macromol. 139 (2019) 199-212. https://doi.org/10.1016/j.ijbiomac.2019.07.133.

[19] R. Jiang, S. Yuan, Y. Zhou, et al., Strategies to overcome the challenges of low or no expression of heterologous proteins in *Escherichia coli*, Biotechnol. Adv. 75 (2024) 108417. https://doi.org/10.1016/j.biotechadv.2024.108417.

[20] S.C. Spohner, H. Müller, H. Quitmann, et al., Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*, J. Biotechnol. 202 (2015) 118-134. https://doi.org/10.1016/j.jbiotec.2015.01.027.

[21] C. Li, J. Zhou, G. Du, et al., Developing *Aspergillus niger* as a cell factory for food enzyme production, Biotechnol. Adv. 44 (2020) 107630. https://doi.org/10.1016/j.biotechadv.2020.107630.

[22] M.L. Abarca, F. Accensi, J. Cano, et al., Taxonomy and significance of black aspergilli, Antonie van Leeuwenhoek 86 (2004) 33-49. https://doi.org/10.1023/B:ANTO.0000024907.85688.05.

[23] J. Chen, L. Gui, B. Chen, et al., Improving expression of pepsinogen A from *Homo sapiens* in *Aspergillus niger* by using a multi-copy gene knock-in strategy, Fermentation 9 (2023) 538. https://doi.org/ 10.3390/fermentation9060538.

[24] F. Ntana, U. Mortensen, C. Sarazin, et al., *Aspergillus*: a powerful protein production platform, Catalysts 10 (2020) 1064. https://doi.org/10.3390/catal10091064.

[25] S. Wang, Y. Xue, P. Zhang, et al., CRISPR/Cas9 system-mediated multi-copy expression of an alkaline serine protease in *Aspergillus niger* for the production of XOD-inhibitory peptides, J. Agric. Food Chem. 71 (2023) 15194-15203. https://doi.org/10.1021/acs.jafc.3c04138.

[26] M.L. Anson, The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin, J. Gen. Physiol. 22 (1938) 79. https://doi.org/10.1085/jgp.22.1.79.

[27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, et al., Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1951) 265-275. https://doi.org/10.1016/S0021-9258(19)52451-6.

[28] X. Liu, Q. Yan, Y. Xue, et al., Biochemical characterization of a novel glycoside hydrolase family 11 xylanase from *Chaetomium* sp. suitable for bread making, Process Biochem. 117 (2022) 1-9. https://doi.org/10.1016/j.procbio.2022.03.017.

[29] S. Liu, H. Sun, M. Nagassa, et al., Enhancing bread anti-staling with glucose-derived Maillard reaction products: In-depth analysis of starches, gluten networks, and moisture status, Food Chem. 455 (2024) 139760. https://doi.org/10.1016/j.foodchem.2024.139760.

[30] P.M. Nielsen, D. Petersen, C.J.J.O.F.S. Dambmann, Improved method for determining food protein degree of hydrolysis. J. Food Sci. 66 (2001) 642-646. https://doi.org/10.1111/j.1365-2621.2001.tb04614.x.

[31] Q. Li, F. Shen, X. He, et al., Modification of soy protein isolate using dielectric barrier discharge cold plasma assisted by modified atmosphere packaging, Food Chem. 401 (2023) 134158. https://doi.org/10.1016/j.foodchem.2022.134158.

[32] J. Cao, X. Tong, J. Cheng, et al., Impact of pH on the interaction between soy whey protein and gum arabic at oil-water interface: Structural, emulsifying, and rheological properties, Food Hydrocolloids 139 (2023) 108584. https://doi.org/10.1016/j.foodhyd.2023.108584.

[33] S. Zhu, Y. Xu, X. Yu, Improved homologous expression of the acidic lipase from *Aspergillus niger*, J. Microbiol. Biotechnol. 30 (2020) 196. https://doi.org/10.4014/jmb.1906.06028.

[34] Y. Xu, Y. Wang, T. Liu, et al., The GlaA signal peptide substantially increases the expression and secretion of α-galactosidase in *Aspergillus niger*, Biotechnol Lett. 40 (2018) 949-955. https://doi.org/10.1007/s10529-018-2540-5.

[35] W. Ji, L. Xu, X. Sun, et al., Exploiting systematic engineering of the expression cassette as a powerful tool to enhance heterologous gene expression in *Trichoderma reesei*, J. Agric. Food Chem. 72 (2024) 5307-5317. https://doi.org/10.1021/acs.jafc.3c07988.

[36] A. Rantasalo, C.P. Landowski, J. Kuivanen, et al., A universal gene expression system for fungi. Nucleic Acids Res. 46 (2018) e111-e111. https://doi.org/10.1093/nar/gky558.

