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Conversion of *Baijiu* distillers' grains to functional peptides: Process optimization and antioxidant activity evaluation

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ABSTRACT

Distiller's grains resourcing is a crucial problem for the sustainable development of the Chinese *Baijiu* industry. In order to improve the protein utilization of distiller's grains. In this study, distiller's grains pretreated by wet crushing combined with amylase/cellulase were further hydrolyzed with neutral protease combined with papain at 50 °C for 3 h. The hydrolysates were decolorized by 1% coconut shell activated carbon for 15 min, and separated and purified by macroporous resin and Sephadex gel to yield peptide fractions (F1, F2, F3 and F4). The F3 fraction with the highest radical scavenging activity was analyzed by nano-LC-MS/MS and peptidomics to identify peptides. Three peptides (especially newly discovered AAHVLAAAFL) displayed strong alcohol solubility and antioxidant activity and were stably bound to key regulatory pathway receptors (Keap1-Nrf2) and myeloperoxidase (MPO), further elucidating molecular mechanism of anti-oxidation. These findings provided knowledge for potential functional peptide candidates and facilitates the high-value commercial development of distiller's grains.

1. Introduction

The brewing of alcoholic beverages is one of the most important agricultural activities worldwide, and distiller's grains are the main by-products of the brewing process (Bravo, Mas-Capdevila, López-Fernández-Sobrino, Torres-Fuentes, Mulero, Alcaide-Hidalgo, et al., 2022; C. Li, Hu, Liang, Wei, & Wang, 2022). *Baijiu* is a traditional Chinese alcoholic beverage and also one of the oldest distilled spirits in the world. It has a wide range of enthusiasts around the world due to its unique taste and aroma produced by microorganisms during grain fermentation (Jie Yang, Zhang, Ding, Chen, Yin, Yang, et al., 2021). According to data from the China Alcoholic Drinks Association, over 7.1563 million kiloliters of *Baijiu* were produced in 2021, along with >100 million tons of distiller's grains produced as waste. *Baijiu* distiller's grains are solid residues of sorghum, corn and other grains. The brew contains 18.2%–31.4% carbohydrates, 12.5%–16.1% protein, 2.0%–4.1% lipids, 0.2%–0.4% minerals and other residual nutrients (Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021; Jie Yang, et al., 2021). At present, distiller's grains are mainly consumed as animal feed, biogas production, composting, microorganisms cultivation, vinegar brewing, etc. These traditional organic solid waste management yield very limited economic benefits and increases environmental risk and pollutants (Y. Liu, Liu, Huang, Ge, Xi, & Mao, 2022). Therefore, it is essential to develop alcohol-soluble antioxidant peptides from Baijiu distiller's grains.

Oxidative stress, as a negative effect of free radicals on biological molecules in the human body, is considered to be an important factor involved in the degenerative or pathological processes of senescence, cancer, atherosclerosis, gastric ulcer and other diseases (Wang, Gong, Li, Yu, Chi, & Ma, 2014). Food-derived natural ingredients was an effective ways to protecting the body from reactive oxygen species (ROS) damage due to their rich sources, convenient extraction, and good biological activity. Functional peptides was one of the key components (composed

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of 2–30 amino acids), mainly derived from fermentation food, protein hydrolyzate, and *in vivo* digestion. It also has hypotensive, hypoglycemic, anti-inflammation, and antioxidant acitivites (Daliri, Oh, & Lee, 2017). Among them, natural antioxidant peptides from grains, vegetables and plant substrates have been the focus of research interest (Y. Zhu, Lao, Pan, & Wu, 2022). *Baijiu* distiller's grains contain various natural components that are not fully hydrolyzed and fermented, which can be used as an ideal source of functional peptides. Antioxidant activity is dependent on specific amino acid sequences of proteins. Notably, it is feasible to release functional amino acid sequences from distiller's grains under controlled enzymatic hydrolysis conditions (Y. Liu, Liu, Huang, Ge, Xi, & Mao, 2022; Wang, Gong, Li, Yu, Chi, & Ma, 2014). The pretreatment method, proteolytic enzyme species, enzyme/substrate ratio and enzymatic hydrolysis conditions (time and temperature) are key parameters that affect the molecular weight and amino acid composition of functional peptides during the processing of distiller's grains (Sila & Bougatef, 2016). Furthermore, adding peptides with antioxidant activity back into *Baijiu* is an ideal approach to enhance its quality function and maximize the value and capacity of distiller's grains resourcing (Yunsong Jiang, Sun, Yin, Li, Sun, & Zheng, 2020). Therefore, it is essential to develop alcohol-soluble antioxidant peptides from *Baijiu* distiller's grains. There have been some attempts to carry out the hydrolysis of distiller's grains using exogenous or endogenous enzymes (Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021; Wang, Gong, Li, Yu, Chi, & Ma, 2014; L. Zhang, Jiang, Yin, Sun, Li, Sun, et al., 2018). However, few systematic investigations have been reported on alcohol-soluble antioxidant peptides in distiller's grains.

The pathway by which antioxidant peptides inhibit oxidative stress involves activation or regulation of related cellular elements, most commonly Kelch-like ECH-related protein 1/nuclear factor erythroid 2-related factor 2 (Keap1-Nrf2) antioxidant responsive element (ARE) (Yin, Han, Liu, Piao, Zhang, Xue, et al., 2022). In the Keap1-Nrf2 pathway, ARE is a specific DNA-promoter binding sequence that can be activated by Nrf2. Exogenous active peptide induces the uncoupling of Nrf2 from Keap1, and then free Nrf2 translocates into the nucleus and activates the ARE, which finally initiates the expression of antioxidant enzyme genes and exerts antioxidant activity (Deshmukh, Unni, Krishnappa, & Padmanabhan, 2017). Therefore, the screening antioxidant activity can be performed *in silico* using molecular docking based on the stability of the degree of binding to Keap1 (Tonolo, Moretto, Grinzato, Fiorese, Folda, Scalcon, et al., 2020; Yin, et al., 2022). In addition, myeloperoxidase (MPO) is another marker that stimulates oxidative stress and is involved in oxidative damage of tissues. It affects lipid metabolism by catalyzing hydrogen peroxide to produce peroxides during inflammation, and has been successfully applied to screen antioxidant peptides from false abalone (*Volutharpa ampullacea perryi*), sea snails (*Turbo cornutus*) and hemp seed protein hydrolysates in a high-throughput process (He, Zhang, Sun, Du, Qiu, Tang, et al., 2019; Kang, Kim, Kim, Lee, & Heo, 2021). It is the current research hotspot to explore possible functions and molecular mechanisms of active peptides from *Baijiu* distiller's grains by docking.

Therefore, in this study, *Baijiu* distiller's grains were enzymatically hydrolyzed to yield hydrolysates with antioxidant activities. Then, a practical method for the isolation and purification of alcohol-soluble antioxidant peptides was established, and two new antioxidant peptides were identified using nano-LC-MS/MS. Furthermore, the peptides were synthesized according to the obtained amino acid sequences, and their structure–activity relationships were further explored by *in vitro* antioxidant activity in combination with *in silico* molecular mechanisms. This study provides a model and strong incentive for the development of added-value products and environmental-friendly utilization of distiller's grains, while adding new knowledge of functional peptides.

2. Materials and methods

2.1. Materials and reagents

Distiller's grains (with fermention time 358, 415, 520 and 937 days, respectively) of Luzhou-flavor and *Baijiu* were provided by Lao Jiao Co., Ltd. (Sichuan, China). The aforementioned materials were stored at −80 °C before use. Trichloroacetic acid (TCA), sodium lauryl sulfate, sodium tetrahydroborate, β-mercaptoethanol, ABTS, DPPH, Sephadex G15 were purechased from Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China. 200 mesh coconut shell (CSAC), lignin phosphate (LPAC), wood physics (WPAC) activated carbon were purechased from Fujian Yuanli Active Carbon Co., Ltd, Fujian, China. Non-polar macroporous resins (XAD16, DA201-C) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China. Resins LXSM83, M90E were gifted by Xi'an Lanxiao Technology Co., Ltd, Xi an, China. Amylase, cellulase, protease (alkaline, acidic and neutral), pancreatin (porcine and bovine origin) and papain were purchased from Solarbio Co., Ltd, Beijing, China. All reagents used in this study were of analytical grade.

2.2. Compositional analysis of distiller's grains

The content analyses of moisture, protein, oligopeptide and alcohol-soluble polypeptide in the distiller's grains were analyzed by referring to previous literature with minor modifications (Ahmed, Sulieman, & Elhardallou, 2013; Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021; C.-Z. Zhu, Zhang, Zhou, Xu, Kang, & Yin, 2013). In detail, the moisture content of the distiller's grains was calculated after drying at 105 °C to constant weight, and its crude protein content was determined using Kjeldahl analysis. In addition, the distiller's grains sample was uniformly mixed with 15% trichloroacetic acid solution at 1:10 (m/v) and the mixture was extracted at 4 °C for 12 h. The supernatant was collected from the extraction solution after centrifugation at 8000 × g for 10 min, and the oligopeptide content of the distiller's grains was calculated as the difference between total nitrogen (determined by Kjeldahl analysis) and amino acid nitrogen (determined by formaldehyde titration). The alcohol-soluble polypeptide content of the distiller's grains was analyzed using *ortho*-phthaldialdehyde (OPA) method with some modifications (C.-Z. Zhu, Zhang, Zhou, Xu, Kang, & Yin, 2013). Briefly, the alcohol-soluble polypeptide was extracted from the distiller's grains using 50% ethanol solution, and the extraction solution was mixed with OPA solution (40 mg/mL) in methyl alcohol at 1:20 (v/v). After 2 min incubation at room temperature (25 °C), absorbance of the mixture was analyzed using a spectrophotometer (A560, AOE Instruments, Shanghai, China), with wavelength at 340 nm and L-glutathione (GSH, 0–1 mg/mL) as standard.

