



UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Sustainable Development and Ecological Transition

First Biochemical Characterization of Seed Storage Proteins in Chinese Originario (*Oryza sativa* L.): A Combined SDS-PAGE and MALDI-TOF Mass Spectrometry Approach

Supervisor: Prof.ssa Maria Cavaletto

Co-supervisor: Dott.ssa Annalisa Givonetti

Candidate Name: Amirhossein Ajam

Matricula Number: 20058977

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Table of Contents

Abstract	4
Chapter 1 – Introduction	6
1.1 Rice as a global crop	6
1.1.1 Agronomic and nutritional importance of <i>Oryza sativa</i>	6
1.1.2 Differences between subspecies, crop groups and traditional Italian varieties	9
1.1.2.1 Subspecies differentiation in cultivated rice	9
1.1.2.2 Diversity among varietal groups and quality classes	10
1.1.2.3 Nutritional and technological differences among rice varieties	11
1.1.2.4 Italian rice varieties as a distinct historical and agronomic heritage	12
1.1.2.5 Molecular markers and cultivar identification	13
1.1.2.6 Importance of varietal diversity for food quality and plant breeding	13
1.1.2.7 Traditional Italian varieties as living genetic resources	14
1.2 Chinese Originario	15
1.2.1 Agronomic and technological characteristics of the Originario group	15
1.3 The Italian rice supply chain	18
1.4 Storage proteins in Rice grains	20
1.4.1 Classification (Albumins, Globulins, Prolamins, Glutelins)	20
1.4.1.1 Albumins	22
1.4.1.2 Globulins	24
1.4.1.3 Prolamins	25
1.4.1.4 Glutelins	27
1.4.2 Biological and nutritional role	29
1.4.3 Potential as precursors of Bioactive peptides	34
1.5 Bioactive peptides in Rice	37
1.6 Enzymatic hydrolysis as peptide release strategy	39
1.6.1 The role of digestive tract digestion (Pepsin)	41
1.6.2 Technological approaches and differences compared to physiological hydrolysis	43
Chapter 2 – Materials and Methods	49
2.1 Study Design	49
2.2 Sample Collection and Flour Production	50
2.3 Protein Extraction	51
2.3.1 Sequential Extraction of Storage Proteins	52

2.4 Determination of Rice Protein Content: Bradford Assay.....	53
2.5 Protein Analysis	54
2.5.1 SDS-PAGE Analysis.....	55
2.5.2 MALDI-TOF Analysis.....	56
2.5.2.1 Matrix Preparation and Sample Preparation	57
2.5.2.2 Spectrum Acquisition Parameters	57
2.6 Statistical Analysis	58
Chapter 3 – Results.....	59
3.1 Assessment of Concentration and Distribution of Storage Proteins	59
3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	59
3.3 MALDI-TOF Analysis.....	63
Chapter 4 – Discussion	67
Chapter 5 – Conclusion	76
References.....	78

Abstract

Seed storage proteins play a crucial role in the nutritional quality, functional properties and germination physiology of rice. The Chinese rice cultivar Originario (*Oryza sativa* L.) has high potential for proteomic studies due to its specific agronomic characteristics and adaptability to environmental conditions. This study aimed to biochemically characterize seed storage proteins in the Chinese rice cultivar Originario (*Oryza sativa* L.) by SDS-PAGE and MALDI-TOF mass spectrometry, and to evaluate its profile in comparison with two traditional Italian cultivars, Roma and Carnaroli.

In the methodology section, seed protein extracts from the three cultivars were separated using specific extraction buffers and subjected to electrophoretic analysis. The obtained bands were examined using image analysis software to determine the molecular weight and relative intensity of the bands. Protein fragments were analyzed for precise identification and identity verification by MALDI-TOF mass spectrometry. With this technique, the precise molecular weight of the subunits was measured with high accuracy and the protein patterns were compared with rice protein data banks.

The findings of this study showed that the protein profile of the Originario cultivar was significantly influenced by glutelins. The analyses revealed the presence of two main subunit groups in glutelins: heavy subunits (H subunits) with molecular weights in the range of 37 to 25 kDa and light subunits (L subunits) with weights of 20 to 23 kDa. The calculated ratio between these subunits was about 3:2, indicating structural equilibrium in the storage proteins of this cultivar. A careful examination of the electrophoretic bands confirmed the absence of abnormal bands with high molecular weights (>120 kDa) which is evidence of the integrity of the seed proteomic structure. Importantly, a comparative analysis revealed that the Chinese Originario cultivar acts as a unique brown rice niche product that possesses a significantly higher nutritional value. This nutritional superiority is demonstrated by its higher total protein content, specifically within the Albumin and Prolamin fractions, when compared to the Roma and Carnaroli varieties, which instead exhibited higher glutelin concentrations optimized for commercial cooking quality.

From a proteomic stability perspective, the MALDI-TOF spectroscopy results were very impressive. The molecular weight deviation of key proteins in the studied samples was calculated to be less than 0.2%. This very high stability indicates that the Originario cultivar has a highly self-regulating and genetically controlled system that maintains a stable protein profile in the face of common environmental changes.

The present study showed that the Originario variety, as a specialized brown rice niche product, is a rich source of high-quality albumins and prolamins, and due to its higher nutritional value and high proteomic stability, it is a very good candidate for plant breeding programs to improve the nutritional value of rice. The identification of this protein profile paves the way for future studies in the field of production of bioactive peptides with antihypertensive and antioxidant properties through enzymatic hydrolysis of seed proteins of this variety. Finally, this study provides the necessary scientific basis for understanding the biochemical mechanisms of protein storage in rice and introduces accurate analytical tools for evaluating new varieties.

Chapter 1 – Introduction

1.1 Rice as a Global Crop

1.1.1 Agronomic and Nutritional Importance of *Oryza sativa*

Rice (*Oryza sativa* L.) is one of the most important agricultural crops in the world and plays an important role in providing food for the human population (Bandumula, 2018). Rice (*Oryza sativa* L.) is a member of the Poaceae family and is considered one of the three major cereals in the world along with wheat and maize. The importance of rice is significant because of its direct role in meeting the nutritional needs of a large part of the world's population (Muthayya et al., 2014). Studies show that rice is the staple food of more than half of the world's population and about 3 to 3.5 billion people in the world directly consume this crop as a major part of their daily diet (Khatun et al., 2019, Jukanti et al., 2025).

In terms of production, rice is one of the largest crops in the world and is widely cultivated annually. The global production volume of white rice is about hundreds of millions of tons per year indicating the economic importance of this international agricultural product (Muthayya et al., 2014). Rice is cultivated in various regions of the world, including Asia, Africa, North and South America, the Middle East and some parts of Europe, but its main focus is on the Asian continent (Jukanti et al., 2025). More than 90% of the world's rice is produced and consumed in Asian countries, indicating the strong dependence of the regions food system on this crop (Muthayya et al., 2014, Bandumula, 2018). China and India play a very important role in global rice production and consumption. These two countries alone supply about half of the world's rice (Bandumula, 2018, Muthayya et al., 2014). Such a position means that any changes in rice production in these countries have significant effects on the world market, food prices and food security in the world. For this reason, many agricultural studies and food policies at the global level pay special attention to the trend of rice production in Asian countries (Sokra et al., 2026).

From a nutritional perspective, rice is of particular importance because of its high contribution to the energy requirements of the human body. Rice grain is considered one of the most important sources of calories in

the human diet due to its significant amounts of carbohydrates and starch. Estimates show that rice provides about 20% of the world's dietary energy (Khatun et al., 2019). In many developing countries, especially in poorer regions of Asia, this share is even higher and may include up to 50% of people's daily calorie intake (Bandumula, 2018). In addition to its economic importance, rice has a very broad social and cultural dimension. In many Asian societies, rice is not only an agricultural product but also an integral part of culture, traditions and dietary patterns. In some Asian languages the word “food” is even used synonymously with “rice” indicating the profound role of this crop in people’s daily lives (Bandumula, 2018).

Starch in rice grains is mainly composed of two main components, amylose and amylopectin. These two components are present in different proportions in different rice varieties, which can affect the physical, textural and nutritional properties of the grain. For example, the amylose content is an important factor in determining the digestibility of starch, glycemic index and cooking properties of rice. Rice with high amylose content tend to have slower digestion and lower glycemic index, while rice with low amylose tend to have a softer texture and faster digestion. In addition to carbohydrates, rice grains also contain other nutritionally important components such as proteins, lipids, dietary fiber, phenolic compounds, phytic acid, vitamins and minerals. Each of these components plays a specific role in determining the nutritional value and functional properties of rice grains. For example, phenolic compounds and some antioxidants can play a role in reducing oxidative stress and improving overall health. Also, the presence of dietary fiber in some parts of the grain, especially in rice bran, can be effective in improving digestive function (Khatun et al., 2019).

However, post-harvest processes, particularly milling and milling, can have a significant impact on the nutritional composition of rice. During these processes, the outer layers of the grain which contain a large number of micronutrients and bioactive compounds, are removed. As a result of the milling process, the content of vitamins, minerals and dietary fiber in white rice is significantly reduced compared to whole rice

(Muthayya et al., 2014). This is particularly important in societies where rice forms a major part of the diet as it can increase the risk of deficiencies in some micronutrients (Khatun et al., 2019).

Protein is another important component of rice grain that is very important in terms of nutritional quality. Although the protein content of rice may be moderate compared to some other grains, its amino acid composition is of particular importance. Studies have shown that rice protein contains essential amino acids such as lysine, methionine and cysteine which are necessary for the growth and maintenance of a healthy body. This feature has led to rice protein being considered as a potential source of quality protein in cereal-based diets. Rice grain storage proteins are important in grain structure and function as well as in determining the nutritional and functional properties of rice. Therefore, studying the structure, composition and properties of these proteins is of great importance in research related to food science and biochemistry (Jukanti et al., 2025).

On the other hand, the increase in global population, climate change and limited natural resources have created new challenges for sustainable rice production. Several factors including water resources, climatic conditions, agronomic management and genetic characteristics, affect rice production (Sokra et al., 2026). In such circumstances, improving production efficiency, increasing nutritional quality and developing rice varieties with desirable characteristics are of particular importance (Vinci et al., 2023). Scientific research in the fields of genetics, plant breeding, biochemistry and food technology play an important role in achieving these goals (Bhandari et al., 2023).

Oryza sativa is one of the most important crops in the world due to its large area of cultivation, its important role in providing energy and protein to humans, its economic importance in many countries and its complex and valuable grain composition. The importance of this crop is significant not only in providing food for the global population but also in areas related to health, agricultural economics and sustainable development (Khatun et al., 2019).

1.1.2 Differences between subspecies, crop groups and traditional Italian varieties

Rice (*Oryza sativa* L.) has a significant genetic, agronomic and phenotypic diversity due to its long history of domestication and wide geographical distribution; adaptation to diverse ecosystems and human selection over centuries have led to this wide diversity. Although botanically classified as a single species, its cultivated forms include a wide range of subspecies, varietal groups and landraces that differ in traits such as plant architecture, grain morphology, amylose content, cooking behavior, milling quality and end-use food applications (Lou et al., 2026, Spada et al., 2004). This diversity is important both from a taxonomic and evolutionary perspective and has important practical implications for plant breeding programs, cropping systems, product processing, nutritional quality and market segmentation of rice. In the context of rice production in Europe and in particular in Italy the differentiation between rice subspecies and traditional cultivars has played an important role in the formation of specialized production systems and high-quality value chains (Chen et al., 2019, Zhang, 2022, Wang et al., 2023).

1.1.2.1 Subspecies differentiation in cultivated rice

In the intraspecific classification of rice, the distinction between the Indica and japonica subspecies is the main feature; however, the existence of intermediate and indigenous forms adds to the complexity and richness of the genetic structure of this crop. These groups are usually distinguished from each other based on a set of morphological traits, physiological characteristics, grain characteristics and molecular markers. Genomic studies of the genus *Oryza* indicate that rice subspecies, despite their common origin, have experienced significant evolutionary divergence during domestication, influenced by selective pressures and adaptation to diverse ecosystems (Chen et al., 2019). These genetic differences have resulted in rice cultivars differing in traits such as flowering time, plant height, panicle shape, grain appearance and cooking quality. These differences ultimately affect rice yield and nutritional quality (Spada et al., 2004).

In plant breeding, the precise boundaries between rice subspecies are not always completely clear. Recent genetic studies show that many rice cultivars due to long-term crossing and human selection, have a complex genetic structure and are a mixture of different species. This is particularly evident in the Mediterranean basin where genetic exchange and past breeding activities have produced local varieties that do not easily fit into standard categories (Chen et al., 2019, Spada et al., 2004).

1.1.2.2 Diversity among varietal groups and quality classes

Beyond the scientific classification of subspecies, rice is divided into different groups based on grain shape, starch type and cooking quality. This classification is of great importance for the sales market because the commercial value of rice is determined not by its scientific name but by characteristics such as its appearance, taste and texture when cooked, all of which are of interest to consumers (Simonelli et al., 2017, Tao et al., 2019). Studies on rice quality show that characteristics such as amylose content, cooking temperature (gelatinization), grain transparency, breakage rate (milling yield) and gypsum content determine the quality and consumer value of rice in the market (Zhang, 2022, Wang et al., 2023).

For example, high-amylose rice tends to have a firmer texture after cooking, are more resistant to digestion and exhibit distinct starch structural characteristics compared to low-amylose or waxy varieties (Tao et al., 2019). In contrast, varieties with lower amylose content often produce softer, stickier grains after cooking and are more valuable in markets where such a texture is desirable (Wang et al., 2023). Starch structure has a major impact on rice quality. Factors such as the branching of amylopectin, the arrangement of starch granules and the bonding between starch and protein directly affect the digestibility of rice and its sensory properties (such as taste and texture). As a result, the classification of rice varieties is not just for classification but also reflects a direct relationship between the plant's genetics, the chemical composition of the grain and what consumers ultimately like (Tao et al., 2019).

Rice diversity is not limited to the common types. There are also varieties such as fragrant, colored, sticky, or high-amylose rice. Each of these groups has a specific chemical structure and is used for specific food applications or markets. The value of these rice is not only due to their appearance or yield but also to their nutritional properties and cooking technology. Therefore, rice classification is directly related to food science, as the chemical composition of the grain affects both its processing and its health properties (Simonelli et al., 2017, Vici et al., 2021, Li et al., 2023).

1.1.2.3 Nutritional and technological differences among rice varieties

The nutritional value of rice varies greatly depending on its type and these differences make each type of rice have specific uses. Studies of different rice cultivars show that the levels of protein, fat, fiber, minerals and starch digestibility differ significantly among different genotypes (Vici et al., 2021). These nutritional differences are important because they can affect postprandial blood sugar levels, feelings of satiety and the use of rice in certain diets. For example, high-amylose rice is more slowly digested and are a better choice for people looking to control blood sugar and absorb energy more slowly. On the other hand, some types of rice are more popular because of their softer texture and better processing characteristics, even if they have different nutritional properties than health-oriented varieties (Tao et al., 2019).

Rice quality is the result of a complex interaction between plant genetics, environmental conditions and postharvest practices (such as storage and processing). Studies show that to truly understand the quality of a rice variety, it is necessary to consider, in addition to the genetic type, factors such as the yield of the rice plant, the physical appearance of the grains and the way the starch cooks. This is of great importance in rice production today, as the main goal of breeding programs is to achieve a balance between yield, cooking quality and environmental compatibility (Wang et al., 2023).

1.1.2.4 Italian rice varieties as a distinct historical and agronomic heritage

In Europe, Italy is the main producer of rice. Over the centuries, the country has developed a wide variety of rice cultivars, including both commercial varieties and traditional and local varieties. Italian rices are the result of a combination of imported breeds, adaptation to the environment and scientific breeding efforts. Historical and genetic studies indicate that these cultivars evolved in this way due to the need to adapt to the specific conditions of the agricultural lands of the Po Valley and to provide the specific characteristics required for traditional Italian cuisine (Spada et al., 2004, Iametti et al., 2025).

Studies of the genetic relationships of Italian rice using molecular markers and pedigrees show that old varieties fall into distinct genetic groups that reflect their history and breeding trajectory. These findings are of great importance because they demonstrate that traditional varieties are not just relics of the past, but are valuable genetic resources that can be used in new breeding projects. Also, combining genealogical information, plant appearance and molecular data provides a better overview of the relationships between cultivars, which helps maintain genetic diversity. These studies are crucial for protecting biodiversity in agriculture, as many old cultivars may contain important genes for disease resistance, adaptation to harsh environments, or improved grain quality (Spada et al., 2004).

