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A comparative secretome analysis of industrial *Aspergillus oryzae* and its spontaneous mutant ZJGS-LZ-21

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ABSTRACT

Aspergillus oryzae koji plays a crucial role in fermented food products due to the hydrolytic activities of secreted enzymes. In the present study, we performed a comparative secretome analysis of the industrial strain of *Aspergillus oryzae* 3.042 and its spontaneous mutantZJGS-LZ-21. One hundred and fifty two (152) differential protein spots were excised ($p < 0.05$), and 25 proteins were identified. Of the identified proteins, 91.3% belonged to hydrolytic enzymes acting on carbohydrates or proteins. Consistent with their enzyme activities, the expression of 14 proteins involved in the degradation of cellulose, hemicellulose, starch and proteins, increased in the ZJGS-LZ-21isolate. In particular, increased levels of acid protease (Pep) may favor the degradation of soy proteins in acidic environments and promote the cleavage of allergenic soybean proteins in fermentation, resulting in improvements of product safety and quality. The ZJGS-LZ-21 isolate showed higher protein secretion and increased hydrolytic activities than did strain 3.042, indicating its promising application in soybean paste fermentation.

1. Introduction

Aspergillus oryzae is an important industrial strain that has been extensively applied in a variety of food manufacturing applications. This organism has a long history in the preparation of traditional fermented condiments and spirits, such as soybean sauce, paste, vinegar, Chinese rice wine, and Japanese Sake (Kitamoto, 2015; Yamane et al., 2002; Zhang et al., 2012). The solid mold preparation, usually called "Qu" in China and koji in Japan, is characterized by large amounts of hydrolytic extracellular enzymes that can be directly used in the hydrolysis of carbohydrates and proteins (Hong et al., 2015;Marui et al., 2013; Wang et al., 2010; Yoshino-Yasuda et al., 2011). *A. oryzae* 3.042 (equivalent to AS 3.951) is regarded as a prime industrial strain because of its high-yield of proteases and it is widely used in most brewery fermentations in China (Zhao et al., 2012). Previous studies have demonstrated that the cooperative function of high hydrolytic activities and a balanced collection of enzymes species secreted allow *A. oryzae* to function more efficiently in the utilization raw materials thus improving the quality of soybean paste fermentation (Hong et al., 2015; Kumazawa et al., 2013). For example, *A. oryzae* mutant A100-8, with enhanced acid and neutral protease activities, promotes the utilization of substrate and improves soy sauce flavor and the quality (Zhao et al., 2014). Recently, Kum et al. (2015) reported that the activities of α-amylase, protease, lipase, and esterase from *Aspergillus* species, commonly increased in fermentation, resulting in the generation of the volatile components of rice-koji (doenjang). While *A. oryzae* mainly secretes high activities of neutral proteases, efforts have been made to enhance its production of acid proteases (Vishwanatha et al., 2010; Xu et al., 2011). Therefore, the abundance and types of enzymes secreted by *A. oryzae* has become a prominent factor for controlling the quality of soybean paste production (de Souza et al., 2015).

Various proteomic techniques have been explored to profile the extracellular proteins of microbes. Differences in the secreted proteins of *Aspergillus*, *Trichoderma*, and *Penicillium* have been detected by SDS-PAGE combined with LC-MS/MS (Borin et al., 2015; Gong et al., 2015). Two-dimensional electrophoresis (2-DE) or isobaric tags for relative and absolute quantification (iTRAQ) methods are often adopted in quantitative assays. Using the iTRAQ technique, many extracellular proteins such as cellulases, hemicellulases, lignin-degrading enzymes, and proteases were identified in the comparative differential secretomes of *Trichoderma reesei* and *A. fumigates* (Adav et al., 2012; Adav et al., 2015). One-dimensional electrophoresis (1-DE) and two-dimensional electrophoresis (2-DE), combined with MALDI-TOF-MS, has allowed the identification of 16 to 41 extracellular protein species in the secretome of *A. oryzae* SU16 koji (Liang et al., 2009; Zhang et al., 2012). Comparative secretome analysis showed a total of 29 identified proteins and indicated the different enzyme species present in solid-state and submerged cultures of *A. oryzae* RIB40, an industrial strain often used to produce sake, soybean paste and soy sauce in Japan

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(Oda et al., 2006). In addition, a genome-sequencing project on *A. oryzae* strain RIB40 has been published and expression sequence tags (ESTs) have been introduced (Machida et al., 2005). In apparent contrast to the less secreted proteins, Zhao et al. (2012) reported 522 unique proteins from whole cell lysate, providing a reference map of the intracellular protein expression profile. These studies, of the genome, transcriptome, and proteome, provide robust data for the analysis of proteins differentially secreted from industrial *A. oryzae* strains (Kitamoto, 2015; Machida et al., 2005; Wang et al., 2010).

In our previous study, we isolated a spontaneous mutant, *A. oryzae* ZJGS-LZ-21, from an *A. oryzae* 3.042 koji sample at Zhejiang Wuweihe Food Co., Ltd., Zhejiang Province, China (Zhu et al., 2016). Based on morphological, genetic, and non-aflatoxigenic characteristics, the ZJGS-LZ-21 isolate is classified as *A. oryzae*. Isolate ZJGS-LZ-21 can produce much higher activities of amylase and acid protease in than its parental strain *A. oryzae* 3.042 and, thus, can enhance soybean paste production. One should be cautious in using the name *A. oryzae* since the non-aflatoxin-producing character is no longer a specific guarantee for *A. oryzae* (Fakruddin et al., 2015; Jorgensen, 2007). *A. flavus*, in *Aspergillus* section *Flavi*, is genetically and functionally almost identical to *A. oryzae* and often isolated from fermented soybean products (Gibbons et al., 2012; Lee et al., 2014; Tao and Chung, 2014). Production of aflatoxins is conditional and often inconsistent within the *A. flavus*/*oryzae* complex (Jorgensen, 2007). Currently, the fact that *A. oryzae* is the currently used name for the non-aflatoxin-producing species of *A. flavus*/*oryzae* associated with oriental fermentations (Hong et al., 2013) relates to economic and societal issues rather than to biology, as suggested by Jorgensen. Here, we investigate the secretome profiles and suggest potential commercial applications for ZJGS-LZ-21 isolates in soybean paste manufacture.

