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Optimization of Extraction Protocols for Seed Storage Proteins in Rice

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Abstract

Seed storage proteins are the principal source of proteins concerning human nutrition. Rice grains are one of the important sources of carbohydrates, proteins and minerals. Thus, the extraction and identification of proteins present in rice seeds are basic step towards the characterization of seed storage proteins. Three methods *viz*., phosphate buffer saline (PBS) method, PBS followed by acetone precipitation (PBS-acetone) method and urea-sodium dodecyl sulphate (urea SDS) buffer method were optimized in this study. Results revealed that, compared to the first and second methods, the urea-SDS method of protein extraction was found to be better in extraction of a greater number of seed storage proteins in rice through SDS-PAGE. Hence, urea-SDS method can be used for extracting seed storage proteins from rice seeds for various proteomic approaches. Furthermore, differences in protein content and protein banding patterns between rice genotypes were observed. These findings underline the importance of method optimization tailored to specific rice genotypes for maximizing protein yield and quality. Such optimization holds promise for enhancing food sustainability and nutritional value, particularly in regions reliant on rice as a staple food.

Keywords : Rice, Protein extraction, SDS-PAGE

More than half of the world's population is currently fed rice (*Oryza sativa* L.), making it one of the most essential staple foods for humans. The demand for rice seeds is rising quickly as a result of the rapidly increasing global population (Khush *et al*., 2005). It is largely consumed in Asian and African countries. Asia is considered a 'Rice Bowl' of the world and more than 90 per cent of rice is produced and consumed in Asia (Santhosh *et al.*, 2018). Area of rice as per the year 2021-22 was 46279 thousand hector, production 129471 thousand tonnes and productivity 2798 kg per hector in India. Rice seeds are used for making edible starches, vinegar, syrups and sweet alcoholic beverages in addition to being eaten as a staple diet and seed bran is utilized as a raw material to extract oil for the cosmetics and pharmaceutical sectors (Deng *et al.,* 2013). In this context, the nutrition of rice is an important key for food sustainability. It is not surprising that about 870 million people are suffering from chronic under nourishment globally (FAO, 2012) and the vast majority of them are from developing countries where rice is closely associated with food security and political stability (Sarma *et al.*, 2018). The nutrient content, pasting and textural qualities of cooked rice have been significantly influenced by seed storage proteins (SSPs), which make up the second-highest prominent part of rice seeds after starch and make up roughly 7 per cent - 10 per cent of the total seed weight (Kawakatsu *et al*., 2010).

Rice with its easily digestible protein content of around 7 per cent, is primarily composed of moisture (12%) and carbohydrates (75 - 80%) (Verma *et al.,* 2017). It also provides essential vitamins such as thiamine, riboflavin and niacin, although it lacks

significant amounts of vitamin C, D or beta-carotene (Fresco, 2005). Rice contains four main types of protein. Out of total 7 per cent seed protein it consists four main types of proteins, albumins (5-10%), globulins (7-17%), glutelin (75-81%) and prolamins (3-6%) (Adebiyi *et al.,* 2009). Efforts to enhance rice protein content have been ongoing for five decades, with limited success achieved by only a few research teams (Shashidhara and Hittalmani, 2019). Increasing rice protein content holds promise for combating protein energy malnutrition, especially in regions where rice is a staple food relied upon by impoverished populations, notably in countries like India.

Rice is staple diet for nearly 90 per cent Asian population. Protein energy malnutrition (PEM) is the important issue in underdeveloped and developing countries due to lesser intake of protein and micronutrients. Consumption of rice with high quality protein is essential to combat the PEM. Another importance of rice protein is its digestibility, the net protein utilization of rice is the highest among the cereals. It contains 65-75 per cent of Glutelin which is easy to digest and however it contains 3 - 6 per cent of prolamin which is difficult to digest (Chandi and Sogi, 2007).

Most human protein needs are met by plant sources, which are cost-effective and easier to transport than animal proteins. Efforts to enhance protein content and quality in food crops have been extensive. However, the focus on increasing grain output during the Green Revolution has led to a decline in micronutrient availability (Hertzler *et al.,* 2020). Seeds primarily accumulate proteins, lipids (often triacylglycerols) (Hsieh *et al.,* 1996) and carbohydrates (often starch) (Gallardo *et al.,* 2003). as storage compounds with seed storage proteins crucial for early plant growth (Baud *et al.,* 2002). Plant seeds contain more proteins than any other plant tissue. However, the presence of carbohydrates and lipids in seeds complicates protein extraction. Seed storage proteins, vital for germination and early growth, dominate 2D gel protein profiles (Wang *et al*., 2008).

