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Proteome Analysis of Metabolic Proteins (p*I* 4−7) in Barley (*Hordeum vulgare*) Malts and Initial Application in Malt Quality Discrimination

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Supporting Information

ABSTRACT: Barley malt is essential for beer production. In the present study, the nonprolamin fractions including proteins with structural functions or metabolic activities were extracted from barley malts of the widely used cultivars Gangpi and Baudin in China. The metabolic proteomes (p*I* 4−7) were constructed and compared using two-dimensional electrophoresis (2DE) followed by matrix-assisted laser desorption/ionization-tandem time-of-flight mass spectrometry (MALDI-TOF/TOF) identification. There were 333 and 354 spots detected in the 2DE gels of Gangpi and Baudin malts, respectively, and about 90% of these spots were shared by the two malts. For all, 377 were successfully identified to 192 proteins, most of which were enzymes and enzyme inhibitors, suggesting important roles in barley malting and the mashing stage of brewing. The Baudin malt was found to contain more spots representing amylases, pathogen-related proteins, and chaperones than the Gangpi malt. In addition, enzymes involved in glycolysis and redox pathways showed significantly different profiles between the two malts, permitting a more in-depth elucidation of the relationship between differential proteins and malt qualities.

KEYWORDS: *barley cultivars, malt quality, metabolic proteins, proteome analysis, 2D gel electrophoresis, mass spectrometry*

INTRODUCTION

Beer has been produced for over 4000 years by a programmed process, including malting, mashing, and fermentation. Key to the beer brewing process is malt, which is prepared from barley malting. Quality of wort and beer as well as production efficiency is affected by the malt quality. During the germination phase of malting, a number of enzymes are synthesized or activated, and in turn, these bring about the degradation of a number of important biopolymers, such as cell wall polysaccharides, proteins, and starch. At the end of the germination stage, kilning or drying temporarily suspends these processes. In the mashing process, substances released during malting are converted into sugars, amino acids, and other nutrients in wort that can be utilized during fermentation. While the biochemistry of malting and brewing has been the subject of extensive study, knowledge on specific molecular processes catalyzed and regulated by metabolic proteins is still limited. This proteome analysis of malt metabolic proteins will be important to the continued clarification of these molecular processes and improvement of the malting quality of barley cultivars, even though the levels of hydrolase activities, which have been thought to be overwhelmingly important, have reached very high levels through breeding and malting technology.[1,2]

It is clear that the isozyme diversity among barley cultivars can be the cause of differences in malt quality. An early study by Görg et al. found significant differences in the pattern of amalyse isozymes between two barley cultivars of contrasting final attenuation values.[3] Aside from the isozymes, it is known that enzyme regulators can have an impact on malt quality. Two types of lipid transfer proteins purified from barley showed inhibition activities on cysteine-class proteases.[4] One of the wheat xylanase inhibitors, which have been proved to inhibit xylanases of invasive bacterial and fungal origin,[5] has been identified in barley.

Thanks to the pioneering exploration on the classification of barley cultivars within different malting grades by comparing the 2DE patterns of seed proteins[3,6] and the recent availability of mass spectrometry for protein identification, the proteomics approach has been successfully introduced to elucidate the relationship between proteins and barley quality and even that of beer.[7,8] Subsequent studies developed the method of extracting and identifying the low-salt soluble fraction consisting of enzymes and enzyme inhibitors from barley seeds and malt.[9] Until recently, more than 400 proteins of barley seeds from the grain filling and mature stages have been identified.[10] Most of these were determined to be involved in stress response and pathogens defense. However, less than 100 protein spots in germinating barley or malt obtained by micromalting have been identified in the previous study,[10,11] and prediction of the relationship between metabolic proteins and malt quality is stranded. No attempt has been undertaken to study the metabolic proteome of barley malts in the Chinese brewing industry, no mention was made on the exploration on the specific metabolic proteins potentially influencing the malt quality by means of proteomics.

Two barley malts of cultivars Gangpi and Baudin frequently used in Chinese breweries were selected for proteome analysis in the present study. The cultivar Gangpi is widely grown in

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Jiangsu Province of China, but its malt quality is generally unsatisfactory by domestic maltsters and brewers,[12] in contrast to the superior malt quality of the imported Australian barley cultivar Baudin.[13,14] One objective of this study was to expand the identification of metabolic proteins in two barley malts of cultivars frequently used in Chinese breweries. In addition, the other one was, for the first time, to investigate the differential metabolic proteins in barley malts with distinct quality differences and in turn to illustrate the potential malt quality related proteins, which would be of great significance in practical applications.