The diversity of rice in Italy is evident, with traditional and local varieties that have retained their indigenous identity. These varieties usually differ from modern, high-yielding rice in terms of height, ripening time, grain shape and flavor and texture. The reason for continuing to cultivate this rice is their association with Italian culinary traditions, their demand in specific markets and the importance of authenticity and traceability for consumers. In addition, some of these varieties are highly valued for their specific qualities in cooking dishes such as risotto, as the starch behavior in this rice has a direct impact on the final quality of the dish. This relationship between variety type and cooking method shows that rice classification in Italy is not just an agricultural issue, but is rooted in the country's culture and food traditions (Simonelli et al., 2017).

1.1.2.5 Molecular markers and cultivar identification

One of the most important advances in identifying rice cultivar diversity is the use of molecular markers to investigate the genetic relationships between different species and cultivars. Studies using markers such as AFLP and SSR have been able to identify subtle differences between similar cultivars and reconstruct the pedigree of Italian rice cultivars with high accuracy. Combining genetic data with morphological and qualitative characteristics allows for more precise classification of rice cultivars. Sometimes two types of rice are quite similar in appearance, but differ greatly in starch composition, nutritional value, or breeding history. In such cases, the use of molecular markers provides more reliable results than visual inspection. This is especially important for ancient Italian rice, as their names are often based on local traditions and do not necessarily correspond to strict scientific classification (Spada et al., 2004).

1.1.2.6 Importance of varietal diversity for food quality and plant breeding

The diversity of rice cultivars is not just a thing of the past, but has a direct impact on improving crop quality, food production processes, and introducing new products. Today, factors such as amylose content, starch molecular structure, water absorption and cooked rice texture are key criteria for determining economic value and breeding goals (Zhang, 2022, Wang et al., 2023, Tao et al., 2019). Older rice cultivars sometimes have lower yields in the field than modern, high-yielding cultivars, but offer much better flavor, cooking quality, or nutritional value. Therefore, maintaining this genetic pool is critical to achieving superior genes in future breeding programs (Simonelli et al., 2017, Li et al., 2023).

In addition, local varieties can be used as suitable genetic material for studying the relationship between genetic structure and plant appearance. The high adaptability of these varieties to local environments allows for detailed investigation of genes affecting quality and the influence of environmental conditions on grain composition. The aim of new breeding methods is to combine the superior quality characteristics of

traditional varieties with the high yielding power and resistance of modern varieties so that the final product contains the best of both groups (Zaghum et al., 2022, Lou et al., 2026).

1.1.2.7 Traditional Italian varieties as living genetic resources

The importance of traditional rice varieties in Italy is due to two fundamental factors: the preservation of cultural heritage and the exploitation of valuable genetic resources. These varieties are the result of centuries of selection by local farmers based on the climatic conditions and food tastes of the region. Furthermore, traditional varieties possess specific genetic characteristics that will be very useful in future breeding, especially to adapt to climate change and new market needs. Accordingly, the conservation of these varieties is not simply a symbolic act, but a vital strategy to maintain agricultural viability and crop diversity in the future (Spada et al., 2004).

Traditional Italian varieties play a significant role in the identity of the country's food products. Their physical characteristics, their reaction to industrial processes and their unique flavor make it possible to produce special products that are often of greater economic value. In fact, this varietal diversity is the link that links agriculture, food science and the preservation of indigenous culture (Vici et al., 2021).

1.2 Chinese Originario

Originario (also known as “Chinese Originario”) was imported from China or Japan in the early 1900s . In particular Chinese Originario also called 105/Originario was introduced in 1904 in Italy, it was characterized by a tall plant height and round grain shape, with high yield and strong resistance to blast. For decades, it represented the primary genetic material for Italian rice cultivation, becoming the progenitor of numerous modern varieties. Chinese Originario had great success in the Vercelli area: it was the most cultivated variety until the 1960s, as stated in many technical reports, and it has been used in breeding programs for many years. Chinese Originario is one of the historical varieties preserved in Italian germplasm banks and is used as a historical reference for biodiversity.

This is supported by long-term genebank conservation programs that have maintained rice genetic diversity for over two decades (Sansoni et al., 2024). Molecular marker studies have further revealed considerable genetic divergence between Italian and Asian rice germplasm, tracing a distinct evolutionary origin for Italian traditional varieties (Faivre-Rampant et al., 2011, Cai et al., 2013). More recently, historical Italian rice accessions preserved in these germplasm banks have also been characterized for valuable disease-resistance traits, including resistance to *Fusarium* species (Ghidoli et al., 2026).

1.2.1 Agronomic and technological characteristics of the Originario group

In the Italian rice classification system, the “Originario” group is defined based on the agronomic and technical characteristics of the grain. Studies show that the identification and differentiation of Italian varieties is not limited to their name or cultivation area, but criteria such as grain size and shape, endosperm quality, cooking method, amylose content, grain hardness after cooking and consumer taste compatibility play a key role in this classification (Simonelli et al., 2017, Tao et al., 2019, Yin et al., 2023).

From a historical and agronomic perspective, the study by Russo & Callegarin (2007) shows that Italian consumers have traditionally preferred round, chalky grains. For this reason, Italian indigenous varieties are mainly classified as japonica, which usually has shorter or medium-grained grains and a soft, crumbly

texture after cooking. In contrast, Indica-type rice are known for their elongated, non-sticky grains, which are more suitable for specific export markets. This classification is of great importance because the “Originario” group, as part of the Italian agricultural heritage, is part of the same genetic and technical characteristics as the japonica type and is completely different from the long and dry-cooked Indica type rice. Improving grain quality is one of the main axes of rice breeding programs in Italy; therefore, in the selection of breeding parents, special attention is paid to the amylose content and its determining role in the texture and cooking characteristics of the grain (Russo & Callegarin, 2007).

According to existing research, the molecular structure of starch and the ratio of amylose to amylopectin are the main factors determining the textural characteristics and cooking quality of rice (Tao et al., 2019). This fact is very important from a technological point of view, as it shows that the quality of Italian rice is not only influenced by agronomic factors, but also that the chemical structure of the grain, in particular the proportions of the starch components, plays a fundamental role in determining its commercial value and consumption pattern. In such a framework, the “Originario” group should be considered not only as an agronomic type, but also as a “technological type” whose quality is evaluated based on the degree to which it complies with the desired cooking and consumption criteria (Russo & Callegarin, 2007).

The cooking quality of rice, especially in the Originario group, is strongly influenced by biophysical parameters such as gelatinization temperature and alkali diffusion rate (Russo & Callegarin, 2007). Any differences in the consumption characteristics of this group, such as stickiness and texture of the final product, are due to differences in the molecular structure of the starch and its behavior under cooking conditions. Therefore, the cooking behavior of Originario varieties can be considered a function of the functional properties of their starch and gelatinization mechanisms, which makes this group a distinct technological identity in the rice classification system (Russo & Callegarin, 2007, Tao et al., 2019).

In a study by Simonelli et al. (2017), ten Italian rice cultivars were evaluated from chemical, physical, textural and sensory aspects and emphasized the necessity of multidimensional evaluation of cultivars. This

study showed that there is a significant correlation between sensory evaluations and instrumental data. Such an approach is of great importance for the Originario group, because a rice group is not identified by laboratory measurements of one or two characteristics; rather, a set of characteristics such as hardness, stickiness, consistency, grain appearance and sensory acceptance must be simultaneously examined. Especially in the case of traditional varieties that are deeply linked to indigenous culinary patterns, sensory quality can be the most decisive criterion for evaluating the final value of the product, even beyond chemical indicators, since the consumer experience is ultimately the main arbiter of the quality and commercial desirability of rice (Simonelli et al., 2017).

A study by Tao et al. (2019) investigated the relationship between starch structure and rice texture. According to this study, increasing amylose content and changing amylopectin structure caused rice to become stiffer after cooking, which reduced its acceptability to consumers. In addition, characteristics such as stickiness, cohesion and softness play a major role in the quality assessment of rice, while excessive hardness is a significant negative factor in product acceptance (Tao et al., 2019).

A review by Yin et al. (2023) also supports this view. According to this study, the texture of cooked rice is the result of the simultaneous interaction of starch, protein, and cell wall and does not depend on a single chemical property. The authors emphasize the importance of structural changes at different scales, thermal behavior and the way the endosperm components react during cooking (Yin et al., 2023). A study by Sultana et al. (2022) shows that in modern rice research, quality assessment is mainly based on a set of objective and standardized indicators. For this reason, concepts such as grain uniformity, visual characteristics, cooking indices and microstructural parameters should be used to analyze each group of varieties, including Originario (Sultana et al., 2022).

1.3 The Italian rice supply chain

The Italian rice supply chain, one of the most organized production networks in Europe, goes beyond on-farm production and encompasses an integrated set of processing, quality control, marketing and regulatory institutions. A descriptive study of this network is important because all stages of production, processing and governance are directly linked. In this regard, the Italian rice research literature can be categorized into four main axes: “market structure and competition”, “governance system and quality certification”, “environmental issues and sustainability” and “post-harvest management, storage and pest control” (Dara Guccione et al., 2021, Trematerra, 2009).

One of the key dimensions of the rice supply chain in Italy is its strategic position in the market and national production. Existing research shows that it is not possible to analyze this market without considering the interaction between producers, policy mechanisms and global competition. In this context, the Ente Nazionale Risi plays an effective role in regulating, monitoring and stabilizing the market, acting as an intermediary between producers, policymakers and commercial actors. This chain is influenced not only by domestic production, but also by export and import flows and international economic pressures, so that the interaction between production, trade and policy plays a decisive role in the formulation of quality strategies, branding and product differentiation. Indeed, in Italy, rice is more than a simple agricultural product, it is a strategic commodity in the structure of the country's food market, its value defined through a network of institutional and commercial relationships (Riccio, 2024).

In addition to market-oriented studies, a detailed understanding of the rice supply chain in Italy requires an analysis of the control, certification and governance system of the chain, especially in the organic production sector. The study shows that the organic rice chain, beyond a simple structure for production

and exchange, is an institutional and regulated space in which consumer trust and the economic value of the product are directly linked to control and certification mechanisms. According to this study, its main objective is to improve control schemes in the organic rice sector through a participatory analysis of the perspectives of different stakeholders, since the chain involves a wide range of actors, including producers, certification bodies, competent authorities and other chain actors and its efficiency depends on the coordination and coherence between these levels. From a descriptive perspective, the challenges of the certification system are not limited to technical aspects, but also include implementation procedures, monitoring guarantees and even the institutional and cultural dimensions of the chain. Thus, the quality and value of organic rice in Italy are the product of a level of trust, coordination and organized compliance that includes all links in the chain (Dara Guccione et al., 2021).

Environmental studies play a crucial role in explaining the place of rice in the Italian supply chain. The study by Blengini & Busto (2009) specifically examines alternative rice production systems using a Life Cycle Assessment (LCA) approach. This study assesses the rice supply chain not only from the perspective of economic or institutional performance, but also as a system with quantifiable environmental consequences. This shows that descriptive studies in the Italian rice sector have gradually moved away from purely market-oriented or production-oriented approaches and towards more integrated analyses that also integrate production, resource consumption and environmental impacts into the chain structure. In fact, each link in the chain, from production systems to management patterns, plays an effective role in moderating or exacerbating environmental impacts (Blengini & Busto, 2009).

From a conceptual perspective, the application of the Life Cycle Assessment (LCA) methodology to the Italian rice supply chain allows for a rigorous comparison of different production systems in terms of sustainability. Rice is considered not simply as a crop, but as a key component of a complex system with measurable environmental consequences (Dara Guccione et al., 2021).

However, any realistic description of the rice supply chain in Italy requires consideration of post-harvest stages, storage and risks from storage pests. Furthermore, experimental studies have assessed the behavioural preferences of a key storage pest towards different Italian commercial rice cultivars; the results of these studies clearly highlight the role of grain quality in attracting or repelling pests (Trematerra, 2009). The post-harvest supply chain enters a critical phase where the quality, quantity and safety of the product are highly dependent on storage conditions and pest management. Therefore, improving the final quality of rice and ensuring its marketability is not limited to measures taken at the field production stage, but is directly influenced by the effectiveness of storage systems and pest control strategies in the post-harvest stages (Dara Guccione et al., 2021). A study by Trematerra (2009) provides important evidence regarding the differences in susceptibility of different Italian commercial rice varieties to storage pests. The results of this study indicate that Superfino and Parboiled varieties are more susceptible to *Sitophilus zeamais*; in contrast, Pre-cooked and Enriched varieties are less susceptible to this pest. The nature of the product and its level of processing are influential variables on storage risks and determine warehouse management requirements. Therefore, the post-harvest phase in the Italian rice supply chain should be considered as an active and strategic element in quality management and risk control (Trematerra, 2009).

1.4 Storage proteins in rice grains

1.4.1 Classification (albumins, globulins, prolamins, glutelins)

Rice grain storage proteins are one of the most important biological components in rice seeds, which, in addition to providing nutritional value, play a decisive role in the physiological processes of grain development, the final quality of the product and the performance indicators of flour and its derivatives. In grain plants, these proteins are the main reservoir of nitrogen, sulfur and essential amino acids for the embryo during the germination stage and are the main supplier of metabolic needs for early growth before the seedling reaches the necessary capacity to absorb nutrients from the environment (Shewry et al., 1995).

In rice, similar to other cereals, storage proteins are mainly accumulated in the endosperm tissue; a structure that accounts for most of the grain volume and is known as the main storage site for carbohydrate (starch) and protein compounds (Yang et al., 2023).

Rice proteins are important not only for understanding the biology of the grain, but also for human nutrition. Since rice is the staple food for billions of people around the world (especially in Asia), its protein quality has a direct impact on people's health (Amagliani et al., 2017b, Amagliani et al., 2017a). Although the amount of protein in rice is not very high compared to some other grains, the type and structure of these proteins determine how easily rice is digested, its nutritional value, how it reacts when cooked and ultimately how our bodies react after consuming it. For this reason, a detailed understanding of the types of rice proteins is essential to understand the chemical properties, nutritional value and even quality improvement of this crop in breeding programs (Amagliani et al., 2017a, Jayaprakash et al., 2022).

The classical classification of grain storage proteins based on solubility properties places them into four main groups: albumins, globulins, prolamins and glutelins (Chandi & Sogi, 2007, Shewry et al., 1995). Although initially proposed solely on the basis of the solubility of proteins in chemicals, this four-fold classification is now used in the structural, molecular and evolutionary fields as the most accurate and valid method for studying rice storage proteins (Yang et al., 2023). In rice grain, these four groups are present simultaneously, but they are not completely identical in terms of quantity, structure, localization and biological role. The transport and accumulation of storage proteins in rice endosperm is carried out through complex intracellular transport pathways and the vacuolar system (Zhu et al., 2021). Among these groups, glutelins are the most abundant proteins, accounting for about 70–80% of the total grain protein (Jukanti et al., 2025). Prolamins follow, while albumins and globulins usually contribute less (Jayaprakash et al., 2022, Amagliani et al., 2017a). However, being small in number does not mean being insignificant and each of these groups can play specific roles in the nutritional quality, grain physiology and endosperm cellular organization (Yang et al., 2023). Storage proteins are synthesized during the stages of grain development and serve as a source of nitrogen required for germination and early plant growth (He et al., 2021).

From a developmental perspective, the synthesis and accumulation of storage proteins in rice occurs mainly during grain filling, a period when the genes encoding these proteins are specifically activated and controlled by a complex network of transcription factors, signaling pathways and environmental conditions (Yang et al., 2023, He et al., 2021). In other words, the final composition of the grain protein fractions is not only the result of the genetic program, but also a reflection of the interaction between genotype, development and environment (Jayaprakash et al., 2022). Therefore, the classification of storage proteins should be considered simultaneously at the chemical, cellular, physiological and nutritional levels. Variation in some genes associated with storage proteins, including the PDIL1-1 gene, can affect the assembly and organization of rice grain proteins (Hori et al., 2022, Liu et al., 2023). Transcriptomic studies have shown that changes in the expression of storage proteins can affect the expression pattern of several genes in rice grain (Kim et al., 2021, Pham et al., 2024).

1.4.1.1 Albumins

Albumins are classically classified as a group of water-soluble proteins that constitute a relatively small fraction of the total protein in rice grains (Amagliani et al., 2017a, Guzmán-López et al., 2025). Despite their lower abundance compared to the glutelin and prolamin fractions, albumins are of considerable importance in terms of nutritional aspects and biological functions. In many plant species, including rice, albumins do not act solely as storage proteins; rather, they can comprise a complex of soluble proteins with diverse functions, including enzymes, enzyme inhibitors, defense proteins and regulatory factors. This special feature places albumins in a unique position at the border between “storage proteins” and “biologically active proteins” and pushes their study beyond quantitative evaluation to qualitative and functional aspects (Shewry et al., 1995, Yang et al., 2023).

