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Research article

Construction and application of a novel genetically engineered *Aspergillus oryzae* for expressing proteases

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ABSTRACT

Background: We aimed to test the possibility of improving polypeptide production from soybean meal fermentation by engineered *Aspergillus oryzae* strains. Four different protease genes were cloned and transformed into wild-type *A. oryzae*, and the engineered *A. oryzae* strains were then used for soybean meal fermentation.
Results: The results showed different degrees of improvement in the protease activity of the four transformants when compared with wild-type *A. oryzae*. A major improvement in the polypeptide yield was achieved when these strains were used in soybean meal fermentation. The polypeptide conversion rate of one of the four transformants, *A. oryzae* pep, reached 35.9%, which was approximately twofold higher than that exhibited by wild-type *A. oryzae*. Amino acid content analysis showed that the essential amino acid content and amino acid composition of the fermentation product significantly improved when engineered *A. oryzae* strains were used for soybean meal fermentation.
Conclusions: These findings suggest that cloning of microbial protease genes with good physicochemical properties and expressing them in an ideal host such as *A. oryzae* is a novel strategy to enhance the value of soybean meal.

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1. Introduction

Aspergillus oryzae is a safe species and listed as generally regarded as safe (GRAS) by the FDA and has been used in the production of fermented foods for many years in Asia [1]. Currently, *A. oryzae* is used in various modern biotechnological applications owing to the development of a transformation system and the ability of this fungus to secrete large amounts of proteins [2,3]. *A. oryzae* is applied in industries as a cell factory to produce a number of hydrolytic enzymes, including nucleases, lipases, and amylases. It is an important industrial workhorse with extensive applications in the production of industrial enzymes, heterogeneous proteins, and organic acids [4,5,6,7].

Bacterial alkaline proteases and fungal acid proteases have commercial value and multiple applications in various industrial sectors [8,9,10,11]. Currently, numerous commercially available alkaline proteases are derived from *Bacillus* strains [12,13]. For instance, *Bacillus licheniformis* and *Bacillus pumilus* are used for the production of alkaline protease that has strong ability to decompose proteins [14,15]. *Aspergillus niger*, which is closer to *A. oryzae* in evolutionary relationship, has excellent performance in acid protease production. It usually mixed with *A. oryzae* to obtain more excellent products in fermentation [16]. The two very important acid proteases produced by *A. niger* are aspartic acid protease (AP) and serine acid protease (PEP).

Soybean meal contains high quantity of soybean protein (40–50%), a superior form of protein [17]. It can be hydrolyzed into soybean polypeptides, which possess better physicochemical properties and physiological functions than soybean protein. They also possess potential broad applications as food, medicine, and feed additives [18]. Microbial fermentation of soybean meal is considered to be one of the more advanced and efficient methods with good application prospect for preparing soybean polypeptides [19,20,21]. This method is inexpensive and can overcome the shortcomings of poor taste and bitter flavor of soybean peptides obtained through enzymatic hydrolysis [22,23]. The critical problem in the fermentation of soybean meal is the method used for selecting excellent strains to improve the utilization efficiency of raw materials and polypeptide yield. In this study, the strain *A. oryzae* 3.951 (Hu Niang 3.042) was chosen as our

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expression system for the construction of four protease-expressing *A. oryzae* transformants, and the ability of these four engineered *A. oryzae* strains to hydrolyze soybean protein was studied.

2. Materials and methods

2.1. Strains, vectors, and chemicals

The strains of *A. niger* and *A. oryzae* (huniang 3.042) were kindly gifted by Dr. Zhang Liang, Shenyang Agricultural University, China. *A. oryzae* was used as the wild-type strain and host for genetic modifications. *B. licheniformis*, *B. pumilus*, and *Agrobacterium tumefaciens EHA105* were obtained from the Laboratory of Plant Genetic Engineering, Shenyang Agricultural University, China. The expression vector pCAMBIA 1304 was gifted by Prof. Zhang Lijun, Shenyang Agricultural University, China. Taq DNA polymerase and PCR reagents were obtained from TransGen Biotechnology Co. (Beijing, China). Restriction endonucleases, T4 DNA ligase, DH5α competent cells, pMD™19 (Simple), and DL2000 DNA marker were purchased from Takara Biotechnology Co. (Dalian, China). Hygromycin B and reverse transcription kit were purchased from Thermo Fisher Technology Co. (Shanghai, China). The primers used in this study were synthetized by Dingguo Biotechnology Co. (Beijing, China).

2.2. Gene amplification and construction of expression vectors

For the construction of the four protease expression vectors, the corresponding PCR-amplified fragments were cloned into the binary expression vector pCAMBIA1304. Two alkaline protease genes *subC* (GenBank accession no. GI487721) and *asp* (GenBank accession no. FJ584420.1) from *B. licheniformis* and *B. pumilus* genomic DNA, respectively, and two acid protease genes *Ap* (NCBI accession no. XM001401056.2) and *pep* (NCBI accession no. XM001392530.2) were cloned using the cDNA of *A. niger* as the template. The primer pairs used for the amplification of these protease genes are listed in Table 1. The PCR products were linked to the pMD19-T vector and sequenced by Dingguo Biotechnology Co.