MATERIAL AND METHODS

Industrial Barley Malt Samples. Barley malt samples of cultivars Gangpi and Baudin were obtained from two different Chinese commercial maltsters which specialize in the malting of domestic and imported barleys, respectively. The malting conditions of cultivars Gangpi and Baudin are shown in Table 1. Barley of cultivar Gangpi (winter barley of two-row regular hulled), developed in the city of Lianyungang in Jiangsu Province since 1994 and widely grown in Jiangsu Province of China currently,[12] was harvested in May 2011 in China. In addition, barley of Baudin cultivar (spring barley of two-row regular hulled), developed at the state of Western Australia, released in 2002[13] and then widely used in Chinese breweries since 2006,[14] was harvested in November 2010 in Western Australia. The quality parameters of the two malts were measured by official analytical methods of the European Brewery Convention.[15]

Table 1. Malting Conditions for Barley of Cultivars Gangpi and Baudin

malting conditions	cultivar of Gangpi	cultivar of Baudin
steeping temperature (°C)	16	16
ex-steep moisture (%)	42	43
germination times (h)	110 ± 5	90 ± 5
germination temperature (°C)	16	16
lowest moisture during germination (%)	46	46.5
kilning procedures	45 °C for 8 h, 65 °C for 5 h, and 85 °C for 3 h	57 °C for 7 h, 65 °C for 6 h, and 85 °C for 3 h

Low-Salt Soluble Protein Extraction. The low-salt soluble fraction of malt protein was extracted as described[9] with minor modifications. Malt (5 g) was milled in liquid nitrogen by a porcelain mortar to essentially homogeneous flour, 4 g of which was added into 20 mL of extraction buffer (5 mmol/L Tris/HCl pH 7.5, 1 mmol/L $CaCl_2$) containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics AG, Rotkreuz, Switzerland). Samples were vortexed for 3 min every 15 min of the 1 h extraction period. Insoluble material was pelleted by centrifugation (10 min, 4 °C, 10 600*g*; 3K-15, SIGMA Laborzentrifugen, Osterode am Harz, German), and then the suspension was filtered through Whatman no. 4 filter paper (Whatman PLC, Maidstone, U.K.) of 70 mm diameter to remove suspended particles. Filtrate was collected and then centrifuged at 17 000*g* for 15 min at 4 °C twice; supernatants were combined and finally filtered through a 0.45 μm filter paper (EMD Millipore, Billerica, MA, USA). Filtrate was collected as low-salt-soluble protein extract and stored at −20 °C until analysis. Protein concentrations in extracts were determined using the Lowery protein assay method with a Bio-Rad kit (Hercules, CA, USA).

2DE. For 2DE analysis, 1000 μg of protein was precipitated with 10 volumes of precooled 10% TCA/acetone solution at −20 °C for 24 h. Precipitates were washed twice with 10 mL of precooled acetone. Protein was dissolved in 345 μL of a rehydration solution containing 8 mol/L urea, 4% (w/v) CHAPS, 0.5% (v/v) IPG ampholytes (pH 4−7), 1% (w/v) DTT, and 0.002% (w/v) bromophenol blue. Sample was then centrifuged at 17 000*g* for 10 min at 4 °C and applied to 18 cm pH 4−7 IPG dry strips (GE Healthcare Biosciences, Uppsala, Sweden) for at least 12 h. Isoelectric focusing (IEF) was performed on an Ettan IPGphor (GE Healthcare Biosciences) with the following operating conditions: 250 V for 45 min, 250−500 V for 45 min, 500−1000 V for 1.5 h, 1000−5000 V for 2 h, 5000−10 000 V for 2.5 h, and finally 10 000 V continuing until the total volt hours reached 45 kVh. After the IPG strips were equilibrated for 15 min in the equilibration buffer (6 mol/L urea, 50 mmol/L Tris-HCl pH 6.8, 2% SDS, 30% glycerol, and 0.002% bromophenol blue) containing 10 mg/mL DTT, followed by 15 min in the equilibration buffer containing 25 mg/mL iodoacetamide, the second dimension vertical SDS-PAGE was carried out on the 12.5% homogeneous polyacrymide gel using an Ettan Daltsix vertical electrophoresis system (GE Healthcare Bioscience). Proteins were visualized using Collidal Coomassie Blue G250.[16] Gels were run in triplicate for each malt sample.

Image Analysis. Destained gels were scanned with Image Master LabScan (GE Healthcare Biosciences), and images were analyzed with PDQuest Advanced 2D Analysis Software (Bio-Rad). According to software analysis, the qualitative differences refer to the protein spots detected only in one 2DE image or a group of 2DE images. The absence of these spots in the other 2DE images is due to the situations that the proteins do not exist in the sample (or the group of samples) or the protein abundance is too low to detect. The qualitative differences reflect the significantly different proteins among the compared samples.

