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# Study on the Extraction of Protein from Aromatic Distillers' Grains and its Activity

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## Abstract

**This paper investigates the extraction of Aromatic Distillers' Grains protein using alcoholic-alkaline, alkaline, and acidic methods, as well as the preparation of distillers' grains peptides through enzymatic hydrolysis. It measures the antioxidant activity before and after hydrolysis, along with the activity of alcohol dehydrogenase. The results demonstrate that the alcoholic-alkaline method yields a higher protein extraction rate compared to the other two methods, optimizing the extraction ratio to (16.44 ± 0.24)%. At a concentration of 1.0 mg/mL, the DPPH radical scavenging rate achieved (88.94 ± 3.36)%, while at 0.4 mg/mL, the ABTS radical scavenging rate achieved (86.92 ± 2.41)%. Utilizing alkaline protease, neutral protease, recombinant protease, and papain for the enzymatic hydrolysis of distillers' grains protein to prepare peptides increase both DPPH and ABTS radical scavenging rates, with alkaline protease demonstrating the most significant effect; the DPPH radical scavenging rate increased from (69.87 ± 0.78)% to (88.97 ± 0.60)%, and the ABTS radical scavenging rate rose from (72.64 ± 3.36)% to (93.82 ± 1.37)%. Furthermore, the alcohol dehydrogenase (ADH) activity of the distillers' grains protein was assessed, revealing that as protein concentration increased, ADH activity also enhanced. Following hydrolysis with the four enzymes, alkaline protease exhibited the best performance, elevating the activity from (2.48 ± 0.307) U/mg prot to (3.32 ± 0.301) U/mg prot. These findings suggest that distillers' grains protein and peptides possess considerable antioxidant properties, providing a valuable reference for subsequent research on the functional attributes of distillers' grains protein.**

## Keywords

**Aromatic Distillers' Grains; Protein; Peptides; Antioxidant Activity.**

## 1. Introduction

Distillers' Grains are primary byproduct in the production of liquor, generating substantial quantities annually during the distillation process. This residue primarily consists of fermented grains and rice husks, rich in nutrients such as starch, protein, and fats[1-3]. Additionally, it harbours a diverse array of microorganisms; if not managed promptly, it is susceptible to decay and mould. Historically, Distillers' Grains have often undergone rudimentary drying processes before being repurposed as either fertilizer or animal feed, leading to resource wastage and significant environmental pollution. Therefore, the reclamation of resources, harmless treatment, and reduction of Distillers' Grains are not only essential for the green manufacturing and sustainable development of liquor but also hold considerable significance in enhancing the economic value of Distillers' Grains and protecting the environment[4,5]. In recent years, research on the resource utilization of Distillers' Grains has yielded

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fruitful results. Distillers' Grains is abundant in phenolic compounds, such as phenolic acids and flavonoids, which are associated with antioxidant properties and are considered potential sources of antioxidant active substances. Furthermore, it can be directly incinerated or used as a raw material for fermentation to produce biogas energy[6], and also serve as a raw material for the production of fuel ethanol and can also serve as a raw material for the production of fuel ethanol and be gasified to generate gas. Additionally, when incorporated into wheat-based products[7], its high fibre content contributes to enhancing immunity, including improving digestion and preventing certain gastrointestinal disorders[8].

The strong-flavoured baijiu is one of the principal varieties of baijiu produced in China, and it has become the most commercially successful aromatic type due to its distinctive flavour and broad appeal. Each year, the annual production of the byproduct of strong-flavoured baijiu-spent grain-can reach approximately 30,000 tons. The primary raw material for strong-flavoured baijiu spent grain is sorghum. However, due to the limitations and inefficiencies of traditional distillation techniques and solid-state fermentation processes, a significant amount of proteins and fibres remains within the spent grain. These components not only provide a solid foundation for the resource utilization of spent grain but also create opportunities for its application in the realms of food, animal feed, and other sectors of the bio-economy[9-13]. Consequently, the byproduct distillers' grains still contain a wealth of nutritional components, including polyphenols, polysaccharides, saponins, and proteins. Sorghum comprises approximately 11% protein by mass, which can be categorized based on its solubility under varying conditions into the following types: albumin (water-soluble), globulin (salt-soluble), alcohol-soluble protein, cross-linked alcohol-soluble protein (soluble in water, alcohol, and reducing agents), and gluten (soluble in detergents, reducing agents, and alkaline environments)[14]. The content of alcohol-soluble protein in sorghum protein is the highest, ranging from 37.5% to 72.9%, with an average of 49.3%. This is followed by gluten, which has a mass fraction of 33.4%, while globulin and albumin account for 7.0% and 5.6%, respectively. These proteins exhibit various biological activities, including antioxidant, anti-obesity, anti-diabetic, cardioprotective, and anti-cancer properties[15,16]. Therefore, extracting the residual proteins from the distillers' grains of strong-flavored liquor can enhance resource utilization and minimize waste.