2. Materials and methods

2.1. Strains and sample preparation of koji

The strain *Aspergillus oryzae* 3.042 (equivalent to AS 3.951) and *A. oryzae* isolate ZJGS-LZ-21 were from Zhejiang Wuweihe Food Co., Ltd., Huzhou City, Zhejiang Province, China. ZJGS-LZ-21 isolate has higher activities of acid protease and amylase than the parent 3.042 strain (Table S2). Seed koji was prepared as the earlier described (Yang et al., 2013). Wheat bran and soybean meal (w/w, 17/3) with 50% (v/w) water were divided into 40 g portions and put into 500 mL Erlenmeyer flasks. After sterilization at 121 °C for 30 min, the autoclaved media was inoculated with fungal spore suspension (10^6 spores/g medium), and incubated at 30 °C for 3 days with flasks shaken every 12 h.

2.2. Protein sample preparation for 2-DE

Koji (25 g) was soaked at 4 °C for 12 h in 100 mL of 50 mM sodium acetate buffer (containing 90 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 4.78) to extract the extracellular protein. The suspension was centrifuged twice at 13,000 × *g* for 10 min to remove large particles. The supernatant was filtered through a 0.45 μm filter (Millipore). With ice-cold acetone precipitation, the filtrate was concentrated and then desalted. Precipitate was dissolved in the lysate buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1 mM PMSF for 2-DE analysis. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.3. 2-DE electrophoresis process and image analysis

2-DE was performed as previously reported with minor modifications (Wu et al., 2009). Before 2-DE, 400 μg of protein was mixed in 300 μL rehydration buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT (dithiothreitol), 0.67% (v/v) IPG buffer. The first dimension of electrophoresis (isoelectrofocusing; IEF) performed on the IPG strips (17 cm with a linear gradient range of pH 4–7; Bio-Rad, USA) in a Protean IEF cell (Bio-Rad, USA) at 20 °C for 80 kV-h. Next, the IPG strips were reduced with 2% DTT for 15 min and alkylated with 2.5% iodoacetamide for 15 min in equilibration buffer (6 M urea, 50 mM Tris-HCl (pH 8.8), 20% glycerol, 2% SDS). The equilibrated strips were capped by low melting agarose (Bio-Rad, USA) and run at 12 °C on 12.5% SDS-polyacrylamide gels by PowerPac Universal electrophoresis system (Bio-Rad, USA). The protein spots were visualized with Coomassie brilliant blue (10% ammonium sulfate, 10% phosphoric acid, 0.12% xylene brilliant cyanine G (G-250), and 20% methanol) (Lima et al., 2010; Zhao et al., 2012a).

The stained gels were scanned using a GS-800 Calibrated Densitometer (Bio—Rad, USA) and the images (300 dpi) were saved as TIFF files. Image analysis was performed with the BIO-RAD PD Quest Basic 2-Dimage processing software (version 8.0.1) according to the protocol provided by the manufacturer. Comparison of spot quantity was performed after background subtraction, normalization, and spot match. Changes in the intensity of protein spots that reached 1.5-fold were considered meaningful in the present work based on statistical analysis (Student's *t*-test). The average ratios, *p*I and Mr of each spot were computed and then exported in Microsoft Excel by the PD Quest software. The abundance of protein spots showing significant differences ($p < 0.05$) also exported and used for further analysis.

2.4. Protein identification by MALDI TOF/TOF tandem MS

Protein spots were excised from the stained gels and were destained in-gel with 50% acetonitrile containing 40 mM ammonium bicarbonate, washed with 100% acetonitrile, and then dried in a SpeedVac. The destained spots were reduced with 10 mM DTT and subsequently alkylated with 55 mM iodoacetamide as previously described (Adav et al., 2013). Finally, the dehydrated gel pieces were thoroughly washed with 100% acetonitrile solution, completely dried by SpeedVac, then digested with trypsin solution (10 ng/μL) by incubating at 37 °C overnight. The supernatant, containing peptides, was mixed with 50% acetonitrile containing 0.1% trifluoroacetic acid and vacuum centrifuged to dryness. The trypsin-digested fraction was reconstituted in matrix solution consisting of α-cyano-4-hydroxycinnamic acid (5 mg/mL) in 100% acetonitrile with 0.1% trifluoroacetic acid. The peptide samples were applied onto the target well, dried at room temperature, and analyzed by AB 4800 MALDI-TOF-TOF Plus mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). The mass spectrum of each protein was collected in a reflective mode with a laser intensity of 4000 and an *m*/*z* range from 800 to 4000 Da. Parent ions with the seven highest signal strengths were analyzed by MS/MS, and their corresponding ion fragments fingerprints were collected using CID collision cleavage at an accelerating potential of 2 kV.

The MS/MS spectra were searched using Mascot software (Applied Biosystems) against the complete proteome NCBI database and Uniprot database based on the annotated *A. oryzae* RIB40 genome information. Search parameters included trypsin digestion, one maximum missed cleavage site, carbamidomethyl of cysteine (fixed modification), methionine oxidation (variable modification), peptide mass tolerance, and fragment mass tolerances of 1000 ppm and ±0.3 Da, respectively. Protein spots were considered identified if the Mascot score and CI % was >60 and 95% respectively with the significance level, $p < 0.05$.

2.5. Separation of the whole protein sample using SDS-PAGE

In addition to protein separation by 2-DE, a second electrophoresis process on 12.5% SDS-polyacrylamide gels, 1-DE, was adopted to analyze the whole protein samples. Briefly, 10 μg of protein was resolved in 2.5 μL loading buffer. After boiling in a water bath for 5 min, the sample was loaded and run at 80 V at 20 °C by electrophoresis system (Bio-Rad, USA). The Band I and II samples obtained were applied to Triple

TOF 5600 system (AB SCIEX) III binding nanospray ion source (AB SCIEX, USA) for analysis (details in the Table S1).

2.6. Bioinformatics analysis

The existence of signal peptide sequences was checked using the signal peptide prediction program SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP), SecretomeP 2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/). The function and composition of identified proteins was analyzed using the database of UniProtKB (http://www.uniprot.org/uniprot/)and QuickGO (http://www.ebi.ac.uk/QuickGO/). Theoretical *p*I and Mr was obtained by using online ExPASy compute *p*I/MW tool (http://www.expasy.org/tools/pi_tool.html)(Vincent et al., 2009). The subcellular location of protein was predicted using the WoLFPSORT program (http://wolfpsort.org/).