[37] J. Deng, W. Huang, Z. Li, et al., Biocontrol activity of recombinant aspartic protease from *Trichoderma harzianum* against pathogenic fungi, Enzyme Microb. Technol. 112 (2018) 35-42. https://doi.org/10.1016/j.enzmictec.2018.02.002.

[38] Y. Guo, X. Li, W. Jia, et al., Characterization of an intracellular aspartic protease (*Ps*APA) from *Penicillium* sp. XT7 and its application in collagen extraction, Food Chem. 345 (2021) 128834. https://doi.org/10.1016/j.foodchem.2020.128834.

[39] Y. Xue, Q. Yan, X. Tian, et al., High-level secretory expression and characterization of acid protease in *Komagataella phaffii* and its application in soybean meal protein degradation, Int. J. Biol. Macromol. 282 (2024) 137011. https://doi.org/10.1016/j.ijbiomac.2024.137011.

[40] E.R. Achmadi, Enzymes as potential source for clean label bakery product: part 1, mechanism and application single enzyme, J. Food Agric. Prod. 2 (2022) 57-76. https://doi.org/10.32585/jfap.v2i2.2708.

[41] Y. Kawamura-Konishi, K. Shoda, H. Koga, et al., Improvement in gluten-free rice bread quality by protease treatment, J. Cereal Sci. 58 (2013) 45-50. https://doi.org/10.1016/j.jcs.2013.02.010.

[42] J. Calle, Y. Benavent-Gil, C.M. Rosell, Development of gluten free breads from *Colocasia esculenta* flour blended with hydrocolloids and enzymes, Food Hydrocolloids 98 (2020) 105243. https://doi.org/10.1016/j.foodhyd.2019.105243.

[43] S. Renzetti, E.K. Arendt, Effects of oxidase and protease treatments on the breadmaking functionality of a range of gluten-free flours. Eur. Food Res. Technol. 229 (2009) 307-317. https://doi.org/10.1007/s00217-009-1048-6.

[44] L. Tebben, Y. Shen, Y. Li, Improvers and functional ingredients in whole wheat bread: A review of their effects on dough properties and bread quality. Trends Food Sci. Tech. 81 (2018) 10-24. https://doi.org/10.1016/j.tifs.2018.08.015.

[45] J.M. Amigo, A. del Olmo Alvarez, M.M. Engelsen, et al., Staling of white wheat bread crumb and effect of maltogenic α-amylases. Part 1: Spatial distribution and kinetic modeling of hardness and resilience, Food Chem. 208 (2016) 318-325. https://doi.org/10.1016/j.foodchem.2016.02.162.

[46] X. Liu, Y. Zhang, X. Qi, et al., Advances of microbial xylanases in the application of flour industries: A comprehensive review, Int. J. Biol. Macromol. 282 (2024) 137205. https://doi.org/10.1016/j.ijbiomac.2024.137205.

[47] S.P. Bangar, A.O. Ashogbon, A. Singh, et al., Enzymatic modification of starch: A green approach for starch applications, Carbohyd. Polym. 287 (2022) 119265. https://doi.org/10.1016/j.carbpol.2022.119265.

[48] G. Ghoshal, U.S. Shivhare, U.C. Banerjee, Rheological properties and microstructure of xylanase containing whole wheat bread dough, J. Food Sci. Technol. 54 (2017) 1928-1937. https://doi.org/10.1007/s13197-017-2627-3.

[49] P.A. Caballero, M. Gómez, C.M. Rosell. Improvement of dough rheology, bread quality and bread shelf-life by enzymes combination, J. Food Eng. 81 (2007) 42-53. https://doi.org/10.1016/j.jfoodeng.2006.10.007.

[50] R. Li, Q. Cui, G. Wang, et al., Relationship between surface functional properties and flexibility of soy protein isolate-glucose conjugates, Food Hydrocolloids 95 (2019) 349-357. https://doi.org/10.1016/j.foodhyd.2019.04.030.

[51] J. Jiang, J. Chen, Y. Xiong, Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes, J. Agric. Food Chem. 57 (2009) 7576-7583. https://doi.org/10.1021/jf901585n.

[52] X. Ma, F. Hou, H. Zhao, et al., Conjugation of soy protein isolate (SPI) with pectin by ultrasound treatment. Food Hydrocolloids 108 (2020) 106056. https://doi.org/10.1016/j.foodhyd.2020.106056.

[53] E. Molina, A. Papadopoulou, D.A. Ledward, Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. Food Hydrocolloids 15 (2001) 263-269. https://doi.org/10.1016/S0268-005X(01)00023-6.