2.3. Optimization of pre-treatment conditions for distiller's grains

2.3.1. Optimization of physicomechanical pre-treatment

The distiller's grains samples were divided into four experimental groups: wet crushing (WC), wet grinding (WG), dry crushing (DC) and dry grinding (DG) group. The dry distiller's grains was obtained with wet raw under 50℃ for 12 h. For WC and DC group, the wet and dry distiller's grains were pulverized with a crusher (Joyoung, Jinan, Shandong, China) for 2 min. For WG and DG group, the distiller's grains were ground with mortar and pestle for 2 min.Wet distiller's grains were stirred in aqueous solution (1:4, m/v) for 30 min and served as a control (NC). After treatment, treatment samples were mixed with Milli-Q water (1:4, m/v), and all groups passed through a 60-mesh sieve to collect the supernatant.

2.3.2. Optimization of single enzymatic pre-treatment

After the above physicomechanical pre-treatment, the distiller's grain samples were further enzymatically hydrolyzed using amylase and cellulose, firstly. After that, a series of single proteases (alkaline

protease, acid protease, neutral protease, porcine pancreatin, bovine pancreatin and papain protease) were also investigated. Before the hydrolysis process, the system pH was adjusted to neutrality by 0.1 mol/L sodium hydroxide solution. All the enzyme were performed under optimal conditions refer to the manufcture's instructional manual (enzyme-substrate ratio, temperature and pH). The enzymatic hydrolysis process lasted for 3 h in a 50 °C water bath and was terminated by inactivating the enzyme in a boiling water bath within 10 min. The supernatant was collected after centrifugation for 10 min at 8000 × g. After the reaction in all groups, and an equal volume of absolute ethanol was added to precipitate insoluble proteins and polysaccharides. The alcoholic solution was collected after centrifugation, followed by vacuum rotary evaporation at 50 °C to obtain alcohol-soluble polypeptides.

2.3.3. Optimization of double enzymatic pre-treatment

Based on the results of single-enzyme optimization, the optimal single enzymes were screened for pairwise compounding. Appropriate concentrations of two enzymes were simultaneously added to the mixed system of the distiller's grains samples and Milli-Q water (m/v, 1:4) for reaction at pH 7.0 and 50 °C for 3 h. After the reaction, the supernatant was collected by centrifugation, and the optimal single enzyme group was used as a control.

The contents of polypeptides, polyphenols and polysaccharides in the supernatants collected from the above treatment groups were analyzed based on the OPA method, Folin-phenol colorimetric method, and phenol–sulfuric acid method, respectively, and the specific steps were carried out as described by Ahmed, Sulieman, and Elhardallou (2013).

2.4. Optimization of protease hydrolysis conditions for distiller's grains

Based on the optimal dual-enzyme combination and pretreatment conditions, the content of alcohol-soluble polypeptides in the hydrolyzate was monitored at different hydrolysis temperatures (35, 40, 45, 50 and 55 °C), different solid–liquid ratios (1:3, 1:5, 1:7, 1:10 and 1:20) and different hydrolysis times (2, 3, 4, 5 and 6 h), respectively. The optimal enzymatic hydrolysis conditions were determined. In addition, the alcohol extraction parameters for optimization were: extraction times (1, 2 and, 3), extraction temperature (25, 30, 35, 40, 45 and 50 °C) and ethanol concentration (25%, 38%, 45%, 52% and 75%). Different levels of ethanol concentrations were refer to the common commercial *Baijiu* alcohol content, aim to explore the flexibility of the extraction process. The optimal parameters for enzymatic hydrolysis and alcohol extraction were determined based on the alcohol-soluble polypeptides content in the hydrolysate.

2.5. Antioxidative ability test of alcohol-soluble peptides in distiller's grains

Antioxidative ability of distiller's grain peptides was analyzed by determining scavenging activities of three radicals: Hydroxyl radical (•OH), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2′-azino-bis (3-ethylbenzothiazo-line-6-sulfonic acid) diammonium salt (ABTS).

For hydroxyl radical (•OH) scavenging activity, in detail, equal volumes of distiller's grains peptides, $FeSO_4$ solution (3 mmol/L), salicylic acid–ethanol solution (3 mmol/L) and H_2O_2 solution (6 mmol/L) were mixed thoroughly. The mixture was incubated at 37 °C in the dark for 30 min and its absorbance was measured at 510 nm (Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021). Reaction mixture without distiller's grain peptides was used as control, and was calculated as follows:

$$\text{Hydroxyl radical scavenging activity } (\%) = (A_c - A_s)/A_c \times 100$$

where A_c and A_s represented the absorbance of the control and sample, respectively.

For determination of DPPH radical scavenging activity, 2 mL DPPH-ethanol solution (0.1 mmol/L) was mixed with 2 mL working standard solution of each distiller's grains peptides or 2 mL Milli-Q water as control. A mixture of 2 mL of absolute ethanol and 2 mL of Milli-Q water was used as a blank. After incubation for 30 min at room temperature, the absorbance of the mixtures was measured at OD517 nm (Yunsong Jiang, Sun, Yin, Li, Sun, & Zheng, 2020). The antioxidative ability was determined as follows:

$$\text{DPPH radical scavenging activity } (\%) = (A_b + A_c - A_s)/A_b \times 100$$

where A_b, A_c and A_s represented the absorbance of the blank, control and sample, respectively.

For determination of ABTS radical scavenging activity, ABTS stock solution (7 mmol/L) and potassium persulfate (2.45 mmol/L) in phosphate-buffered saline (PBS, pH 7.4) were allowed to stand in the dark for 16 h to generate ABTS radical cations ($ABTS^{\bullet+}$). Before use, the $ABTS^{\bullet+}$ solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm using 10 mmol/L PBS (pH 7.4). Distiller's grain peptides (2 mL) were mixed with 2 mL of the $ABTS^{\bullet+}$ solution and incubated in a water bath at 37 °C for 30 min. The antioxidative ability of the samples was calculated by detecting their absorbance at 734 nm. Milli-Q water (instead of distiller's grains peptides) mixed with ABTS•+ solution was used as control. The calculation formula was as follows:

$$\text{ABTS radical scavenging activity } (\%) = (A_c - A_s)/A_c \times 100$$

where A_c and A_s represented the absorbance of the control and sample, respectively.

2.6. Decolorization process of distiller's grains peptides

2.6.1. Activated carbon decolorization

The distiller's grains hydrolyzate was prepared according to the optimal enzymatic hydrolysis conditions, and then filtered with Waterman 1 filter paper to remove impurities after leaching with 50% ethanol. The distiller's grains hydrolysate was decolorized by adding different proportions of 200-mesh coconut shell activated carbon (0.5%, 1%, 1.5%, 2% and 2.5%) at 30 °C for 5, 10, 15, 20 and 25 min, respectively. In addition to quantitative distiller's grains peptides yield, absorbance (OD380nm, determined by full-wavelength scanning) of the samples were also compared between different treatment groups to characterize the decolorization effect.

2.6.2. Resin decolorization

The distiller's grains hydrolysate was decolorized with resin M90E. After the atmospheric pressure wet column packing was completed, the distiller's grains hydrolysate was loaded into the column at a flow rate of 3 BV/h for decolorization. The decolorization solution was collected every 1 BV to determine the peptide content and absorbance value.

2.7. Resin purification of distiller's grain peptides

2.7.1. Static adsorption parameter optimization

The pre-treatment of XAD16, DA201-C and LXSM83 resin was as follows: Resin was soaked in 95% ethanol for 24 h to fully swell. It was washed with ethanol until the absorption peak at 220 nm was zero, followed by washing with deionized water to neutrality. Next, the resin was soaked in 5% HCl solution for 3 h and washed with deionized water until neutral. It was soaked with 5% NaOH solution for 3 h and washed to neutral.

The enzymatic hydrolysis concentrate (250 mL, peptide concentration 12.18 mg/mL) was mixed with different types of resins (10 g dry weight) in a beaker, and then adsorbed at room temperature for 8 h (interval 30 min sampling to monitor the adsorption effect) by shaking at a rate of 160 times/min. The precipitate was collected by vacuum filtration, then divided into seven portions and mixed with equal volume (30 mL) of ethanol solution (concentrations of 20%, 30%, 40%, 50%,

60%, 70% and 80%, respectively) for desorption. After 8 h of desorption, the content of total phenols, total sugars and total peptides in the eluates were analyzed. The adsorption and desorption rates were calculated using the following fomulas:

$$\text{Adsorption rate} = (C_i - C_a)/C_i \times 100\%.$$

$$\text{Desorption rate} = (C_d)/(C_d - C_a) \times V_e/V_p \times 100\%.$$

where C_i is the protein concentration of the initial solution (mg/mL); C_a is the protein concentration of the adsorption solution (mg/mL); C_d is the protein concentration of the desorption solution (mg/mL); V_p is the volume of peptide solution and V_e is the volume of ethanol.