Currently, the primary methods for extracting protein from distillers' grains include alcohol-alkali extraction, alkaline extraction, and enzymatic extraction[17-20]. The alcohol-alkali method and alkaline extraction primarily utilize the principle of acid-base precipitation, where the distillers' grains protein is dissolved in a dilute alkaline solution. The pH of the solution is then lowered to the isoelectric point of the protein, causing the distillers' grains protein to precipitate out. In contrast, the enzymatic method employs enzymes to disrupt the cell wall structure, thereby increasing the solubility of the protein in the extraction solvent and enhancing the yield of crude protein[21-23]. Some researchers have extracted proteins from fermented coffee residues [24] and then digested them using pepsin and trypsin to obtain hydrolyzed peptides. This experiment demonstrates that distillers' grains also contain a certain amount of peptides. Moreover, some researchers have found that peptide compounds possess certain antioxidant properties and can also treat inflammation caused by alcohol. For example, phenolic acids extracted from beer spent grains exhibit good antioxidant activity and can effectively manage hypertension[25]. Additionally, studies on distillation waste grains have shown that fermented grain proteins can inhibit the activity of angiotensin-converting enzyme (ACE), as well as glucose and amylase. However, there is relatively little research focused on the extraction and activity of proteins from distillers' grains of strong-flavored liquor [26-31].

This study is based on single-factor experiments and selects the alcohol-alkali method, subtraction method, and acid method, using protein yield as the indicator to determine the optimal extraction method and solid-to-liquid ratio for proteins from strong-flavored liquor distillers' grains. Additionally, it compares the changes in antioxidant activity and alcohol dehydrogenase activity before and after the proteolysis of the distillers' grains protein, aiming to recover protein from the distillers' grains. The development of this method not only helps improve the extraction efficiency of

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proteins from strong-flavored liquor distillers' grains but may also provide new insights for the resource utilization of these by-products.

## 2. Materials and Methods

### 2.1 Materials and Chemicals

Fragrant white liquor distillers' grains, Shenjiu Group Co., Ltd.; Alkaline protease, neutral protease, compound protease, papain, bovine serum albumin, Beijing Solabio Technology Co., Ltd.; L-ascorbic acid USP Grade, total antioxidant capacity assay kit (ABTS method), ethanol dehydrogenase activity assay kit, Coomassie Brilliant Blue G-250, Amicon(R) Ultra-15 Centrifugal Filter, 15 ml sample volume, 10 kDa MWCO, Shengong Biochemical Engineering Co., Ltd.; DPPH, Shanghai Ruji Biotechnology Development Co., Ltd. All chemicals were of analytical grade.

### 2.2 Instruments and Equipment

Full-wavelength microplate reader ReadMax1200, Shanghai Shinepo Bio-Technology Co., Ltd.; ESJ110-413 electronic analytical balance, Shenyang Longteng Electronics Co., Ltd.; 101-3AB electric blast drying oven, Tianjin Tester Instruments Co., Ltd.; vacuum freeze dryer FD-1C-50, Chengdu Yiheng Technology Co., Ltd.; medical centrifuge JW-3021HR, Anhui Jiawen Instrument Equipment Co., Ltd.; intelligent pH meter PHS-3C, Chengdu Century Ark Technology Co., Ltd.; constant temperature water bath HH-3K3W, Jiangsu Jiamei Instrument Manufacturing Co., Ltd.

### 2.3 The Extraction Process of Distiller's Grains Protein

#### 2.3.1 Methods for the Extraction of Distiller's Grains Protein.

Place the fresh distiller's grains into an oven to evaporate moisture, then crush and pass through a 20-mesh sieve for later use[20].

