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EDITED BY
B. N. Dar,
Islamic University of Science and Technology, India

REVIEWED BY
Marina Mefleh,
American University of Rome, Italy
Maria Simona Chiș,
University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania

*CORRESPONDENCE
Mohammed Alsafran
✉ m.alsafran@qu.edu.qa

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RETRACTED: RuBisCO: a sustainable protein ingredient for plant-based foods

Malik Adil Nawaz, Deepak M. Kasote, Najeeb Ullah, Kamal Usman and Mohammed Alsafran*

Agricultural Research Station, Qatar University, Doha, Qatar

Ribulose bisphosphate carboxylase/oxygenase (RuBisCO), is a widely available plant protein receiving great interest because of its nutritional and functional properties. It can be a valuable source of protein for vegetarians. However, it has not received commercial significance due to the lack of a streamlined extraction process at the industrial scale, including its potential health benefits. In this review, we have summarized the literature on the biochemical characteristics of RuBisCO and compared its nutritional value with other plant proteins, as well as highlighted its digestibility, allergic traits, and potential health benefits. Moreover, the existing literature on the extraction of RuBisCO, associated challenges in industrial-scale RuBisCO purification, and recent innovations that occurred in this context are compiled. We believe this review will provide insights into RuBisCO's nutritional value and techno-functionality. Altogether, RuBisCO can be a sustainable source of protein in the future, especially for vegetarians.

KEYWORDS

leaf protein, RuBisCO, sustainability, food processing, nutritional value, functional properties

1 Introduction

The growing global population and increasing demand for protein led to major increases in concerns about food security and environmental sustainability. Concerns about the impact of traditional protein production on the environment have led to a substantial increase in research efforts in recent years to explore more environmentally friendly alternative protein sources (Tan et al., 2023). Therefore, plant proteins are gaining popularity as a replacement for animal proteins, largely driven by increasing consumer demand influenced by factors such as health concerns, vegetarian diets, and religious dietary restrictions (Pingali et al., 2023). The demand for plant-based proteins is currently at an all-time high, and it is estimated to reach a global worth of $162 billion by 2030 (Nawaz et al., 2023). Traditionally, legumes such as soybeans (*Glycine max* L. Merr.), lentils (*Vicia lens* L. and *V. culinaris* L.), and peas (*Lathyrus oleraceus* Lam.) are the most important plant protein sources, and the food industry has launched a completely new range of food products in response to the rising demand for vegan protein. However, these commercial plant-based proteins are usually considered nutritionally inferior to their animal counterparts due to the lack of essential amino acids, and the presence of allergens, anti-nutrients, and off-flavors (Gu et al., 2023). Furthermore, the difficulties in the processability of these plant proteins end up in low-quality plant-based products (Giampieri et al., 2022).

In response to these nutritional inferiorities and poor techno-functionalities of commercial plant proteins, a growing scientific interest in the exploration of proteins from agricultural waste has been observed in recent years. These materials are being explored for their potential valorisation, which involves converting them into proteins with diverse functional and biological activities. This approach not only reduces waste but it offers innovative solutions for sustainable

protein production (Wong et al., 2020). Among these new sources of plant proteins, the photosynthetic enzyme, ribulose bisphosphate carboxylase/oxygenase (EC.4.1.1.39; RuBisCO) has been given great attention (Di Stefano et al., 2018), due to its wide distribution in plants, eukaryotic algae, cyanobacteria, and photosynthetic bacteria. In addition, the excellent nutritional value of RuBisCO, which is readily digestible and contains a balanced essential amino acids composition per international (e.g., FAO and WHO) standards for human consumption.

In this article, we reviewed RuBisCO's biochemical characteristics and different forms of RuBisCO. Moreover, we critically reviewed the nutritional and biochemical advantages of RuBisCO as a food component and drew comparisons with the most commonly used plant and animal proteins. Additionally, we also highlighted the physicochemical and functional properties of RuBisCO as a potential food ingredient. We also summarised various extraction interventions of RuBisCO from different plant sources, including their limitations. Lastly, we have discussed the potential commercial application of RuBisCO. We believe that this review will significantly contribute to the existing knowledge about RuBisCO, a valued and sustainable source of plant protein.