Albumins, despite their small contribution to total grain protein, play a key role in determining the nutritional value of the crop (Amagliani et al., 2017b). This fraction is particularly important because

albumins contain a more balanced pattern of essential amino acids and are more digestible than other storage proteins (Amagliani et al., 2017a). Accordingly, even with a small mass fraction in total protein, the presence of albumins can improve the biological quality of rice protein. In nutritional analyses, a distinction should always be made between “total protein quantity” and “protein quality” because the role of albumins is not to increase the mass fraction, but to improve the qualitative indicators and nutritional value of protein (Amagliani et al., 2017b, Amagliani et al., 2017a).

From a structural and evolutionary perspective, a recent review suggests that albumins are likely to share evolutionary links with prolamins and have undergone significant structural diversification during the evolution of seed plants (Yang et al., 2023). These findings suggest that albumins should not be considered a simple subgroup, but rather represent part of a key evolutionary pathway in plant storage proteins. On the other hand, due to their lower molecular weight and higher solubility, this group of proteins exhibits distinct behavior during extraction, processing and digestion (Amagliani et al., 2017a).

At the cellular level, albumins have a more diverse distribution pattern in the grain tissue, unlike some major storage proteins that are deposited in specific organelles (protein bodies). This heterogeneous distribution complicates the process of their identification and quantification in proteomic and chemical studies (Amagliani et al., 2017b, Jayaprakash et al., 2022). However, the presence of albumins, along with other fractions, constitutes an important part of the balance of the endosperm protein network. Under certain conditions, any change in the accumulation rate of the major fractions can affect the relative contribution of albumins, indicating the existence of some kind of interaction and regulatory mechanism in the synthesis pathways of grain proteins (Yang et al., 2023).

From a practical perspective, albumins have a special place. Given the higher digestibility and more balanced amino acid profile of this fraction, increasing its relative contribution or improving its composition is considered desirable from a human nutritional perspective (Amagliani et al., 2017a). However, any intervention in the seed regulatory network may affect the homeostatic balance between different protein

fractions; therefore, corrective measures to improve protein quality require a deep understanding of the interactions between albumins and other storage proteins (He et al., 2021).

1.4.1.2 Globulins

Globulins are a class of storage proteins that are classically soluble in dilute salt solutions and are recognized as one of the main forms of nitrogen storage in many seed plants (Lee et al., 2015, Shewry et al., 1995). However, in rice and some other cereals, the contribution of this fraction is more limited compared to glutelins and prolamins and is classified as minor proteins (He et al., 2021, Jayaprakash et al., 2022). Despite this quantitative contribution, the position of globulins in structural and evolutionary studies is important because this group of proteins has a long history in seed evolution and in many plant species, they constitute the dominant form of storage proteins. Globulins are among the most ancient families of storage proteins in seed plants from an evolutionary perspective (Yang et al., 2023). In other words, the small quantitative contribution of globulins in rice grain does not necessarily mean a marginal role for this fraction, but rather reflects an evolutionary shift in cereals in favor of the dominance of other fractions such as prolamins and glutelins (Shewry et al., 1995).

Structurally, globulins are often identified as multi subunit proteins with a regular spatial organization that are transformed into their final structure by precise biosynthetic, folding and post-translational processes (Shewry et al., 1995). This structural complexity extends the role of this fraction beyond a purely passive reservoir of nitrogenous substances; in many plant species, globulins, in addition to their nutritional function, also affect the physical properties and stability of the seed storage structure. In the case of rice, although the quantitative contribution of globulins is limited, their presence is considered a key component in the molecular and functional organization of the endosperm (Jayaprakash et al., 2022).

Recent scientific evidence suggests that in rice grain, the reduction in the accumulation of specific fractions such as globulins can be compensated by the increase in other protein groups (Yang et al., 2023). This

phenomenon is of great importance, as it indicates the existence of compensatory mechanisms in the process of storage protein synthesis. Accordingly, the different protein fractions do not act in isolation, but rather as part of an interconnected and intertwined regulatory network. Therefore, any change caused by genetic factors or environmental conditions that causes fluctuations in the number of globulins can indirectly change the quantitative and qualitative dimensions of other protein fractions (He et al., 2021).

From a nutritional point of view, globulins are of higher value in some plants due to their better quality of amino acids than proteins rich in proline and glutamine. In rice, although their contribution is limited, they cannot be ignored in a comprehensive assessment of the protein quality of the grain (Amagliani et al., 2017a, He et al., 2021). Furthermore, in proteomic and compositional studies, the identification of globulins is essential for accurately mapping the protein profile of the grain (Amagliani et al., 2017b, Jayaprakash et al., 2022). Consequently, globulins should be considered part of the molecular architecture of rice protein storage, which, although not quantitatively dominant, has high scientific value from a developmental, regulatory and comparative perspective (Shewry et al., 1995, Yang et al., 2023).

1.4.1.3 Prolamins

Prolamins, as one of the most important storage proteins in cereals, account for a significant proportion of total grain nitrogen in many members of the Poaceae family (Shewry et al., 1995). This group, which is distinguished on the basis of solubility in aqueous alcohols, is known for being rich in the amino acids' proline and glutamine. In rice, prolamins are the second major fraction of storage proteins in the endosperm after glutelins. The importance of these proteins in rice is not limited to their amounts, but they have a special place in studies related to grain proteins due to their participation in the organization of protein bodies, their influence on the physical properties of the grain and their nutritional quality (Amagliani et al., 2017a, He et al., 2021, Jayaprakash et al., 2022).

Prolamins are a highly diverse family of proteins with specific structural domains, repetitive sequences and distinct patterns of evolutionary divergence (Yang et al., 2023). This protein family has followed different evolutionary paths to specialization in various cereals, with rice being a prime example of this diversity. Although prolamins account for a smaller proportion of the total protein stores in rice compared to cereals such as maize, they are still essential components of the grain storage structure (He et al., 2021).

From a cellular perspective, one of the most important features of rice prolamins is their assembly into structures called protein bodies (Yang et al., 2023, He et al., 2021). These protein bodies are formed in the developing endosperm and are specialized sites for the deposition, condensation and storage of a portion of the grain proteins. The protein bodies are located adjacent to starch granules and form part of the complex spatial organization of the grain storage tissue (Yang et al., 2023). This spatial arrangement is important for both efficient nitrogen storage and can indirectly influence grain processing and ripening behavior (Jayaprakash et al., 2022).

A study by Yamagata et al. (1982) on developing rice grains showed that the synthesis of storage proteins is a timed and stepwise process. Similarly, prolamins are synthesized and accumulated during specific stages of grain development; this accumulation occurs in a coordinated manner with endosperm filling and gradual grain maturation (Yamagata et al., 1982). A study by He et al. (2021) on the biosynthesis of rice storage proteins emphasizes that the accumulation of these proteins is controlled by a complex network of genes, transcription factors and environmental conditions. Thus, the final amount of prolamins is not a function of a single gene or developmental stage, but rather the result of extensive interactions between different molecular pathways (He et al., 2021).

Mutations in genes involved in the production of prolamins can alter the composition of proteins and how they are stored in the rice grain (Guzmán-López et al., 2025). Nutritionally, prolamins are often problematic because of their specific amino acid composition; they often lack essential amino acids such as lysine, which reduces overall protein quality (Amagliani et al., 2017a). In rice, the ratio of prolamins to other

proteins, especially glutelins, is an important indicator of the nutritional quality of the grain (Amagliani et al., 2017b, Jayaprakash et al., 2022).

1.4.1.4 Glutelins

Glutelins are the most important and abundant fraction of storage proteins in rice grain and are often considered the dominant endosperm protein (He et al., 2021, Jayaprakash et al., 2022, Amagliani et al., 2017a). Based on their extraction properties, this group is extracted in alkaline solutions or in the presence of solubilizing compounds. From a structural perspective, glutelins usually originate from precursors that, after synthesis, undergo complex post-translational processing to become active functional subunits (Shewry et al., 1995, Yamagata et al., 1982). The predominance of glutelins over other fractions is one of the distinguishing features of the protein profile of rice compared to some other cereals (He et al., 2021, Jayaprakash et al., 2022).

Glutelins not only account for the largest share of total grain protein, but also play a crucial role in influencing functional and nutritional properties (Amagliani et al., 2017b). Given that this group constitutes the most abundant protein reserve in rice, any change in its amount, structure, or subunit pattern can significantly affect the final grain quality. In proteomic studies, differences in the expression and accumulation patterns of glutelins have been linked to changes in grain quality, endosperm composition and nutritional properties. Thus, glutelins occupy a privileged position among rice storage proteins, both in terms of quantity and functional impact (Jayaprakash et al., 2022).

In the biosynthesis process, rice glutelins are synthesized as preproteins or large precursors and after entering the cell secretory pathway, are converted to their mature form through folding, transport and proteolytic cleavage processes (He et al., 2021). A study by Yamagata et al. (1982) showed that the synthesis of storage proteins in developing grains is a tightly controlled process and depends on the developmental stage. He et al. (2021) also emphasized that the biosynthesis pathway and accumulation of

glutelins are influenced by genetic and environmental factors and changes in temperature, growth conditions, or plant nutritional status can affect their accumulation (He et al., 2021). In recent years, CRISPR/Cas9 technology has been used to create targeted mutations in glutelin genes and change the protein composition of rice grains (Alhusnain et al., 2024).

From a cellular perspective, glutelins accumulate in specific endosperm storage structures and together with prolamins, play a role in the formation of protein bodies and the spatial organization of protein storage (He et al., 2021). This organization is biologically important because the way proteins are packaged and stored can affect their stability during the dry period of the seed and the speed of their mobilization during germination (Yamagata et al., 1982, Yang et al., 2023). In addition, the pattern of glutelin accumulation can also affect some physicochemical properties of the grain, because the ratio of protein to starch in the endosperm is one of the factors determining the processing behavior and texture of the final product (Amagliani et al., 2017a, Jayaprakash et al., 2022).

Nutritionally, glutelins, although the predominant protein in rice, are not the only measure of protein quality. Their high abundance makes them a major contributor to total protein content, but the biological quality of the final protein still depends on the amino acid composition and their ratio to other fractions (Amagliani et al., 2017b). However, since glutelins are the most abundant, any improvement in their structural or amino acid quality can have a significant impact on the nutritional quality of rice (He et al., 2021, Jayaprakash et al., 2022). In cereals such as rice, the expression of genes encoding storage proteins is specifically induced during grain filling and glutelins play a major role in the transcriptional pattern and final accumulation (Yang et al., 2023). Editing glutelin genes can affect not only the composition of grain proteins but also the accumulation of storage materials and grain properties (Chandra et al., 2023). Glutelins are at the heart of the endosperm developmental program. Thus, glutelins are not only the most dominant protein fraction in rice, but also one of the most important elements in the storage biology of this grain (He et al., 2021).

1.4.2 Biological and nutritional role

Rice grain storage proteins are among the most important determinants of the biological, physiological and nutritional quality of this grain. These proteins play a fundamental role not only at the plant level in providing the necessary materials for continued embryo and seedling growth after germination, but also at the human nutritional level, they are considered to be the main determinants of the protein value of rice (Chen et al., 2018, Kim et al., 2013). Given that rice is the staple food of a large part of the world's population, the importance of its protein composition is directly related to food security, diet quality and even plant breeding programs (Zhao et al., 2021, Li et al., 2023). From the perspective of seed biology, storage proteins accumulate mainly in the endosperm during development and remain as a concentrated source of nitrogen, carbon and amino acids for the post-germination period at the end of seed maturity. In fact, the rice seed at maturity enters a state in which it must be able to maintain the reserves necessary to resume metabolic activity in its structure until germination. In this process, storage proteins act as strategic reservoirs and during controlled degradation during the early stages of seedling development, provide the raw materials required for the synthesis of enzymes, structural proteins, regulatory compounds and other vital molecules (Lee et al., 2015, Jukanti et al., 2025).

The biological role of these proteins goes beyond providing food for the embryo and depends on how they are organized into protein bodies, the time and place of synthesis and their interactions with other cellular components. In rice, storage proteins are assembled into specific protein bodies, the formation, stability and composition of which are important for the health of the endosperm tissue. Any change in the balance between different protein groups can alter the structure of these bodies and affect the cellular arrangement, stability of the stores and even the physical properties of the grain (Kim et al., 2013, Lee et al., 2015).

From a human nutrition perspective, the importance of rice storage proteins is mainly due to the fact that this grain contributes a high proportion of daily energy intake in many societies and is also an important source of protein intake in some populations. However, the nutritional value of rice protein does not depend solely on the total amount of protein in the grain, but also on the composition of protein fractions, the type of amino acids, their location in the protein structure and their digestibility, which are also factors determining the final quality. In other words, two rice varieties may not differ significantly in terms of total protein content, but they may differ significantly in terms of nutritional quality, because the predominant protein type, the ratio of different protein groups and the amount of essential amino acids in them can differ (Zhao et al., 2021, Li et al., 2023).

A study by Zhao et al. (2021) showed that glutelins comprise about 80% of the storage proteins of rice seeds. Furthermore, this study showed that not all types of glutelins are nutritionally equivalent and type B glutelins are richer in lysine content than type A. This difference is an important point in assessing the protein quality of rice, because lysine is one of the limiting amino acids in cereals and its deficiency reduces the biological value of dietary protein. Therefore, the difference in the ratio between different types of glutelins could be one of the determining factors of the nutritional quality of rice (Zhao et al., 2021).

In contrast, prolamins are generally less nutritionally desirable. Due to their structural characteristics, this group of proteins is usually lower in essential amino acids, especially lysine and in many cases is less digestible (Zhao et al., 2021, Li et al., 2023). Because prolamins aggregate in protein bodies and have a dense, hydrophobic structure, they are difficult for digestive enzymes to access. . This reduces the body's ability to utilize this protein fraction. Therefore, in nutritional analyses, a high proportion of prolamins usually limits protein quality. A study by Kim et al. (2013) showed that reducing 13-kDa prolamins in rice improved the nutritional value. In this study, inhibition of this type of prolamins caused a change in the overall composition of storage proteins and through compensatory mechanisms, increased the amount of glutelins and some other protein fractions. In this study, the lysine content of the modified grains was reported to be about 28% higher than the control. This increase is not merely a minor or marginal change,

but has important nutritional implications. Lysine is a limiting nutrient in many cereals and any increase in its richness can improve the biological value of the protein consumed. The reduction in prolamins also affects not only nutritional aspects, but also changes the biological organization of storage proteins and the pattern of protein body formation. By purposefully adjusting the composition of storage proteins, it is possible to improve the nutritional quality of the grain without necessarily increasing the total protein content. This is of great importance for plant breeding and genetic engineering programs, since the ultimate goal in many of these programs is not to increase the quantity of protein, but to improve its quality in terms of human nutritional needs (Kim et al., 2013).

Along with glutelins and prolamins, globulins are also of biological and nutritional importance. Their contribution to rice is not as great as that of glutelins, but their presence is important in the balance of grain protein storage. Lee et al. (2015) have shown that these proteins play a role in the normal process of storage protein accumulation and possibly in the stability of the internal organization of the endosperm. Globulins are part of the regulatory network of grain development and the organization of protein stores. From a nutritional perspective, globulins generally have a more balanced amino acid composition than prolamins and are considered higher-quality fractions in many plants. Consequently, although their amounts are lower in rice, their presence can contribute to improving the nutritional balance of protein (Lee et al., 2015, Li et al., 2023).

Albumins, although a minor fraction in rice, are of considerable nutritional value. This group of proteins is usually more soluble and due to their structure, has a higher digestibility. Their amino acid composition is also often more balanced than prolamins. Although the amount of albumin in rice is low, this small amount can also help improve the overall quality of the protein. The final value of dietary protein is not only a function of the predominant fraction, but also low abundance but high-quality groups can influence the final quality (Li et al., 2023).

One of the most important aspects of the nutritional role of storage proteins is the issue of digestibility and bioavailability of amino acids. A protein may theoretically contain desirable amino acids, but if its structure is such that it is resistant to digestive enzymes, its practical nutritional value will be reduced (Jukanti et al., 2025). A study by Zhao et al. (2021) showed that the storage and nutritional quality of proteins cannot be analyzed without considering the three-dimensional structure, molecular interactions and changes during storage or processing. This means that the nutritional role of rice proteins is not only a function of their initial chemical composition, but also depends on their shape and packaging in the grain (Zhao et al., 2021).

In this context, the role of protein bodies in determining nutritional quality is crucial. In rice, prolamins are mainly stored in type I protein bodies, while glutelins are mostly found in type II protein bodies. This difference in location and structure can affect how these proteins are digested (Kim et al., 2013). Protein bodies that contain prolamins are usually denser and more resistant which limits the access of digestive enzymes. In contrast, proteins that are stored in more accessible or less dense structures are more digestible (Zhao et al., 2021).