Each protein spot on a malt gel image was considered as present if the corresponding spot with the same molecular weight and p*I* was present on triplicate gels of the malt sample. By comparing the gel images of Gangpi and Baudin malts, the spots appearing separately in the gel image of Baudin or Gangpi malt were selected as the differential protein spots between the two malts. The master gel was created by compiling every shared spot on the 2DE gel images of Baudin and Gangpi malts to a virtual gel with PDQuest software.

Protein Identification. Each spot in the 2DE gels was cut out and subjected to in-gel trypsin digestion.[17] The peptide solution (1 μL) was applied to an Anchorchip target (Bruker-Daltonics, Bremen, Germany) with the same volume of matrix (10 mg/mL, α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) and analyzed on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics). Tryptic peptides were analyzed in the positive ion reflector mode, and spectra were calibrated using Bruker peptide calibration standard II (Bruker-Daltonics). At least 10 peptide fragments were selected to be analyzed in lift mode. After spectra were processed by FlexAnalysis software and analyzed by BioTools software (Bruker-Daltonics), an in-house Mascot server (http://www.matrixscience.com) was used for database search in the Green Plant taxonomy of NCBInr at the National Center for Biotechnology Information. The following criteria were used for the database search: tryptic digestion; monoisotopic peptide values; at least four matching peptide masses; a maximum of one missed cleavage per peptide; fragment mass tolerance of 0.5 Da and peptide mass tolerance of 100 ppm, together with the acceptance of cysteine carbamidomethylation (fixed modifications) and methionine oxidation (variable modifications). For a positive identification, a score calculated by the Mowse scoring algorithm in MASCOT was considered as significant ($p < 0.05$). The sequences encoding predicted proteins of unknown function were subjected to BLAST search in NCBI.

RESULT AND DISCUSSION

Malt Quality. To reflect the industrial production, the malt samples used in the present study were obtained from malt factories. Both of the malts were produced under their own optimum conditions controlled by expert maltsters, who ensured the malt quality reached the optimum level. The malt quality parameters of the samples are shown in Table 2.

Table 2. Comparison of Malt Quality Parameters between Baudin and Gangpi

malt parameter	Baudin	Gangpi
moisture content (%)	4.5 ± 0.1	4.7 ± 0.1
saccharification time (min)	8.6 ± 0.2	13.2 ± 0.3
filtration rate (min)	47.0 ± 2.0	110.0 ± 5.0
viscosity (8.6%) (mPas)	1.31 ± 0.03	1.53 ± 0.01
extract (%, dry)	82.1 ± 0.6	76.0 ± 0.3
wort color (EBC)[a]	5.20 ± 0.04	5.00 ± 0.09
turbidity (EBC)[b]	1.51 ± 0.00	4.86 ± 0.03
free amino nitrogen (mg/100g, dry)	170.0 ± 5.3	155 ± 2.1
total malt protein (g/100g, dry)	10.1 ± 0.2	12.6 ± 0.1
KI (%)	44.8 ± 0.2	43.3 ± 0.3
diastatic power (°WK)[c]	290 ± 6	221 ± 4

[a]Wort color was analyzed by EBC turbidity meter. [b]According to Analytica-European Brewery Convention method, 1 EBC is defined as the turbidity of standard solution (mixture of 1% hydrazine sulfate solution and 10% hexamethylenetetramine solution with equal volume) diluted 1000 times. [c]Windisch-Kolbach unit: 1 WK is defined as 1 g of maltose produced by 100 g of dry malt decomposing soluble starch for 30 min at a temperature of 20 °C, pH 4.3.

Quality indicators, including KIs, moisture content, and wort color, showed minor differences between the two malts. The Kolbach Index (KI, soluble nitrogen/total nitrogen) is a measure of endosperm protein modification (or solubilization) that occurred during malting.[7] The KIs of the two malts were ideal for brewing (KI 43−45%[18]) and very near to each other, which proved the proteins in both malts were developed to their optimum levels during malting. The similar kilning intensities, indicated by moisture contents and wort color between the two malts, suggested the proteins experienced almost the same degrading course during kilning following the barley germination stage.

The saccharification time is measured by calculating malt starch degradation time during mashing, and diastatic power (DP) is a general measure of carbohydrate hydrolase activity.[18] As previously reported, malt DP varied with barley cultivars and environment and was strongly correlated with β-amylase and α-amylase activities.[19] The lower DP and saccharification time of Gangpi was assumed to result from the β-amylase or α-amylase difference between the two malts. Both wort viscosity and turbidity of Gangpi malt were much higher than that of Baudin, and the wort lautering time of Gangpi is much longer, which agreed with the pronounced filterability problems in domestic breweries when using malt of some barley cultivars[20] and were often attributed to the nonstarch polysaccharide (NSP) and prolamine not fully degraded during malting.[21] Peroxidases could also contribute to the malt filterability.[22] The wort extract is the yield of all degraded micromolecules, and the low extract was always accompanied with the low DP and filterability problems.[20] Thus, the gaps of saccharification time, DP, filterability, and extract from the Gangpi malt to the Baudin suggested that metabolisms in Gangpi malt were not as active as that in Baudin, which was a result of the differences in metabolic proteins.