Many extraction methods have been discussed in literature over time. The alkali extraction method involves exposing proteins to 1M NaOH solution at pH 11 for 3 hours at 40°C. After stirring, the solution is centrifuged at 3000 x g for 15 minutes to separate the protein from other seed components, yielding a protein-containing supernatant (De *et al.,* 1969; Nashef *et al.,* 1977 and De *et al.,* 2016).

Another method for protein extraction from rice bran and broken rice have been described by Chittapalo and Noomhorm (2009), where they used an ultrasound - assisted alkali method for extraction of rice protein, rice bran suspension in water is subjected to ultrasound treatment and alkali adjustment (pH 11), followed by traditional protein extraction steps: centrifugation, filtering, isoelectric precipitation, cleaning, freeze-drying and storage.

According to Xia *et al.* (2012), microfluidization treatment involves wet milling of broken rice in water, followed by microfluidization to break up protein-starch agglomerates. Centrifugation separates the supernatant, where the protein layer is isolated, treated with enzymes for purity and stored.

Fujiwara *et al.* (2010) utilized an electric stone mill to grind rice seeds, followed by filtration through a 355-micrometer sieve. The rice powder was then extracted in pre-extraction buffer (50 mM Tris-HCl pH 7.4, 0.5 M NaCl) on a magnetic stirrer for over 3 hours. After centrifugation at 14,000 g for 10 minutes, the pellet was re-suspended in extraction buffer containing 50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 1 per cent CTAB and 10 mM β-mercapto ethanol.

Rice seed protein extraction and analysis are crucial for understanding the characteristics of rice seed storage proteins. SDS-PAGE is a commonly used method to separate complex protein mixtures with high resolution (Nowakowski *et al.,* 2014). Optimizing extraction methods for SDS-PAGE analysis is vital for protein characterization. In this study, three extraction methods were examined: phosphate buffer saline (PBS) extraction with acetone precipitation and urea-SDS buffer. PBS and PBS-acetone methods are popular extraction methods but the problem is only salt soluble proteins are extracted here, so we used urea-SDS buffer to solubilize most of the proteins as the urea is strong denaturant. Standardized extraction methods will facilitate

comparative studies and help researchers elucidate the roles of specific rice seed proteins in physiology and biochemistry.

Material and Methods

Rice Seed Samples

Matured rice seeds of BPT-5204 and HPR-14 were provided by the Genomics and Gene Editing Laboratory, Department of Genetics and Plant Breeding, University of Agricultural Sciences, GKVK, Bengaluru. Experiment was conducted in Plant Biotechnology department in the year 2022-2023.

Preparation of Defatted Flour

Rice samples were dehusked with palm dehusker and brown rice was ground to fine powder. Fine powder was sieved through 60-mesh size. The rice flour was defatted using hexane in the ratio of 1:4 shaken for 1 h in a rotary shaker and centrifuged at 10,000 rpm for 10 min. to separate the fat containing hexane. The flour was vacuum-dried to remove traces of hexane and the resulting flour was stored in airtight glass containers at ambient temperature for further use.

Extraction of Proteins with Phosphate Buffer Saline (PBS)

The process involved grinding 100 mg of rice flour with 2 ml. PBS buffer having 7.4 pH. The composition of the buffer was referred from Chazotte (2012) after some modifications (composition given in Table 1), to create a homogeneous mixture in a pestle and mortar. The homogenized protein mixture was centrifuged at 20,000 rpm for 20 min at 4°C. The supernatant was collected and centrifuged again for 20 min and then stored at -20°C until used.