2.7.2. Dynamic loading parameter optimization

LXSM83 wet resin (10 g) was packed into a column (Φ1.2x18cm) and equilibrated with deionized water to zero absorbance at UV 220 nm. The optimization parameters were as follows: Dynamic loading concentration (40, 30, 20 and 10 mg/mL), flow rate (0.5, 1 and 1.5 BV/h).

2.7.3. Isolation crude peptides by ethanol gradient elution and ultrafiltration

Based on the optimizated resin load parameters, the peptide was isolated at columm (Φ2.6x40cm) by ethanol gradient elution with 0, 20%, 40%, 60% and 80% concentraction. The elutions (ASP-0, ASP-1, ASP-2, ASP-3, ASP-4) was collected and ultrafiltered by cut off 3 kDa (Pall Life Sciences, New York, USA) for antioxidant activities evaluation respectively.

2.8. Separation resin elution fraction ASP-2 by Sephadex G15

The 5 mL ethanol elution active fraction ASP-2 at 20 mg/mL was load to column (Φ1.6x100cm) packed with Sepadex G15. The target peptides was obatined by deionized water elution at a flow rate of 1.0 mL/min under UV detection wavelength 220 nm. Fractions (F1, F2, F3, F4) were collected by peaks and lyophilized for antioxidant activities evaluation.

2.9. Identification and synthesis of alcohol-soluble peptides in distiller's grains

The fraction F3 powder were reduced by 10 mmol/L DL-dithiothreitol (DTT, dissolved in double-distilled water) at 56 °C for 1 h and then alkylated by 50 mmol/L iodoacetamide (IAA) at room temperature in the dark for 40 min. The extracted peptides were lyophilized to near dryness and resuspended in 2–20 μL of 0.1% formic acid. Identification of peptides was performed using an ultra-high performance liquid chromatography with Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (LC-MS/MS, Thermo Fisher Scientific, Waltham, USA) as previously outlined with slight modification (Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021). Briefly, the chromatography was carried out using a reversed-phase C18 nanocolumn (150 μm × 15 cm, 1.9 μm, Dr. Maisch GmbH, Germany) with 5 μL injection volume, 600 nL/min flow rate and 20 °C column temperature. Eluent A was 0.1% formic acid in water, and eluent B was 0.1% formic acid in acetonitrile–water (4:1, v/v). The linear gradient was performed as follows: 0 min, 6% B; 5 min, 9% B; 20 min, 14% B; 50 min, 30% B; 58 min, 40% B; 60 min, 95% B. The MS analyzer was operated at a 2.2 kV spray voltage, 270 °C capillary temperature, a resolution of 60,000 at 400 *m/z* and a precursor range of 400.0–1200.0 *m/z*, and the MS/MS analyzer was set at a product ion scan range of 50.0–1200.0 *m/z* and collision energy of 28 eV. Data acquisition and processing were carried out using Peaks Viewer 4.5 software program (Bioinformatics Solutions; Waterloo, Canada) combined with *de novo* sequencing and candidate peptides were chemically synthesized by GL Biochem Ltd. (Shanghai, China) for antioxidant activity assessment.

2.10. Molecular docking and virtual screening

The potential antioxidant active peptides were screened by online tools Peptide Ranker(https://distilldeep.ucd.ie/PeptideRanker/) and AnOxPePred (for free radical scavenging and chelating ability, https://services.healthtech.dtu.dk/service.php?AnOxPePred-1.0) collaborate with local docking program. The interaction of distiller's grain peptides with Keap1 protein (PDB ID:2FLU, https://www.rcsb.org/structure/2FLU) and human MPO (PDB ID: 3F9P, https://www.rcsb.org/structure/3F9P) was selected for docking calculation by Autodock Vina (Version 1.1.2, Scripps Research Institute, La Jolla, CA, USA), and the three-dimensional structure of selected peptides were generated using ChemBio 3D (Version 18.0, PerkinElmer Informatics, Waltham, MA, USA). The binding sites coordinates of 2FLU and protein 3F9P was x: −2.310, y: 6.232, z: 1.639 and x: −27.919, y: 11.742, z: −28.552, respectively. The cavity radius was set at 30 Å. The biotoxicity and bitterness of peptides were evalutated by ToxinPred(https://crdd.osdd.net/raghava/toxinpred/) and iBitter-Fuse (http://camt.pythonanywhere.com/iBitter-Fuse). Visualization of protein–ligand interactions was performed using Ligplot (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/).

2.11. Statistical analysis

All the analyses were carried out in triplicate and the data were subjected to analysis of variance (ANOVA) and Duncan's test using the Statistical Package for the Social Sciences software (Version 23.0, SPSS Inc., Chicago, IL, USA). The diagrams were drawn using Origin software (Pro 9.1, OriginLab Corp., Northampton, MA, USA). Results were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Pretreatment of Baijiu distiller's grains

Moisture and peptide contents in the *Baijiu* distiller's grains after 358, 415, 520 and 937 days of fermentation, respectively, are shown in Supplementary Table S1. The contents of moisture, protein, oligopeptides and alcohol-soluble peptides in the four samples corresponded to the range of 57.1% − 58.3%, 12.4% − 14.2%, 1.7% − 2.2% and 2.1% − 2.7% (w/w, dry basis, d.b.), respectively, which were consistent with previous distiller's grains composition analysis reports (Garzón, Veras, Brandelli, & Drago, 2022; Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021; Ratanapariyanuch, Shim, Wiens, & Reaney, 2018). Among them, the samples of distiller's grains after 520 days of fermentation contained significantly ($p < 0.05$) higher levels of protein (14.2%), oligopeptides (2.1%) and alcohol-soluble peptides (2.7%). This may related to the balance of distiller's grains protein decomposition and production by microorganisms that use small molecular metabolites (amino acids, monosaccharides and other nutrients) during the long-time fermentation. Thus, it is considered as a good source of functional peptides for resourcing "waste" materials from the brewing industries.

There is also a large amount of lignocellulosic materials in *Baijiu* distiller's grains, such as rice bran, rice husk, etc., implying physical and enzymatic methods are necessary steps to ensure the extraction efficiency of high-value components (Y. Liu, Liu, Huang, Ge, Xi, & Mao, 2022). Four physicomechanical processes (wet crushing, wet grinding, dry crushing and dry grinding) and two enzymatic methods (amylase and cellulase) were used to pretreat the distiller's grains. The contents of total protein, carbohydrates and phenols in the distiller's grains processed by dry and wet crushing were higher than those by dry and wet grinding (Fig. 1a). Crushing had a greater degree of physical damage than grinding, which facilitated the release of functional ingredients from the distiller's grains. Some other physical approaches (i.e. steam explosion, shear emulsifying, ultrasonic and microwave) were also reported to destroy husk structures, which improved the extraction