2 Biochemical characteristics of RuBisCO

RuBisCO is the carboxylase enzyme, which plays a key role in one of the critical steps of photosynthesis *viz.*, carboxylation of ribulose-1,5-biphosphate (RuBP) by assimilation of CO_2 during the first reaction of Calvin cycle (Tomar et al., 2017). This series of reactions commences with CO_2 assimilation and ends in carbohydrate synthesis (Stitt et al., 2010). Hence, RuBisCO plays a critical role in capturing inorganic carbon (~90% of the total) and transforming it into organic form (Liu et al., 2020). Nonetheless, RuBisCO can also interact with O_2 instead of CO_2, initiating a series of reactions known as photorespiration. This energy and O_2-dependent process releases CO_2 and NH_3 as by-products (Busch, 2020). This makes RuBisCO a somewhat inefficient catalyst, as it spends at least 25% of its time reacting with O_2 in the presence of both gases (Andersson, 2008).

RuBisCO is the major fraction (30–65%) of soluble leaf protein, making it the most abundant protein in nature (Bagheri et al., 2017). Apart from plants, RuBisCO is also present in algae, cyanobacteria, various photosynthetic bacteria, and even some non-photosynthetic alga (e.g., *Euglena longa*) and non-photosynthetic chemoautotrophic bacteria (e.g., *Thiobacillus denitrificans*) (Maeda et al., 1999; Tabita et al., 2008; Záhonová et al., 2016). Approximately, 5 kg per person of RuBisCO is globally available, most of which is synthesized annually. This abundance emphasizes how vital RuBisCO is to the global carbon cycle, which is necessary to keep life on Earth (Rae et al., 2021).

3 Structure of RuBisCO

The basic functional unit of RuBisCO is a homodimer of large subunits (LSUs), with each subunit consisting of an N-terminal α/β domain and a C-terminal $(\beta/\alpha)_8$ triosephosphate isomerase (TIM) barrel. With a molecular weight of 550 kDa, RuBisCO comprises of eight LSUs, with individual molecular weight ranging from approximately 51–58 kDa. Additionally, it includes eight small subunits (SSUs), with molecular weight between 12 and 18 kDa. Four forms of RuBisCO have been identified. All of these forms are made of dimers of catalytic LSUs (Tabita et al., 2008). The LSU8, octameric core of Form I is decorated at the top and bottom by four dimers of SSU. SSUs are only seen in type I. Depending on the source, dimers of LSU ranging from LSU2 to LSU8 make up Form II. Only a few archaea include Form III, which is made up of dimers of LSU arranged as above in either an LSU2 or (LSU2)5 configuration. The RuBisCO-like Protein, or Form IV, seems to always have an LSU2 structure thus far (Figure 1).

While the amino acid sequence of LSU is relatively consistent across various higher plant species, with more than 80% homology. Conversely, amino acid sequence primarily exists in SSU, which has around 70% homology (Andersson and Backlund, 2008). Consequently, different crop species may showcase minor variations in RuBisCO amino acid composition, leading to discrepancies in specific biochemical attributes such as isoelectric point, molecular weight, bioactivities, etc., (Pérez-Vila et al., 2022).

LSU is encoded by a single gene located in the chloroplast genome, inherited exclusively from the female progenitor (Cheng et al., 2017). In contrast, SSU, a product of a gene family is inherited from both maternal and paternal progenitors. These genetic attributes significantly influence the overall quality and quantity of RuBisCO protein extracted from plant tissues (Sun et al., 2021). Therefore, these genetic factors must be considered during large-scale extraction of this protein for food purposes.

In chloroplast, RuBisCO primarily resides in the soluble fraction, constituting more than half of the total leaf proteins of C3 plants (Pérez-Vila et al., 2023). In C4 plants, however, RuBisCO percentage ranges from 8 to 23% (Kubien et al., 2008). The effective RuBisCO concentration in plant leaves is influenced by factors such as nitrogen supplies, plant type, plant age, and light intensity (Kubien et al., 2011). These factors collectively determine the abundance of RuBisCO in leaves, impacting its contribution to overall plant protein content.

4 RuBisCO as a potential food ingredient

Commercially available proteins may be inadequate to meet the nutritional criteria, primarily because most of them are either allergenic, lack essential amino acids, or have sensory issues such as unappealing tastes (Zhang et al., 2023). Expanding the production and refinement of RuBisCO, a non-allergenic, complete, and easily digestible protein found abundantly in photosynthetic tissues, holds the potential to address global challenges, including protein deficiency, a well-documented public health concern (Tanambell et al., 2023). RuBisCO seems to possess all the essential qualities for a protein that can be used as either food or feed while resolving the limitations mentioned above of commercial protein ingredients. However, it raises an important question of why it has not been introduced into the market for commercial use.