Table 1

Composition of phosphate buffer, Chazotte (2012)

Composition: Chazotte (2012)	Modified
Potassium dihydrogen phosphate (KH_2PO_4) 1.8 mM	Sodium dihydrogen phosphate (NaH_2PO_4) (0.2 M)
Disodium hydrogen phosphate (Na_2HPO_4) 10 mM	0.2 M
Sodium chloride (NaCl) 137 mM	No change
pH 7.4	No change

Extraction with Phosphate Buffer Saline and Acetone Precipitation

This protocol was based on the work of (Wongpia *et al.*, 2015) after some modifications (composition given in Table 2) all the components were same as PBS, only the acetone was used to precipitate the protein. 100 mg of rice powder was homogenized with 2.0 mL of PBS buffer containing 1mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 20,000 rpm for 20 min at 4°C, the supernatant was transferred to a new tube and centrifuged again to obtain a supernatant. The proteins in the supernatant were precipitated by adding six volumes of cold acetone. The sample was incubated at -20°C overnight to achieve complete precipitation before being centrifuged at 20,000 rpm for 20 min at 4°C. The resulting protein pellet was washed with ice-cold acetone and the pellet was freeze-dried. The dried pellet was re-suspended in PBS buffer. The protein solution was centrifuged for 10 min to eliminate insoluble substances and then kept at -20°C until used.

Table 2

Composition of phosphate buffer and acetone precipitation (Wongpia *et al.*, 2015)

Chemicals	Concentration
NaH_2PO_4	0.2 M
Na_2HPO_4	0.2 M
NaCl	0.15 M
Acetone	Six volumes
pH	7.4

Extraction with Urea-Sodium Dodecyl Sulphate (SDS) Buffer

This method was referred from the work of (Gan *et al.*, 2021), after some modifications (composition given in Table 3), in this method 100 mg of rice flour was homogenized with 2 ml of urea-SDS buffer in pestle and mortar. The homogenized mixture was

Table 3
Composition of urea-SDS buffer (Gan *et al.*, 2021)

Composition (Gan *et al.*, 2021)	Modified
Tris HCl 250 mM	50 mM
Urea 8 M	No change
SDS 4 %	No change
2-mercaptoethanol 5 %	No change
Glycerol 20 %	No change
6PMSF 100 µg/mL	1 mM
pH 6.8	No change

centrifuged at 20,000 rpm for 20 minutes at 4°C. The supernatant was collected and centrifuged again for 20 min and then stored at -20°C until used. This step may be repeated to remove traces of carbohydrate debris which may disturb SDS-PAGE analysis.

Protein Estimation

Rice proteins extracted from all three methods were estimated by Bradford protein estimation kit (Product Code ML106, HiMedia Laboratories Pvt. Ltd. Maharashtra, INDIA) based on the manufacture protocol with the bovine serum albumin (BSA) as a protein standard.

SDS-PAGE Analysis of Rice Seed Storage Proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical slab gel of 1 mm thickness according to the method of Laemmli (1970). The protein profile of all samples was determined by SDS-PAGE under reducing conditions. 10 µl of each sample was mixed with reducing sample buffer, consisting of 0.3 M Tris-HCl, 5 per cent SDS, 50 per cent glycerol and 100 mM DTT, before being heated at 95°C for 5 min while mixing at 300 rpm using mini spin. Solutions were allowed to cool and sample volumes corresponding to 30 µg were loaded onto 12 per cent acrylamide gel in a mini gel unit (TARSONS Mini dual electrophoresis unit for two slabs gel 10×8 cm). BlueRAY, prestained protein ladder (Cat. No. SP006-0500) molecular weight standards ranging from 11 to 180 kDa were used as molecular weight marker. Electrophoresis was performed at 70 V until samples crossed the stacking gel and at 100 V for resolving gel until the ladder separated. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. Staining solution containing 1 per cent Coomassie Brilliant Blue R-250, 10 per cent acetic acid, 40 per cent methanol and 50 per cent distilled water. The gels were destained by 10 per cent acetic acid, 40 per cent methanol and 50 per cent distilled water until a clear background was obtained. Subsequently, the gel was documented in a gel documentation unit using white light.

Statistical Analysis

Statistical analysis of the experimental data was carried out by Analysis of Variance in Completely Randomized Design using Duncan's Multiple Range test (DMRT) in the online statistical tool OPSTAT (Sheoran *et al*., 1998). Experimental results were represented as means ± standard error from three replications.