The amylase promoter (PamyB) and α-glucosidase terminator (TagdA), both of which are efficient expression elements in *A. oryzae*, were used to construct the expression vector pCAMBIA1304 through *EcoRI*/*BamHI and SpeI*/*BstEII* to construct the framework of the expression vector named paa. The alkaline protease genes *subC* and *asp* and the acid protease gene *Ap* were inserted into the vector paa through the *SalI*/*SpeI* site and named paa-*subC*, paa-*asp*, and paa-*Ap*, respectively. The remaining acid protease gene *pep* was cloned into paa using homologous recombination PCR (One Step Cloning Kit, Novoprotein) and named paa-*pep*. In each of the four constructed expression constructs, the target genes were placed downstream of the amylase gene promoter and upstream of the α-glucosidase gene terminator. The primer pairs used for the construction of these protease genes and transcription elements are listed in Table 2, and the expression vectors constructed are illustrated in Fig. 1.

Table 1
Primers used for protease gene amplification in this study.

Gene name	Gene length (bp)	Primer name	Primer sequences (5′–3′)
subC	1140	subC-F	GCTCTAGAGCATGATGAGGAAAAAGAGT
(GI 487721)		subC-R	ACGCGTCGACTTATTGAGCGGCAGCTTC
asp	1152	asp-F	GCTCTAGAGCATGTGCGTGAAAAAGAAAAAT
(FJ 584420)		asp-R	ACGCGTCGACTTAGTTAGAAGCTGCTTG
Ap	1185	Ap-F	TGCTCTAGAATGGTCGTCTTCAGCAAAAC
(XM 001401056)		Ap-R	CCCAAGCTTCTAAGCCTGAGCGGCGAA
pep	1527	pep-F	TGCTCTAGAATGCGTTCCTTCTCCGTTGTCG
(XM 001392530)		pep-R	CCCAAGCTTTCAAGCATAATACTCCTCCA

2.3. Transformation of the protease genes into A. oryzae using A. tumefaciens

The four constructed plasmids were transformed into *A. tumefaciens* EHA105 cells using the freeze–thaw method [24]. Subsequently, genetic transformation of *A. oryzae* was performed according to the method given by Groot [25]. By adding acetosyringone (AS) as an inducer, the conidiospores of *A. oryzae* were incubated with induced *A. tumefaciens EHA105* containing protease expression plasmid at 28°C for 3 d. After co-cultivation, the transformants were screened on the basis of hygromycin B resistance. The selected transformants were subcultured thrice on Czapek-Dox agar plates to obtain stable transformants.

2.4. Detection of A. oryzae transformants

Single flat colonies of different *A. oryzae* transformants were picked and cultured in Czapek liquid medium at 170 rpm and 30°C for 3 d. Subsequently, the thallus of four positive transformants and wild-type *A. oryzae* was collected, and the genomic DNA was extracted. Hygromycin B gene (NCBI accession no. XM003071606.1) was amplified by PCR using the genomic DNA of the different *A. oryzae* transformants as the template. The amplified products were subjected to agarose gel electrophoresis to detect hygromycin B gene in positive transformants. The protease genes were amplified using the genome DNA of recombinant *A. oryzae* subC, *A. oryzae* asp, *A. oryzae* Ap, and *A. oryzae* pep as the templates to detect the target genes in the positive strains. RT-PCR was used to examine the expression level of the target gene. After synthesizing cDNA from the isolated mRNA of the four positive transformants and the wild-type strain, the protease genes of interest were amplified using specific primers for the genes. The reaction mixture contained 1 μl cDNA products, 0.5 μl Taq polymerase, 1 μl forward primers, 1 μl reverse primers, 2 μl dNTPs (2.5 mM), and 19.5 μl PCR buffer. The reaction cycle settings were as follows: 94°C, 30 s; 55°C, 30 s; 72°C, 1.5 min (28 cycles). The primers used for hygromycin B gene amplification and RT-PCR are listed in Table 3.

2.5. Measurement of protease activities of the A. oryzae transformants

The protease activities of the *A. oryzae* transformants were measured using a modified procedure based on the Folin–Ciocalteu method [26]. All the transformants and wild-type *A. oryzae* used in this study were cultivated at an inoculum size of 1×10^6 spores/ml in Sakaguchi flasks (500 ml) containing 100 ml of soybean residue medium at 170 rpm and 30°C on an orbital shaker for 4 d. The composition of the liquid fermentation medium is as follows: 2.5% soybean meal, 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, and 1.0% soluble starch. The supernatant was obtained by centrifugation at 4000 rpm for 10 min. Then, 1 ml of the supernatant was appropriately diluted and incubated in a water bath

Table 2
Primers used for the construction of protease expression vectors.