Overall, the close data of moisture contents, wort colors, and KIs between the two malts were significant for proteome comparison because these parameters indicated that the metabolic proteins in both malts were developed to their own optimum status for industrial production, they experienced almost the same degrading course at the end of malting, and the proportions of extractable protein were near. In addition, the qualities of both malts are suitable for brewing;[18] thus, the following metabolic proteome analysis could be on behalf of the fundamental metabolic proteins composition of malt used in large-scale production, and differential proteins are responsible for the malt quality differences.

Metabolic Proteins Identified in Both Gangpi and Baudin Malts. A study by Weiss et al. showed that Tris-HCl buffer (50 mmol/L, pH 8.8) extracted proteins from barley mainly consisted of albumins, globulins, and part of the hordeins.[23] To avoid hordeins masking the albumin and globulin portions which are a mixture of metabolic proteins, Ostergaard et al. modified the protein extraction method using a low-salt solution (5 mmol/L Tris/HCl pH 7.5, 1 mmol/L $CaCl_2$) to omit the hordeins.[9] The low-salt solution extraction was adopted in the study to investigate the malt metabolic proteome.

There were 333 and 354 spots detected in the 2DE gels of Gangpi and Baudin malts, respectively. The shared spots accounted for 93% and 87% of all protein spots in the 2DE gels of Gangpi and Baudin malts, respectively. A total of 410 spots (as shown in Figure 1) was selected for MALDI-TOF/TOF

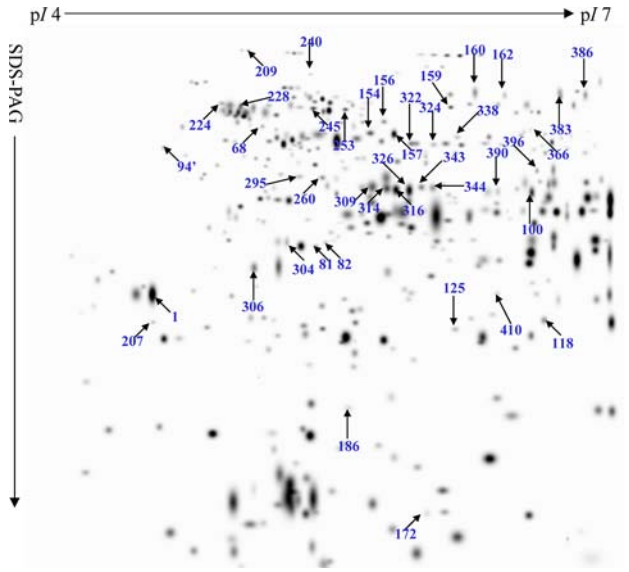

Figure 1. Master gel representing spots present in both Baudin and Gangpi malts. Assigned numbers indicate hydrolases. Each arrow directs a spot, and the corresponding number is labeled in the tail of the arrow.

analysis, and 377 spots were successfully identified to 192 proteins (Supporting Information Table 1). Of the identified 192 proteins, eight groups were classified according to their functions (Table 3), and the distribution probability of protein spots in each group was shown. It is similar to proteome analysis of barley seed[10] that a protein was identified in multiple spots, with serpins (Protein Z) of 36 spots as representatives in the present study (Supporting Information Table 1). It is suggested that post-translational modifications and protein degradations caused the same protein to occur in different spots in malts.

The most abundant proteins (approximate 30% of protein spots) identified in both malts were proteins involved in carbohydrate metabolisms (Table 3). Four spots of low p*I* α-amylase (AMY1), three spots of high p*I* α-amylase (AMY2),

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Table 3. Classification of All Proteins Identified in Both Malts of Cultivars Baudin and Gangpi

protein categories	protein spots of each category/total spots (%)	identified proteins number
carbohydrate metabolism	29	44
proteins, peptides and amino acids metabolism	13	33
lipid metabolism	2	9
nucleic acids metabolism	5	13
pathogenesis-related proteins	19	17
other stress-related proteins	14	31
chaperones	8	17
others	10	28

five spots of β-amylase, and one spot of limit-dextrinase were identified in both malts. α-Amylase initiates the breakdown of starch by endohydrolysis of α-1,4 glucosyl linkages. β-Amylase catalyzes the release of maltoses from nonreducing chain end of starch and dextrin, and limit-dextrinase facilitates the activity of both α- and β-amylases by cleaving α-1,6 glucosyl linkages.[18] AMYl is remarkably thermostable under acidic conditions and has a higher affinity to starch than AMY2 and thus is able to more effectively hydrolyze starch granules.[24] It has been reported that there were four genes encoding AMY1 and six genes encoding AMY2 isozymes in barley,[25] but only one AMY1 and two AMY2 gene products were found in green malt, which suggested that the other AMY-encoding genes were not significantly expressed.[26]