Results and Discussion

Three methods were used to optimize the protein extraction method *viz.,* PBS, PBS-acetone and urea-SDS method for SDS-PAGE analysis. The results of the urea-SDS method found to be revealing more protein bands in SDS-PAGE analysis.

Extraction with Phosphate Buffer Saline (PBS)

Extraction of total proteins from phosphate buffer revealed only a few proteins compared to urea-SDS buffer (Plate 1, Lanes 1 and 2). We were able to obtain bright bands of salt-soluble proteins, which are globulins, as PBS contains salts (Amagliani *et al.,* 2017). Due to their lower solubility in a salt solution, protein fractions such as albumin, glutelin and prolamin appear as slightly lighter bands compared to globulin when visualized on SDS-PAGE gel. Comparing the protein profiles between HPR-14 and BPT-5204, there is not much difference in banding patterns except for one protein band near 63 kDa, which is brighter in HPR-14 than in BPT-5204. This may be due to the concentration of that particular protein being higher in HPR-14, as its total protein content is found to be high as reported by Shashidhara and Hittalmani (2019).

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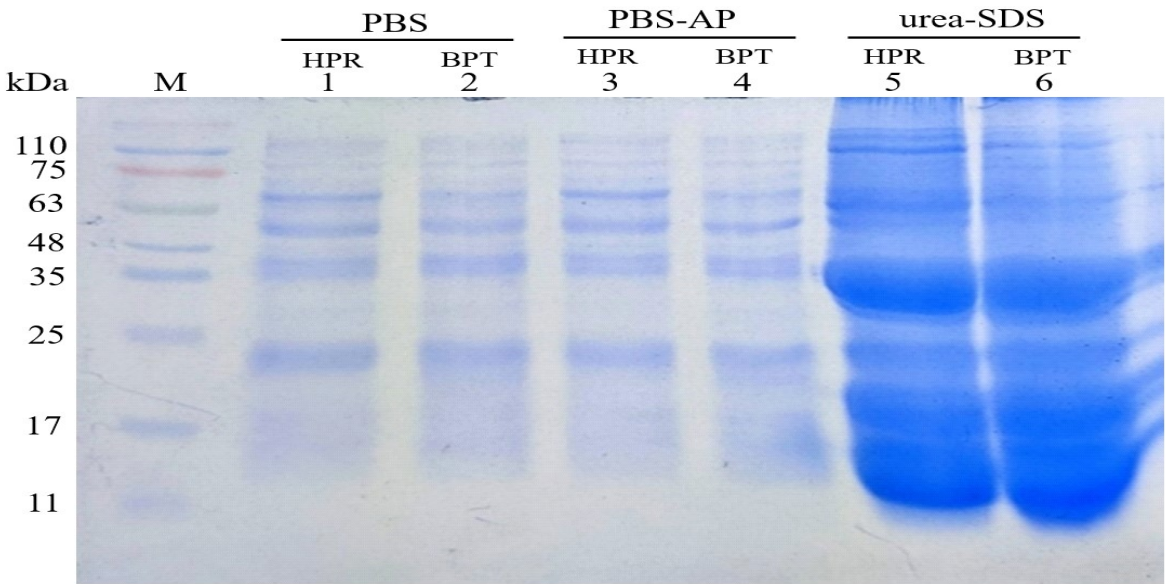

Plate 1 : SDS-PAGE protein profile of total protein of HPR-14 and BPT-5204 extracted with different buffers
Legends : M : protein marker, PBS : Phosphate buffer saline, PBS-AP: PBS-acetone

In this method, the protein concentration was found to be 3.2 per cent and 3.1 per cent in BPT-5204 and HPR-14, respectively as shown in Fig. 1 and Table 4, which was estimated using the Bradford protein estimation method (Bradford, 1976). The estimated protein value of BPT-5204 varies from the previously reported studies (Deepa and Singh, 2010) due to differences in the method of protein estimation, where they used the Micro Kjeldahl method and were able to obtain 7.4 per cent protein content. HPR-14 showed