Omics technologies have made significant advances in the investigation of genetic and molecular pathways affecting the composition of storage proteins and the nutritional quality of rice. From a genetic perspective, variation in the composition of storage proteins has great potential to improve the nutritional quality of rice (Zaghum et al., 2022). A study by Chen et al. (2018) showed that genetic differences between genotypes can influence the pattern of accumulation of these proteins. This is of great importance for the development of cultivars with higher nutritional quality, as it allows breeders to pursue desirable traits such as reduced prolamins, increased lysine-rich glucoretins, or improved balance between protein fractions within the framework of genetic programs (Chen et al., 2018).

At the physiological level, it is essential that the role of storage proteins in the grain is not considered as a static and unchangeable factor. These proteins undergo qualitative and quantitative changes in close relation to environmental conditions during growth, the grain maturation process and even post-harvest storage.

Studies have shown that storage conditions, together with the structural changes that proteins experience over time, can significantly influence the final quality of rice (Zhao et al., 2021). These potential changes can have significant effects on enzymatic accessibility, protein-starch interactions and consequently on the nutritional quality and sensory characteristics of the final product (Zhao et al., 2021, Jukanti et al., 2025).

From a practical perspective, one of the most important points in assessing the protein quality of rice is that the total protein content is not a sufficient indicator for judging nutritional value. A rice variety may have higher total protein but, due to the predominance of lower quality fractions such as some prolamins, have lower nutritional value than a variety with lower total protein but a more balanced composition (Kim et al., 2013, Li et al., 2023).

The nutritional role of rice storage proteins is meaningful in the context of the general human diet. Rice is commonly consumed with other foods and in mixed diets, deficiencies in some of its amino acids can be partially compensated for by other food sources. However, in societies with a high dietary reliance on rice, any improvement in the intrinsic quality of its protein has a significant impact on the nutritional status of the population (Li et al., 2023). In such circumstances, increasing essential amino acids, improving digestibility and reducing the contribution of low-quality proteins could be effective in reducing protein malnutrition and improving diet quality. For this reason, improving the composition of rice storage proteins is not only a scientific goal but also a practical priority in the field of food security (Zhao et al., 2021, Li et al., 2023). Rice grain storage proteins have a dual but highly intertwined role. On the one hand, they are essential for the plant itself, as they serve as a reserve source for germination, early growth and survival. On the other hand, for humans, they determine part of the nutritional quality of the most important staple food in many regions of the world (Chen et al., 2018, Lee et al., 2015).

1.4.3 Potential as precursors of bioactive peptides

In addition to their primary role in providing nitrogen and amino acids for embryo development and establishment, rice grain storage proteins are of considerable nutritional, functional and biotechnological importance (Cavazos & Gonzalez de Mejia, 2013, Dei Più et al., 2014). One of the most important aspects is their capacity to act as precursors of bioactive peptides, meaning that protein sequences present in the grain are broken down into smaller fragments by enzymatic hydrolysis, digestion, fermentation, or other technological manipulations that can exert specific physiological activities in the body. Accordingly, the value of rice proteins is not limited to their overall protein content or storage role, but also lies in their potential to release peptides with health-promoting effects (Mojumder et al., 2024, Scarabattoli et al., 2025, Selamassakul et al., 2018).

One of the most important biological activities of dietary peptides is their ability to inhibit angiotensin-converting enzyme (ACE), which can be effective in lowering blood pressure and preventing cardiovascular diseases (Daza-Rodríguez et al., 2023). Some peptides derived from dietary proteins can inhibit the enzyme dipeptidyl peptidase-IV (DPP-IV), which can be effective in the management of type 2 diabetes (Liu et al., 2019). Cavazos and Gonzalez de Mejia's study identified potentially bioactive peptides in cereals such as wheat, oats, barley and rice. This study showed that the proteins of these cereals have a high frequency of sequences associated with various biological activities, including ACE inhibitory peptides, dipeptidyl peptidase inhibitory peptides, antioxidants, antithrombotic, hypotensive and opioid peptides. Rice, along with other cereals, has been reported as a potential source for the production of bioactive peptides and sequences with anticancer potential have been reported for rice proteins (Cavazos & Gonzalez de Mejia, 2013).

The stability of bioactive peptides in the gastrointestinal tract depends on their structural features, including peptide chain length, amino acid composition and peptide sequence (Ahmed et al., 2022). Bioactive peptides can be released during the digestive process or through enzymatic hydrolysis of dietary proteins

(Iametti et al., 2025). Enzymatic hydrolysis of rice bran protein can lead to the production of bioactive peptides with significant antioxidant activity (Adebiyi et al., 2009). Several breeding programs have been developed with the aim of producing rice cultivars with low glutelin content and improving the nutritional properties of the grain (Chen et al., 2023). Various transcription factors are involved in regulating the expression of genes responsible for the synthesis of storage proteins in rice grain (So et al., 2025).

ACE inhibitor peptides can be effective in regulating blood pressure, because angiotensin-converting enzyme is involved in the production of angiotensin II and as a result, increased vascular resistance. Inhibition of this enzyme, in turn, can lead to a decrease in blood pressure and a decrease in the risk of cardiovascular diseases. Antioxidant peptides can also play a role in neutralizing free radicals and reducing oxidative stress. This process is of fundamental importance in the prevention of cell damage and many chronic diseases. The presence of antithrombotic, opioid and DPP inhibitor sequences also indicates that cereal peptides can have diverse functions in the regulation of physiological systems (Cavazos & Gonzalez de Mejia, 2013).

From a structural perspective, this potential is dependent on the composition and organization of the rice protein fractions. Each of these fractions can carry sequences that can be converted into active peptides upon hydrolysis. Therefore, the capacity of rice to produce bioactive peptides depends not only on the total protein content but also on the molecular characteristics and sequence of the amino acids present in each fraction. The distribution of hydrophobic, aromatic, charged and branched amino acids, together with the type of available linkages, determines which peptides will be released during hydrolytic processes (Mojumder et al., 2024, Scarabattoli et al., 2025, Selamassakul et al., 2018).

The paper by Dei Più et al. (Dei Più et al., 2014) showed that even a by-product of the rice starch industry can be converted into a source for the production of antioxidant peptides after hydrolysis with enzymes or microbial cells. In this study, hydrolyzed rice protein was investigated using commercial enzymes such as Alcalase, Neutrase and Flavourzyme as well as microbial hydrolysis with *Bacillus* spp. and the results

showed that this route can lead to the production of compounds with high biological activity. This finding is of considerable industrial importance, as it demonstrates that even low-value by-products and side streams of the food industry have the potential to be converted into valuable raw materials for the production of functional peptides (Dei Più et al., 2014). A study by Scarabattoli et al. (2025) also showed that the production of bioactive peptides from rice could be industrially scalable and applicable.

The study by Mojumder et al. (2024) also highlights the multifunctional nature of bioactive peptides, focusing on antioxidant, antidiabetic and antihypertensive activities. This study showed that peptides derived from the hydrolysis of dietary proteins may not have a single effect, but can simultaneously affect multiple physiological pathways. For example, an antioxidant peptide can be effective in reducing oxidative stress and indirectly contribute to improving glucose metabolism and cardiovascular protection (Mojumder et al., 2024). On the other hand, the study by Selamassakul et al. (2018) emphasizes the necessity of moving from the “general activity identification” stage to the “active molecule identification” stage. In fact, until the peptide responsible for the biological activity is precisely identified, the relationship between its structure and function will not be fully elucidated. This is also crucial for rice proteins, because only by precisely identifying the sequences responsible for the activity can their latent potential be exploited in the design of functional products (Selamassakul et al., 2018).

From a nutritional perspective, bioactive peptides derived from rice storage proteins may contribute to improving human health indicators. Some of these peptides may be effective in lowering blood pressure, others in inhibiting oxidative processes and some in regulating glucose metabolism or reducing inflammation (Cavazos & Gonzalez de Mejia, 2013, Mojumder et al., 2024, Selamassakul et al., 2018). Thus, rice and its protein products can be considered beyond a mere energy source and can be evaluated as a platform for the production of compounds with health-related value. This perspective is especially important in a situation where the food industry seeks to more fully utilize resources, reduce waste and convert by-products into high-value-added products (Scarabattoli et al., 2025).

It should also be noted that the potential for producing bioactive peptides is not a static and fixed characteristic, but depends on several factors such as the type of protein, the extraction method, the hydrolysis conditions, the type of enzyme and even the pretreatments applied to the raw material. Therefore, rice protein in its natural state may not have obvious biological activity, but after controlled hydrolysis it can become a rich source of active peptides. This is very important from the point of view of food technology, because it shows that with the appropriate design of the extraction and hydrolysis processes, innovative functional products can be produced from common plant sources (Dei Più et al., 2014, Selamassakul et al., 2018).

1.5 Bioactive peptides in rice

Among plant sources, rice is of particular importance as one of the most widely consumed foods in the world. In addition to carbohydrates, rice grain contains a set of storage proteins including glutelins, prolamins, globulins and albumins, each of which plays an important role in nitrogen storage and plant embryo development (Amagliani et al., 2017b, Amagliani et al., 2017a, He et al., 2021, Shewry et al., 1995). These proteins are not only important in terms of nutritional value, but can also serve as precursors for the production of bioactive peptides. Genetic and biochemical studies have shown that the structure and distribution of these proteins in rice endosperm are complex and regulated by multiple biological and genetic pathways (Chen et al., 2018, Yamagata et al., 1982). Furthermore, changes in the expression of genes involved in the synthesis of storage proteins can alter the composition and functional properties of these proteins (Kim et al., 2013, Lee et al., 2015).

Rice bran has also attracted considerable attention as one of the richest parts of the rice grain in terms of bioactive compounds. This part of the grain contains significant amounts of proteins, lipids, dietary fiber and phenolic compounds, making it a suitable source for the extraction of bioactive compounds (Amagliani

et al., 2017a, Sultana et al., 2022). Various studies have shown that enzymatic hydrolysis of proteins in rice bran can lead to the production of peptide fractions with diverse biological activities. For example, Scarabattoli et al. (2025) showed that sequential hydrolysis of defatted rice bran using proteolytic enzymes such as Alcalase and Flavourzyme, followed by fraction separation by ultrafiltration, resulted in the production of peptides with molecular weights below 1 kDa. These peptide fractions showed significant inhibitory activity against ACE (>85%) as well as significant antioxidant capacity in DPPH and ABTS assays (Scarabattoli et al., 2025).

In addition, some studies have succeeded in identifying specific peptide sequences responsible for biological activities in rice protein hydrolysates. Adebiyi et al. (2009) identified several peptides with significant antioxidant activity by enzymatic hydrolysis of rice bran protein by pepsin followed by chromatographic separation. Among these sequences, the peptide Tyr–Leu–Ala–Gly–Met–Asn was shown to have significant free radical scavenging ability (Adebiyi et al., 2009). Rice protein-derived peptides may play an important role in reducing oxidative stress. This phenomenon is associated with aging processes and many chronic diseases, including cardiovascular and metabolic diseases (Zaky et al., 2022).

In addition to white rice and rice bran, pigmented rice varieties have also been investigated as potential sources for the production of bioactive peptides. These varieties usually contain higher levels of bioactive phenolic compounds and phytochemicals, which can also affect the biological properties of protein hydrolysates. Selamassakul et al. (2018) showed that the hydrolysis of proteins extracted from black rice can lead to the production of peptides with significant antioxidant activity, which have the potential to be used in the development of functional foods (Selamassakul et al., 2018).

In recent years, several review articles have also emphasized the broader importance of bioactive peptides derived from food for human health. According to Zaky et al. (2022), peptides derived from food proteins can exhibit a wide range of biological activities, including antioxidant, antihypertensive, anti-inflammatory, antimicrobial and immunomodulatory activities (Zaky et al., 2022). For this reason, these compounds have

increasingly been considered as key components in the development of functional foods, dietary supplements and nutraceutical products (Amigo & Hernández-Ledesma, 2020, Chourasia et al., 2023).

1.6 Enzymatic hydrolysis as a peptide release strategy

One of the best and most effective methods for isolating bioactive peptides from food proteins is enzymatic hydrolysis. This method has received much attention in the fields of food science and technology, nutrition and biotechnology in recent decades. In this method, enzymes that break down proteins (proteolytic enzymes) break the bonds between amino acids in the protein structure. As a result, smaller peptide fragments with lower molecular weight are produced. These resulting peptides can often exhibit various biological properties, such as antioxidant, anti-inflammatory, antimicrobial, antihypertensive and antidiabetic properties (Cavazos & Gonzalez de Mejia, 2013, Chourasia et al., 2023, Zaky et al., 2022). The reason this process is important is that many bioactive peptides exist hidden or “encoded” in the primary structure of proteins and can only display their biological activity when the protein structure is broken in a controlled manner (Iametti et al., 2025, Li-Chan, 2015).

From a biochemical perspective, food proteins are inherently complex molecules with secondary, tertiary and sometimes quaternary structures that limit direct access to their amino acid sequences. By breaking these spatial structures, enzymatic hydrolysis allows for the release of sequences that were previously biologically inactive. As such, this process is not only a degradative method but also a means of potentially activating functional compounds. Many of the health benefits of food proteins are actually due to the peptides released during hydrolysis, rather than the intact protein itself (Amigo & Hernández-Ledesma, 2020, Chakrabarti et al., 2018, Zhu et al., 2023).

From a biotechnological and industrial perspective, enzymatic hydrolysis offers significant advantages over chemical methods. It is performed under milder conditions in terms of temperature and pH, has higher specificity for target linkages and is less likely to destroy bio sensitive compounds or generate unwanted byproducts. Furthermore, the ability to precisely control parameters such as enzyme type, reaction time, temperature, pH and substrate-to-enzyme ratio allows for a highly tailored peptide profile and optimizes the production of targeted peptides (Mora & Toldrá, 2023, Zaky et al., 2022, Zhu et al., 2023).

Among the various enzymes, human digestive enzymes and especially pepsin, are of particular interest in hydrolysis studies. This is because these enzymes are naturally responsible for the digestion of dietary proteins in the body. The use of these enzymes in in vitro experiments simulates natural digestion conditions and provides a more accurate view of how peptides are released in the body (Yang et al., 2025). For this reason, enzymatic hydrolysis is considered in many studies as a bridge between “crude dietary protein” and “active, absorbable peptides” (Amigo & Hernández-Ledesma, 2020, Chakrabarti et al., 2018, Iametti et al., 2025).

In recent scientific research, enzymatic hydrolysis is recognized not only as a simple step in the production process, but also as a smart tool for converting proteins into health-promoting compounds. After enzymatic digestion, plant proteins produce peptides that have important biological properties, such as angiotensin-converting enzyme (ACE) inhibition, DPP-IV inhibition and antioxidant properties. This is doubly important for plant proteins and cereals, because these sources have a high capacity to produce these valuable peptides due to their great diversity in amino acid composition, structural form and location in the food web (Iametti et al., 2025, Zhu et al., 2023). In fact, the three-dimensional structure of the protein, the type and properties of the enzyme used, as well as the conditions of the digestion process, determine which parts of the protein are converted into active peptides. From this perspective, enzymatic hydrolysis is no longer just an extraction method, but an essential tool for “unlocking” the biological potential of food proteins (Tao et al., 2019).

Furthermore, reviews of bioactive peptides have emphasized that assessing the properties of these peptides without taking into account the digestion process provides incomplete and sometimes misleading results regarding their stability and absorption. Peptides produced *in vitro* may undergo changes or even be completely degraded and inactivated during passage through the gastrointestinal tract (Iametti et al., 2025). For this reason, simultaneous examination of *in vitro* hydrolysis and real digestion (or simulated models thereof) is essential to accurately understand the behavior of peptides in the body (Amigo & Hernández-Ledesma, 2020, Chakrabarti et al., 2018).

From an applied perspective, the use of enzymatic hydrolysis in the food industry is a way to produce raw materials or formulations with significantly higher nutritional value and functional properties. Vegetable and cereal proteins, if hydrolyzed under controlled conditions, become a valuable source of peptides beneficial to health (Zaky et al., 2022, Zhu et al., 2023). For this reason, enzymatic hydrolysis plays a key and essential role in the production of functional foods, protein supplements and health-oriented products (Cavazos & Gonzalez de Mejia, 2013, Chourasia et al., 2023).