(1)Alcohol-Alkali Method: Take a specified amount of fresh distiller's grains powder and add it to an alcohol-alkali mixture [V (95% ethanol): V (0.5 mol/L NaOH) = 1:2]. Combine the distiller's grains powder and the alcohol-alkali mixture in a ratio of 1:30 (g/mL) and stir for 240 minutes. After centrifugation, collect the supernatant and adjust the pH of the filtrate to 4.7 using 1 mol/L hydrochloric acid. Allow it to stand for precipitation, then centrifuge again to obtain a crude protein precipitate. Adjust the precipitate with water to neutrality, collect the filtrate, and centrifuge to yield another crude protein precipitate, which is then freeze-dried.

(2)Alkali Method: Take a specified amount of fresh distiller's grains powder and add it to 0.5 mol/L NaOH, maintaining a solid-to-liquid ratio of 1:30 (g/mL). Stir for 240 minutes. After centrifugation, collect the supernatant and adjust the pH of the filtrate to 5.2 using 1 mol/L hydrochloric acid. Allow it to stand for precipitation, then centrifuge to obtain a crude protein precipitate. Adjust the precipitate with water to neutrality, collect the filtrate, and centrifuge to yield another crude protein precipitate, which is then freeze-dried.

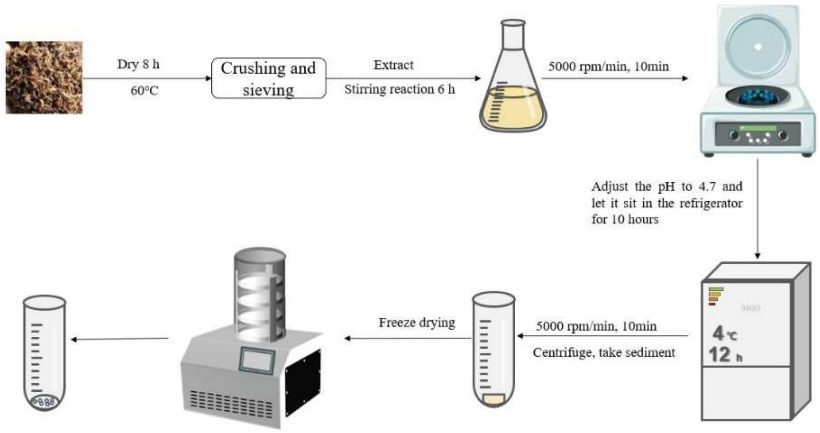


**Figure 1.** Protein extraction flowchart

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(3)Acid Method: Take a specified amount of fresh distiller's grains powder and mix it with 70% anhydrous ethanol in a solid-to-liquid ratio of 1:10 (g/mL). Add hydrochloric acid to adjust the pH to 2.0 and include 0.25% sodium bisulfite according to the volume ratio, stirring for 240 minutes. After centrifugation, collect the supernatant and concentrate it under vacuum. Allow it to stand for precipitation, then centrifuge to obtain a crude protein precipitate. Wash the precipitate with water, collect the filtrate, and centrifuge to yield the protein precipitate, which is then freeze-dried. The extraction process is illustrated in Figure 1.

#### 2.3.2 Determination of Protein Content

Following the method established by Jiang Huiping[32], the protein content is determined using a modified Coomassie Brilliant Blue assay. The modified Coomassie Brilliant Blue solution is prepared, and a standard curve is generated using bovine serum albumin at different concentrations as a reference. A quantity of 10 mg of the distiller's grains protein powder is dissolved in 10 mL of PBS buffer, resulting in a protein solution of 1 mg/mL. This solution is then mixed thoroughly with the Coomassie Brilliant Blue working solution, allowed to stand for 2 minutes, and the absorbance is measured at 595 nm. PBS buffer is used as a blank reference in place of the protein solution to calculate the protein content in the solution.

### 2.4 Fourier Transform Infrared (FTIR) Analysis

Conduct infrared characterization of the distiller's grains protein to observe its characteristic functional groups.