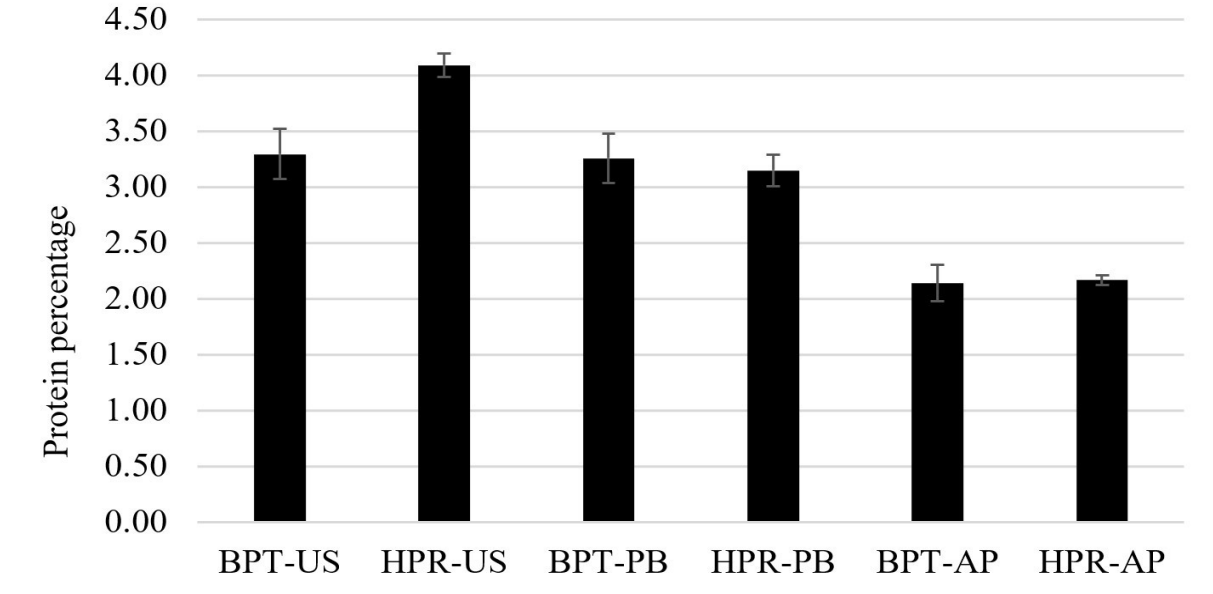

Fig. 1 : Protein estimation of seed proteins of BPT-5204 and HPR-14 from the three extraction methods
Legends : US- Urea-SDS, PB- phosphate buffer saline, AP- Acetone precipitation

Table 4

The protein percentage of seed proteins from BPT-5204 and HPR-14 determined using three extraction methods

Genotype with different buffers	Protein percentage mean
BPT-US	3.297 [b]
HPR-US	4.094 [a]
BPT-PB	3.259 [b]
HPR-PB	3.152 [b]
BPT-AP	2.141 [c]
HPR-AP	2.169 [c]
C.D.	0.260
SE(m) ±	0.083

Values in the same column with different subscript letters (a, b, c, d & e) differ significantly at p<0.01 (DMRT)

a difference in protein estimation values compared to previous studies (Shashidhara and Hittalmani, 2019) reported it to be 14 per cent. This difference may be due to the environmental conditions as Shashidhara and Hittalmani (2019) grew rice in aerobic conditions and protein was estimated by the Kjeldahl method. The observed augmentation in the total protein content may be attributed to variations in the genetic makeup inherent to the *indica* subspecies of rice. This suggests a potential correlation between genetic diversity and protein levels, necessitating further investigation into the underlying genetic factors influencing protein synthesis in these genotypes.

Extraction with Phosphate Buffer Saline Followed by Acetone Precipitation

In this method, pelleted protein was resuspended in PBS and used as a sample for SDS-PAGE. The number of bands obtained in this method (Plate 1, Lanes 3, and 4) resembles the results of the PBS extraction method. The outcomes obtained from this method were not satisfactory when compared to the urea-SDS method. The protein concentration for this method was determined to be 2.1 per cent for both BPT-5204 and HPR-14 (Fig. 1 and Table 4). These values are slightly lower compared to the PBS method. This may be due to the loss of some proteins during acetone precipitation and subsequent resolubilizing in PBS for protein estimation. In this method, the banding pattern was also similar when compared between both genotypes, except for one protein band in HPR-14 near 63 kDa, which is brighter compared to BPT-5204, similar to the results of the above experiment with PBS.