3. Results and discussion

3.1. Differentially expressed proteins in A. oryzae 3.042 and ZJGS-LZ-21isolate

The secreted enzymes are regarded as the basal factors affecting the quality of soybean paste in the process of fermentation (Yoshino-Yasuda et al., 2011). Samples of secreted proteins were prepared as described in the materials and methods to profile the extracellular proteins of *A. oryzae* 3.042 and the ZJGS-LZ-21isolate. Each strain was analyzed in parallel triplicates and the analysis was repeated three times. The secreted protein content from the ZJGS-LZ-21 isolate had a mean value of 255.38 ± 29.93 (standard error) μg/g dry koji, approximately 1.5-fold higher than that of strain 3.042 (Fig. 1A). This indicates that the isolate ZJGS-LZ-21 has a higher capability for protein secretion as is evidenced by the band density in Fig. 1B. Moreover, the results of 1-DE showed the clear differences in the profiles of the protein expression patterns of the two strains. 2-DE gels were run in triplicate and the high-resolution gels from each sample were used as the reference map to depict the secretome difference between *A. oryzae* 3.042 and the ZJGS-LZ-21isolate. Here, 552 ± 21 (in strain 3.042, Fig. 2A) and 971 ± 85 (in isolate ZJGS-LZ-21, Fig. 2B) protein spots were detected on the gels by PD Quest software, respectively (Table S3). The representative spots from 2-DE gels are displayed in Fig. 2C. A total of 152 statistically different protein spots was obtained and excised. After analysis by MALDI TOF/TOF tandem MS, 27 protein spots were finally identified (Tables 1 and S4). The amount of identified proteins was similar to those of previous reports, 16 secreted proteins from soy sauce koji by *A. oryzae* 3.402, 29 secreted proteins from solid and submerged fermentation by *A. oryzae* RIB40, and 41 from Shaoxing rice wine koji by *A. oryzae* SU16, respectively (Liang et al., 2009; Oda et al., 2006; Zhang et al., 2012). Many spots could not be identified due to low content, high level of protein glycosylation, or their absence in the reported database for *A. oryzae* (Wu et al., 2009). Multiple protein isoforms were likely derived from post-translational modification or proteolytic processing (Lessing et al., 2007; Wang et al., 2012). Spots 13, 21, and 39 were identified as α-amylase A type-3 (P0C1B4); and spots 94 and 95 as neutral protease 2 (Q2UP30) on the 2-DE gels. Similar results were observed in *A. oryzae* RIB40 where 29 protein species out of 195 spots were identified (Oda et al., 2006). Since 2, 3-dihydroxybenzoate decarboxylase (spot 81) and fructose-bisphosphate aldolase (spot 117) were both detected as the cytoplasm they were not included in the comparative secretome of *A. oryzae*.

Among the 27 identified protein spots, the molecular mass of most of the corresponding proteins ranged from 20.03 to 135.95 kDa. The presence of signal peptide was checked using a signal peptide prediction program (SignalP 4.1 and SecretomeP 2.0 server) to assess the secretion pathway of these proteins. The results showed that 20 proteins possessed secretory signal peptides, indicating their secretions through the classical secretory pathway in *A. oryzae*. Yet another three proteins (spots 89, 186 and 187), without signal peptide, are suggested to follow a non-classical secretory mechanism or are found outside the cells due to cell death or cell lysis (Liu et al., 2014). The current results were similar to a previous report in which 17 extracellular protein species followed the classical secretory pathway and 10 protein species followed a non-classical secretory mechanism (Zhang et al., 2012).

In ZJGS-LZ-21 isolate the expression of proteins corresponding to 15 spots were increased and those corresponding to 11 spots was decreased (Table 1). Further analysis revealed that spots 67 (Q2UCU3), 89 (Q2UKD0), 186 (Q2U8Y), and 187 (Q2U9M7) were detected exclusively in ZJGS-LZ-21 isolate, while Q2UP30 (neutral protease 2) was only detected in strain 3.042. There were seven proteins (corresponding

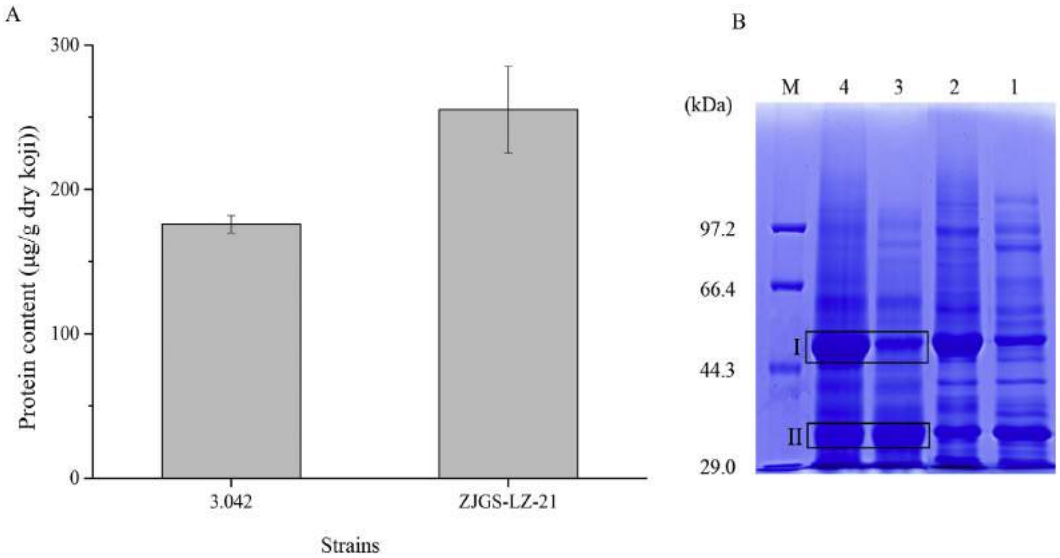

Fig. 1. Determination of the secreted proteins by *A. oryzae* 3.042 and its ZJGS-LZ-21isolate. A. The contents of secreted proteins from *A. oryzae* 3.042 and isolate ZJGS-LZ-21 koji. B. One-dimensional electrophoresis image of extracellular proteins on PAGE gel. Lane 1 and 2: the raw secreted proteins (10 μg) from strain 3.042 and ZJGS-LZ-21isolate; Lane 3 and 4: the secreted proteins treated with acetone (proteins 10 μg) from strain 3.042 and ZJGS-LZ-21isolate; M lane: standard molecular weight marker. Band I and II were excised from the gel and subjected to in-gel digestion with trypsin prior to MALDI TOF/TOF tandem MS analysis