### 2.5 Preparation of Distiller's Grains Peptides

Removal of Impurities by Water Washing Before Enzymatic Hydrolysis: Take 1.0 g of the distiller's grains protein powder and add water at a ratio of 1:6 (g/mL). After maintaining a constant temperature in a water bath at 60°C and oscillating for 30 minutes, centrifuge the mixture.

Enzymatic Hydrolysis: Take 1 g of the distiller's grains protein and add 30 mL of distilled water. Adjust the pH using a 1 mol/L NaOH solution, then add an appropriate amount of enzyme. Hydrolyze for 240 minutes, followed by inactivation in a water bath at 90°C for 15 minutes. Centrifuge at 4000 r/min for 15 minutes, collect the supernatant, and filter using a 10 kDa ultrafiltration membrane to obtain the peptide solution. Subsequently, determine the protein concentration. The optimum pH and temperature for different proteases are shown in Table 1.

**Table 1.** The optimal pH values and temperatures for various proteases.

| Type | pH | Temperature |
|---|---|---|
| Alkaline protease | 9.0~11.0 | 45~60 |
| Neutral protease | 6.8~7.0 | 45~50 |
| Papain | 6.0~7.0 | 55~65 |
| Composite protease | 6.0~7.0 | 45~55 |

### 2.6 In Vitro Activity Assay

#### 2.6.1 DPPH Radical Scavenging Activity

Accurately weigh DPPH radical powder and dissolve it in anhydrous ethanol to prepare a working solution of DPPH radical at a concentration of 0.3 mmol/L. Prepare a series of different concentrations of distiller's grain protein solutions ranging from 0.1 to 2.0 mg/mL, using varying concentrations of vitamin C solution as a control. Add the DPPH radical working solution, allow it to stand for 30 minutes, and then measure the absorbance at 517 nm using a microplate reader. The DPPH scavenging rate can be calculated using the following formula[17]:

DPPH Scavenging Rate (%) = $(1 - (A_1 - A_2) / A_0) \times 100\%$

Where:

$A_0$: Absorbance value of the control group (100 μL anhydrous ethanol + 100 μL DPPH solution);

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$A_1$: Absorbance value of the sample group (100 μL sample solution + 100 μL DPPH solution);

$A_2$: Absorbance value of the blank group (100 μL sample solution + 100 μL anhydrous ethanol).

#### 2.6.2 Determination of ABTS Scavenging Activity

Measurement according to the operational guidelines of the kit.

#### 2.6.3 Determination of Ethanol Dehydrogenase Activity

Measurement according to the operational guidelines of the kit.

## 3. Results

### 3.1 Results of Single-factor Experiments on the Extraction of Protein from Distiller's Grains

Using bovine serum albumin as the standard, a linear equation was derived: $y = 0.073x + 0.021$, with $R^2 = 0.996$, indicating a strong linear relationship. The protein content was calculated based on the standard curve. As illustrated in Figure 1. A, the yield of protein extracted from the distiller's grains using the alcohol-alkali method surpassed that of both the alkaline and acid methods, with the alcohol-alkali method yielding approximately twice the amount obtained through the acid method. These results are generally consistent with those of Hou Mengyuan et al[22]., although slight discrepancies exist. The primary reason for this variation is likely the substantial addition of strong acid solutions during the acid extraction process, which leads to protein hydrolysis and consequently reduces the protein yield.

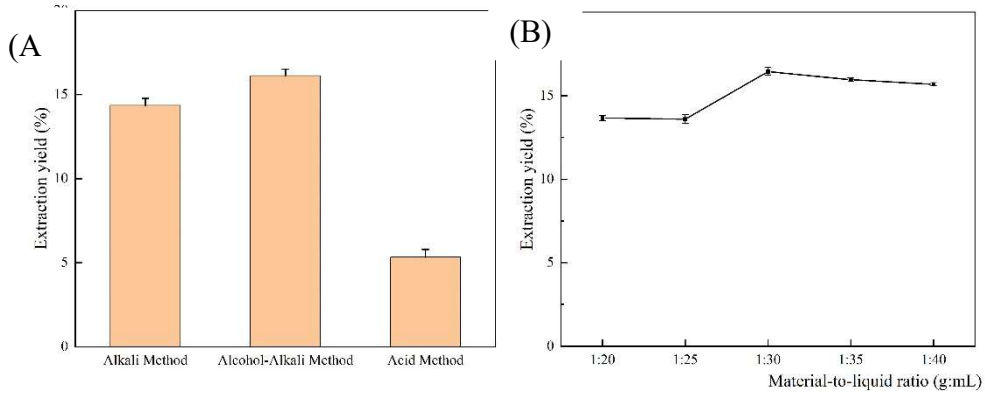


**Figure 2.** Results of single-factor experiments on protein extraction; (A)The effect of extraction methods on protein yield; (B)The effect of material-to-liquid ratio on protein yield.