Four enzymes or conserved domains involved in NSP hydrolysis were identified in both malts, an arabinoxylan arabinofuranohydrolase isozyme AXAH-I, a β-D-xylosidase, a chain A of β-D-glucan glucohydrolase isozyme exo1, and a 1,4-β-D-mannan endohydrolase precursor (Supporting Information Table 1). During malting, NSP hydrolases form channels for release of starch and protein degrading enzymes from aleurone and allow access to their contacts with endosperm.[2] Due to the high viscidity and molecular weight of NSP, their degradation levels affect the lautering performance and clarity of wort.[27] The types of NSP hydrolases identified in the malt in this study were fewer than those of germination,[28] but none of NSP hydrolases found in the study appeared in the proteome analysis of barley seed during filling and maturation.[29]

The other proteins involved in carbohydrate metabolism were identified as enzymes implicated in glycolysis, the tricarboxylic acid cycle (TCA cycle), the pentose shunt, and saccharide anabolism. During aerobic respiration, the TCA cycle in the mitochondria is a hub for oxidative degradation of carbohydrates, proteins, and other molecules. It provides energy for dynamic metabolisms, but excessive respiration during barley germination can result in the superfluous consumption of substances, resulting in decreased malt yields.[30] The effect of glycolysis, pentose shunt, and saccharide anabolism on malt quality, however, is not clear.

Pathogenesis-related proteins (PRs) were the second most abundant category of proteins identified in the present study (Table 3). PRs are involved in the protection of dormant and germinating cereal seeds against pathogenic microorganisms and insects.[31] Chitinases are expressed to hydrolyze the cell wall chitin of fungi.[32] Serpin superfamily proteins probably inhibit exogenous serine proteases that break down storage protein of barley seed.[33] Thaumatin-like proteins (TLPs) in barley leaves were found to be induced by infection by *Drechslera teres*,[34] and TLP4 was identified in this study. Bifunctional inhibitors (BFIs) of amylase and proteinases, including trypsin/amylase inhibitors pUP13 and pUP18, and α-amylase/trypsin inhibitors CMa, CMb, and CMd, were also identified in the two malts. BFIs inhibit amylases and proteinases from micro-organisms, insects, and nonarthropod invertebrates.[35]

Moreover, 2DE maps of beer proteome revealed the dominant presence of PRs.[22,36] Nine of the PRs identified in the study have been previously reported in beer, and PRs are of considerable technological importance in brewing. Protein Z is the most abundant protein in malt and beer and has been identified as a major beer antigen. Its glycated form can improve beer foam stability.[8,22] The ns-LTP1 is known to be the second most abundant protein in beer after protein Z.[22] It has been shown that addition of ns-LTP1, of either cereal or microbial origin, improved both the foam potential and the foam half-life.[37] A recent proteomic study of beer haze suggested that CMb was one of the growth factors contributing to colloidal haze in beer.[36]

A large number of stress-related proteins were also found in the two malts. Eleven oxidative-stress-related proteins were identified in the both malts (Supporting Information Table 1). Oxygen is necessary for barley germination, but incomplete oxidation of oxygen produces oxygen radicals during malting, which can damage biological systems, inactivate enzymes, and result in development of wort and beer hazes and stale flavors in beer.[38,39] These oxidoreductases can regulate and maintain the intracellular redox environment. The most abundant desiccation stress proteins in the malts are heat shock proteins, expression of which is induced primarily by heat shock factors, and their translation increases when the plant is exposed to elevated temperature.[40] The kilning stage following barley germination during malting was considered as the most possible inducing factor in the study.

Protein spots identified to enzyme catalyzing storage protein metabolisms were not very abundant, but the variety of enzyme types was great, preceded only by enzymes involved in carbohydrate metabolism (Supporting Information Table 1). Degradation of storage protein, which typically accounts for 9–13% of the dry weight of barley seeds, plays a significant role in influencing the lautering performance and clarity of wort, nearly equal to that of the NSP.[41] It was reported that cysteine endopeptidases played the most important role in barley protein hydrolysis.[42] Four types of cysteine proteinases or conserved domains: an endopeptidases EP-A, a cysteine proteinase EP-B isoform 1 precursor, a cysteine endoprotease B isoform 2, and a cathepsin B were identified in both malts; the rest were identified to enzymes involved in peptide and amino acid metabolism. An important indicator of malt quality and protein modification during malting is the KI, and it is mainly determined by enzymes involved in the protein metabolisms.[41]

The less abundant protein categories identified were enzymes involved in lipid and nucleic acid metabolism, chaperones, and proteins with unidentified functions (Table 3 and Supporting Information Table 1). All shared proteins identified in the present study preliminarily constituted the main metabolic proteins (p*I* 4–7) of barley malt in large-scale production, indicating their key roles in barley malting and mashing.