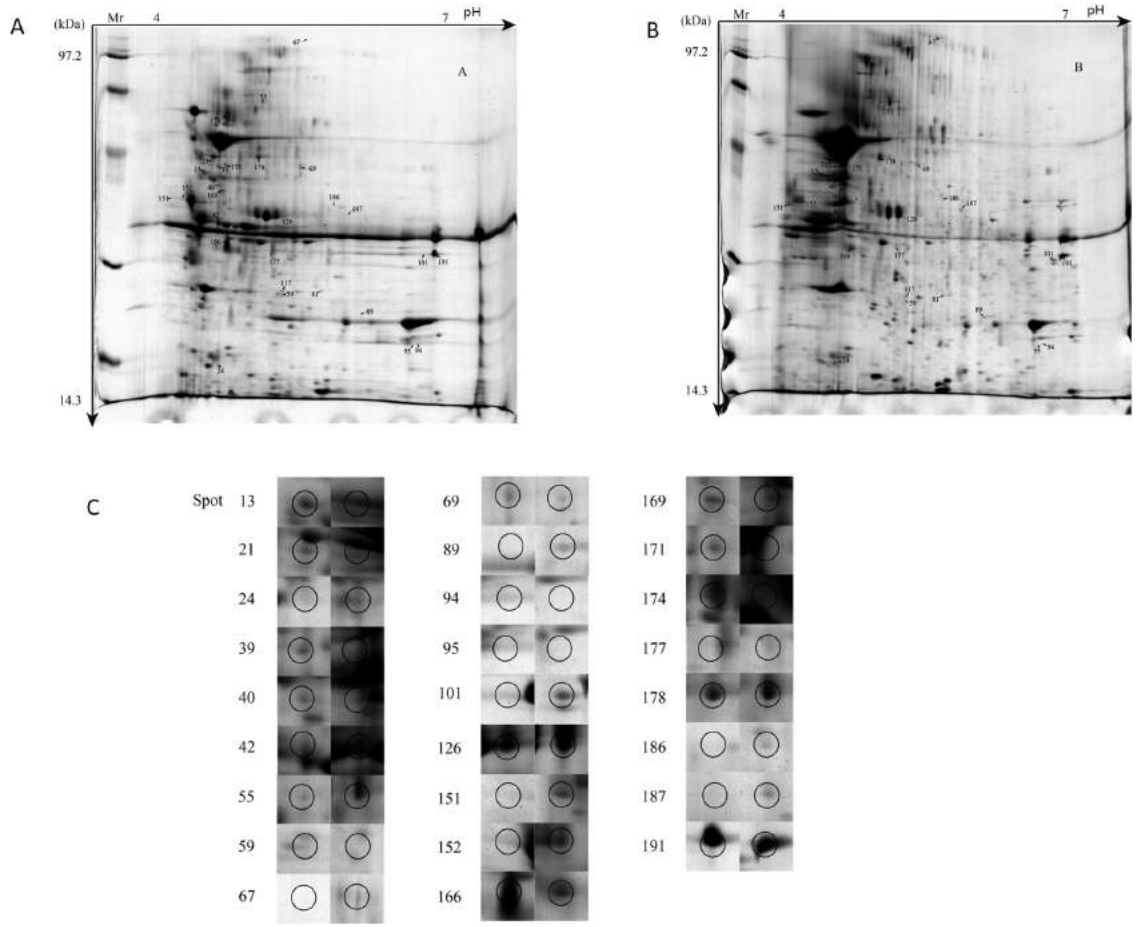

Fig. 2. Comparative secretome profiles of *A. oryzae* 3.042 and strain ZJGS-LZ-21. A and B. 2-DE diagrams of secretome of *A. oryzae* 3.042 koji and ZJGS-LZ-21 isolate, respectively. The identified differential proteins are marked by arrows and numbers and listed in Table 1. C. Representative secretome spots analysis of *A. oryzae* koji from 2-DE resolution (p*I* 4–7 gel), a closer view of differentially protein spots (strain 3.042, left; isolate ZJGS-LZ-21, right) are shown.

to spots 152, 55, 42, 101, 151, 24, and 69) in which expressions regulated at least 5-fold higher in ZJGS-LZ-21 isolate. Specifically, the proteins (spots 152 (P0C1B3) and 42 (O94163)) displayed over a 10-fold increase in expression. Meanwhile, there were seven proteins in which expressions were down-regulated by 2-fold in ZJGS-LZ-21 isolate. The differential expressions of these proteins were further classified according to biological process in the GO and Uniprot database (Fig. 3). In terms of species amounts, 65.22% of total identified proteins were involved in the carbohydrate metabolism, 26.09% in proteolysis, and 4.35% in redox process. Notably, 91.3% of these proteins have roles in carbohydrate metabolism and proteolysis, which are consistent with the formation of koji through hydrolysis processes (Kitamoto, 2015). Previously, a comparative proteome of *A. oryzae* RIB40 was carried out to analyze extracellular proteins under solid-state and submerged culture conditions to determine protein secretion profiles. In the solid-state growth condition, glucoamylase B, alanyl dipeptidyl peptidase, xylanase G1, α-amylase, and β-glucosidase were detected, consistent with the current study(Oda et al., 2006). The greater abundance and types of secreted enzymes from the ZJGS-LZ-21 isolate may afford better hydrolysis performance and produce more sugars to support microbial metabolism during the soybean paste fermentation.

3.2. Proteins involved in carbohydrate hydrolysis

The increased accumulation of α-amylase A type-1/2 (spot 152, 12.4-fold) and glucoamylase (spot 178, 1.56-fold) were observed in ZJGS-LZ-21 isolate. Amylase is one of prominently secreted enzymes of *A. oryzae* (Oda et al., 2006). Both α-amylase A type-1/2 and glucoamylase catalyze the degradation of starch to glucose by acting on 1,4- and 1,6-glycoside bonds (Fig. 4A). This catalysis improves the utilization of substrate and, thus, the quality of the resulting soybean paste. Importantly, increased glucose production also results in the unique flavor generation by yeast and bacteria growth in the post-fermentation of soybean paste (Hong et al., 2012; Ng'ong'ola-Manani et al., 2014; Roh, 2014).