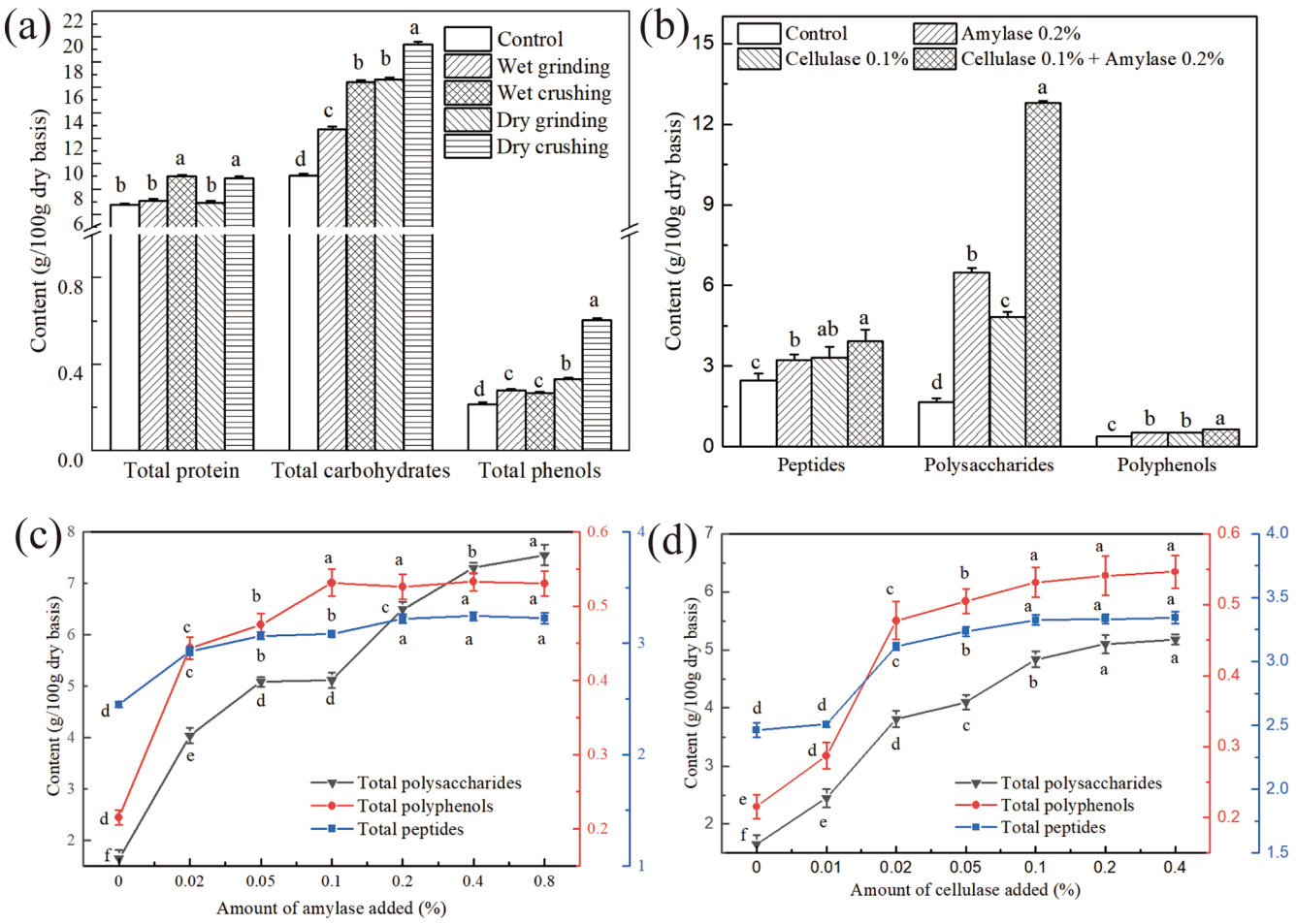

Fig. 1. Optimizing physical (a) and enzymatic (b, c and d) pretreatment of Baijiu distiller's grains. Amylase (c) and cellulase (d) single-enzyme pretreatment effects and their synergistic effects (b). Note, Results are mean ± SD (n = 3), different labeled letters represent significant differences ($P < 0.05$).

efficiency by affecting the surface and inside of the substances and ultimately destroying the matrix or cell walls based on high energy, pressure or heat (Goodman, 2020; Yongli Jiang, Zhou, Zheng, Wang, Deng, & Zhao, 2021). Dry crushing required additional energy to predry the distiller's grains, implying that wet crushing is potentially environmentally friendly, can serve as an easy-to-operate and industrially applicable method and can be chosen as the subsequent machining process to achieve efficient extraction of the target components.

After wet crushing pretreatment, the effect of amylase and cellulase on distiller's grains were shown in Fig. 1c and 1d. As for the treatment with amylase only, total phenols and total peptides reached the highest at the addition of 0.1% and 0.2%, respectively, while total polysaccharides increased with amylase addition over the entire range of 0–0.8%. As for the addition of cellulase between 0 and 0.4%, both total phenols and total peptides reached the highest at 0.1%, while total polysaccharides were at 0.2%. Therefore, amylase and cellulase at 0.2% and 0.1%, respectively, were considered for the treatment of the distiller's grains to obtain functional peptides. Moreover, compared with single enzyme treatment and co-treatment with 0.1% cellulase and 0.2% amylase, the yields of total peptides, total polysaccharides and total phenols of the latter were significantly ($p < 0.05$) higher (Fig. 1b). Due to the complex composition of distiller's grains, dual-enzyme co-processing was favored in many studies because a single enzyme did not provide excellent enzymolysis efficiency for distiller's grain utilization (Yunsong Jiang, Wang, Yin, Sun, Wang, Zhao, et al., 2021; Pan, Liu, Xu, Chen, & Cheng, 2021; Peng, Kong, Wang, Ai-lati, Ji, & Mao, 2021). Overall, these results indicate that the physical processing of wet grinding combined with dual-enzymatic hydrolysis of exogenous complex enzymes can achieve efficient release of functional peptides from distiller's grains due to the better substrate dissolution and enzymolysis efficiency.

3.2. *Enzymatic hydrolysis and alcohol extraction of Baijiu distiller's grains*

To obtain biologically functional peptides, six commercial proteases were further used to hydrolyze crude protein extracted from the distiller's grains under the optimal reaction conditions specified by the manufacturer. The results are shown in Supplementary Table S2. Compared with the water-extracted control group, the addition of all single enzymes increased the hydrolysis of crude protein from the distiller's grains, accompanied by an increase in alcohol-soluble peptides content. Among them, two pancreatins (porcine source and bovine source, respectively) showed strong ability to hydrolyze distiller's grains (hydrolysis degree>22%). In a study about sorghum spent grain antioxidant peptides production, the neutral protease and flavourzyme gives the hydrolysis degree 10.9%(Garzón, Veras, Brandelli, & Drago, 2022). This indicated that convert distiller's grains into functional peptides by enzymatic hydrolysis was one of the effective ways to utilize residual proteins. Proteolytic enzymes break down peptide bonds to produce protein hydrolysates consisting of peptides and amino acids, and the specificity of the enzymes determines the size and the sequence of the peptides, as well as their biological activity (He, et al., 2019). Gliadin is the dominant protein in *Baijiu* distiller's grains, accounting for >40%. It is an alcohol-soluble protein comprising α/β-, γ-, and ω-gliadin, which can be hydrolyzed to a large extent by neutral proteases with low

specificity on the amino acids residues and pancreatin with high activity and purity (He, et al., 2019; Pourmohammadi & Abedi, 2021). Furthermore, considering the flavor-enhancing effect of papain (Y. Li, Yu, Goktepe, & Ahmedna, 2016; You, Zhao, Regenstein, & Ren, 2011), it was preferable to carry out the subsequent optimization with pancreatin (porcine source), neutral protease and papain. The dual-enzyme hydrolysis results indicated that all the "papain + pancreatin", "pancreatin + neutral protease" and "neutral protease + papain" exhibited a higher peptide yield than the single-enzyme hydrolysis (Table S3). The interaction of neutral protease and papain had the highest hydrolysis efficiency among the five combinations, which suggested that it was suitable for digesting protein complexes extracted from the distiller's grains. Both papain and neutral protease are endopeptidases, the former being from cysteine protease preparation and the latter being from *Bacillus subtilis* protease preparation, and both of them had broader specificity than pancreatin (Y. Li, Yu, Goktepe, & Ahmedna, 2016; Pourmohammadi & Abedi, 2021). Besides, there was no significant difference ($p > 0.05$) in peptide content between the one-step simultaneous addition and two-step batch addition of the pretreatment enzyme combination (amylase + cellulase) and proteolytic enzyme combination (papain + neutral protease), but the degree of hydrolysis was slightly higher in the one-step addition (Table S4).

Based on the double-enzyme combination screened above (0.2% amylase, 0.1% cellulose, 0.1% papain, and 0.1% neutral protease under pH 7.0 and 50 ℃), the enzymatic hydrolysis conditions (liquid/solid ratio, hydrolysis temperature and hydrolysis time) and alcohol