1.6.1 The role of digestive tract digestion (pepsin)

Digestion in the gastrointestinal tract, especially in the stomach, plays a key role in the release of bioactive peptides from dietary proteins. Pepsin, the main gastric enzyme, is responsible for initiating protein breakdown (Yang et al., 2025). This enzyme, which is activated in the acidic environment of the stomach, breaks down complex protein structures into smaller, more absorbable fragments by breaking specific peptide bonds. These fragments may function as bioactive peptides in the digestive tract or be further hydrolyzed to simpler molecules (Iametti et al., 2025).

The importance of pepsin in studies of bioactive peptides is not limited to its physiological role. One of the main reasons researchers have focused on this enzyme is the attempt to recreate the real conditions of the human digestive tract in *in vitro* digestion models. In these models, simulated gastric digestion using pepsin

and acidic pH adjustment is an important part of the laboratory process. Especially in standard protocols such as INFOGEST, the gastric phase is a key component for understanding the structural changes of proteins and the release of bioactive peptides. Bioactive peptides in the gastric environment can have different fates: some of them are released at this stage, others are unstable to acidic conditions and protease activity and are converted into smaller and even inactive compounds. Therefore, the gastric phase is not only a destructive step, but also a selective and decisive step in the formation of the final peptide profile (Iametti et al., 2025).

Plant proteins can produce a wide range of bioactive peptides during digestion. Since pepsin is the first enzyme involved in the human digestive tract, its role in predicting the release of these peptides is essential. Any comprehensive assessment of the potential for producing functional peptides should also seriously consider the gastric stage, as this stage can determine the initial hydrolysis pattern and consequently, the biological fate of the peptides (Yang et al., 2025).

From a methodological perspective, the use of pepsin as a reference enzyme in in vitro digestion models allows for comparability of results and a move towards standardization of the assessment of bioactive peptides (Iametti et al., 2025). Furthermore, pepsin produces cleavage patterns that are different from other proteases due to its specific properties and interactions with pH and protein structure. This property makes it a suitable tool for investigating the ability to release peptides embedded in food proteins (Yang et al., 2025).

In in vitro digestion models, the gastric phase, in addition to simulating the physiological state of the body, plays the role of an efficient tool for screening and identifying rich sources of bioactive peptides. As has been suggested in systematic reviews of plant proteins, the ability to release these peptides in the digestive tract is directly related to the type of initial food processing. In fact, pepsin and the gastric digestion phase play a productive role in the release and formation of functional compounds beyond simple degradation (Iametti et al., 2025). Pepsin can be considered the starting point of a chain in which dietary proteins are

first broken down into smaller fragments in the stomach, then these fragments are further hydrolyzed in the small intestine by pancreatic enzymes and finally some of them are converted into bioactive peptides with health-promoting effects (Iametti et al., 2025, Yang et al., 2025). Therefore, the role of pepsin is not limited to the production of primary products of digestion, but also affects the quality, quantity and diversity of the final peptides, a matter that is particularly important for sources rich in plant and cereal proteins (Tao et al., 2019, Zhu et al., 2023).

1.6.2 Technological approaches and differences compared to physiological hydrolysis

In recent years, bioactive peptides have become a leading area of research in the food and pharmaceutical industries due to their unique functional properties. These compounds, which are usually released as a result of the controlled breakdown of proteins (by enzymes, microorganisms, or chemical methods), have been shown to have numerous biological roles, including antioxidant properties, blood pressure regulation, diabetes management, inflammation reduction and modulation of immune responses. What makes these molecules particularly valuable is their “latent” nature. This means that these peptides are confined within the primary structure of proteins and only after being released from the protein matrix do they have the opportunity to affect biological targets in the body (Barati et al., 2020).

From a technological perspective, the production of bioactive peptides is often achieved through three methods: enzymatic hydrolysis under controlled conditions (in vitro), microbial fermentation and chemical hydrolysis (Mora & Toldrá, 2023). Each method differentially affects the pattern and severity of bond breakage, the type and size distribution of peptides and consequently the biological properties of the final products. These technological approaches differ in terms of design and controllability compared to physiological hydrolysis, i.e., the natural digestion of protein in the gastrointestinal tract. Physiological hydrolysis is a complex process influenced by a complex set of digestive enzymes (from pepsin and trypsin to carboxypeptidases), physicochemical conditions of the stomach and intestines, and other food

components. For this reason, it is difficult to exert direct control over the protein degradation pathway and achieve the expected results in the body environment (Li-Chan, 2015).

In vitro enzymatic hydrolysis is considered the reference method for the production of bioactive peptides from various proteins. This approach is superior to other methods due to its features such as high specificity, precise controllability and compatibility with industrial production. In this method, specific proteases (such as commercial types of alcalase, flavorase, and digestive enzymes) are used, each of which creates a different pattern of peptides by cutting specific points of the protein chain (based on the characteristics of the amino acids). In fact, the right choice of enzyme (or combination of enzymes) along with careful management of conditions such as temperature, pH and enzyme-to-substrate ratio allows researchers to purposefully design and produce the physicochemical properties of the final peptides (such as chain length, electrical charge and hydrophobicity) (Mora & Toldrá, 2023).

Mora & Toldrá (2023) showed that recent advances in this field are not limited to enzyme selection alone, but also to the development of “process-assisted technologies.” For example, the use of ultrasound-assisted hydrolysis facilitates the penetration of the enzyme into the protein matrix, accelerating the unfolding of secondary and tertiary structures and thus increasing the rate and depth of hydrolysis. Similarly, the use of microwave energy (microwave-assisted hydrolysis) can result in more uniform heating and reduced process time. The use of high pressure, enzymes immobilized on solid substrates and continuous bioreactors are other significant advances in this area, which allow for the continuous and uniform production of protein hydrolysates (Mora & Toldrá, 2023).

The production of peptides with low molecular weight (often below 3 and especially in the range of 500–1500 Daltons) is one of the important achievements of advanced enzymatic hydrolysis that is of great importance. On the one hand, these small peptides have a higher penetration power through the intestinal epithelial barrier and consequently, their bioavailability is improved. On the other hand, the short peptide chain increases their stability against enzymatic degradation in the digestive tract and increases the

likelihood of their transfer to the systemic circulation in an active form. This aspect plays a crucial role in the development of functional foods and nutraceutical products. Because real success in this field is not only the production of the peptide in the laboratory, but also ensuring the delivery of the active compound to the target tissue in the body (Barati et al., 2020).

In vitro enzymatic hydrolysis has another advantage, namely the possibility of multifaceted process optimization. This means that researchers can create an optimal balance between factors such as the degree of hydrolysis, organoleptic properties (e.g., bitter taste), solubility, thermal stability and biological function. The use of “design of experiments” strategies and multivariate optimization methods has greatly helped to precisely guide the hydrolysis process towards the production of desired peptides (Barati et al., 2020). In this regard, the integration of the hydrolysis process with advanced identification techniques such as peptidomics and LC-MS/MS allows for precise monitoring of the products and understanding of their structure–activity relationship (Mora & Toldrá, 2023).

Compared to physiological hydrolysis, the fundamental distinguishing point of technological enzymatic hydrolysis is “controllability”. In physiological hydrolysis (in vivo), parameters such as the type, concentration and sequence of enzyme action, pH and residence time are not under the control of the researcher; whereas in vitro systems allow for the precise definition and reproducibility of these variables. Hence, technological hydrolysis is recognized as a strategy for producing “targeted peptides” and standards, while physiological hydrolysis is mainly used in the form of “tests to assess the stability and pharmacokinetic fate of peptides in the body” (Li-Chan, 2015).

Microbial fermentation, as a natural method for producing bioactive peptides, is carried out through the proteolytic activity of microorganisms such as lactic acid bacteria, yeasts and molds. This process, which plays a key role in the production of traditional products such as yogurt, tempeh and miso, is considered a “biocompatible” and popular approach due to its roots in ancient human experiences. However, from a process engineering perspective, controlling hydrolysis in fermentation is more difficult than in pure

enzyme therapy. Because fermentation systems have more unpredictable dynamics due to metabolic complexities (such as pH changes, production of secondary metabolites and strain coexistence). In practice, the high sensitivity of this process to environmental conditions (temperature, substrate composition and culture density) causes the smallest fluctuations in production parameters to lead to large changes in the peptidome composition and final product quality (Barati et al., 2020).

Barati et al. (2020) showed that although microbial fermentation can produce a variety of bioactive peptides, several key limitations prevent it from being considered a “standard technique” for the production of targeted peptides. First, the lack of the ability to precisely control the degree of hydrolysis and peptide chain length; second, the high variability in enzymatic activity of different strains and even strains of the same species under different conditions; and third, the risk of generating undesirable compounds from a sensory (off-flavors, intense bitterness) or safety (production of biogenic amines in some fermentation systems). Thus, although microbial fermentation is a valuable complementary tool, it is considered less “programmable” and “repeatable” than enzymatic hydrolysis from a technological and industrial perspective (Barati et al., 2020).

Chemical hydrolysis, based on the use of strong acids or bases at high temperatures and times, is a traditional method for protein degradation that has been popular due to its ease of implementation and low cost; however, this method has serious limitations for the production of precise bioactive peptides (Mora & Toldrá, 2023). Mora & Toldrá (2023) emphasize three main shortcomings of this process: first, the lack of “specificity-driven” properties, which leads to the production of a random mixture of small fragments and amino acids instead of targeted peptides. Second, the destruction of essential and sensitive amino acids, which effectively reduces the biological potential and nutritional value of the product. Finally, the formation

of unwanted by-products such as cross-chains or chlorinated compounds makes this method challenging for modern nutraceutical applications from a public health and sensory perspective (Mora & Toldrá, 2023).

Chemical hydrolysis is the opposite of physiological hydrolysis. Chemical hydrolysis is a harsh, non-selective process that is foreign to the biological environment, while hydrolysis in the digestive tract is a precise, gentle and stepwise process based on enzyme activity. Therefore, despite the continued use of chemical hydrolysis in some laboratory analyses (such as determining the amino acid composition of proteins), this method does not meet the necessary standards for the production of bioactive peptides for the development of nutraceutical and functional products and has given way to enzymatic methods (Mora & Toldrá, 2023).

In conclusion, the physiological hydrolysis process in the human digestive tract is a complex, multistep mechanism that proceeds in a self-regulating manner. In the first phase, dietary proteins are denatured in the acidic environment of the stomach (pH 2–3) and broken down into smaller fragments by pepsin. This process is then completed in the small intestine by the action of pancreatic enzymes and peptidases present in the brush border membrane. A precise understanding of the outcome of this process requires consideration of environmental and physiological variables; factors such as food residence time, the presence of other food components in the food matrix, as well as specific host characteristics such as digestive health status, age, and composition of the gut microbial community (microbiota) all affect the function of the enzymes and the pattern of final peptide production (Li-Chan, 2015).

Barati et al. (2020) emphasize that although physiological hydrolysis can “naturally” lead to the release of bioactive peptides, the highly dynamic and unpredictable nature of this system makes it an unsuitable method for “targeted production” of peptides. In other words, a protein may be converted into peptides with specific activity in vitro or in a simple in vitro model, but in the real digestive tract, these peptides may not

be formed at all, may be present only transiently, or may be completely degraded to dipeptides, tripeptides, or amino acids during later stages of digestion (Barati et al., 2020).

Li-Chan (2015) criticizes the current state of research, noting that many of the results on bioactive peptides are based solely on controlled laboratory settings and lack clinical support. According to him, in order to exploit the potential discovered in the laboratory in nutraceutical products, it is necessary to carefully monitor the pharmacokinetic profile of these compounds (including absorption, distribution, metabolism and excretion) as they pass through the human gastrointestinal tract. This view clearly shows that there is a fundamental difference between “technological hydrolysis” (which is a means of optimal production) and “physiological hydrolysis” (which determines the actual fate and stability of peptides in the body) and the two should not be considered equivalent (Li-Chan, 2015).

Common peptide production techniques, such as enzymatic hydrolysis or fermentation, although efficient for initial identification and screening, are not suitable “on their own” to fully simulate gastrointestinal digestion. These methods cannot be considered as suitable techniques to “further stimulate digestion in the gastrointestinal tract,” but should be used in conjunction with standard in vivo or simulated digestion models (such as the INFOGEST protocols) to obtain a more realistic picture of peptide stability and bioavailability (Barati et al., 2020).

The main challenge in this area is to establish a link between the “technological production” and the “physiological fate” of peptides. This means that bioactive peptides must not only be produced in controlled systems, but also must maintain their stability against the complex conditions of internal digestion and reach the target tissue in an active form. For this reason, the current research approach has moved towards the use of integrated models. An approach in which in vitro enzymatic hydrolysis is combined with standard models of simulated digestion of the gastrointestinal tract and using powerful peptidomics tools, bioinformatics modeling (computer simulation) and finally clinical trials, paves the way for achieving health-oriented products (Mora & Toldrá, 2023).

Chapter 2 – Materials and Methods

2.1 Study Design

The main objective of this study is to identify the storage proteins of rice grains and analyze their biochemical and proteomic properties. To achieve this goal, the study was designed based on the step-by-step separation of the main protein fractions and their quantitative and qualitative measurement. For this purpose, after preparing rice grain samples and preparing uniform flour from them, this material was used as the basic substrate for protein extraction and analysis.

In the next step, rice grain storage proteins were separated using sequential extraction method based on the solubility differences of protein groups. This standard approach, which is used to study the protein profile of cereals, allows the separation of the main fractions including albumins, globulins, prolamins and glutelins. For this purpose, each fraction was extracted using specific solvents and in sequential steps to provide a separate and pure medium for proteomic and biochemical analyses.

In the next step, the Bradford colorimetric method was used to quantify the extracted proteins in each fraction. The mechanism of this method is based on the specific binding of the dye Coomassie Brilliant Blue to proteins. Bradford is considered a reference method for measuring protein concentration in laboratory studies due to its advantages such as appropriate sensitivity, ease of implementation and compatibility with the microplate format.

In the next step, the structural features and protein patterns of the samples were evaluated by combining electrophoresis and mass spectrometry. First, the protein profile of the fractions was determined by SDS-PAGE to separate the proteins based on molecular weight. Then, to achieve more accurate identification and analysis of the molecular mass distribution of the proteins, the advanced mass spectrometry technique with matrix-assisted laser desorption/ionization and time-of-flight (MALDI-TOF) was used.

The extracted data were finally processed using statistical software. In order to ensure the validity of the results, statistical analyses were performed to compare the means and determine significant differences between different groups, which paved the way for a more accurate interpretation of the characteristics of rice storage proteins.

2.2 Sample Collection and Flour Production

For the purpose of this study, rice grain samples were collected from several sampling points to provide a representative sample of the studied material. To reduce the effect of possible heterogeneity between samples and increase the generalizability of the results, sampling was carried out from 10 different points. After collection, all samples were combined to obtain a composite and uniform sample that could represent the general characteristics of the studied samples.

To prepare the sample for biochemical experiments, rice grains were first hulled to remove their outer shell. Removing the shell makes the edible part of the grain, which contains the biological compounds under study, available. After this step, the hulled grains were prepared for flour production.

In the next step, the prepared grains were ground using a mixer. The grinding process was carried out in four consecutive steps, each time for 30 seconds. The grinding was carried out in several short-term steps with the aim of preventing excessive increase in the temperature of the sample during the grinding process, because excessive heating can lead to structural changes or destruction of some biomolecules, especially protein compounds. Using this method made it possible to produce flour with relatively fine and uniform particles, while maintaining the stability of the compounds in the sample.

Finally, the prepared flour was collected in suitable containers and stored in suitable conditions until the next experiments. The produced flour was used as a raw material for various laboratory steps.

In addition to the Chinese Originario cultivar, two Italian rice varieties, Roma and Carnaroli, were included in this study for comparative purposes. The hulling, milling, and flour preparation processes for these two cultivars were performed under identical conditions to those described for the Chinese Originario cultivar.

2.3 Protein Extraction

In order to investigate the protein composition of rice grain, storage proteins were extracted from the prepared flour. In cereal grains, proteins are divided into several main groups based on their solubility characteristics, including albumins, globulins, prolamins and glutelins. Each of these groups is soluble in different solvents and therefore can be separated from each other using sequential extraction methods.

In this study, rice grain storage proteins were extracted using a sequential extraction method based on the differences in solubility of different protein groups. This method was based on the protocol presented by Givonetti et al. (2021). In this approach, all protein fractions were extracted from a given amount of flour and after each extraction step, the remaining precipitate was used to isolate the next group of proteins.

In the sequential extraction process, water-soluble proteins (albumins) were first separated from the sample. After separation of this fraction, the remaining precipitate was used to extract proteins soluble in salt solutions (globulins). In the next step, proteins soluble in alcoholic solvents (prolamins) were extracted and finally, proteins soluble in alkaline media (glutelins) were separated from the remaining precipitate.