Extraction with Urea-SDS Buffer

Total proteins obtained from the extraction using urea-SDS buffer revealed more protein bands compared to PBS buffer, as the urea-SDS method of protein extraction is more capable of solubilizing a greater number of proteins due to its strong protein denaturing properties. In terms of banding patterns, there is not much difference in both the genotypes. However, when comparing both the genotypes for their band thickness, some of the bands obtained in HPR-14 are brighter when compared to BPT-5204 *viz*., the protein bands at 110 and 63 kDa. Except for these two, all the bands are similar in both genotypes in terms of brightness (Plate 1, Lanes 5 and 6).

The reason why the urea-SDS method extracts more protein is that by denaturing proteins, urea helps to unfold and solubilize them, making them more accessible. Urea also disrupts non-covalent interactions, such as hydrogen bonds and hydrophobic interactions, which are important for protein stability and folding. By disrupting these interactions, urea aids in the dissociation of protein complexes and helps to solubilize proteins. Urea increases the solubility of proteins by reducing the hydrophobic interactions between protein molecules and water molecules. Additionally, urea can function as a buffering agent, helping to maintain a stable pH during protein extraction. Protein-protein contact is less stable than protein-urea contact when urea is present, which is the exact opposite of the situation in pure water, where protein-protein contact is more stable than protein-water contact. This causes the equilibrium to shift from the native to the denatured ensemble (Das *et al*., 2009).

The protein concentration of extracted proteins from urea-SDS was found to be 3.2 per cent and

4.0 per cent quantified by Bradford assay, in BPT-5204 and HPR-14, respectively (Fig. 1 and Table 4). Compared to the other two methods, the urea-SDS method showed a slightly higher protein concentration. This may be because of the denaturation ability of urea, as it acts as a strong protein denaturant, disrupting the native structure of proteins.

Comparison of the Number of Protein Bands Between the Extraction Methods

A comparison of the number of protein bands and their brightness was made to determine differences in protein extraction methods among the three methods. In the PBS and PBS-acetone methods, a total of 10 bands were observed at molecular weights of 17 kDa, 22 kDa, 28 kDa, 35 kDa, 37 kDa, 48 kDa, 63 kDa, 67 kDa, 76 kDa and 112 kDa, among which five bands were bright (67 kDa, 53 kDa, 35 kDa, 37 kDa and 22 kDa) and the other five were lighter bands (Plate 1, Lanes 1, 2, 3 & 4). However, in the urea-SDS method, a total of 16 bands were observed at molecular weights of 11 kDa, 14 kDa, 17 kDa, 22 kDa, 28 kDa, 35 kDa, 37 kDa, 48 kDa, 53 kDa, 63 kDa, 67 kDa, 74 kDa, 76 kDa, 100 kDa, 112 kDa and 114 kDa) among which nine were bright (11 kDa, 14 kDa, 17 kDa, 22 kDa, 28 kDa, 35 kDa, 37 kDa, 63 kDa and 100 kDa) and the other seven were lighter (48 kDa, 53 kDa, 67 kDa, 74 kDa, 76 kDa, 112 kDa and 114 kDa) (Plate 1, Lanes 5 and 6).

Although the same number of bands was observed among all the genotypes with their respective methods, the brightness of the bands was not consistent. This discrepancy is attributed to the higher protein content in HPR-14, as it is land race which contains a greater number of defence proteins. This comparison enables us to discern differences in protein extraction methods and the number of protein bands among the genotypes.

In conclusion, our study investigated three different extraction methods for rice seed storage proteins, namely phosphate buffer saline (PBS), PBS followed by acetone precipitation and urea-sodium dodecyl sulphate (SDS) buffer. The results demonstrated that the urea-SDS method yielded the highest protein concentration and the greatest number of protein bands, attributed to its strong denaturing properties. While PBS extraction provided satisfactory results, acetone precipitation following PBS extraction led to a slight reduction in protein yield. Additionally, our findings suggest that the protein content and banding patterns may vary between rice genotypes. Overall, study contributed to understanding of the selection and optimization of extraction methods for rice seed storage proteins, with implications for food sustainability and nutritional enhancement strategies. Further research focusing on refining extraction protocols and exploring the functional properties of extracted proteins is necessary to fully harness the potential of rice seed storage proteins for diverse applications.

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