Qualitative Differences between Baudin and Gangpi Malts. When the proteome maps of the two barley malts were compared (Figure 2), 23 spots were found only in the 2DE gel

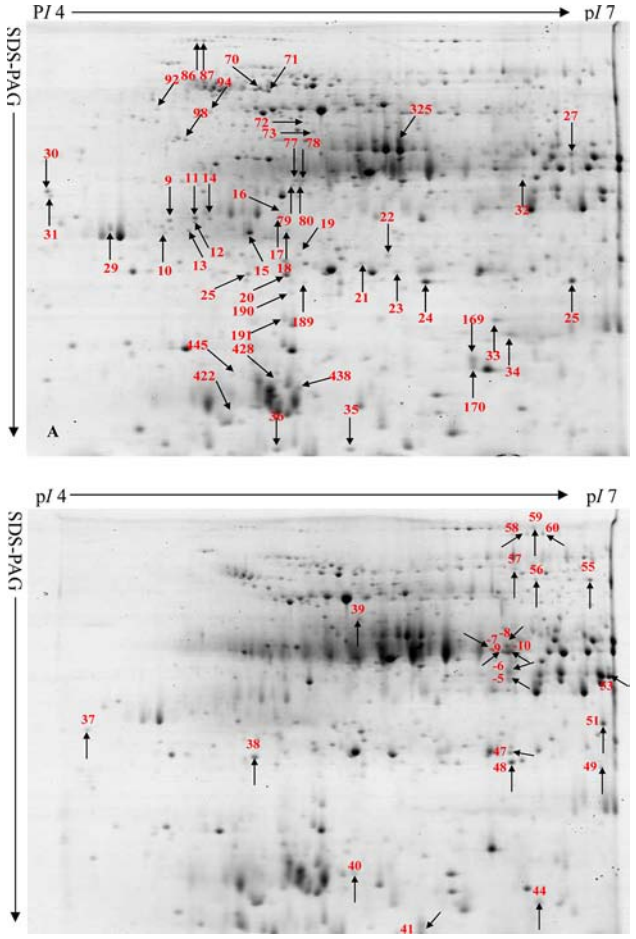

Figure 2. Representative 2DE gels of Baudin (A) and Gangpi (B). Spots present only in one malt are indicated with arrows.

image of Gangpi (Table 4) while 44 spots were present only in the Baudin malt (Table 5). The most significant difference was more spots representing amylases in the Baudin malts, including one spot of α-amylase isozyme precursor (spot 325) and three spots of β-amylase spots (spots 94, 169, and 170) (Figure 3). Lesser amounts of amylases that play a key role in starch hydrolysis during mashing could greatly contribute to the long saccharification time, low diastatic power, and low extract of the Gangpi malt.

Enzymes involved in glycolysis appeared in different profiles between the two malts. In addition to the shared enzymes in the master gel, a fructose-bisphosphate aldolase (spots −7, −8, and −10) and a glyceraldehyde-3-phosphate dehydrogenase (spot 43) were found in Gangpi, but enolases (spots 11, 12, and 14), fructose-bisphosphate aldolase (spot 16), glyceraldehyde-3-phosphate dehydrogenase (spot 27), triosephosphate isomerase (spot 28), glyceraldehyde-3-phosphate dehydrogenase (spot 77), and cytosolic phosphoglycerate kinase (spot 79) were found only in the Baudin malt. Dihydrolipoamide dehydrogenase (spot 55), one of enzymes catalyzing conversion of pyruvate to acetyl-CoA, was only identified in Gangpi malt. However, there are few report on the relationship between enzymes involved in glycolysis and malt quality.

Proteins involved in amino acid metabolism, such as glutamine synthetase (spot 18), putative leucine aminopeptidase (spot 22), and cystathionine β-synthase pair (spot 72), were found to be present only in Baudin malt, which might have indirectly improved the quality of Baudin malt, as the germinating barley grain requires amino acid as nutrients.