In addition to starch, there are large quantities of cellulose, hemicellulose and pectin in the soybean paste substrate. The plant cell wall degradation machinery in *A. oryzae* has been extensively deciphered over the past decade. Cellulolytic, xylanolytic and pectinolytic enzymatic activities are coordinately responsible for this process (de Vries and Visser, 2001; Khosravi et al., 2015; Udatha et al., 2015). The derived sugars are not only utilized as carbon source for microbial growth during the fermentation, but also contribute to color and flavor formation. In the current study, five cellulases were identified, *endo*-β-1,4-glucanase B (EG) (spot 151), 1,4-β-D-glucancellobiohydrolase A (CBH) (spot 171), β-glucosidase A, I and H (βG) (spots 55, 186 and 187), respectively (Fig. 4B). EG has endoglucanase activity to cleave β-1,4 glycosidic bond, which occurs in complex natural cellulosic substrates such as carboxymethylcellulose (CMC), hydroxyethylcellulose (HEC) and β-glucan. CBH releases the disaccharide cellobiose from the non-reducing end of the cellulose polymer chain, and its expression was 7-fold lower in the ZJGS-LZ-21isolate. In addition, βG catalyzes the last step releasing

Table 1
Identification of differential secreted proteins between *A. oryzae* ZJGS-LZ-21 isolate and strain 3.042 by MALDI TOF/TOF tandem MS.

Spot no.[a]	Accession number	Protein description	CAZy family	Change fold	*p*-Value[b]	Exp.pI/MW [kDa]	Theor.pI/MW [kDa]	Coverage (%)/score[c]	Cellular location[d]	SignalP[e]
Carbohydrate metabolism										
13	P0C1B4	Alpha-amylase A type-3	GH13	−1.71	0.003	4.33/54.93	4.52/54.80	100/500	extr	Y
21	P0C1B4	Alpha-amylase A type-3	GH13	−5.68	0.009	4.39/59.16	4.52/54.80	100/500	extr	Y
39	P0C1B4	Alpha-amylase A type-3	GH13	−7.39	0.015	4.46/57.92	4.52/54.80	100/500	extr	Y
152	P0C1B3	Alpha-amylase A type-1/2	GH13	12.40	0.014	4.13/46.08	4.48/54.81	100/254	extr	Y
178	P36914	Glucoamylase	GH15	1.56	0.014	4.82/61.07	4.99/65.47	99.549/80	extr	Y
55	Q2UUD6	Probable beta-glucosidase A	GH3	6.29	0.001	4.89/86.24	4.86/93.41	100/482	extr	Y
171	Q2UBM3	Probable 1,4-beta-D-glucan cellobiohydrolase A	GH7	−7.94	0.038	4.52/58.05	4.27/48.13	99.978/93	extr	Y
42	O94163	Endo-1,4-beta-xylanase F1	GH10	11.19	0.000	4.47/42.47	4.73/35.40	100/463	extr	Y
101	Q96VB6	Endo-1,4-beta-xylanase F3	GH10	9.61	0.000	6.58/34.47	6.3/34.70	100/226	extr	Y
166	Q75P26	Acetylxylan esterase A	CE1	−2.52	0.048	4.52/35.18	4.89/33.29	100/678	extr	Y
186	Q2U8Y5	Probable beta-glucosidase I	GH3	Up[f]	0.012	5.41/47.43	5.3/92.14	100/372	extr	N
187	Q2U9M7	Probable beta-glucosidase H	GH3	Up[f]	0.013	5.86/42.35	5.97/90.68	99.286/78	extr	N
67	Q2UCU3	Probable beta-galactosidase A	GH35	Up[f]	0.003	5.19/135.95	5.33/109.87	100/433	extr	Y
151	Q2UPQ4	Probable *endo*-beta-1,4-glucanase B	GH5	8.57	0.005	4.05/45.51	4.41/36.96	100/132	extr	Y
89	Q2UKD0	Probable NAD(P)H-dependent D-xylose reductase xyl1	NA	Up[f]	0.014	5.82/23.15	5.78/35.87	91.606/67	extr	N
177	Q2U1X8	Probable arabinan endo-1,5-alpha-L-arabinosidase C	GH43	1.51	0.000	4.95/31.78	4.94/34.19	100/143	extr	Y
40	Q2U8R6	Probable pectate lyase A	PL1	−2.26	0.011	4.46/50.81	4.74/34.12	100/589	extr	Y
Proteolysis										
126	Q2U1F3	Leucine aminopeptidase A		1.51	0.001	5.02/42.45	5.03/41.10	100/917	extr	Y
169	Q2ULM2	probable leucine aminopeptidase 2		−2.81	0.005	4.47/49.18	4.94/53.56	100/171	extr	Y
24	Q06902	Aspartic protease pep1		5.60	0.013	4.4/17.44	4.85/42.31	100/142	extr	Y
174	Q9Y8E3	Dipeptidyl-peptidase 5		−11.36	0.002	4.52/78.7	4.66/80.38	100/214	extr	Y
94	Q2UP30	Neutral protease 2		Down[g]	0.000	6.2/20.07	4.92/37.10	100/203	extr	Y
95	Q2UP30	Neutral protease 2		Down[g]	0.013	6.13/20.03	4.92/37.10	100/203	extr	Y
191	P12547	Alkaline protease 1		1.71	0.002	6.48/37.76	5.95/42.57	100/734	extr	Y
Oxidation-reduction										
69	Q877A8	Catalase B		9.83	0.002	5.2/57.09	5.29/79.87	99.939/89	extr	Y
Aromatic compound metabolism										
81	P80402	2,3-Dihydroxybenzoate decarboxylase		−11.36	0.002	5.41/26.56	5.25/38.86	100/203	cyto	N
Glycolysis										
117	Q9HGY9	Fructose-bisphosphate aldolase		2.00	0.001	5.03/26.74	5.8/39.72	100/217	cyto_mito,nucl	N