As illustrated in Figure 2. B, when the solid-to-liquid ratio ranges from 1:20 to 1:40 (g/mL), the extraction of protein from the distiller's grains increases with the volume of the alcohol-alkali solution. The maximum protein extraction rate of 17.93 mg/g is achieved at a solid-to-liquid ratio of 1:30 (g/mL). Beyond a ratio of 1:35 (g/mL), the protein from the distiller's grains is sufficiently solubilized, and the extraction yield does not increase further. Therefore, it can be preliminarily concluded that the optimal solid-to-liquid ratio for extracting protein from the distiller's grains is 1:30 (g/mL).

### 3.2 Fourier Transform Infrared (FTIR) Analysis

As shown in Figure 3, there is a strong absorption peak at 3400 $cm^{-1}$, which corresponds to the O-H stretching vibration. A weak absorption peak at 2927 $cm^{-1}$ is attributed to C-H stretching. The absorption peak at 1656.57 $cm^{-1}$ corresponds to C=O, and the peak at 1542 $cm^{-1}$ is associated with the stretching vibrations of peptide bonds and N-H bonds in amino groups in the second harmonic region. Therefore, it can be inferred that the sample contains functional groups such as N-H bonds and peptide bonds, which are consistent with the main functional groups found in proteins.

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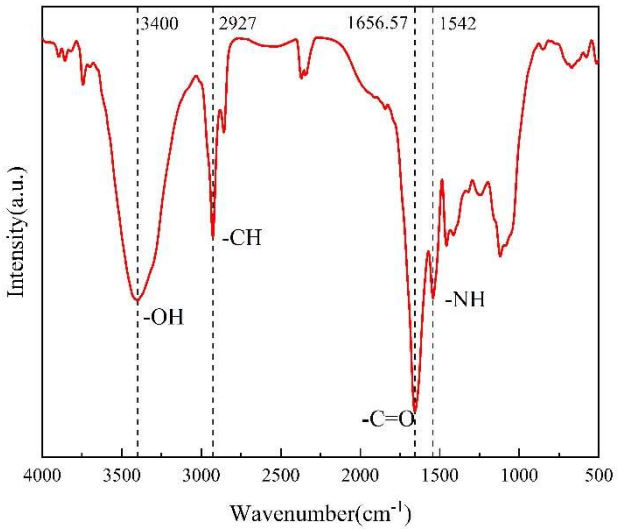


**Figure 3.** FTIR spectra.

## 3.3 Results of in Vitro Activity Assessment

### 3.3.1 The Efficacy of DPPH Free Radical Scavenging Rate

As illustrated in Figure 4, the protein derived from sake distillers' grains demonstrates a commendable efficacy in scavenging DPPH free radicals. Furthermore, the scavenging effect of the sake distillers' grains protein on DPPH free radicals intensifies progressively with increasing concentration, stabilizing at a protein concentration of 1.0 mg/mL, where the scavenging effect reaches (88.94 ± 3.36)%. This indicates a reduction in the number of unpaired electrons present in the DPPH free radicals, resulting in a decreased absorbance at 517 nm. It is hypothesized that the sake distillers' grains protein may capture the single electrons within the DPPH free radicals; however, the precise mechanism underlying the scavenging action of the sake distillers' grains protein on DPPH free radicals warrants further investigation.