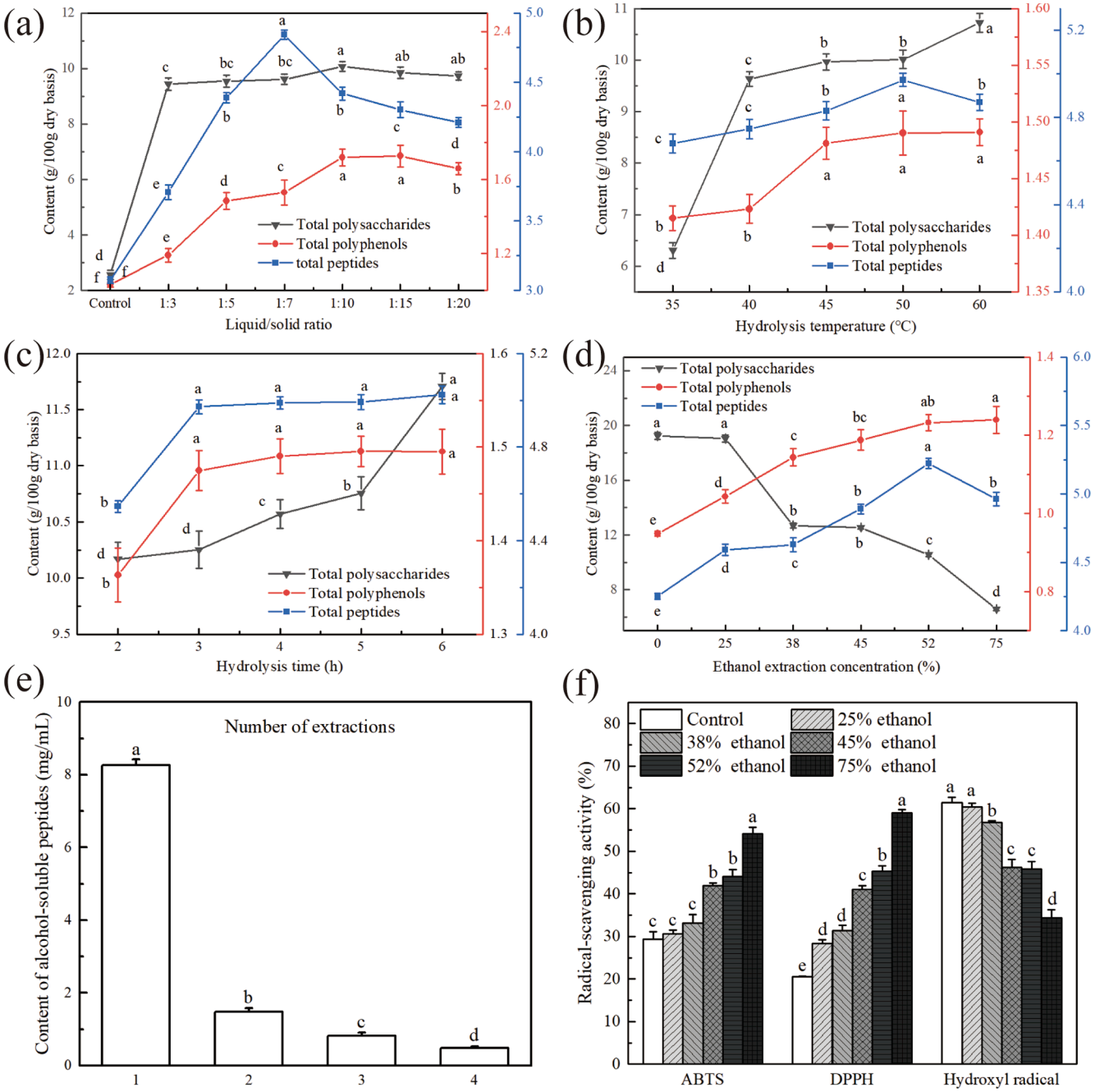

Fig. 2. Optimizing enzymatic hydrolysis and alcohol extraction of Baijiu distiller's grains. Solid-liquid ratio (a), hydrolysis temperature (b), hydrolysis time (c); final ethanol concentration (d), extraction times (e), anti-oxidation of alcohol extracts(f). Note, Results are mean ± SD (n = 3), different labeled letters represent significant differences ($P < 0.05$).

extraction conditions (extraction times and extraction concentration) were further optimized (Fig. 2). When the solid–liquid ratio was 1:7, the yield of peptides was the highest. The polyphenols and polysaccharides increased with solid–liquid ratio, and the interference was greater. Furthermore, polyphenols and polysaccharides increased with solid–liquid ratio which resulted in interference with the separation and purification of peptides (Fig. 2a). As for the enzymatic hydrolysis temperature, the polypeptide yield was highest at 50 °C (Fig. 2b). Subsequently, the distiller's grains were enzymatically hydrolyzed at 50 °C with a solid–liquid ratio of 1:7 for 2–6 h. The results indicated that the yields of polysaccharides and polyphenols increased gradually with the enzymatic hydrolysis time, while the content of peptides reached equilibrium and did not change significantly ($p>0.05$) after enzymatic hydrolysis for 3 h. Therefore, the optimal enzymatic hydrolysis conditions

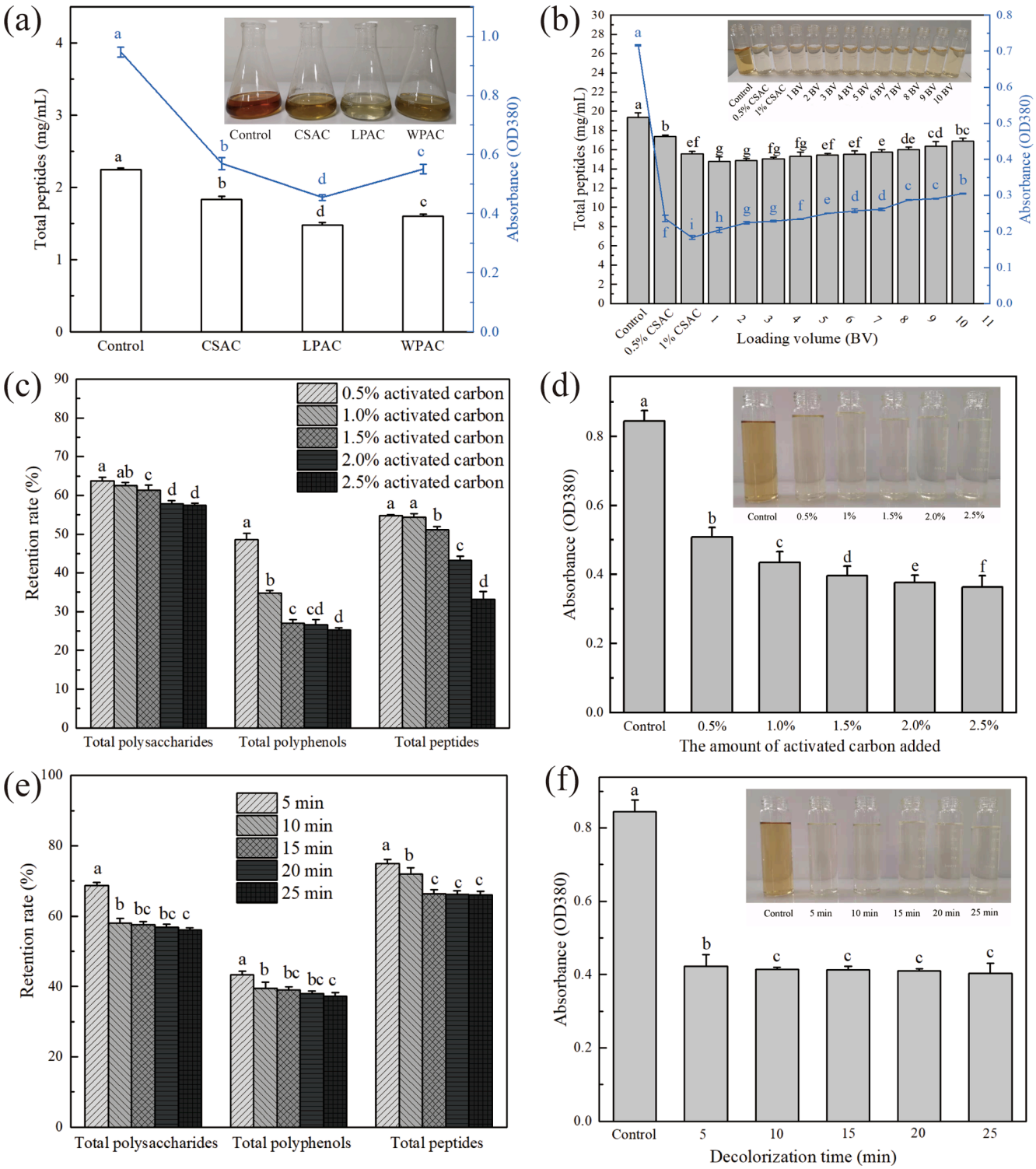

Fig. 3. Optimizing the decolonization of Baijiu distiller's grains. Different types of activated carbon (a), resins with different loading volumes (b); retention rate (c) and absorbance (d) of activated carbon with different additions; retention rate (e) and absorbance (f) of activated carbon with different adsorption time. Note, Results are mean ± SD (n = 3), different labeled letters represent significant differences ($P < 0.05$).

were set at solid–liquid ratio of 1:7 and the enzymatic hydrolysis at 50 °C for 3 h.

After the distiller's grains were treated under the optimal enzymatic hydrolysis conditions, the alcohol-soluble crude peptide solutions were obtained by extraction with different final concentrations of ethanol (Fig. 2d). The peptide content gradually increased with increase in ethanol concentration. However, peptide content decreased when the ethanol concentration was >52% due to competitive dehydration of ethanol to achieve precipitation. Cookman and Glatz (2009) also found that protein extraction with higher concentration of ethanol was not as effective, which may be related to β- and γ-gliadins in distiller's grains being soluble in low-concentration aqueous ethanol but not in high-concentration aqueous ethanol. In addition, the content of alcohol-soluble peptides in the supernatant after enzymatic hydrolysis of distiller's grains decreased with increase in extraction times (Fig. 2d). That is, the more alcohol extraction time, the higher the recovery rate of peptides. Considering energy saving and low cost, a satisfactory recovery rate can be obtained with twice the extraction of the distiller's grains enzymatic hydrolysates. In this present study, >90% of the alcohol-soluble polypeptides were effectively recovered in the residue. Subsequently, the antioxidant activity of the crude peptide solution was further analyzed, and the results showed that the free radical scavenging rate of ABTS and DPPH increased with increase in ethanol extraction concentration, while the hydroxyl radical scavenging rate was the opposite. This may be because the addition of ethanol produced new hydroxyl radicals, resulting in false positive results. It was worth noting that both the 52% and 75% ethanol extraction groups showed good scavenging abilities of ABTS and DPPH free radicals. Considering the peptide content and antioxidant activity, a final concentration of 52% ethanol was extracted twice as the optimal alcohol extraction parameter.