Primer name	Sequences (5′–3′)[a]	Temperature (°C)
subC-F1	ACGCGTCGACATGATGAGGAAAAAGAGT	54
subC-R1	CGGACTAGTTTATTGAGCGGCAGCTTC	
asp-F1	ACGCGTCGACATGTGCGTGAAAAAGAAAAAT	54
asp-R1	CGGACTAGTTTAGTTAGAAGCTGCTTG	
Ap-F1	ACGCGTCGACATGGTCGTCTTCAGCAAAAC	55
Ap-R1	CGGACTAGTCTAAGCCTGAGCGGCGAA	
pep-F1	ATCCTCTAGAGTCGAATGCGTTCCTTCTCCGTTGTCG	58
pep-R1	CTTCTCCTTTACTAGTCAAGCATAATACTCCTCCA	
PamyB-F	CCGGAATTCATGGTGTTTTGATCATT	55
PamyB-R	CGCGGATCCCATAAATGCCTTCTGT	
TagdA-F	CTAGACTAGTTGAAGGAAGCGTAACAGGATAGC	55
TagdA-R	GGGTCACCGGCAGTAACCCATTCCCGGT	

[a] Restriction sites are underlined.

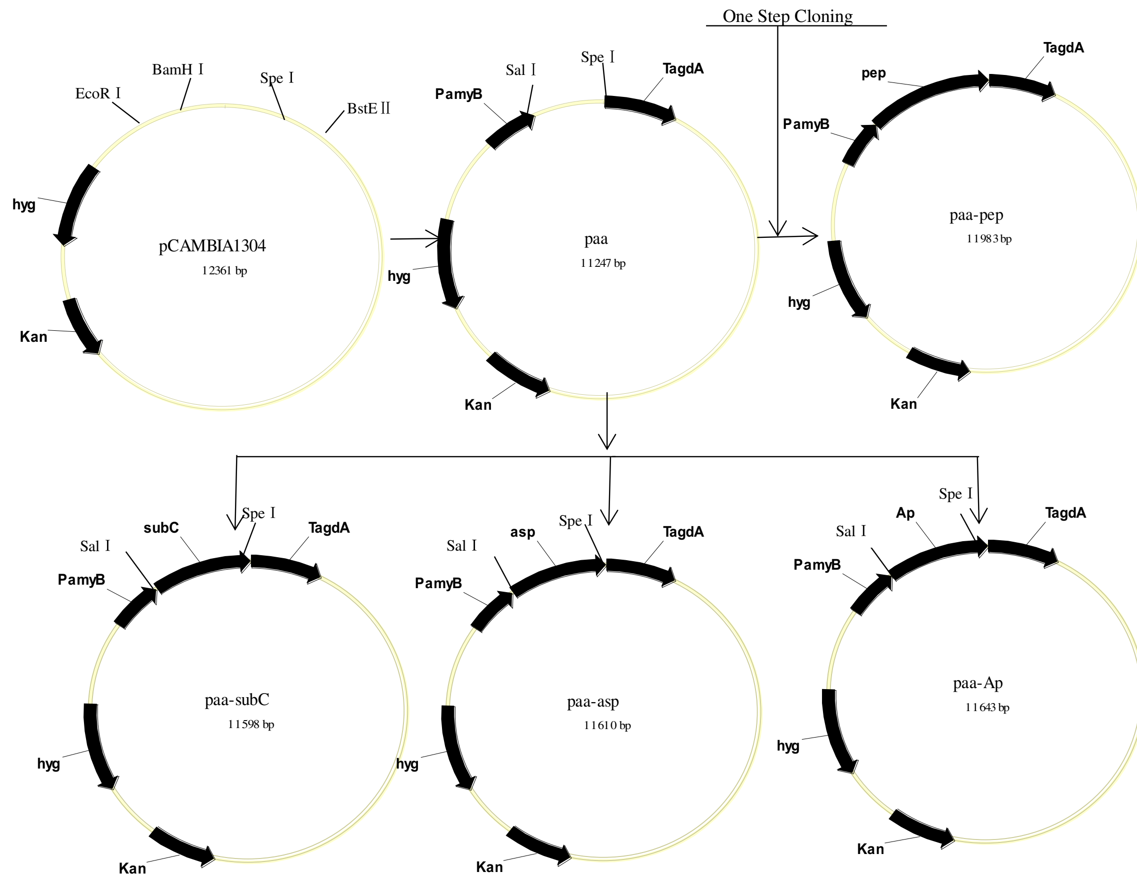

Fig. 1. Construction process of the four vectors for the expression of different proteases in *A. oryzae.*

at 40°C for 2 min, and 1 ml of 2% casein was added to it and heated for exactly 10 min. Immediately after, 2 ml of trichloroacetic acid solution (0.4 mol/l) was added to the mixture to terminate the reaction, and the mixture was kept in the water bath at 40°C for 20 min to precipitate the residual protein. The reaction supernatant formed after residual protein precipitation was extracted by centrifugation. To 1 ml of the reaction supernatant, 5 ml of sodium carbonate solution (0.4 mol/l) and 1 ml of Folin reagent were added, and the mixture was kept in a water bath at 40°C for 20 min to complete the color reaction. The absorbance was measured at 680 nm using a spectrophotometer (type 7200; Unico, Shanghai, China) and then converted to the amount of tyrosine equivalent on the basis of a standard curve. One unit (U/ml) of protease activity is defined as the amount of enzyme required to obtain 1 μg of tyrosine equivalent per minute per ml of fermentation broth at 40°C [Equation 1]. Borax-sodium hydroxide buffer (0.05 mol/l, pH 10) and lactate-sodium lactate buffer (0.05 mol/l, pH 3) were used for the determination of alkaline and acid protease activity, respectively. The same buffers with appropriate pH values were used for the dilution of the fermentation broth supernatant.