NA: no available. up: only produce in isolate ZJGS-LZ-21; down: only produce in *A. oryzae* 3.042.
[a] Spot numbers match those marked in Fig. 2.
[b] Significantly difference as calculated by Student's *t*-test.
[c] Protein score ≥ 60 indicate identity.
[d] Distribution of proteins according to localization: extr: extracellular; cyto: cytoplasm; mito: mitochondrial; nucl: nuclear.
[e] The presence of signal peptides was predicted by using the signal v.4.1 program (http://www.cbs.dtu.dk/services/SignalP/).
[f] Only detected in *A. oryzae* ZJGS-LZ-21.
[g] Only detected in *A. oryzae* 3042.

glucose from the inhibitory cellobiose and its expression is up-regulated 6-fold (Barr et al., 2012). Notably, utilization of carbon sources, such as cellulose, can be limited by various environmental factors even the existence of cognate genes in their genome, which are tightly controlled at the transcription or post transcriptional levels (Daynes et al., 2008). A complex network of these transcriptional modulations has been extensively evaluated and reviewed (Tani et al., 2014; Udatha et al., 2015). Overexpression of transcription factor *xlnR* promotes the elevation of cellulolytic and xylanolytic activities in *A. oryzae*, about 40 secreted proteins being increased (Noguchi et al., 2009). Clr-1/2 positively regulates the increase of 50% cellulose-responsive genes in *N. crassa* (Coradetti et al., 2012). Additionally, a variety of small metabolites, including lactose in *T. viride*, cellobiose in *A. oryzae* and xylose in *A. niger*, have been identified as inducers of cellulase gene expression. In the koji preparation for soybean paste fermentation, the components are complex including various nutrients from raw material and/or its derived hydrolysates, which will collectedly induce xylanase, pectinase and cellulose hydrolysis of cell wall components (Sapna and Singh, 2014). The representative secreted enzymes activities are listed in Table S2, although their activities were not high.

There were six differentially expressed proteins involved in the degradation of hemicellulose, β-galactosidase A (Gal) (spot 67), endo-1,4-β-xylanase *F*1/F3 (EX) (spots 42 and 101, respectively), acetylxylan esterase A (AXE) (spot 166), arabinan endo-1,5-α-L-arabinosidase C (ABN) (spot 177), and NAD(P)H-dependent D-xylose reductase xyl1 (XR) (spot 89) (Fig. 4C). β-D-galactosidase hydrolyzes the non-reducing terminal β-D-galactose residues. Both EXs and AXE participate xylan degradation process. EXs catalyze the hydrolysis of xylan by randomly splitting the β-1,4 backbone to release xylose (Moukouli et al., 2011). In the ZJGS-LZ-21 isolate, the expression of EXs was up-regulated by at least 9-fold, resulting in greater hydrolysis of xylan-containing substrates. AXE removes the acetyl group from the backbone of the xylan chain, accelerating the hydrolysis of xylan by endoxylanases (Barr et al., 2012). The release of xylose, a pentose sugar, can contribute to the formation of the characteristic color and luster in soybean paste (Davidek et al., 2008; Nomi et al., 2011). XR and XDH (NAD-dependent xylitol dehydrogenase) can convert xylose into xylitol or ethanol, and these alcohols may strengthen flavor formation of soybean paste (Dmytruk et al., 2008; Jeffries, 2006). ABN is a glycosyl hydrolase that has the ability to cleave the α-1,5-L-arabinan glycosidic bonds to release arabino-oligosaccharides and L-arabinose (de Sanctis et al., 2010). L-Arabinose plays a key role in the color and flavor production in the soybean paste fermentation, similar to xylose, and is used as a substrate by lactic acid bacteria to produce lactate and acetate, conferring sauce aroma components (Rodas et al., 2006). We also observed reduced levels of a pectate lyase (PNL, spot 40) (Fig. 4D) in ZJGS-LZ-21 isolate.

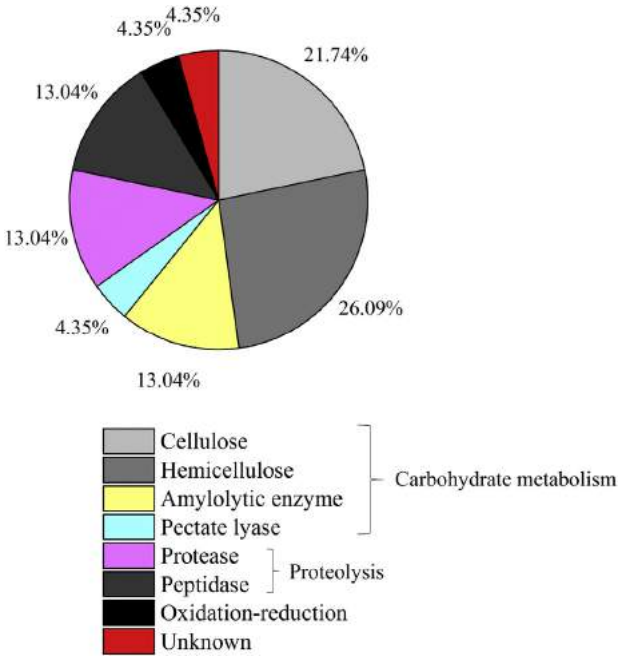

Fig. 3. The diagram of functional attributions of the significantly expressed proteins in *A. oryzae* ZJGS-LZ-21 isolate and strain3.042koji.

PNL can catalyze pectin degradation by β-elimination (Lara-Marquez et al., 2011), helping to degrade cell wall pectin for fermented soybean paste. The reduced role of PNL may not be significant due to the low levels of pectin in the substrate used in soybean paste fermentation, but there is a cocktail interaction among the enzymes degrading cellulose, hemicellulose, and pectin (Udatha et al., 2015). Nonetheless, one should note that these hydrolytic enzymatic activities on plant cell walls are significantly lower than amylase and protease in the koji preparation, but their effects on flavor formation seem much more important due to the release of various sugars. In addition to *A. oryzae*, a large variety of bacteria or other fungi also participate in the fermentation process of soybean paste. Strains belonging to genera *Leuconostoc*, *Lactococcus*, *Bacillus*, *Citrobacter*, and some uncultured bacteria, along with fungi *Geotrichum*, *Candida*, *Pichia*, *Mucor*, and Zygomycota and so on, have been isolated as the predominant microbial flora from traditional soybean paste made in China, Korea and Japan (Gao et al., 2013; Hong et al., 2015; Kumazawa et al., 2013). These microorganisms can utilize the various sugars and produce their metabolites (such as amino acids, organic acids, alditols and the like), and, thus, comprise these characteristics of fermented soybean paste.