The use of this method allows for a relatively accurate separation of different protein fractions and is therefore widely used in studies on the composition of cereal storage proteins. The protein extracts obtained from each extraction step were pooled to be used in subsequent steps to determine the protein content and investigate the molecular characteristics of the proteins. Details on sample preparation and the different extraction steps are provided in the following subsections.

2.3.1 Sequential Extraction of Storage Proteins

Sequential extraction of rice grain storage proteins was performed according to the method reported by Givonetti et al. (2021). In this method, different groups of storage proteins including albumins, globulins, prolamins and glutelins were extracted from a given amount of flour in a stepwise manner based on their differences in solubility. At each step, after separation of the soluble fraction, the remaining precipitate was used to extract the next fraction.

In the first step, albumins that are soluble in water were extracted. For this purpose, the flour sample was suspended in ultrapure water at a ratio of 1:10 (w/v). Then, the samples were vortexed for 5 minutes to create a uniform dispersion in the solvent medium. After that, the samples were stirred for 55 minutes at 250 rpm at 4 °C to complete the extraction process. Next, the samples were centrifuged at $12,000 \times g$ for 10 minutes at 4 °C. The supernatant containing the albumin fraction was collected. To remove the remaining albumins, the resulting pellet was washed once with water; the sample was vortexed again for 5 minutes and centrifuged under the same conditions, then the washing solution was discarded.

In the second step, globulins were extracted. For this purpose, the remaining precipitate from the previous step was suspended in a 5% NaCl solution at a ratio of 1:10 (w/v). The samples were first vortexed for 5 min and then stirred at 250 rpm for 55 min at 4°C. After that, the samples were centrifuged at $12,000 \times g$ for 10 min at 4°C and the supernatant containing the globulin fraction was collected. In order to completely remove the remaining proteins, the precipitate was washed once more with a 5% NaCl solution, which included vortexing and centrifugation under the same conditions.

In the third step, prolamins were extracted. For this purpose, the precipitate obtained from the previous step was treated with 60% ethanol at a ratio of 1:5 (w/v). The samples were first vortexed for 5 min and then stirred at 250 rpm for 55 min at room temperature. After that, centrifugation was performed at $12,000 \times g$ for 10 min at room temperature and the supernatant containing the prolamins fraction was collected. 50 µL of this extract was kept for MALDI-TOF analysis. The remaining extract was dried under vacuum and the

resulting protein pellet was reconstituted in 50 μ L of 2D electrophoresis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DTT and IPG buffer with a pH range of 3 to 10.

In the final step, glutelins were extracted. For this purpose, 0.1 M sodium hydroxide solution was added to the remaining sediment from the previous step at a ratio of 1:10 (w/v). The samples were vortexed for 5 min and then stirred at 250 rpm for 55 min at room temperature. Subsequently, centrifugation was performed at $12,000 \times g$ for 10 min and the supernatant containing the glutelins fraction was collected. This extract was used for subsequent analyses.

2.4 Determination of Rice Protein Content: Bradford Assay

In order to determine the amount of protein in each of the extracted fractions, the Bradford colorimetric method was used. This method is one of the most common methods for quantitative protein measurement and is based on the binding of the dye Coomassie Brilliant Blue G-250 to protein molecules. In this process, the binding of the dye to the amino groups and aromatic amino acids present in the protein structure causes a change in the optical absorption of the dye, the intensity of which is proportional to the concentration of protein in the sample.

In this study, the Bradford assay was adapted for microplate readings. Bovine Serum Albumin (BSA) was used as a calibration standard to prepare the standard curve. The standard used was prepared from the Quick Start™ BSA Standard kit (Cat. No. 5000206, Bio-Rad). Standard solutions with specific concentrations were prepared and used to plot the standard curve to allow accurate calculation of protein concentrations in the samples.

In each test, 10 μ L of protein samples was mixed with Bradford reagent and their optical absorbance at a specific wavelength was measured using a microplate reader. The optical absorbance obtained for each sample was converted to protein concentration using a BSA standard curve.

In order to increase the accuracy and reliability of the results, all measurements were performed in three technical replicates and the average values obtained were used for subsequent analyses. This method allowed for the comparison of the amount of protein present in different fractions extracted from rice grains and provided a basis for subsequent analyses.

2.5 Protein Analysis

In order to investigate the structural features and molecular pattern of proteins extracted from rice grains, electrophoresis and mass spectrometry methods were used. These analyses allow the investigation of the molecular weight distribution of proteins, the identification of protein bands, and the accurate determination of the molecular mass of peptides and proteins. In this study, the protein pattern of the samples was first investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, in order to more accurately identify the protein and peptide compounds present in the samples, MALDI-TOF mass spectrometry was used.

The simultaneous use of these two analytical methods allows for a more comprehensive assessment of the protein composition of samples; SDS-PAGE provides information about the molecular weight distribution and pattern of protein bands, while MALDI-TOF allows for precise determination of molecular mass and identification of protein compounds at a higher level of accuracy.

2.5.1 SDS-PAGE Analysis

In order to investigate the protein pattern and determine the molecular weight distribution of proteins extracted from rice grains, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. This method is one of the most common techniques for separating proteins based on their molecular weight and, by denaturing proteins and creating a uniform negative charge, allows them to be separated in an electric field.

To perform electrophoresis, 10 ug of the extracted protein samples were first mixed with sample buffer containing SDS and reducing agents to break the tertiary and quaternary structures of the proteins and to convert the proteins into linear polypeptide chains. The samples were then heated for a short time to completely denature the proteins. After preparation, a specified volume of each sample was loaded into the wells of a precast 4–20% polyacrylamide gradient gel.

Electrophoresis was performed in a vertical gel system and a standard protein molecular weight marker was used to determine the relative molecular weight of the protein bands. During the electrophoresis process, proteins moved in an electric field within the gel matrix and were separated from each other based on molecular size, such that proteins with lower molecular weight migrated faster and larger proteins migrated slower.

After electrophoresis, the gels were stained using an appropriate stain to make the protein bands visible. The resulting band patterns were examined to compare the protein composition of the samples and assess possible differences between the different fractions.

2.5.2 MALDI-TOF Analysis

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry was used to accurately determine the molecular mass of protein and peptide compounds in the samples. This method is considered one of the advanced techniques in proteomics that allows for rapid and accurate identification of the molecular mass of peptides and proteins.

In this method, protein samples were first mixed with a suitable matrix. The matrix plays an important role in absorbing laser energy and transferring it to the analyte molecules, causing their gentle ionization without extensive destruction of the molecular structure. The resulting mixture was placed on the target plate and after drying at room temperature, prepared for analysis in the MALDI-TOF instrument.

During the analysis process, the samples were irradiated with laser pulses, which resulted in the desorption and ionization of molecules from the surface of the target plate. The ions produced were accelerated in the electric field and moved in the flight tube of the device. The time it took for the ions to reach the detector depended on their molecular mass; so that lighter ions reached the detector faster and heavier ions reached the detector more slowly. Based on the time of flight of the ions, the mass spectrum of the sample was recorded and the molecular mass of the compounds present was determined.

Mass spectrometry data were used to examine the protein composition of the samples and identify possible peptides and proteins present. This method allowed for more detailed information about the molecular characteristics of the protein compounds present in the samples under study.

2.5.2.1 Matrix Preparation and Sample Preparation

To perform MALDI-TOF analysis, a suitable matrix solution was first prepared. The matrix used was sinapinic acid 10 mg/ml in 30:70 ACN/0.1% TFA, v/v, selected in such a way that it could absorb the laser energy and provide efficient transfer of energy to the protein and peptide molecules. The matrix solution was prepared in a suitable solvent and thoroughly mixed before use to obtain a uniform solution.

To prepare the sample, a certain amount of protein extract was mixed with an equal volume of matrix solution. The mixture was gently stirred to create a uniform dispersion of sample molecules in the matrix medium. Then, a small amount of the prepared mixture was placed on the MALDI target plate.

The droplets placed on the target plate were dried at room temperature to form matrix-analyte crystals. The formation of these crystals is essential for the successful desorption and laser ionization process. After the samples were completely dried, the target plate was placed inside the MALDI-TOF instrument and prepared for mass spectrum recording.

2.5.2.2 Spectrum Acquisition Parameters

Mass spectra of the samples were recorded using a MALDI-TOF instrument in a 2,000 – 20,000 m/z ratio. In this process, the samples were irradiated with laser pulses, which resulted in the desorption and ionization of molecules from the surface of the target plate. The ions produced were accelerated in an electric field and moved through the instrument's flight tube and the molecular mass was calculated based on their time of flight.

To increase the accuracy and quality of the obtained data, 6,000 laser shots were recorded from different points of each sample spot and their average was used as the final spectrum. The molecular mass range studied was selected based on the type of sample and the nature of the protein compounds.

After recording, the mass spectrometry data were processed and analyzed using the device's proprietary software. The spectra obtained were used to investigate the presence of peptides and proteins and determine their relative molecular mass.

2.6 Statistical Analysis

All experiments were performed at least in triplicate and the results were reported as mean $\pm$ standard deviation. Appropriate statistical software was used to analyze the data and examine statistical differences between samples.

One-way analysis of variance (ANOVA) was used to compare the means between different groups. In cases where significant differences were observed between groups, appropriate post hoc tests were used to determine pairwise differences between means. The level of statistical significance in all tests was considered to be $p < 0.05$.

The results of statistical analyses were used to interpret data related to protein content and other parameters studied.

Chapter 3 – Results

3.1 Assessment of Concentration and Distribution of Storage Proteins

In order to accurately determine the concentration and relative contribution of four protein fractions (albumin, globulin, prolamin and glutelin) in Chinese Originario rice, quantitative analyses were performed, and the distribution of storage proteins was evaluated. According to the findings, storage proteins constitute 59.3% of the total protein content of this cultivar. The relative distribution of these fractions shows that glutelins constitute the dominant fraction with 72.2%, followed by albumin (13.2%), globulin (9.8%) and prolamin (4.8%) (N=3). The concentration obtained are presented in Table 3.1.1.

Table 3.1.1. Protein concentrations of different fractions. Results are reported as mean concentration (mg/mL) $\pm$ standard deviation (N=3).

Carnaroli	Roma	Chinese originario	Fraction
0.106 $\pm$ 0.006	0.072 $\pm$ 0.005	0.512 $\pm$ 0.013	Albumins
0.364 $\pm$ 0.014	0.364 $\pm$ 0.012	0.378 $\pm$ 0.012	Globulins
0.057 $\pm$ 0.004	0.066 $\pm$ 0.007	0.187 $\pm$ 0.004	Prolamins
4.842 $\pm$ 0.140	3.737 $\pm$ 0.105	2.794 $\pm$ 0.057	Glutelins

3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein profiles of the four extracted fractions are shown in Figure 3.2.1. The pattern of protein bands in each fraction indicates their distinct molecular characteristics.

Chinese originario: This fraction shows a wide range of molecular weights from 10 to 100 kDa. The most prominent bands were observed at 50 kDa, between 37 and 25 kDa, and between 15 and 10 kDa.

Globulin: In this fraction, the indicator bands appeared at 50 kDa, 20 kDa, and two bands in the range of 15 to 10 kDa.

Glutelin: This fraction is characterized by three clear bands: one band in the range of 37 to 25 kilodaltons, one band around 20 kilodaltons and another band in the range of 15 to 10 kilodaltons.

Prolamin: The profile of this fraction includes a band at 20 kDa, a band immediately below it, and two very dense bands in the range of 15 to 10 kDa.

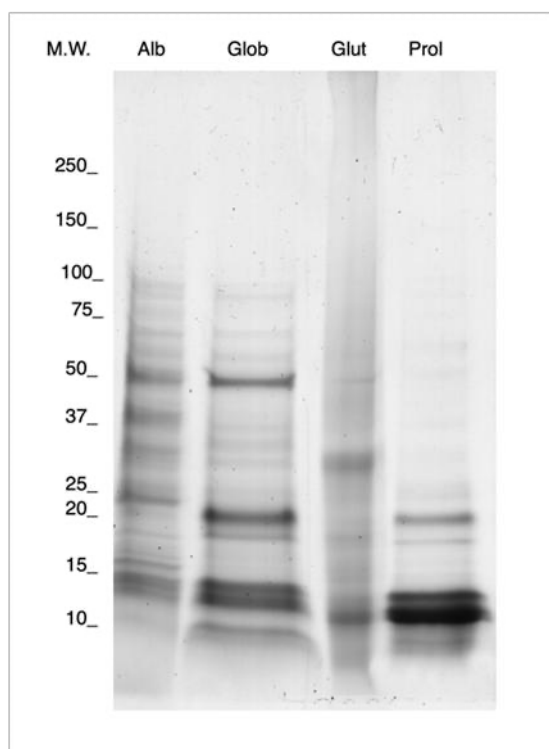


Figure 3.2.1. Protein profile of 10 μ g of albumin (Alb), globulin (Glob), glutelin (Glut) and prolamin (Prol) fractions with molecular weight (M.W.) markers.

Roma rice cultivar

Albumin: This fraction shows a wide range of molecular weights from 10 to 100 kDa. The most prominent bands were observed at 50 kDa, between 37 and 25 kDa, and a highly dense band in the range of 15 to 10 kDa.

Globulin: In this fraction, the indicator bands appeared at 50 kDa, 20 kDa, and two dense, interconnected bands in the range of 15 to 10 kDa.

Glutelin: This fraction is characterized by very low-intensity, faint bands in the range of 37 to 25 kilodaltons and two distinct, separate bands in the range below 10 kilodaltons.

Prolamin: The profile of this fraction includes a band in the 25 to 20 kDa region and an extremely dense, dark, and broad zone in the range of 15 to 10 kDa.

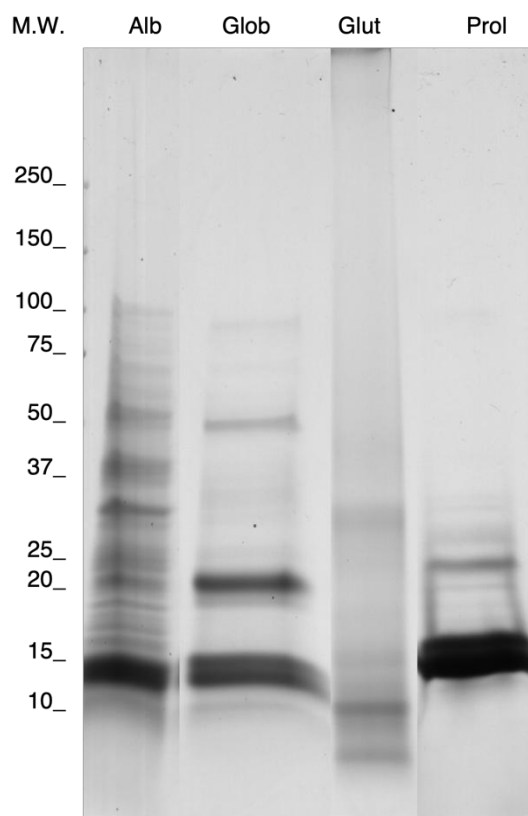


Figure 3.2.2. Protein profile of 10 μ g of albumin (Alb), globulin (Glob), glutelin (Glut) and prolamin (Prol) fractions with molecular weight (M.W.) markers of Roma cultivar.

Carnaroli rice cultivar

Albumin: This fraction shows a diverse fraction of molecular weights between 10 and 100 kDa. Prominent bands were detected at 50 kDa, between 37 and 25 kDa, and a highly dense, dark band in the range of 15 to 10 kDa.

Globulin: In this fraction, the representative bands appeared at 50 kDa, a very distinct band at 20 kDa, and two dense, separate bands in the range of 15 to 10 kDa.

Glutelin: This fraction is characterized by low-intensity bands in the range of 37 to 25 kilodaltons and several distinct, medium-intensity bands in the region below 15 kilodaltons (between 10 and 15 kDa and below 10 kDa).

Prolamin: The profile of this fraction includes a clear band in the 25 kDa region and an extremely dense, dark, and broad zone in the range of 15 to 10 kDa, exhibiting a smeared appearance in the region below 10 kDa.