3.3. Decolorization of Baijiu distiller's grains

The decolorization effect of different activated carbon on distiller's grains peptides were compared as shown in Fig. 3 (a). Compared to the control, the distiller's grains peptide solution treated with LPAC had the lightest color, followed by WPAC and CSAC. In addition, the loss of peptides decreased sequentially after the three activated carbon treatments. Specifically, the decolorization effect of LPAC (52.0%) was enhanced by 10.2% compared with WPAC (41.8%) and accompanied by a 15.54% increase in the amount of peptide loss. Overall, CSAC treatment had the highest peptide retention rate (81.7%), as well as an effective decolorization effect (57.1%). Therefore, CSAC treatment was considered for the subsequent decolorization adsorption of distiller's grapeptides.

Resin decolorization also has a variety of applications in the food industry such as the production of sugar and fruit juice beverage (Achaerandio, Güell, & López, 2002). M90E resin with ethanol tolerance was used for decolorization of distiller's grains peptides at different loading volumes, and the comparative analysis with CSAC is shown in Fig. 3. With increase in loading volume during resin treatment, the color of distiller's grains peptide gradually became darker and the content of peptide gradually increased. The decolorization rates were 72.2% and 55.6% and the loss rates of peptides were 23.7% and 12.9%, respectively, at 1BV and 10BV loading volumes. Overall comparison of the treatment effect of activated carbon (total addition: 0.5% and 1.0%) and resin (loading volume: 1BV ~ 10 BV) revealed that both 0.5% and 1.0% activated carbon had better decolorization effect and lower loss of peptides. Therefore, activated carbon was preferred for decolorization in terms of efficiency and cost in this current study. The advantages of resins are that they can be regenerated, thus reducing the amount of solid residues, and they allow the decolorization process to be carried out in a continuous mode (Achaerandio, Güell, & López, 2002) Therefore, resin decolorization can be considered in industrial processes.

The peptide retention and decolorization efficiency of activated carbon with different additions were subsequently investigated, as shown in Fig. 3 (c) and (d), respectively. Absorbance of the solution decreased significantly ($p < 0.05$) with addition of activated carbon, implying that decolorization efficiency of activated carbon depended on the amount of activated carbon added. There was no significant ($p > 0.05$) difference in the adsorption capacity of peptides and polysaccharides with the addition of 0.5% and 1.0% activated carbon, but the latter significantly ($p < 0.05$) reduced the content of polyphenols. Furthermore, the additions of 1.5%, 2.0% and 2.5% resulted in about 34-46% loss of peptides while removing polysaccharides and polyphenols. Therefore, the addition of 1.0% activated carbon had good peptide retention and removal of polyphenols and polysaccharides.

Different decolorization adsorption times were further optimized on the basis of 1% optimal activated carbon addition (Fig. 3e and f). After activated carbon adsorption for>5 min, the absorbance of the solution decreased significantly ($p < 0.05$) from light yellow to colorless. The contents of polysaccharides, polyphenols and peptides in distiller's grains decreased significantly ($p < 0.05$) with the extension of adsorption time in the first 15 min, and tended to be stable after 15 min. In general, the optimal decolorization condition for *Baijiu* distiller's grains was 1.0% activated carbon CSAC for 15 min, which resulted in near colorless decolorization and a peptide retention rate of 66.1%.

3.4. Resin purification of Baijiu distiller's grains

Static adsorption of the decolorized distiller's grains enzymatic hydrolysate was accomplished on three different resins (Fig. 4a), and then desorbed using different concentrations of ethanol (Fig. 4b). The non-polar resin LXSM83 had the highest adsorption rate of peptides in the distiller's grains hydrolysate (69.6%), followed by resin D2A01-C (66.8%) and XAD16 (66.8%). All three resins were able to achieve a desorption rate of about 95% at ethanol concentrations of 60%, 50% and 70%, respectively. Macroporous resin adsorption is mainly based on electrostatic force, hydrogen bond interaction, complexation and size sieving for the separation and purification of biologically active compounds (Xiong, Zhang, Zhang, Shi, Jiang, & Shi, 2014). Resin LXSM83 exhibited strong adsorption and desorption capacity, which may be related to its large surface area, pore structure, unique adsorption properties and surface functional groups, etc (Zhuang, Zhao, Lin, Dong, Chen, Feng, et al., 2016). Thus, it was chosen for the subsequent dynamic adsorption/desorption experiments.

The dynamic loading concentration and flow rate of the decolorized distiller's grains enzymatic hydrolysate were further optimized using resin LXSM83, as shown in Fig. 4c and d, respectively. When the distiller's grains enzymatic hydrolysate were loaded at 10, 20, 30, 40 and 50 mg/mL, the adsorption capacities were 97.5, 139.1, 153.6, 103.0 and 78.5 mg/g resin, respectively. In addition, when the sample loading flow rate was 1, 2 and 3 BV/h, the adsorption capacities were 184.7, 153.6 and 126.2 mg/g resin, respectively. The dynamic adsorption process of macroporous resin was divided into three stages, including 0–30 min (boundary layer diffusion), 30–90 min (gradual adsorption stage, which was the rate-limiting intraparticle diffusion) and 90–180 min (final equilibrium stage) (Zhuang, et al., 2016). Overall, the time required for the resin to reach adsorption equilibrium decreased as the loading concentration and flow rate increased. Considering the adsorption capacity and adsorption equilibrium time, the appropriate loading concentration and flow rate were determined to be 30 mg/mL and 1 BV/h, respectively.

Based on the above-optimized parameters, gradient elution was performed with deionized water and different concentrations of ethanol to separate and obtain one water-soluble and four alcohol-soluble fractions, which are named as ASP-0, ASP-1, ASP-2, ASP-3 and ASP-4, respectively (Fig. 4e). All the peaks were single and symmetric, indicating that peptides derived from Baijiu distiller's grains were separated into five fractions according to different alcohol solubility by resin LXSM83. The eluted peptide proportion of ASP-0, ASP-1, ASP-2, ASP-3

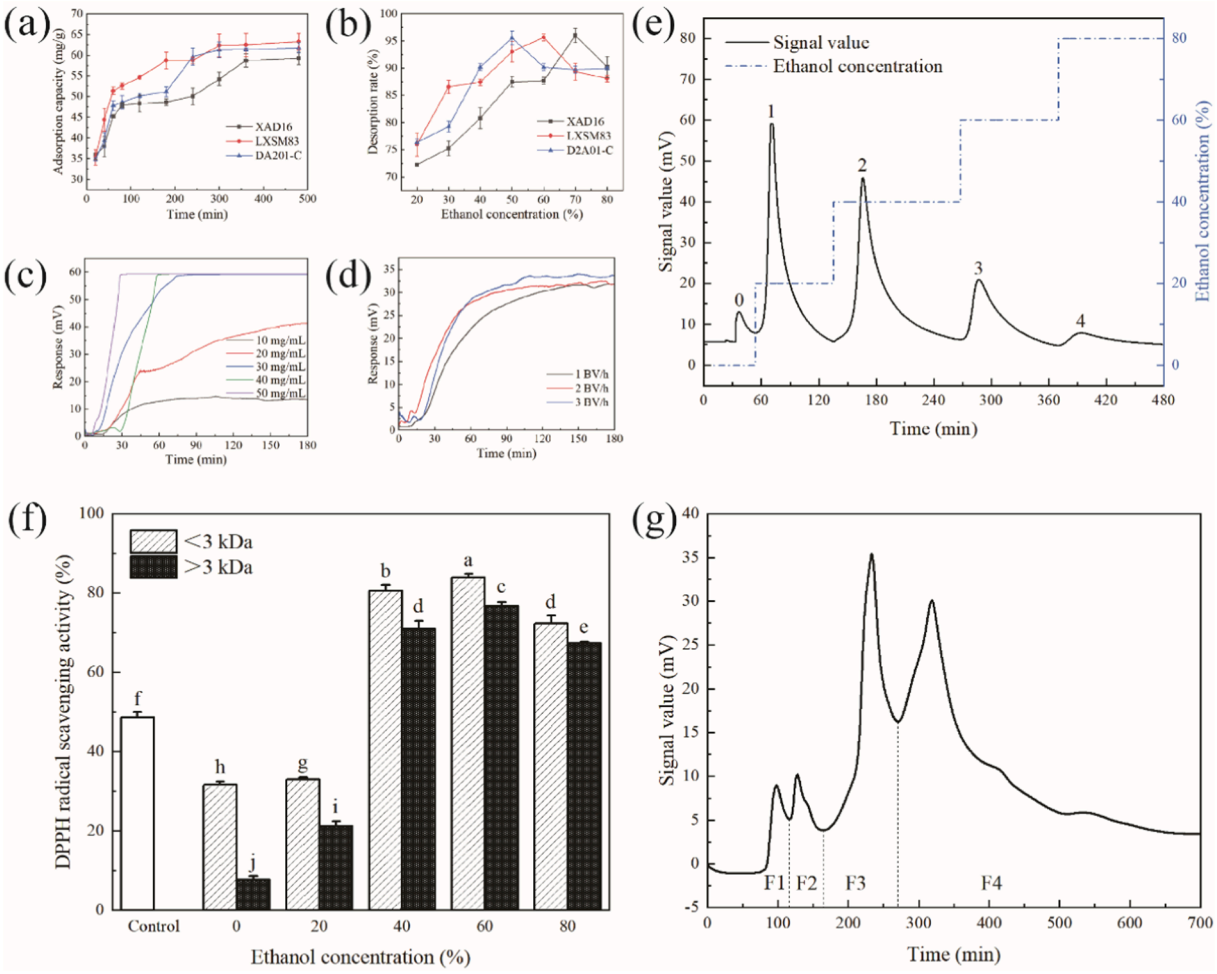

Fig. 4. Isolation and purification of Baijiu distiller's grains. Optimization of static adsorption (a) and desorption (b) of peptides from distiller's grains; Optimization of dynamic loading concentration (c) and flow rate (d) of distiller's grain peptides; ethanol gradient elution isolation of distiller's grain peptides (e); DPPH radical scavenging rate of different fractions eluted with resin (f); G15 gel separation of crude distiller's grains peptides (g). Note, Results are mean ± SD (n = 3), different labeled letters represent significant differences ($P < 0.05$).