3.3. Proteins involved in protein degradation

Typically, the fermentation process of soybean paste is carried out under a low pH, and acid proteases are often required to completely hydrolyze proteins. Alkaline and neutral proteases are the predominant proteases produced by *A. oryzae* (de Souza et al., 2015; Sriranganadane et al., 2010). Therefore, improving acid protease production is a goal of industrial strain development (Zhao et al., 2012a). By physical N^+ ion implantation mutagenesis, *A. oryzae* strain A100-8 was screened and its secreted acid protease was improved up to 1.44-fold. In the current study six types of proteins involved in protein degradation were identified, including aspartic protease pep1 (Pep1) (spot 24), alkaline protease 1 (Alp1) (spot 191), neutral protease 2 (Ntp2) (spots 94 and 95), leucine aminopeptidase A (LapA) (spot 126), leucine aminopeptidase 2 (Lap2) (spot 169), and dipeptidyl-peptidase 5 (DppV) (spot 174). The expressions of Pep1, Alp1 and LapA were upregulated in the ZJGS-LZ-21isolate, but the rest of the degrading enzymes were produced at reduced levels (Table 1).

Pep1 (Q06902) is an endopeptidase that belongs to the acid proteases family. It maintains activity to hydrolyze the peptide bonds to aspartic acid residues of soy proteins even at pH 3.5 (Sriranganadane et al., 2010). In the current report, Pep1 expression increased by 5.6-fold, which benefits protein degradation in soybean paste fermentation under acidic conditions. This result is consistent with the high acid protease activity of the ZJGS-LZ-21 isolate. More interestingly, Pep1 may also act on the aspartic acid residue in immunodominant epitopes of soybean proteins, thus, its increased levels can promote the reduction of allergic proteins such as Gly m Bd 30 K (Helm et al., 2000), Gly m Bd 28 K (Xiang et al., 2004) and Gly m Bd 60 K (Sun et al., 2013). Pep1 may actually improve the safety of the fermented soybean paste by reducing these allergens (Fig. S1). Currently, only microbial fermentation or enzymatic hydrolysis can decrease food allergenicity affording products generally recognized as safe (GRAS; Verhoeckx et al., 2015). According to the process of paste fermentation, neutral and alkaline protease preferably function at the initial stage before the medium acidification by accumulation of organic acids, and are still the most predominant enzymatic activities in koji. Alp1 (P12547) is a serine protease that is stable and activated at alkaline pH value, and this protease is an important secreted enzyme applied in the leather and detergent industries (Mushtaq et al., 2015). Alp1 also acts on the serine residue of immunodominant epitopes (Fig. S1) and may play a role in the degradation of allergenic soy proteins during the process of soybean paste fermentation. LapA/Lap2 and DppV are peptidases that catalyze the hydrolysis of polypeptides at the N-terminal position of peptides and proteins to release various amino acids. These peptidases play a key role in amino acid generation in the fermentation process of soybean paste (Lin et al., 2007; Sriranganadane et al., 2010). However, Lap and DppIV are neutral proteases and the acidic pH in the soybean paste fermentation limits their performance. Increased levels of acid protease Pep1 and its improved activity on soybean protein allergen confer the ZJGS-LZ-21 isolate with improved quality characteristics.

3.4. Other differentially enhanced proteins

In the ZJGS-LZ-21 isolate, the expression level of catalase B was upregulated 10-fold. Catalase B is a mono-functional heme-catalase and most of fungi contain several types of catalases, including CatA, CatB, CatP, CatC, and CatD (Hansberg et al., 2012; Wang et al., 2013). Catalase quenches hydrogen peroxide eliminating oxidative cell damage. In the process of mycelia growth, *A. oryzae* may respond to a variety of environmental factors, such as nutrient supplements, temperature, moisture content, and light intensity, to produce a large quantity of reactive oxygen species (ROS) that damages cell organelles and compromises cell functions. The up-regulation of catalase B in the ZJGS-LZ-21 isolate may help cells survive exposure to these ROS, resulting in greater stability and adaptability in the koji preparation process. Mold growth stops in the highly salted soybean paste and the secreted enzymes dominate the late hydrolysis process. Thus, the contribution of catalase B in the koji to the soybean paste fermentation requires further study.

3.5. Resolution of 54 kDa and 32 kDa bands by MALDI-TOF MS

In the 2-DE gels (Fig. 2), one obvious gathering spot and one horizontal stripe near the 54 kDa and 32 kDa were observed, and were referred to the Band I and II (Fig. 1B), respectively. The 2-DE process adopted here poorly distinguished these two group proteins, despite our extensive efforts on adjusting the protein loading and electrofocusing parameters. Therefore, we tried to combine 1-DE with MALDI-TOF MS methods to resolve the protein species inside these two heavy bands from the ZJGS-LZ-21 isolate. The results obtained indicate that Band I and II contain 53 and 55 protein species, respectively (Tables 2 and S1). Moreover, the theoretical *p*I values of all these protein