Table 3
Primers used for hygromycin B gene amplification and RT-PCR.

Primer name	Sequences (5′–3′)
hygB-F	ATGCCTGAACTCACCGCGAC
hygB-R	CTATTCCTTTGCCCTCGGAC
subC-F_2	ATGAGGAAAAAGAGT
subC-R_2	TTATTGAGCGGCAGCTTC
asp-F_2	ATGTGCGTGAAAAAGAAAAAT
asp-R_2	TTAGTTAGAAGCTGCTTG
Ap-F_2	ATGGTCGTCTTCAGCAAAAC
Ap-R_2	CTAAGCCTGAGCGGCGAA
pep-F_2	ATGCGTTCCTTCTCCGTTGTCG
pep-R_2	TCAAGCATAATACTCCTCCA

$$\text{Enzyme activity unit (U/ml)} = \frac{A \times 4 \times N}{10} \quad \text{[Equation 1]}$$

where A is the concentration of tyrosine equivalent. The average absorbance value of the parallel test tube is obtained from the spectrophotometer after the addition of the color reagent, and the concentration of tyrosine equivalent is calculated from the standard curve of tyrosine. N is the fermentation broth dilution multiple.

2.6. Extraction of polypeptides from engineered A. oryzae-fermented soybean meal and calculation of polypeptide conversion rate

Soybean meal was steamed at 121°C for 25 min using a steam sterilizer. After cooling, the spore suspensions (1×10^6 spores/ml) of the four transformants and wild-type *A. oryzae* were inoculated into the soybean meal medium and incubated for 4 d. The composition of the solid fermentation medium was as follows: 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, and 1.0% soluble starch (ratio of material:water = 1:1). Subsequently, 5 g of the fermentation product was mixed with 10 ml of deionized water and placed in a water bath at 50°C for 6 h. Then, the mixture was allowed to settle for 30 min after adding an equal volume of trichloroacetic acid solution (15%) to precipitate macromolecular proteins. The supernatant was obtained by centrifugation at 4000 rpm for 10 min and stored in a 25-ml tube after appropriate dilution. The polypeptide content was determined using the biuret method [27]. In brief, to 1 ml of the appropriately diluted supernatant, 4 ml of biuret reagent was added, and the absorbance was measured at 540 nm after placing the reaction mixture at room temperature for 30 min. The polypeptide content was determined by

contrasting with the standard curve obtained using casein (Sigma, USA) as a standard protein, and the conversion rate of polypeptides in the fermented product was calculated as shown in [Equation 2]

$$\text{Conversion rate of polypeptides} = \frac{\text{content of polypeptides}}{\text{soybean meal protein content}} = 100\% \qquad \text{[Equation 2]}$$

where soybean meal protein content is calculated using the formula $M \times 98.9\% \times 42.6\%$. M is the amount of sample, 98.9% corresponds to soybean meal content of dry matter, and 42.6% corresponds to the nitrogen content of soybean meal determined by following the Kjeldahl method.

2.7. Analysis of free amino acids

A total of 5 g of the fermented product was mixed with 10 ml of deionized water and placed in a water bath at 50°C for 6 h. Then, an equal volume of sulfosalicylic acid solution (15%) was added to the mixture and centrifuged at 4000 rpm for 10 min, and the supernatant was stored in a 25-ml tube. The obtained supernatant was then filtered through a 0.22-μm membrane to remove impurities and diluted appropriately before detection. The free amino acid contents were measured using an automatic amino acid analyzer (Hitachi L-8800) with 17 amino acid standard solutions. The experimental conditions were as follows: temperature of the separation column, 57°C; temperature of the reaction column, 136°C; buffer flow rate, 0.4 ml/min; ninhydrin flow rate, 0.35 ml/min; detection wavelength of channel 1, 570 nm; detection wavelength of channel 2, 440 nm; and sample volume, 20 μl.

2.8. Scanning electron microscopy examination of the fermented soybean meal

After incubation for 4 d, the surface structure of the fermented soybean meal was examined by scanning electron microscopy (SEM). The samples were coated with gold and visualized under a scanning electron microscope at 1.0 kV × 3.5 k magnifications (Hitachi S-4800).