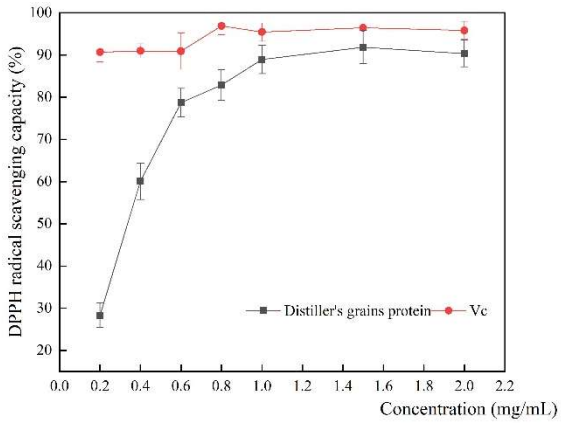


**Figure 4.** The scavenging effects of distiller's grains protein at varying concentrations on DPPH free radicals

### 3.3.2 The Efficacy of ABTS Free Radical Scavenging Rate

As depicted in Figure 5, the protein extracted from sake distillers' grains exhibits a notable capacity to scavenge ABTS free radicals. At a protein concentration of 0.4 mg/mL, the scavenging effect on ABTS free radicals reaches (86.92 ± 2.41)%, indicating that the sake distillers' grains protein can react with the oxidized ABTS+ ions, leading to a decolorization effect that consequently lowers the absorbance at 734 nm. The scavenging rate increases with the elevation of the sake distillers' grain protein concentration.

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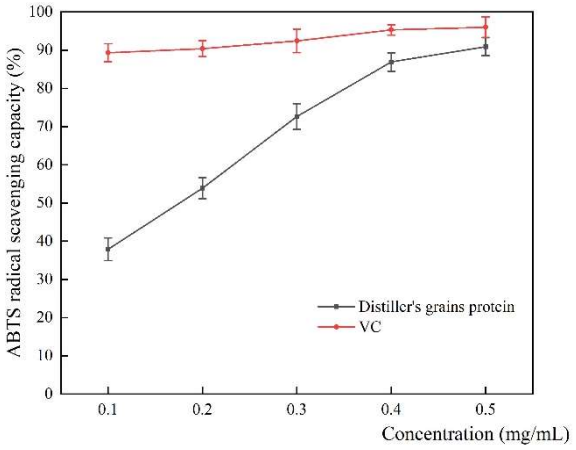


**Figure 5.** The scavenging effects of distiller's grains protein at varying concentrations on ABTS free radicals

### 3.3.3 The Impact of Enzymatic Hydrolysis on the DPPH Free Radical Scavenging Rate

By employing alkaline protease, neutral protease, papain, and composite protease to enzymatically hydrolyze the sake distillers' grains protein, and subsequently determining the concentration of peptides to 0.5 mg/mL using an improved Bradford assay, the variations in DPPH free radical scavenging activity were observed. According to the results presented in Figure 6, the enzymatically treated sake distillers' grains protein exhibited a marked enhancement in its capacity to scavenge DPPH free radicals. Notably, the optimization achieved with alkaline protease was the most effective, with the DPPH free radical scavenging rate increasing from (69.87 ± 0.78)% to (88.97 ± 0.60)%. The effects of composite protease and papain were found to be comparable, while the improvement conferred by neutral protease was rather negligible.

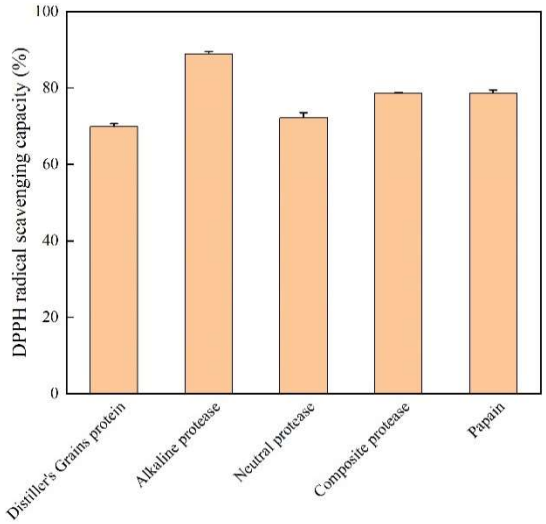


**Figure 6.** The scavenging effects of DPPH free radicals following protein hydrolysis

### 3.3.4 The Impact of Enzymatic Hydrolysis on the ABTS Free Radical Scavenging Rate

In this study, alkaline protease, neutral protease, papain, and composite protease were employed to enzymatically hydrolyze the protein from distillers' grains. The concentration of the resulting peptides was quantified using a modified Bradford assay, achieving a concentration of 0.5 mg/mL, followed by an assessment of the variations in ABTS radical scavenging activity. The results, as illustrated in Figure 7, indicate a significant enhancement in ABTS radical scavenging activity post-hydrolysis. Notably, alkaline protease exhibited the most pronounced optimization effect, with the ABTS radical scavenging rate increasing from (72.64 ± 3.36%) to (93.82 ± 1.37%), while the improvements observed with the other three proteases were relatively comparable.