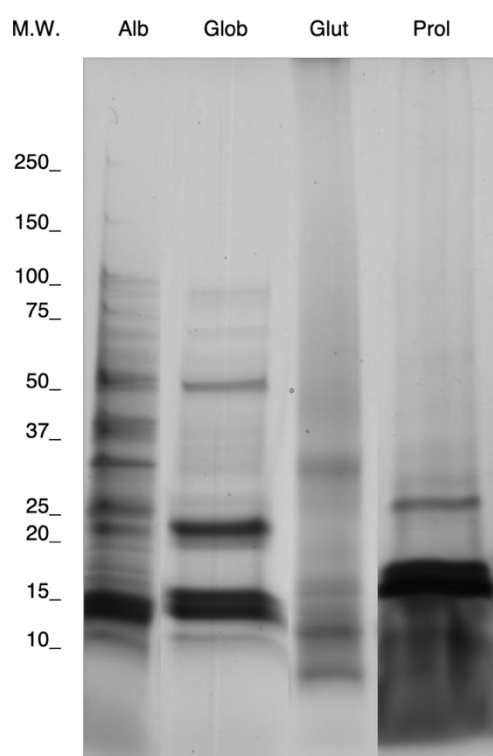


Figure 3.2.3. Protein profile of 10 µg of albumin (Alb), globulin (Glob), glutelin (Glut) and prolamin (Prol) fractions with molecular weight (M.W.) markers of Carnaroli cultivar.

3.3 MALDI-TOF Analysis

Chinese originario

The MALDI-TOF spectra of the protein fractions are shown in Figure 3.3.1. In all four fractions analyzed, a significant distribution of molecules in the range of 2–20 kDa was observed and all contained proteins with a mass of less than 10 kDa.

Albumin: This fraction has a prominent peak at 3,734.923 Daltons and a medium intensity peak at 10,076.710 Daltons along with a population of protein in the range of 5 to 8 kDa.

Globulin: This fraction has a peak at 18,890.842 daltons and a population of protein in the range of 13 to 16 kDa.

Glutelin: Low intensity peaks are visible in this fraction in the range of 14 to 15 kDa.

Prolamin: A peak at 18,892.848 Daltons is present in this fraction along with a population of peaks in the range of 13 to 16 kDa.

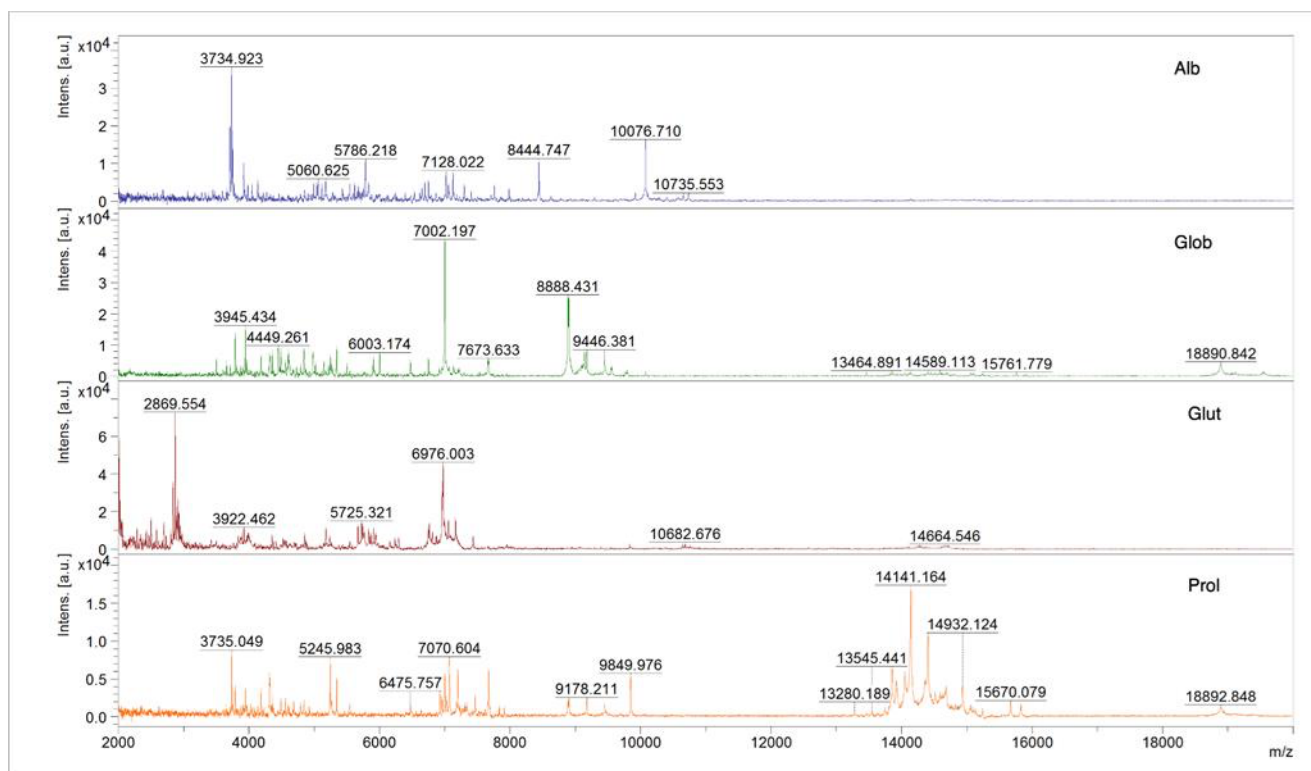


Figure 3.3.1. MALDI-TOF spectra of albumin (Alb), globulin (Glob), glutelin (Glut) and prolamins (Prol) fractions.

Roma

Albumin: This fraction has a peak at 3,734.837 Daltons and a population of peaks in the range of 5 to 8 kDa along with a medium intensity peak at 14,687.912 Daltons.

Globulin: A prominent peak at 5,342.404 Daltons is present in this fraction along with a population of protein in the range of 7 to 9 kDa and a peak at 18,889.724 Daltons.

Glutelin: Low intensity peaks are visible in this fraction in the range of 3 to 5 kDa and a peak at 6,964.999 Daltons.

Prolamin: A peak at 14,138.730 Daltons is present in this fraction along with a population of peaks in the range of 14 to 15 kDa and a peak at 5,341.830 Daltons.

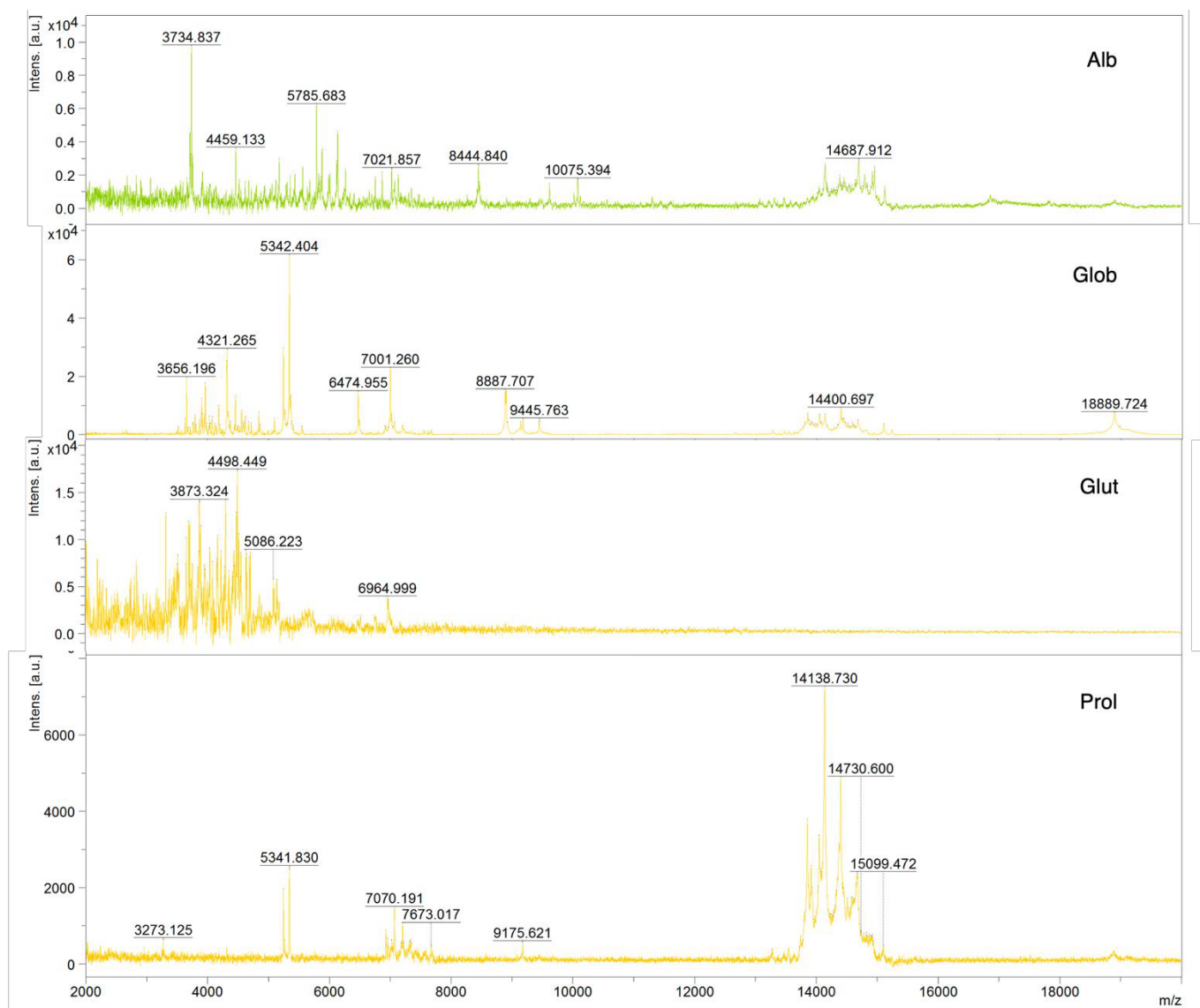


Figure 3.3.2. MALDI-TOF spectra of albumin (Alb), globulin (Glob), glutelin (Glut) and prolamins (Prol) fractions of Roma cultivar.

Carnaroli

Albumin: This fraction has a peak at 3,735.355 Daltons and a population of protein in the range of 5 to 8 kDa along with a peak at 14,949.012 Daltons.

Globulin: A prominent peak at 5,342.671 Daltons is present in this fraction along with a population of peaks in the range of 7 to 9 kDa and a peak at 18,889.349 Daltons.

Glutelin: Low intensity peaks are visible in this fraction in the range of 3 to 5 kDa along with a distinct peak at 4,304.676 Daltons.

Prolamin: A strong peak at 14,139.418 Daltons is present in this fraction along with a population of peaks in the range of 14 to 15 kDa and a peak at 5,342.470 Daltons.

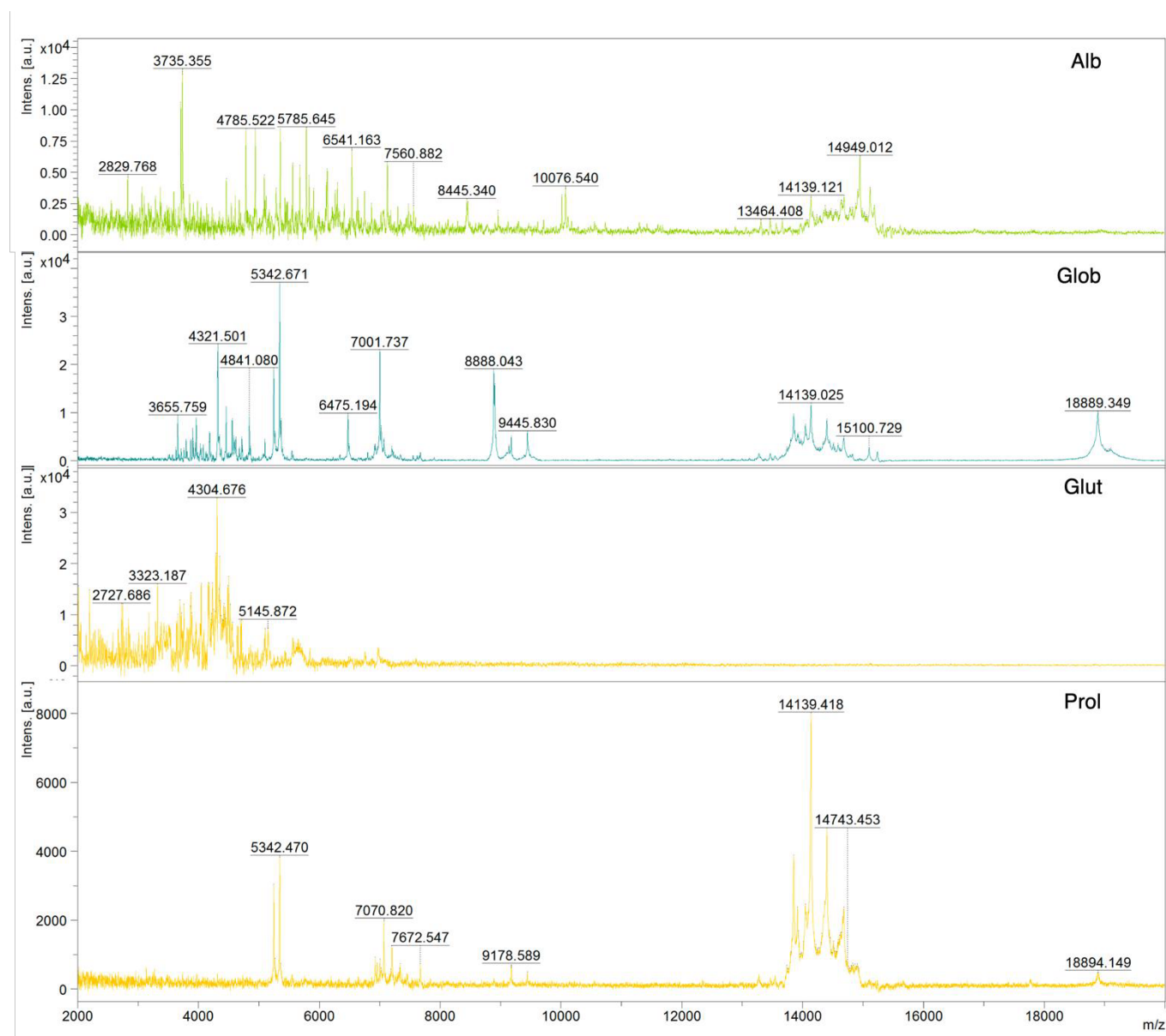


Figure 3.3.3. MALDI-TOF spectra of albumin (Alb), globulin (Glob), glutelin (Glut) and prolamin (Prol) fractions of Carnaroli cultivar.

Chapter 4 – Discussion

Rice (*Oryza sativa* L.) is a major source of energy and protein for a large portion of the world's population, and has an important place in food security and biochemical studies. Although rice is primarily known as a source of starch, its technological quality, nutritional value and health-related potential are strongly influenced by its protein profile and molecular organization of storage proteins. According to classical solubility protocols, rice grain proteins are classified into four groups: albumin, globulin, prolamin and glutelin, each of which has different functions in terms of physicochemical properties and roles in germination, endosperm structural stability and nutritional value (Bandumula, 2018). Although the composition of these proteins is considered a relatively stable genetic trait, environmental conditions and genotype during grain development can directly affect their final quantity and quality (He et al., 2021).

The results of the present study showed that storage proteins constituted 59.3% of the total grain protein content of this cultivar. Among them, glutelins were identified as the dominant fraction with a contribution of 75.15% (with a concentration of 2.794 ± 0.057 mg/mL), followed by albumins with 12.94% (0.512 ± 0.013 mg/mL), globulins with 9.55% (0.378 ± 0.012 mg/mL) and prolamins with 2.36% (0.187 ± 0.004 mg/mL) (N=3).

This distribution reflects a functional biochemical pattern observed in high-quality Japonica rice cultivars (Muthayya et al., 2014). The predominance of glutelins in Chinese Originario indicates favorable nitrogen accumulation in the endosperm, which both ensures the mechanical stability of the grain and facilitates the production of bioactive peptides in subsequent enzymatic hydrolysis processes (Amagliani et al., 2017b). The differences in concentration in the other fractions are consistent with their physiological role. Albumins and globulins, which constitute a large part of the protein reserves, play a key role as metabolic compounds

in the regulation of enzymatic reactions and responses to environmental stresses (Zhao et al., 2021). The study by Kim et al. (2013) showed that such a distribution profile in Chinese Originario could indicate its high potential as a protein source. On the other hand, the preservation of protein structure, especially disulfide bonds, probably allows for better protein extraction and purification efficiency.