3. Results and discussion

3.1. Cloning of protease genes and sequence analysis

Two alkaline protease genes *subC* and *asp* and two acid protease genes *Ap* and *pep* were amplified by PCR, and the nucleotide sequences of the four genes were determined. Then, the PCR products were subjected to sequencing and homology analysis, and the sequences were compared with those in the NCBI database. The results revealed that the *subC* gene sequence exhibited 98% homology with protease gene from *B. licheniformis* (GenBank accession no. GI487721); the *asp* gene sequence exhibited 98% homology with protease gene from *B. pumilus* (GenBank accession no. FJ584420); the *Ap* gene sequence exhibited 95% homology with acid protease gene from *A. niger* (NCBI accession no. XM001401056); and the *pep* gene sequence exhibited 95% homology with protease gene from *A. niger* (NCBI accession no. XM001392530). The N-terminal signal sequence of the four protease genes was predicted online (http://www.cbs.dtu.dk/services/SignalP/). The result of the analysis showed that the N-terminal signal sequence was present in each protease.

Subsequently, the amino acid sequences encoded by these genes were analyzed. The length of the alkaline protease gene *subC* was 1140 bp, which encoded a 379-amino acid polypeptide belonging to the class of serine protease Subtilisin Carlsberg. The length of the gene *asp* was 1152 bp, which encoded a 383-amino acid polypeptide belonging to an alkaline serine protease class. The length of the acid protease gene *Ap* was 1185 bp, which encoded a 394-amino acid polypeptide belonging to acid protease of aspartic acid class. The length of the gene *pep* was 1527 bp, which encoded a 508-amino acid polypeptide belonging to the acid protease of serine class.

3.2. Transformation of protease expression plasmid into A. oryzae and detection of transformants

With the backbone of the binary expression plasmid pCAMBIA1304 and the efficient expression elements of *A. oryzae*, two alkaline protease expression vectors, paa-*subC* and paa-*asp*, and two acid protease expression vectors, paa-*Ap* and paa-*pep*, were constructed. The results of endonuclease digestion and DNA sequencing confirmed that all the genes were correctly inserted into the expression vector. The prepared protease expression plasmids were subsequently introduced into *A. oryzae* using *A. tumefaciens*.

A. tumefaciens-mediated transformation (ATMT) is an efficient method for the transformation of plants and fungi [28,29,30,31], and it has been successfully used for transforming exogenous genes into *Saccharomyces cerevisiae*, *A. niger*, and *Trichoderma reesei* [25,32]. In the present study, ATMT was used to transform four protease expression plasmids into the host *A. oryzae*. Positive transformants of *A. oryzae* were selected and confirmed by PCR amplification. Detection of hygromycin B gene (NCBI accession no. XM003071606.1) amplification is shown in Fig. 2a, and the bands (1020 bp) of different *A. oryzae* transformants are shown in lanes 2–6, with wild-type *A. oryzae* as control in lane 1. In Fig. 2b, lanes 2–6 show the detection result of the target protease genes in different *A. oryzae* transformants. PCR results confirmed the integration of T-DNA into the *A. oryzae* genome. The transcription levels of the four inserted protease genes in *A. oryzae* transformants were determined by RT-PCR analysis, as shown in Fig. 3. The results clearly revealed that the expression levels of the four inserted genes (*subC*, *asp*, *Ap*, and *pep*) in the transformants were higher than those in the wild-type *A. oryzae*.

3.3. Improved protease activities of the engineered A. oryzae

The protease activity of *A. oryzae* is critical for the utilization of nitrogen source. By introducing an exogenous protease gene into the host strain, the protease activity can be significantly improved [33]. In a previous study, *Bacillus stearothermophilus* subtilizing J gene was transferred into *Bacillus subtilis*, and the resulting enzyme activity increased 46-fold [34]. Genetic engineering has previously been used to construct *A. oryzae* strains with enhanced protease activity [35]. In the present study, highest alkaline and acid protease activities of 165 and 143 U/ml, respectively, were achieved when soybean meal was fermented for 4 d using engineered *A. oryzae* asp and *A. oryzae* pep. In

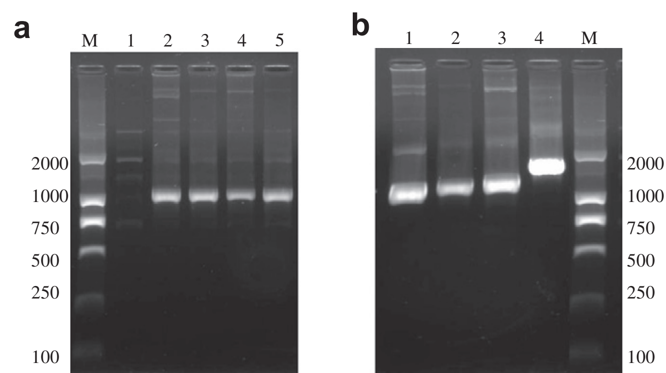

Fig. 2. PCR analysis of *A. oryzae* transformants. (a) PCR analysis of the hygromycin B gene (*Hyg*) in four *A. oryzae* transformants M. DL 2000 DNA Marker 1. *Hyg* gene in wild-type *A. oryzae* 2–5. *Hyg* gene in *A. oryzae* subC, *A. oryzae* asp, *A. oryzae* Ap, *A. oryzae* pep. (b) PCR analysis of the target proteases in four *A. oryzae* transformants M. DL2000 DNA Marker 1–4. Target protease gene in *A. oryzae* subC, *A. oryzae* asp, *A. oryzae* Ap, and *A. oryzae* pep.