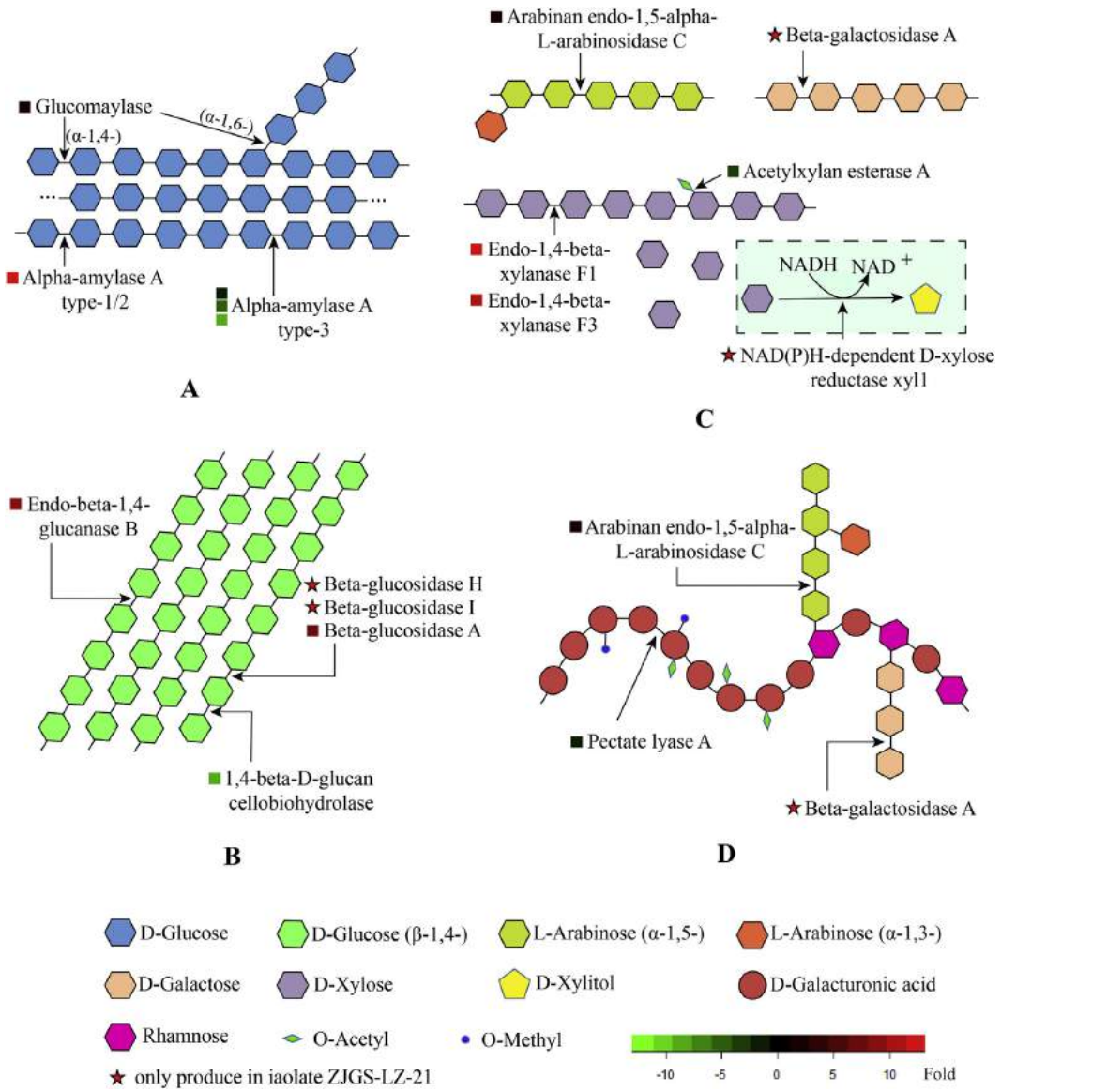

Fig. 4. Diagram of polysaccharide degradation by various differential proteins produced by *A. oryzae*. A, B, C and D: starch-, cellulose-, hemicellulose- and pectin-degradation, respectively. The change magnitudes of identified protein are indicated by the intensity of the color of the blockage (red and green token represent increased or decreased expressions in the ZJGS-LZ-21isolate, respectively). The rows symbolize the enzyme active sites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Identification results of representative extracellular proteins in 54 kDa and 32 kDa bands from *A. oryzae* Koji by MALDI-TOF MS/MS.[a]

	No.	Unused	%Cov	%Cov(50)	%Cov(95)	Accession	Protein description	Theor.pI	Peptides (95%)
Band I[b]	1	133.81	98.400003	97.799999	88.58	P0C1B3	Alpha-amylase A type-1/2	4.48	654
	2	38.89	42.590001	35.02	31.439999	Q2UCU3	Probable beta-galactosidase A	5.33	32
	3	31	46.759999	38.069999	31.45	Q9Y8E3	Dipeptidyl-peptidase 5	4.66	19
	4	27.06	50	50	41.010001	P36914	Glucoamylase	4.99	30
	5	22.34	53.960001	46.86	45.030001	Q2ULP9	Predicted protein	4.82	24
Band II[b]	1	99.35	72.210002	72.210002	70.469999	P12547	Alkaline protease 1	5.95	518
	2	45.39	69.340003	64.130002	62.120003	P0C1B4	Alpha-amylase A type-3	4.52	261
	3	35.06	70.950001	70.950001	68.809998	O94163	Endo-1,4-beta-xylanase F1	4.73	97
	4	22.71	27.309999	18.48	17.1	Q9Y8E3	Dipeptidyl-peptidase 5	4.66	38
	5	20.71	63.859999	59.939998	44.580001	Q2UHY1	Aldo/keto reductase family proteins	5.96	25

[a] The analytic method and detail data were presented in Table S1.
[b] Both of Band I and Band II were excised from the Fig. 1B.

species range from 4.11 to 5.96, consistent with their locations on the electrofocusing strip in 2-DE process (Fig. 2). The high similarities in molecular mass and *p*I values explain the isolation difficulty and the low resolution by the current 2-DE method. Thus, much narrower pH IPG strips for 2-DE process or new method such as iTRAQ proteomic analysis may be required to resolve these issues in the future studies. In the Band I, α-amylase A type-1/2 was identified with the highest unused score (133.81) and the most matching peptides amounts (95%) (654), the same as alkaline protease 1 in Band II. The results are consistent with previous studies of the soy sauce koji, where amylase A type-1/2 and alkaline protease 1 were the two dominant enzymes in the secreted proteins of *A. oryzae* (Kitamoto, 2015; Liang et al., 2009).

4. Conclusions

In this study, we performed a comparative secretome analysis of industrial *A. oryzae* koji to investigate its characteristics. A novel isolate from *A. oryzae* 3.042 koji, *A. oryzae* ZJGS-LZ-21, has a unique extracellular enzyme profile with high activities and diverse protein species and may be more suitable for soybean paste fermentation. Consistently, *A. oryzae* ZJGS-LZ-21 koji not only would promote better raw material utilization, but also should improve the quality of products, including color or flavor. The improved ability of cleaving immune-dominant epitopes in soybean proteins is noted, which should greatly contribute to the safety of the derived paste. Therefore, ZJGS-LZ-21 isolate demonstrates promising potential for the manufacture of soybean paste.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2017.02.003.

Conflict of interests

The authors declare that they have no competing interests.

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