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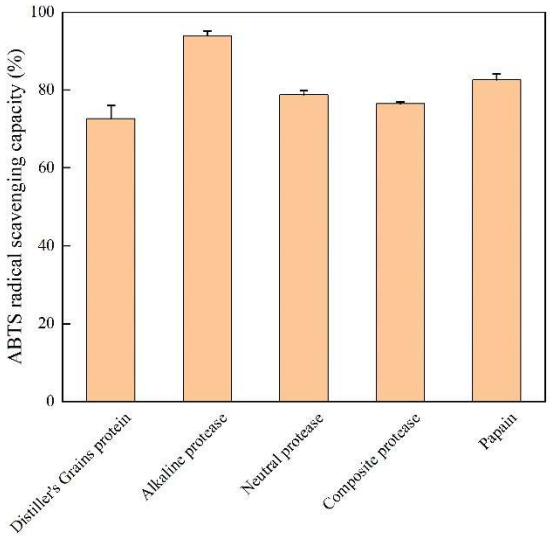


**Figure 7.** The scavenging effects of ABTS free radicals following protein hydrolysis

### 3.3.5 The Effect of Enzymatic Hydrolysis on ADH Enzyme Activity before and after Treatment

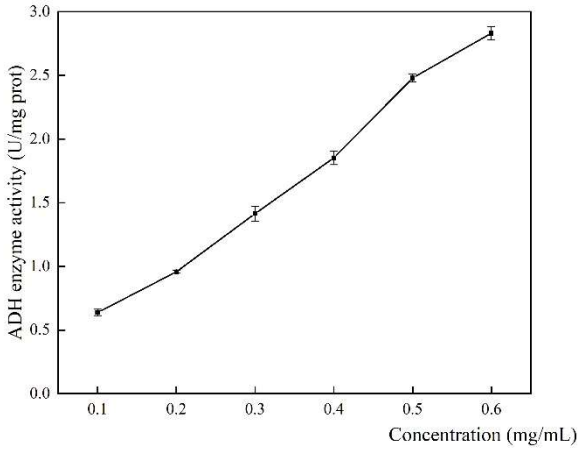


**Figure 8.** The ADH enzyme activity of distiller's grains protein at different concentrations

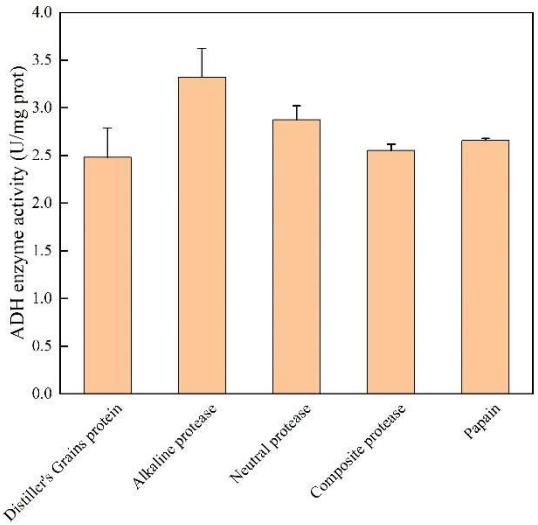


**Figure 9.** The variations in ADH enzyme activity following protein hydrolysis

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As shown in Figure 8, sake distillers' grains protein exhibits a robust ADH activity, which strongly indicates that it contributes to the stable production of $NAD^+$, demonstrating commendable ethanol dehydrogenase activity. The higher the concentration, the more pronounced the effect, suggesting its potential as a hangover remedy. Furthermore, Figure 9 illustrates that the effects of alkaline protease are superior to those of other types of hydrolytic enzymes. This superiority may be attributed to the higher degree of hydrolysis of the products resulting from this particular protease, which contains a greater abundance of low molecular weight peptides.