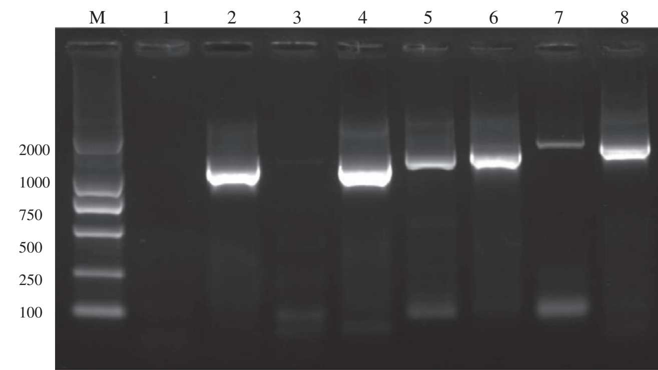

Fig. 3. RT-PCR analysis of inserted protease genes in the four *A. oryzae* transformants; M: DL2000 DNA Marker; 1: *SubC* gene in wild-type *A. oryzae*; 2: *SubC* gene in *A. oryzae* subC; 3: *Asp* gene in wild-type *A. oryzae*; 4: *Asp* gene in *A. oryzae* asp; 5: *Ap* gene in wild-type *A. oryzae*; 6: *Ap* gene in *A. oryzae* Ap; 7: *Pep* gene in wild-type *A. oryzae*; 8: *Pep* gene in *A. oryzae* pep.

contrast, the alkaline and acid protease activities of the wild-type *A. oryzae* were only 118 and 59 U/ml, respectively (Fig. 4). In particular, the alkaline protease activity of *A. oryzae* asp was 140% higher than that of the wild-type *A. oryzae*, whereas the acid protease activity of *A. oryzae* pep was 242% higher than that of the wild-type *A. oryzae*. Wild-type *A. oryzae* mainly secretes neutral protease and produces a relatively low amount of alkaline protease, acid protease, amylase, and cellulase, leading to inefficient degradation of raw materials such as protein, starch, and cellulose [36]. The improvement in acid protease activity of *A. oryzae* has practical significance in the soy sauce brewing industry [37]. The results of the present study indicated that the protease activity of the engineered *A. oryzae* increased after being transformed by genetic engineering, and these strains may show significant improvement in their fermentation efficiency when used in the fermentation of protein substrates.

3.4. Effect of engineered A. oryzae on polypeptide conversion rate in solid-state fermentation of soybean meal

The soybean meal fermentation capability of the engineered *A. oryzae* for the production of soybean peptides was examined. The polypeptide conversion rate exhibited by the engineered *A. oryzae* in solid-state fermentation of soybean meal is shown in Fig. 5. When compared with the wild-type *A. oryzae*, the polypeptide conversion rate of the different engineered *A. oryzae* strains presented different degrees of enhancement. The polypeptide conversion rate of soybean meal fermented by *A. oryzae asp* was 32.3%. The highest polypeptide conversion rate, reaching up to 35.9%, was exhibited by *A. oryzae* pep, which was about twofold higher than that of the wild-type *A. oryzae*. Therefore, an increase in the protease activity of *A. oryzae* by the introduction of exogenous protease may be beneficial to enhance the yield of soybean peptides. Moreover, the results also revealed that this transformation method can improve the ability of *A. oryzae* to utilize raw materials effectively. Currently, mixed fermentation is often used to improve the utilization of raw material proteins and enhance polypeptide yields [38]. However, this process is limited by the need for different culture conditions for different strains. Therefore, it is very important to construct new engineered strains to achieve better production performance. With soybean meal as raw material, the soybean polypeptide conversion rate of nine different strains was examined [39], and the polypeptide conversion rate had reached 26.4% after fermentation with *A. oryzae*. A higher polypeptide conversion rate of 31.5% was obtained by *B. subtilis* in fermentation of soybean meal with optimized fermentation process [40]. These results suggested that engineered *A. oryzae* in the present study can be used in the fermentation of soybean meal to obtain higher quantities of polypeptides.

3.5. Effects of engineered A. oryzae on the free amino acid content

The contents of 17 amino acids, including seven essential amino acids, in fermented soybean meal obtained with different engineered *A. oryzae* strains are shown in Table 4. The highest total free amino acid content of 152 ± 0.1 mg/g was presented in the soybean meal fermented with *A. oryzae* Ap, and the next highest of 115 ± 0.2 mg/g was presented by *A. oryzae* pep. The total free amino acid contents were 59.6 ± 0.3 and 56.6 ± 0.1 mg/g of soybean meal fermented by *A. oryzae* asp and *A. oryzae* subC, respectively. The highest essential amino acid content of 76.0 ± 0.1 mg/g was exhibited by *A. oryzae* Ap; the essential amino acid contents by *A. oryzae* asp and *A. oryzae* subC were slightly higher than wild-type *A. oryzae*. It can be surmised that after the insertion of an exogenous acid protease gene, engineered *A. oryzae* Ap and *A. oryzae* pep exhibited better acid protease activity to compensate for the deficiency of acid protease, which contributed to more efficient hydrolysis of proteins in the raw materials to amino acids.