and ASP-4 was 45%, 30%, 19%, 3.7%, 0.2%. Molecular weight distributions of the four alcohol-soluble elution peaks were analyzed using GPC (Table S5). All of them were mainly composed of peptides with molecular weight <3 kDa (>98%). Replete studies have revealed that the molecular weight of food-derived antioxidant peptides are mainly concentrated at <3 kDa (Yunsong Jiang, et al., 2021; W.-Y. Liu, Zhang, Miyakawa, Li, Gu, & Tanokura, 2021; C.-Z. Zhu, Zhang, Zhou, Xu, Kang, & Yin, 2013), which may be attributed to the ethanol-soluble crude peptide of distiller's grains containing higher content of potentially functional active peptides. Subsequently, each elution peak was separated by ultrafiltration to obtain two components with molecular weights > 3 kDa and < 3 kDa, and the DPPH radical scavenging rate of each component was investigated as shown in Fig. 4(f). Compared to the control, fractions eluted with 40%, 60% and 80% ethanol exhibited stronger DPPH radical scavenging capacity, while those eluted with deionized water and 20% ethanol exhibited weaker DPPH scavenging. This indicated that antioxidant peptides in distiller's grains had good alcohol solubility, which was consistent with existing literature (Yunsong Jiang, Sun, Yin, Li, Sun, & Zheng, 2020). Due to the high proportion of ASP-2 fraction (eluted with 40% ethanol) and its strong antioxidant activity, it was further separated using gel filtration chromatography, and then four sub-fractions, F1, F2, F3 and F4, were collected respectively, as shown in Fig. 4 (g) and Table S6. The separation results of ASP-2 showed that distiller's grains peptides were mainly concentrated in sub-fractions F3 and F4, and the purity of peptides and DPPH scavenging activity of F3 were higher than those of other sub-fractions. Therefore, it was selected for subsequent analysis and identification.

3.5. *Identification of peptides from Baijiu distiller's grains*

Sub-fraction F3 was loaded in Nano-LC-MS/MS and all identified peptides were composed of 6–17 amino acids residues. Previous studies revealed that functional peptides typically contain 2–20 amino acid residues (He, et al., 2019; Ngoh & Gan, 2016). *Baijiu* distiller's grains peptides ranged in the molecular mass from 798 to 1500 Da, which also demonstrated the effective fractionation to yield peptides with MW < 3 kDa using ultrafiltration. The potentially active peptides sequence with intensity > 2×10^8 were screened by machine learning discrimination tools Peptide Ranker (>0.5) and AnOxPePred (free radical scavenging ability > 0.35, chelating ability > 0.25) firstly. A total of 26 peptides with antioxidant residues were obtained according to the BIOPEP database, and then five candidate peptides were further selected and determined based on docking score (affinity energy) with 2flu and 3f9p protein (Table 1). The calculation results of biotoxicity and bitterness indicated that all other peptide sequences had no potential biotoxicity and bitterness. The activity prediction analysis of the BIOPEP database suggested that the five candidate peptides not only had potential antioxidant activities but also had potential hypoglycemic and hypotensive activities, which was consistent with the results of strong potential

Table 1
Potential alcohol-soluble active peptides (selected based on free radical scavenging ability > 0.35, chelating ability > 0.25, Ranker value > 0.5, Vina score (2flu) < -7 kcal/mol, and Vina score (3f9p) < -7 kcal/mol).

Sequence		Score Intensity ($\times 10^8$)	Scavenging ability	Chelating ability	Ranker value	Vina score (2flu) (kcal/mol)	Vina score (3f9p) (kcal/mol)	Source (Uniprot database)
LAPWAGQP	85	10.20	0.5142	0.2561	0.6427	−8.4	−8.4	Chloroflexi bacterium, *Deltaproteobacteria*
LLPFYPQ	97	3.03	0.5532	0.2919	0.7367	−7.3	−8.4	Sorghum bicolor (Qin, et al., 2020)
LLPFYPQG	85	2.77	0.5614	0.2639	0.7865	−8.9	−7.7	Sorghum bicolor, *Acidobacteria bacterium*
LMFPYPQ	80	2.33	0.5779	0.2768	0.7776	−8.3	−7.8	*Paenibacillus lautus*
AAHVLAAAL	80	2.13	0.3923	0.2555	0.5882	−7.7	−8.7	Not yet identified

biological activities predicted by the Peptide Ranker. The source of five peptide sequences was further traced based on the Uniprot protein library and literature report. Among them, the peptide LLPFYPQ has been reported (Qin, Zhang, Li, Cai, Lu, Gu, et al., 2020). Microbes (LAPWAGQP, LLPFYPQG, LMFPYPQ), sorghum bicolor (LLPFYPQG, LLPFYPQ) were the main peptide sources, and one unreported potentional functionally active peptides (AAHVLAAAFL) were newly discovered in *Baijiu* distiller's grains. Hydrophobic amino acids such as Ala (A), Val (V), Leu (L), Pro (P), Tyr (Y), Met (M), Gln (Q) and Gly (G), and aromatic amino acids such as Phe (F), Tyr (Y), Trp (W) and His (H), were considered to be effective in enhancing the antioxidant activity of peptides (J. Chen, Yan, Zhang, Zheng, Guo, Li, et al., 2021; Gao, Li, Chen, Gu, & Mao, 2021; Zhao, Zhao, & Lu, 2020). The former enhanced the solubility of peptides in the lipid and promoted reactivity with hydrophobic radical species such as DPPH radicals (Juanjuan Yang, Hu, Cai, Chen, Ma, Yang, et al., 2018). The latter was linked to antioxidant capacity by donating an electron through a resonant structure that converted radicals to stable molecules (Q. Liu, Yang, Zhao, & Yang, 2020). Furthermore, the position of amino acid in the peptide sequence had a great influence on its antioxidant activity, such as the presence of Leu (L), Pro (P) and Tyr (Y) at the N-terminus or C-terminus of the peptide has been shown to contribute to its antioxidant activity (Gao, Li, Chen, Gu, & Mao, 2021; He, et al., 2019; Yin, et al., 2022). These features were consistent with the structures of the five candidate peptides identified in this study, thus they were inferred as functional peptides with potential antioxidant activity.

3.6. Evaluation and molecular mechanism of antioxidant peptides from Baijiu distiller's grains

The five candidate peptides were ranked based on their intensity, exclude the reported LLPFYPQ, finally the four LAPWAGQP, LLPFYPQG, AAHVLAAAFL, LMFPYPQ was prepared for antioxidant activity validation with glutathione as controls (Fig. S1). The results showed that all peptides had appropriate DPPH and ABTS free radical scavenging rates (Fig. S1). And the IC50 values (Table S7) shows that the LLPFYPQG, AAHVLAAAFL, LMFPYPQ (Fig. S2) has the strong radical scavenging activity, with 0.87, 0.64, 0.75 mmol/L for DPPH and 0.33, 0.49, 0.65 mmol/L for ABTS scavenging, respectively.

The furthermore explanation of three antioxidant peptides (LLPFYPQG, AAHVLAAAFL, LMFPYPQ) with Nrf2 and 3f9p residues was conducted via molecular docking (Fig. 5). The Keap1/Nrf2 system is considered to be the most important endogenous signaling pathway involved in antioxidant capacity, and myeloperoxidase 3f9p (MPO) is the main enzyme involved in the *in vivo* generation of reactive oxygen species (ROS) (S. Chen, Chen, Du, & Shen, 2020). Therefore, the stability of binding to Nrf2 and 3f9p residues based on molecular docking is widely used to evaluate the antioxidant activity of peptides as well as to illustrate the interaction mechanisms (Deshmukh, Unni, Krishnappa, & Padmanabhan, 2017; Gao, Li, Chen, Gu, & Mao, 2021; Yin, et al., 2022).