In a comparative analysis among the studied varieties, it is evident that the Chinese Originario cultivar should be classified as a distinctive niche product in the rice market. Unlike traditional Italian cultivars such as Roma and Carnaroli, which are predominantly white rices rich in glutelins (with concentrations of 3.737 and 4.842 mg/mL, respectively) optimized for the cooking texture of risotto, Chinese Originario represents a specialized brown rice with significantly higher nutritional value. This superiority is driven by its higher total protein content, and specifically, its elevated concentrations in the albumin (0.512 mg/mL) and prolamin (0.187 mg/mL) fractions compared to Roma and Carnaroli. The elevated albumin fraction in Chinese Originario, known for its high solubility and digestibility, enhances the grain's biological potential to release functional bioactive peptides. MALDI-TOF analysis further revealed clear structural variations; the prolamin fraction in Roma and Carnaroli exhibits a highly intense peak around 14 kDa, whereas Chinese Originario displays a more diverse peak distribution and a higher abundance of low-molecular-weight peptides below 10 kDa (such as the 3734 Da and 6976 Da peaks). This distinct molecular architecture underscores its unique nutritional and functional value as a premium brown rice product.

Electrophoretic analysis of rice storage proteins is a fundamental tool for understanding the organization of protein subunits (Jayaprakash et al., 2022), which both defines the biochemical properties of the grain and determines the functional behavior and the ability of proteins to be hydrolyzed to produce bioactive peptides (Peighambardoust et al., 2021). In the present study, the electrophoretic profiles of four protein fractions extracted from the cultivar Chinese Originario were mapped with high accuracy. SDS-PAGE results showed that albumins, as the most diverse fraction, exhibited a wide range of molecular weights from 10 to 100 kDa. Prominent bands at 50 kDa, the 37–25 kDa region and the 10–15 kDa range indicated a rich and complex albumin proteome. This diversity in albumin molecular weights is in complete agreement with

the findings of Jayaprakash et al. (2022). According to their study, rice albumins have diverse polypeptide structures ranging from 10 to 200 kDa due to their enzymatic and metabolic roles; a diversity that ensures the functional stability of these proteins under different environmental conditions (Jayaprakash et al., 2022). In the present study, the observation of bands in the 50 kDa range is consistent with some reports on rice seed proteins. According to the study of He et al. (2021), some storage proteins and their associates play a role in the response to environmental stress, especially heat stress and ER stress.

In contrast, the globulin fraction of Chinese Originario showed a distinct band pattern, including prominent peaks at 50 kDa, 20 kDa and two dense bands in the 10–15 kDa range. In the present study, the 20 kDa bands were consistently present, which, according to Chernukha et al. (2021), is a qualitative indicator of the health of storage proteins. These findings indicate that Chinese Originario, despite its genetic differences with Indica cultivars, is very close to global standards in terms of globulin protein composition. The intensity of the bands is slightly different from that of Lee et al. (2015).

In the present study, glutelins accounted for the dominant 75.15% of the total storage proteins. The SDS-PAGE profile of our glutelin showed three distinct and strong bands in the range of 37–25 kDa, about 20 kDa and 10–15 kDa. This pattern is directly consistent with the acidic and basic subunits of glutelin, which are the main structural components of the endosperm (Shewry et al., 1995). Jayaprakash et al. (2022) showed that the acidic (30–35 kDa) and basic (19–25 kDa) subunits of glutelin together form a cross-linked network that determines the viscoelasticity of the rice grain. The observation of similar bands in Chinese Originario is a proof of the high technological quality of this cultivar.

The analysis of the prolamin fraction is of great importance. Although this fraction accounts for a small proportion (2.36%) of the total storage proteins, it plays a crucial role in the internal architecture of the proteins due to its hydrophobic nature and specific oligomeric structure. In SDS-PAGE gels, prolamin bands were mainly observed in the 10–15 kDa range. This finding is consistent with the study by Kim et al. (2013) who showed that the decrease in prolamin content could be compensated by changes in the

organization of other fractions. For Chinese Originario, low prolamin concentration compared to the other fractions indicates a biochemical modification that probably facilitates the germination process and enzymatic hydrolysis, by increasing the accessibility of digestive enzymes to the glutelin core during subsequent processing steps, which is an important quality advantage (He et al., 2021).

The role of disulfide bonds in the stability of the protein structure of Chinese Originario is important in SDS-PAGE data. Under reducing conditions, the disappearance of some high molecular weight bands (above 60 kDa) and the appearance of dense bands in the 20–37 kDa region provide strong evidence for the presence of interchain disulfide bonds that hold the acidic and basic subunits together in the glutelin network. According to a study by Yin et al. (2023), the structural stability of rice proteins is directly related to the disulfide bond network. Proteins lacking these linkages are more susceptible to hydrolysis. For Chinese Originario, this molecular arrangement provides a favorable balance between physical stability and biodegradability potential. This finding is consistent with the study by Sokra et al. (2026), who believe that controlling the quality of proteins (especially the chemical linkages within them) is the most important factor in producing rice that is both nutritionally superior and more suitable for cooking or baking.

Furthermore, the present study shows that the bands observed in SDS-PAGE of this cultivar have a very close structural similarity to the proteomic patterns of Italian cultivars reported by Spada et al. (2004) and Russo and Callegarin (Russo & Callegarin, 2007). This indicates that Chinese Originario is both a quality specimen in terms of protein content and phylogenetically and biochemically superior among cultivars adapted to similar climates. The presence of 16 and 18 kDa subunits in some of the analyzed fragments, reported by Yamagata et al. (1982), indicates the preservation of genetic and biochemical purity in this cultivar.

SDS-PAGE data in the present study showed that Chinese Originario has a coherent set of protein subunits that are ideal for industrial applications such as the extraction of antioxidant or antihypertensive peptides, as the pattern of bands observed is similar to those reported in the study of Adebisi et al. (2009) and the

study of Cavazos and Gonzalez de Mejia (Cavazos & Gonzalez de Mejia, 2013) as “potential precursors for biological activities.” Chinese Originario has great potential to redefine quality standards in the rice value chain, not only in terms of quantity (total protein amount), but also in terms of quality (distribution of subunits and molecular connections).

In the present study, MALDI-TOF mass spectrometry was used as a high-resolution tool to accurately map the proteome of Chinese Originario. While SDS-PAGE provides a general view of the subunits, MALDI-TOF reveals hidden layers of protein structure by providing precise molecular weights (Da). Analysis of the mass spectra extracted from this cultivar revealed a set of distinct peaks at distinct mass ranges, all of which reflect the biological and structural diversity of rice storage proteins. Our results identified characteristic peaks at 8, 12, 15, 22 and 34 kDa, which is in good agreement with the reports of Zhao et al. (2021) on the rice proteome. These precise molecular weights constitute the biochemical identity of Chinese originario.

The peaks identified in the range of 10 to 15 kDa (especially 12 and 14 kDa) confirm the presence of sulfur-rich prolamins in the studied cultivar. According to Yamagata et al. (1982), these proteins play an important role in the formation of the structural core of endosperm proteins. In the present study, the intensity of these peaks indicates the appropriate abundance of these subunits, which, according to Chen et al. (2018), is a stable genetic indicator in rice cultivars with favorable cooking quality. The peak observed at 22 kDa is identified as the α -glutelin subunit, a structure that is primarily responsible for the formation of the viscoelastic network in rice dough, according to a study by Shewry et al. (1995). The similarity of this molecular mass in Chinese Originario with the world standard values indicates that the structural stability of the proteins in this variety is fully optimized at the genetic level (Shewry et al., 1995).

In addition, the present study showed strong peaks in the 30–35 kDa range in MALDI-TOF spectra, which correspond to the acidic subunits of glutelin. According to Jayaprakash et al. (2022), Chinese Originario has a high capacity to release bioactive peptides during enzymatic hydrolysis and proteins with molecular

weights in this range have amino acid sequences that, upon digestion by pepsin or trypsin, release peptides with strong antioxidant activity. This finding is consistent with the studies of Adebisi et al. (2009) and Cavazos and Gonzalez de Mejia (Cavazos & Gonzalez de Mejia, 2013). In these studies, the precise molecular weight of these subunits determined the accessibility of the active sites of proteases to generate angiotensin-converting enzyme (ACE) inhibitory peptides. Therefore, the data from the present study on Chinese Originario provide a basis for predicting the bioactive activities of this cultivar.

The peaks detected in the 4–8 kDa region in the MALDI-TOF data are likely to be small peptides and low molecular weight (LMW) proteins. These proteins, which have been identified as direct precursors of bioactive peptides in the studies of Mora and Toldrá (Mora & Toldrá, 2023) and Zhu et al. (2023), were specifically identified in our samples. The studies show that the protein structure of this variety, unlike other cereals studied by Chourasia et al. (2023), has specific features that make it very suitable for bio-applications (advanced processing).

In the present study, data from SDS-PAGE and MALDI-TOF mass spectrometry provided a more comprehensive view of the subunit structure in Chinese Originario. While SDS-PAGE focused on the separation of protein bands based on molecular weight under denaturing conditions, MALDI-TOF provided more detailed molecular mass details of smaller polypeptides. A remarkable convergence between the two methods was observed in the identification of low molecular weight components, with all four protein fractions in MALDI-TOF showing molecules with a mass of less than 10 kDa, which was in good agreement with the bands observed in the 10–15 kDa range in SDS-PAGE.

In the case of the globulin fraction, MALDI-TOF data recorded a specific peak at 18,890.842 Daltons, which matched the 20 kDa band observed on SDS-PAGE. This match showed that what appeared as a band of about 20 kDa on electrophoresis was actually a collection of proteins of the same mass that were separated with high precision by mass spectrometry.

In the glutelin fraction, the presence of low intensity peaks in the 14-15 kDa range in MALDI-TOF made the details of the 10-15 kDa bands in SDS-PAGE clearer. The observed difference in the 14-15 kDa peaks versus the 20 kDa values could be due to subunit differences in the Chinese Originario samples, which mass spectrometry was able to reveal more accurately than classical electrophoresis.

The findings of the present study showed that the tools used, namely SDS-PAGE to outline the overall proteome and MALDI-TOF to identify precise mass peaks, confirmed that glutelins, with their dominant structure, along with the participation of albumin, globulin and prolamin, form a complex molecular profile.

Bioactive peptides derived from the hydrolysis of rice storage proteins are considered as an important source for health-related applications due to their multiple functional abilities, including antioxidant, anti-inflammatory, antihypertensive and enzyme-inhibiting activities (Amigo & Hernández-Ledesma, 2020, Chakrabarti et al., 2018). Chinese Originario, due to its diverse and rich protein profile, is a powerful source for extracting a wide range of these bioactive peptides that can play a key role in promoting human health. According to the results of MALDI-TOF spectroscopy and SDS-PAGE electrophoresis, glutelin, globulin, albumin and prolamin proteins showed a wide range of low molecular weight subunits (between 2 and 20 kDa), which are known as the main precursors for the production of bioactive peptides. In particular, the peaks below 10 kDa observed in MALDI-TOF indicate the presence of short and medium-length peptides with very high biological potential (Dei Più et al., 2014).

In the science of “seed proteomics,” the pattern that each variety exhibits reflects the complex synthesis, accumulation, and processing of storage proteins. The Chinese Originario cultivar, based on SDS-PAGE and MALDI-TOF analyses in the present study, has an exceptionally stable profile dominated by glutelins (approximately 75% of the total protein reserve). Such stability can only be the result of a “systemic balance” between genetic capacity and controllable environmental pressures, a balance that is considered an indicator of molecular health and endemic adaptation for this cultivar.

From a genomic perspective, the proteomic pattern of each cultivar is a direct function of the SSP (Seed Storage Protein) gene set and its regulatory networks. Amagliani et al. (2017a) showed that intervarietal variation in rice is often caused by polymorphisms in promoter regions and differences in copy number of GluB1–GluB5 genes (encoding glutelin). In the present study, the MALDI-TOF results showed that the genetic sequence of Chinese Originario was consistent with the study of Yamagata et al. (1982). This suggests that the parts related to gene reading and protein synthesis in this plant function in a highly regulated and coordinated manner. Chen et al. (2018) showed that even point changes in cis-regulatory motifs (such as the G-box and ACGT motif) can alter the glutelin-to-prolamin ratio by up to 40%. The lack of such abnormalities in our samples suggests long-term selective pressure to maintain the efficiency of the ER/Golgi-dependent folding pathway. Also, electrophoretic data from the present study showed that the ratio of the 25–37 kDa (heavy subunits H-subunits) to 20–23 kDa (light subunits L-subunits) bands in this strain is approximately 3:2 (Lee et al., 2015). This constant ratio indicates both the synthesis rate and is an indicator of the stability of the transcripts and the function of the specific chaperones BiP and PDIL in the endoplasmic reticulum.

Environmental factors, especially in cereals, play a “soft regulator” role on protein structure. According to the study of Shewry et al. (1995), nitrogen and temperature stresses during the grain filling phase directly affect the rate of aggregation and interaction between glutelin and prolamin. In the study of Bhandari et al. (2023), it was reported that plants grown under temperature stress above 35°C had a 25% decrease in total protein content and their SDS-PAGE bands showed aspartame cleavage. According to the findings of the present study, the absence of abnormally heavy bands (>120 kDa) and low-intensity MALDI peaks suggests that the growth conditions of Chinese Originario were within the suboptimal temperature and nutritional range, an environment that allowed post-translational processing pathways, especially the formation of disulfide bonds in the ER network, to proceed without disruption (Yin et al., 2023).

The analyzed seed proteins of Chinese Originario has a dual advantage due to its relatively high genetic stability and controlled environmental influence: on the one hand, the high abundance of storage proteins can increase the nutritional value and yield of this variety; and on the other hand, the stability and predictability of the structure of proteins and peptides derived from them makes this variety a more suitable and reliable option for industrial applications, especially in the production of bioactive peptides.

The findings of Mojumder et al. (2024) and Scarabattoli et al. (2025) showed that low-Mw fractions of classical rice glutelin have the highest ACE and nitric oxide synthase inhibitory activity. Since the subunit profile of Chinese Originario exactly matches the weight range of those studies, it can be predicted that this strain will be a valuable donor for the industrial extraction of antihypertensive and antioxidant peptides.

From a biotechnological perspective, the core glutelin genes (such as GluA1 and GluB4) in this cultivar can be used as a genetic template (backbone) for metabolic engineering projects to improve protein quality and stability in rice cultivars.

Future research directions should include 1) applying controlled stress schemes (heat, N deficiency, salinity) to determine the threshold for proteomic stability, 2) combining mRNA profiling with MALDI data to quantitatively model transcript-to-protein elasticity and 3) employing quantitative proteomics techniques (MS/MS) for comparison across generations. The maintenance of stability at the protein level is a prerequisite for standardization of protein-based industries in health supplements and nutraceutical formulations.

Chapter 5 – Conclusion

The present study aimed to elucidate the biochemical properties of seed storage proteins in the Chinese Originario cultivar. Using a combined and synergistic analytical approach of SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), the profile of the proteins of this rice cultivar was investigated and mapped with much greater accuracy than traditional methods. The results obtained from these molecular analyses revealed how this plant stores its proteins and why its seeds are consistent in terms of yield and quality.

One of the key findings of this study was the demonstration of the structural stability of the storage protein system of Chinese Originario. Electrophoretic and mass spectrometry analyses showed that glutelins, as the main component of seed storage proteins, have a well-defined and balanced subunit pattern. Although environmental factors and external stresses could affect plant physiological variables, the "proteomic identity card" of Chinese Originario seed is a conserved system. This feature is of great importance for the modern agricultural industry because it will guarantee the stability of the biochemical quality and nutritional value of the product in different cultivation climates. In a comparative evaluation, it was revealed that while the traditional Italian cultivars Roma and Carnaroli exhibit significantly higher concentrations of glutelins to support their commercial cooking and technological profiles, Chinese Originario presents a distinctly alternative protein profile. From an industrial perspective, the findings of this study showed that Chinese Originario rice is much more than a common food and should be highlighted as a unique brown rice niche product that possesses a potential nutritional value. This nutritional potential is directly attributed to its higher total protein content, particularly within the Albumin and Prolamin fractions, when compared to the Roma and Carnaroli varieties. By accurately identifying the structure of the proteins of this rice, it became

possible to produce "bioactive peptides" compounds that have antioxidant properties and can help control blood pressure. The abundant albumin fraction in this cultivar, characterized by high solubility and superior digestibility, offers an exceptional substrate for releasing these functional short-chain peptides. In addition, the exceptional stability of the proteins of this variety is also a great advantage for plant breeders. This rice can be used as a suitable genetic base in breeding projects to produce new, higher-quality varieties with higher nutritional value without altering its taste or cooking quality. This study was an important step in accurately identifying the proteins of this rice variety. It is suggested that future studies investigate the effects of harsh environmental conditions (such as drought, soil salinity and temperature changes) on the genes responsible for producing these proteins. Also, the use of more advanced laboratory methods (such as LC-MS/MS technique) will help us identify very small and specific proteins and ensure what therapeutic or biological benefits these compounds really have. Finally, we can say that proper management of Chinese Originario can greatly contribute to ensuring food security and producing high-quality and valuable food products in the future.

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