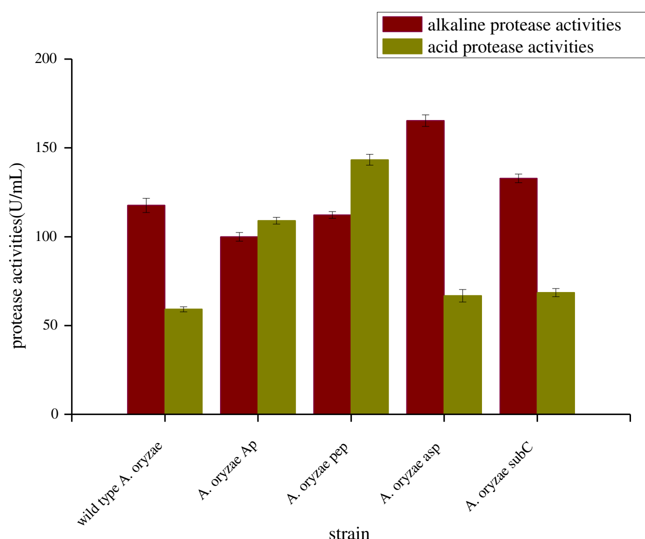

Fig. 4. Protease activities of four engineered *A. oryzae* and wild-type *A. oryzae*.

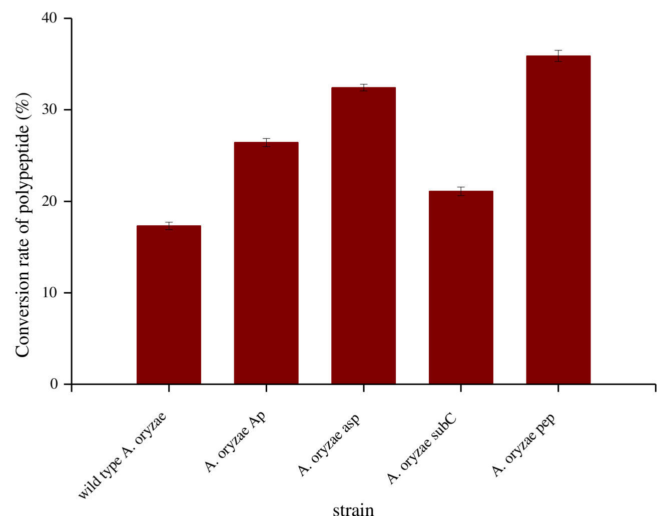

Fig. 5. Polypeptide conversion rate of four engineered *A. oryzae* and wild-type *A. oryzae* in soybean meal fermentation.

Table 4
Free amino acid content of soybean meal fermented by engineered *A. oryzae* and wild-type *A. oryzae* (mg/g)[b].

	Wild type *A. oryzae*	*A. oryzae* subC	*A. oryzae* asp	*A. oryzae* Ap	*A. oryzae* pep
Asp	1.56 ± 0.03	2.41 ± 0.01	2.24 ± 0.01	6.53 ± 0.07	3.09 ± 0.02
Thr[a]	2.23 ± 0.01	2.07 ± 0.01	2.19 ± 0.003	7.86 ± 0.01	4.54 ± 0.03
Ser	1.29 ± 0.003	1.54 ± 0.05	1.77 ± 0.003	5.11 ± 0.02	3.73 ± 0.03
Glu	5.34 ± 0.04	9.04 ± 0.05	9.43 ± 0.07	18.8 ± 0.02	13.5 ± 0.01
Gly	0.53 ± 0.003	0.46 ± 0.004	0.87 ± 0.01	3.67 ± 0.07	1.88 ± 0.01
Ala	1.10 ± 0.001	2.82 ± 0.05	4.14 ± 0.07	12.7 ± 0.02	8.53 ± 0.08
Cys	3.93 ± 0.001	4.18 ± 0.01	0.82 ± 0.001	–	–
Val[a]	6.53 ± 0.04	5.97 ± 0.03	4.52 ± 0.01	9.46 ± 0.02	8.24 ± 0.04
Met[a]	2.91 ± 0.01	3.77 ± 0.03	2.06 ± 0.002	4.31 ± 0.03	3.66 ± 0.07
Ile[a]	1.17 ± 0.002	1.16 ± 0.01	3.59 ± 0.03	9.87 ± 0.02	8.88 ± 0.05
Leu[a]	2.55 ± 0.01	3.77 ± 0.02	8.88 ± 0.01	19.5 ± 0.1	16.4 ± 0.01
Tyr	–	0.43 ± 0.003	1.07 ± 0.01	10.1 ± 0.04	8.10 ± 0.003
Phe[a]	6.61 ± 0.01	9.29 ± 0.01	8.01 ± 0.003	16.7 ± 0.02	16.9 ± 0.03
Lys[a]	2.77 ± 0.01	3.46 ± 0.04	2.93 ± 0.07	8.36 ± 0.05	4.99 ± 0.01
NH3	1.30 ± 0.01	1.28 ± 0.03	1.82 ± 0.01	3.05 ± 0.02	2.04 ± 0.01
His	0.73 ± 0.01	1.07 ± 0.01	0.93 ± 0.002	3.73 ± 0.03	2.03 ± 0.04
Arg	0.81 ± 0.001	3.84 ± 0.02	4.35 ± 0.05	12.7 ± 0.02	8.46 ± 0.03
Total amino acids	41.4 ± 0.1	56.6 ± 0.1	59.6 ± 0.3	152 ± 0.1	115 ± 0.2
Total essential amino acids	24.8 ± 0.1	29.5 ± 0.01	32.2 ± 0.1	76.0 ± 0.1	63.6 ± 0.2