Two-dimensional residue interaction analysis of the peptides and Nrf2 showed that the peptide AAHVLAAAFL interacted with Arg415, Ser508, Asn387, Asp389 and Arg380 of 2flu residues to form five hydrogen bonds, and interacted with residues Tyr572, Ala556, Tyr334, Ser602, Tyr525, Phe478, His436, Ile46 and Gly433 to form hydrophobic van der Waals forces. Peptide LLPFYPQG formed five hydrogen bonds with Arg336, Tyr334, Arg380, Gln530 of 2flu residues and formed van der Waals hydrophobic interactions with residues Tyr525, Gly574, Tyr572, Ser555, Ala556, Arg415, Gly364, Asn382 and Phe577. Peptide LMFPYPQ formed six hydrogen bonds with His575, Tyr572, Ser363, Arg380 and Arg483 of 2flu residues and formed van der Waals hydrophobic interactions with residues Gly574, Phe577, Tyr334, Tyr525, Arg415, Phe478, Ile461 and Ala556. In addition, the affinity energy of LLPFYPQG, AAHVLAAAFL and LMFPYPQ was −8.9, −7.7 and −8.3 kcal/mol, respectively, which indicated that all three peptide sequences stably combined with 2flu residues (Table 1). It was known that Nrf2 mainly bound to the cytoskeletal anchoring protein Kelch-like ECH-related proteins in the cytoplasm under normal circumstances, and the elevated levels of ROS and electrophiles led to dissociation and release of Nrf2 and transfer to the nucleus, thereby promoting the expression of cellular antioxidant-related protective genes. Therefore, the inhibitory effect of functional peptides on Nrf2 dissociation was key in exerting antioxidative function (Deshmukh, Unni, Krishnappa, & Padmanabhan, 2017; Yin, et al., 2022).

The active sites of MPO were Asp94, Glu242, Met243, Gln91, His95, Arg239 and His336 (Gao, Li, Chen, Gu, & Mao, 2021). A total of seven hydrogen bonds formed between MPO and peptide AAHVLAAAFL, which contained amino acids Arg323 and Arg31 on the B chain, Arg323, Lys505 and Asp508 on the C chain. Meanwhile, there were hydrophobic interactions with Arg31, Trp32 and Val30 on the A chain, Val30, Trp32 and Pro34 on the B chain, Ile160, Phe439, Asp321 and Arg438 on the C chain, Thr501, Arg504 and Val320 on D chain. This was similar to the results of the antioxidant peptides screened from sea cucumbers, which also acted on Arg31, Trp32 and Pro34 on the B chain and Asp321 and Arg323 on the D chain (Y. Zhang, He, Bonneil, & Simpson, 2020). Furthermore, there were four hydrogen bonds (with Arg31 on B chain, Arg321 on C chain and Lys505 on D chain) and twelve hydrophobic interactions (with Arg31, Val30 on A chain, Val30, Pro34 and Trp32 on B chain, Phe439 and Arg438 on C chain, Cys440, Phe439, Thr501, Arg504 and Val320 on D chain) were observed for peptide LLPFYPQG, four hydrogen bonds (with Arg31 and Trp32 on A chain, Ile160 on C chain, and Arg323 on D chain) and eight hydrophobic interactions (with Ala35, Pro34, Leu33, Val30 on A chain, Val30 on B chain, Asn162, Phe439 on C chain, and Asp321 on D chain) were observed for peptide LMFPYPQ. All three peptides were able to hinder the substrate compounds to be oxidized by docking in the hydrophobic pocket at the crevice entrance to the MPO cavity (Davies, 2010).

4. Conclusion

Functional peptide fractions from Baijiu distiller's grains were prepared and purified by a novel strategy combining enzymatic hydrolysis as well as macroporous resin separation, and validated by radical scavenging analysis and molecular docking. Peptidomics analysis of the

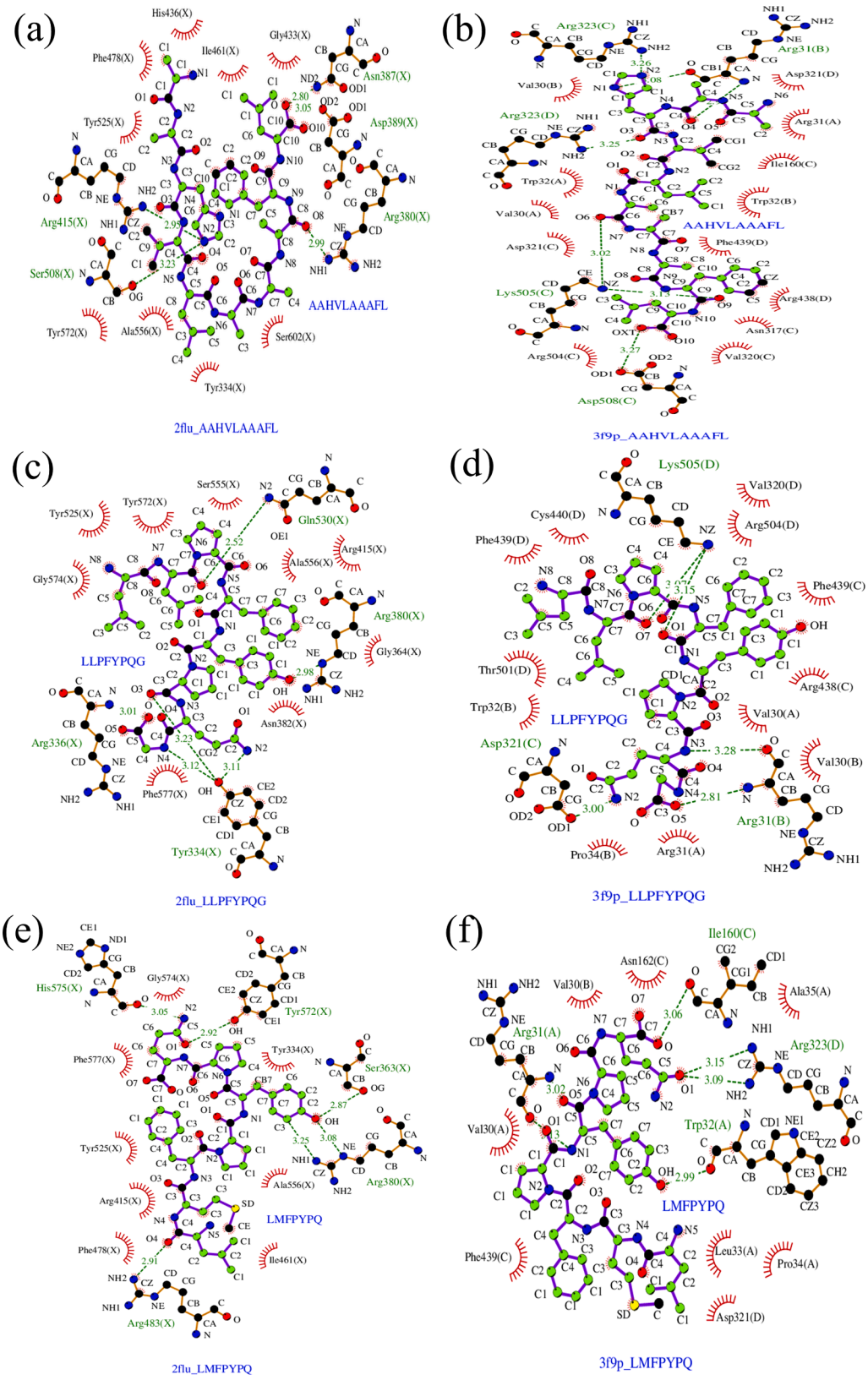

Fig. 5. Molecular interaction of distiller's grain peptides with 2flu and 3f9p residues. Interaction of peptide AAHVLAAAFL with 2flu (a) and 3f9p (b) residues, peptide LLPFYPQG with 2flu (c) and 3f9p (d) residues, and peptide LMFPYPQ with 2flu (e) and 3f9p (f) residues. Note, the green lines represent hydrogen bonding interactions and arc-shaped amino acid residues represent hydrophobic interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

active fraction revealed five alcohol-soluble antioxidant peptides, three of which (peptide sequences LLPFYPQG, AAHVLAAAFL and LMFPYPQ) showed high oxidative activity involving the Keap1/Nrf2 and MPO pathways. This work confirmed that distiller's grains, a typical organic solid by-product of the alcoholic beverage fermentation industry produced in large quantities, can be considered as a potential source of functional peptides for high-value waste resourcing. In the future, it would be beneficial to conduct *in vivo* studies or clinical trials to further evaluate the safety of these peptide-based products prior to commercialization, especially after extensive processing that may change the

natural integrity and quality of the constituent peptides. Nevertheless, this current work not only provides an effective sustainable development approach to reduce environmental pollutants and bioorganic waste, but also a reasonable strategy to explore suitable candidates for functional foods or drugs to treat diseases associated with oxidative stress.

Ethical Statements

I would like to declare on behalf of my co-authors that the work described was original research and it was not been published elsewhere and submitted simultaneously for publication elsewhere. All the authors listed have approved the manuscript that is enclosed.

CRediT authorship contribution statement

Xiaogang Liu: Data curation, Formal analysis, Methodology, Resources, Writing – original draft. **Rui Chang:** Formal analysis, Software, Writing – review & editing. **Zhilei Zhou:** Software, Project administration, Writing – review & editing. **Qingxi Ren:** Software, Formal analysis, Software, Writing – review & editing. **Caihong Sheng:** Resources. **Yu Lan:** Resources. **Xiaonian Cao:** Resources. **Jian Mao:** Conceptualization, Funding acquisition, Project administration, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105722.

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