Another major difference was that Baudin malt contained more PRs, such as serpin-Z7 (spots 73, 78, and 80), serpin-Z2B (spot 190), CMb (spot 438), CMd (spot 428), pUP13 (spot 422), pUP38 (spot 445), and Chitinase (spots 20, 23, and 34). However, Chitinase was present in the Gangpi malt in different forms (spots 37 and 38). The differential profiles of PRs between the two malts may result from the difference of growing environments, invasive pathogens, as well as differences in disease resistance between the barley cultivars. The grown region Jiangsu Province of Gangpi cultivar is as rainy as the grown region West Australia for the Baudin cultivar; thus, both malting barleys are likely to have a similar disease pressure. The larger number of unique PRs in the Baudin malt seemed to be related to the low content of nitrogen, as it has been reported that increased fungi infection occurred in barley and wheat with lower nitrogen fertilizer[43,44] and that Baudin barley was susceptible to most leaf diseases.[13] However, the different profiles of PRs can provide different protection mechanisms of endogenous enzymes during barley growing and malting and have a different impact on the barley and malt quality. In addition, Baudin contained more chaperones including heat shock protein (spots 71, 98), and low-temperature-induced proteins (spots 87, 88) suggested the intensive protection system for other protein activities against temperature fluctuation during kilning of the Baudin malt.

More oxidative stress-related proteins were detected in the Gangpi malt, such as peroxidase BP 1 (spots −5 and −6) (Figure 3), glutathione-*S*-transferase (spot 47), and sulfite reductase (spot 57). Peroxidase has a positive effect in keeping the system redox balance, but it may pose a threat to the filterability and clarity of wort. It is well known that peroxide removal catalyzed by peroxidase could cause polyphenol

Table 4. Proteins Identified Only in the Gangpi Malt

spot id	protein names	accession no.	spot id	protein names	accession no.
60	β-D-xylosidase fructose-bisphosphate	gil18025342	41	glyoxalase dihydrolipoamide	gil326520285
−7, −8, −10	aldolase glyceraldehyde-3-phosphate dehydrogenase, cytosolic	gil226316443	55	dehydrogenase	gil326517553
43		gil120668	53	aldose reductase	gil728592
39	*S*-adenosylmethionine synthase 4	gil122220776	57	sulfite reductase	gil326507694
−5	peroxidase BP 1 chain A, crystal	gil167081	51	proteasome_β_type5	gil326524101
−6	structure of barley grain peroxidase 1	gil157830301	44	B3-hordein	gil123459
47	glutathione-*S*- transferase	gil75674128	58	Glycinin	gil5712199
37, 38	chitinase	gil563489	42	hypothetical protein	gil326520285
−9	protein Z (180 AA)	gil19079	48	hypothetical protein	gil112821176
40	glyoxalase	gil326529043	49	hypothetical protein	gil2266666

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Table 5. Proteins Identified only in the Baudin Malt

spot id	protein names	accession no.	spot id	protein names	accession no.
325	α-amylase type B isozyme, precursor	gil2851583	445	trypsin/amylase inhibitor pUP38	gil225103
94, 169	β-amylase	gil113786	73, 78, 80	serpin-Z7	gil75282567
170	β-amylase	gil11322499	190	serpin-Z2B	gil75279909
11	enolase	gil326490934	191	glyoxalase_I	gil326514208
12	enolase 1-like	gil357110857	15	lactoylglutathione lyase	gil326493416
14	predicted: enolase	gil326493636	33	aldose reductase	gil110590879
16	fructose-bisphosphate aldolase	gil226316443	35, 36	late embryogenesis abundant protein	gil547817
77	glyceraldehyde-3-phosphate dehydrogenase	gil28172913	87, 88	low-temperature-induced protein; provisional	gil326487522
79	phosphoglycerate kinase, cytosolic	gil326522650	70	RuBisCO large subunit-binding protein subunit β	gil2493650
27	glyceraldehyde-3-pho sphate dehydrogenase	gil34787348	71	heat shock 70 kDa protein like	gil115448989
28	triosephosphate isomerase	gil2507469	98	heat shock 70 kDa protein	gil326497219
18	glutamine synthetase	gil326507474	29	seed maturation protein	gil326531218
22	putative leucine aminopeptidase	gil18652402	30	seed maturation protein	gil326519240
72	cystathionine β-synthase pair	gil326519614	9	S-adenosylmethionine-dependent methyltransferases I	gil326489477
21	chain A, crystal structure of barley grain peroxidase 1	gil157830301	19	glycosyl hydrolase family 1	gil326493626
20, 23, 34	Chitinase	gil563489	92	glycosyl hydrolase family 1	gil326488897
428	α-amylase/trypsin inhibitor CMd	gil585291	32	2-nitropropan dioxygenase-like	gil326487674
438	α-amylase/trypsin inhibitor CMb	gil585290	189	cupin_2	gil326529599
422	trypsin/amylase inhibitor pUP13	gil225102	24, 25	hypothetical protein	gil112821174