Data expressed as mean values (n = 3) ± SD; SD-standard deviation. The experiments were carried out in triplicate, and data were subjected to analysis of one way analysis of variance (ANOVA) using SPSS.
[b] Data expressed as mg/g sample dry matter.
[a] Essential amino acids.

3.6. Structure of the fermented soybean meal

SEM images of the surface structure of soybean meal subjected to solid-state fermentation by different *A. oryzae* strains are shown in Fig. 6. As illustrated in Fig. 6a, the wild-type *A. oryzae* could achieve only surface degradation of the soybean meal. In contrast, fermented soybean residue was largely destroyed when the engineered *A. oryzae* were used, particularly *A. oryzae* Ap and *A. oryzae* pep, as shown in

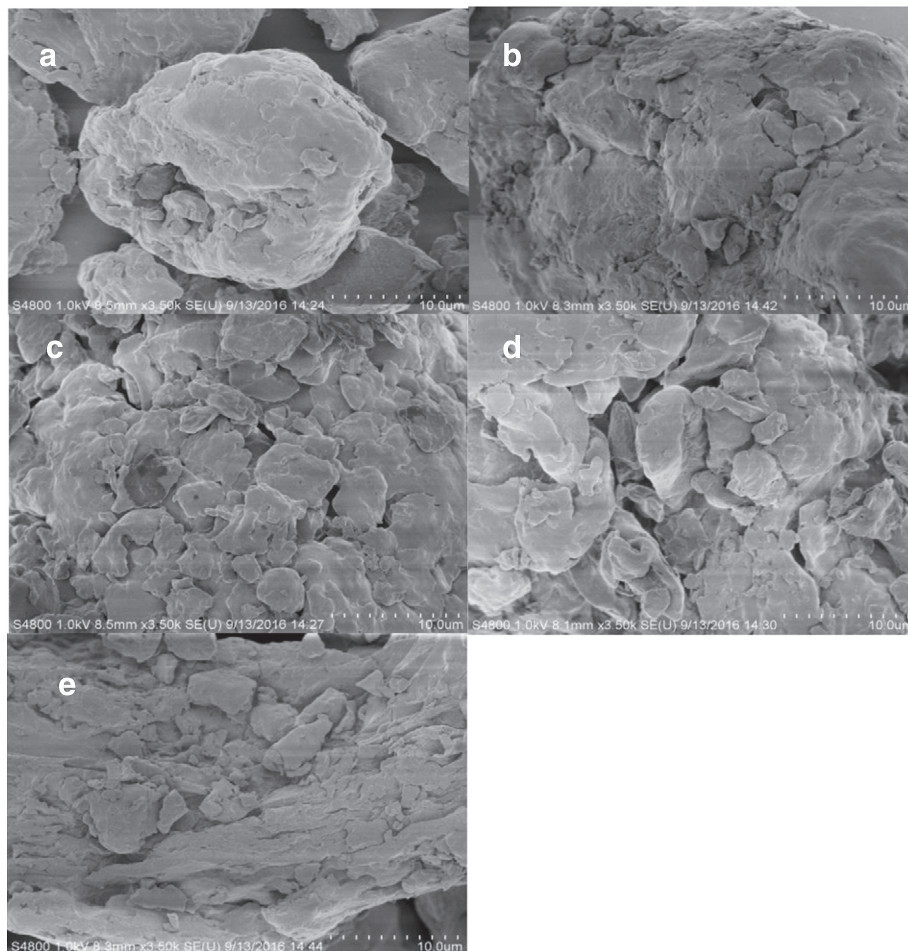

Fig. 6. Surface structure of soybean meal fermented by four engineered *A. oryzae* and wild-type *A. oryzae.* (a) Soybean meal fermented by wild-type *A. oryzae.* (b) Soybean meal fermented by *A. oryzae* subC. (c) Soybean meal fermented by *A. oryzae* asp. (d) Soybean meal fermented by *A. oryzae* Ap (e) Soybean meal fermented by *A. oryzae* pep.

Fig. 6d and Fig. 6e, respectively. Accordingly, fermentation with engineered *A. oryzae* resulted in the more severe degradation of soybean meal substrate and smaller particle size of soybean residue compared with wild type *A. oryzae*.

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Conflict of interest

The authors have no conflict of interests.

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