## 4. Discussion

This experiment compared the effects of three methods-alcohol-alkaline, acid, and alkaline-on the extraction of protein from aromatic liquor distillers' grains. The results indicated that the alcohol-alkaline method yielded the most favourable extraction results, consistent with the findings of Yang Tingting and others. Subsequently, the extraction ratio of the alcohol-alkaline method was optimized, revealing that a ratio of 1:30 (g/mL) resulted in the highest extraction yield, which differed somewhat from the results obtained by Hou Mengyuan[20-22]. Furthermore, the DPPH and ABTS radical scavenging activities of the liquor distillers' grains protein were assessed, demonstrating that as the concentration increased, its antioxidant activity was also enhanced. At a concentration of 1.0 mg/mL, the DPPH radical scavenging rate reached (88.94 ± 3.36)%, while at 0.4 mg/mL, the ABTS radical scavenging rate was (86.92 ± 2.41)%, indicating that the liquor distillers' grains protein possesses significant antioxidant activity[28-33].

Subsequently, alkaline protease, neutral protease, composite protease, and papain were employed to hydrolyze the liquor distillers' grains protein and the variations in DPPH and ABTS radical scavenging rates before enzyme hydrolysis were measured. The results revealed that the antioxidant activity of the resulting liquor distillers' grains peptides increased following proteolytic hydrolysis, consistent with the findings of Hou Mengyuan and others[14], with alkaline protease demonstrating the most significant enhancement effect. Experimental results indicated that the liquor distillers' grains protein exhibits ethanol dehydrogenase activity. This study found that the extracted protein from aromatic liquor distillers' grains also possesses ethanol dehydrogenase activity, which intensifies with increasing protein concentration. Furthermore, the formation of smaller peptides through hydrolysis further enhances this activity. Additionally, the liquor distillers' grains peptides extracted by Wei Dong and colleagues showed ACE inhibitory properties and the ability to lower blood pressure[25]. The peptides prepared in this study await further in-depth investigations into their bioactive properties[34-36].

## 5. Summary

The experimental results indicate that the alcohol-alkaline method yields a higher extraction rate of liquor distillers' grains protein compared to the other two methods. Through single-factor experiments, the optimal extraction ratio for the alcohol-alkaline method was determined to be 1:30 (g/mL). Furthermore, infrared spectroscopy characterization revealed that the extracted liquor distillers' grains protein exhibited characteristic peaks corresponding to specific protein functional groups. Consequently, different extraction methods influence protein yield, with the alcohol-alkaline method being more suitable for the extraction of liquor distillers' grains protein, warranting further investigation into its bioactivity. The findings demonstrate that the liquor distillers' grains protein possesses significant antioxidant capacity, which increases with concentration; at 1.0 mg/mL, the DPPH radical scavenging rate reached (88.94 ± 3.36)%, and at 0.4 mg/mL, the ABTS radical scavenging rate achieved (86.92 ± 2.41)%.Utilizing alkaline protease, neutral protease, composite protease, and papain to hydrolyze the liquor distillers' grains protein for peptide preparation, the protein concentration was subsequently diluted to 0.5 mg/mL for measuring changes in DPPH radical scavenging rate and total antioxidant capacity. The results indicated that both the DPPH radical scavenging rate and total antioxidant capacity increased post-hydrolysis, with alkaline protease demonstrating the most pronounced effect; the DPPH radical scavenging rate rose from (69.87 ±

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0.78)% to (88.97 ± 0.60)%, while the ABTS radical scavenging rate increased from (72.64 ± 3.36)% to (93.82 ± 1.37)%. Additionally, the ADH enzyme activity of the liquor distillers' grains protein was assessed, revealing that as protein concentration increased, ADH activity was also enhanced. After hydrolysis by the four enzymes, alkaline protease exhibited the most significant effect, increasing from (2.48 ± 0.307) U/mg protein to (3.32 ± 0.301) U/mg protein, while the enhancement effects of the other three enzymes were not as pronounced. This provides a theoretical reference for the subsequent utilization of aromatic liquor distillers' grains.

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