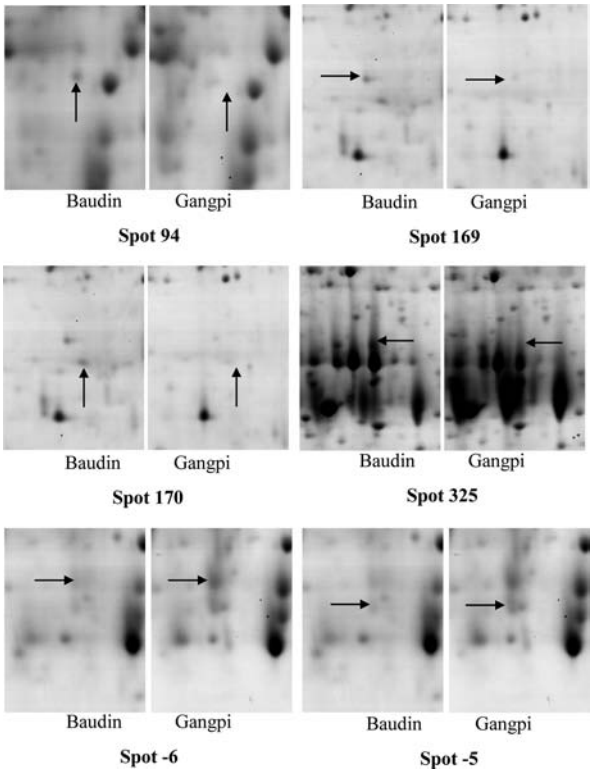

Figure 3. Enlarged 2DE maps of differential amylase spots and peroxidase spots between Gangpi and Baudin malts (spots 94, 169, 170, 325, −5 and −6).

oxidation and the cross-linking of oxidized polyphenols and proteins which is a considerable contributor of beer haze.[45] This also could contribute to the low lautering rate and high wort turbidity in the Gangpi malt.

In barley, it is well known that for each protein there is only one or a small number of encoding genes transcribed. Therefore, the differential electrophoretic mobility of every protein is assumed to be the result of various post-translational modifications, which have important implications in the properties of proteins that influence malt quality. In the two malts, chain A of barley grain peroxidase 1 (spot −6 in Gangpi, spot 21 in Baudin) and Chitinase (spots 37, 38 in Gangpi; spots 20, 23, 34 in Baudin) showed different profiles. Additional study on the modifications of malt quality-related proteins that occur during the malting process is needed.

In conclusion, the present study targeted a comprehensive survey of the metabolic proteins over the p*I* range 4−7 present in barley malt of two cultivars frequently used by the Chinese breweries. A total of 377 spots representing 192 proteins were identified in the two malts, and 310 spots representing 149 proteins were shared by both. Of all these, only 40 spots were identified as 26 hydrolases. Hydrolases have been traditionally considered as the most important factors of malt quality for brewing. Approximately 33% of the spots were identified as stress-related proteins, mainly related to pathogen and oxidative resistance. Proteins related to oxidative stress beneficially balance the redox environment in vivo; otherwise, some of them, such as peroxidases, have a potential to result in wort turbidity through oxidation of polyphenols. PRs protect barley against pathogens by inhibiting invasive enzymes. This could be significant during barley germination when temperature and humidity are suitable for microbrial growth.[46] The importance of PRs in malting and brewing should be the subject of future study. Many of the proteins related to desiccation resistance were likely to be induced by kilning and related to protection of other metabolic proteins. Nearly one-half of the spots were identified as proteins involved in the housekeeping processes, and about 10% were hypothetical proteins. Their specific effects on the malt quality remain to be investigated.

Among the proteins identified, 44 spots of 38 proteins were present only in the gel of the Baudin malt whereas 23 spots of 20 proteins were found only in that of the Gangpi malt. Some of the qualitatively different proteins would be expected to be associated with the differences in malt quality. Less amylase spots were found in the Gangpi malt, which was of lower quality. The importance of amylases for liberation of extract and formation of fermentable sugars during mashing has long been recognized. Less PRs and chaperones appearing in Gangpi

malt might indicate its relative lack of response to environment fluctuations.

Genetic backgrounds, growth environments, and malting processes of the two cultivars are different. Barley cultivars and growth environments which mutually choose are the congenital factors of malt qualities. The malt quality meeting brewing requirements is gained by maltsters optimizing malting conditions and taking advantage of the congenital traits.[47,48] The diversity in metabolic proteins between the two malts used in the current study may result from barley cultivars as well as these factors mentioned above. However, the different proteins found in the study were responsible for and represent the status of malt quality in industrial use. Combined with the quantitatively different proteins (ongoing research), the qualitatively different proteins found in the malts are potential markers for malt quality discrimination. They may also provide important clues for barley breeding improvement for breeders as well as malting condition optimizing for maltsters. In addition, with all these improvements in malt quality, there will be more time saved, less output cost used, and better beers produced by brewers.

■ ASSOCIATED CONTENT

Supporting Information

Protein identification of all the shared spots in the 2DE gels (overlapping pH 4−7) of the two malts investigated. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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