5 Physico-chemical properties of RuBisCO

5.1 Solubility

Protein solubility is the amount of protein that remains dissolved in a solution under specific conditions like ionic strength,

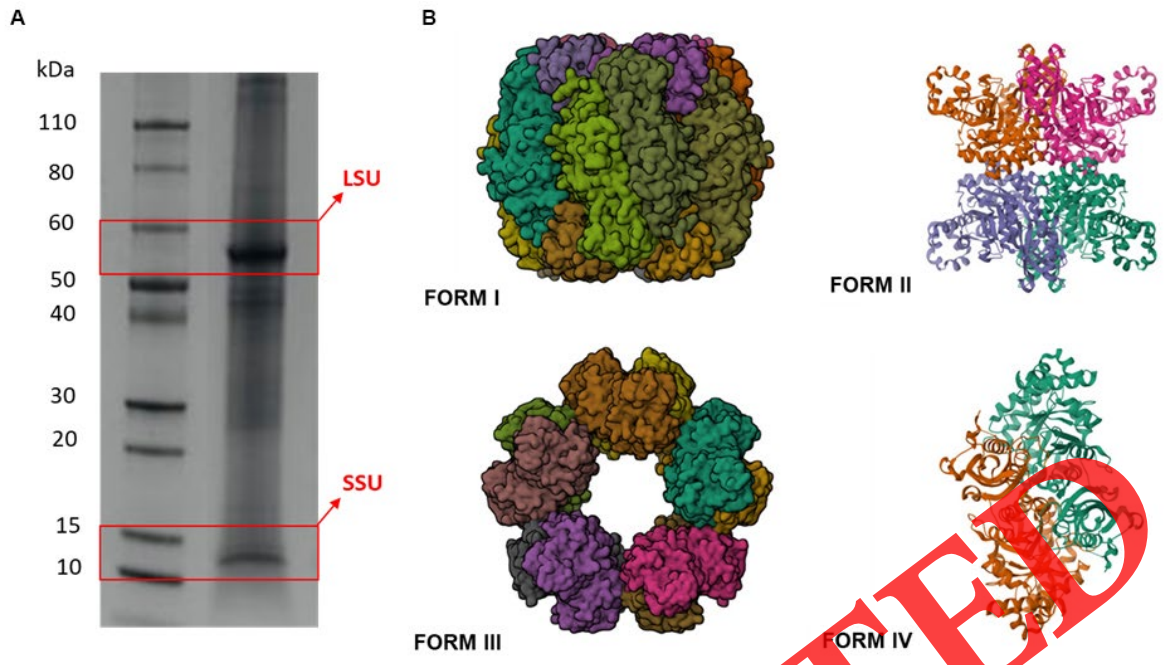

FIGURE 1
Electrophoretic separation of RuBisCO subunits and its different forms based on the number of subunits. **(A)** RuBisCO subunits from sweet potato (*Ipomoea batatas* L. Lam.) leaves separated by SDS-PAGE under reducing conditions. The estimated molecular weight of the marker standard on the left is expressed in kDa. LSU and SSU are highlighted by red boxes. **(B)** Representative structures of various RuBisCO forms. Structures are obtained from RCSB Protein Data Bank web portal (https://www.rcsb.org). Form I (PD ID: 8RUC), form II (PD ID: 7T1C), form III (PD ID: 3A12), and form IV (PD ID: 1YKW).

pH, protein concentration, and temperature (Tanger et al., 2022). It plays a crucial role in food systems like thickening, gelling, emulsifying, and foaming (Day et al., 2022; Nawaz et al., 2022; Tan et al., 2023). Key determinants include the balance of interactions between protein–protein, protein-water, and water–water interactions, and the effects of entropy related to mixing (Ismail et al., 2020). Proteins become more soluble in water when they have increased surface hydrophilicity and decreased molecular weight (Huang et al., 2021). Smaller proteins exhibit greater solubility than complex-structured larger proteins (Nawaz et al., 2021).

Various studies have reported the solubility of leaf proteins mainly RuBisCO as a function of pH (Famuwagun et al., 2020b; Ma et al., 2022). Some of the reported solubility data of RuBisCO from various plant sources *viz.*, sugar beet (*Beta vulgaris* L.), alfalfa (*Medicago sativa* L.), duckweed (*Lemna minor* L.), and spinach (*Spinacia oleracea* L.) under various pH levels is presented in Figure 2. Kobbi et al. (2017) and Kiskini (2017) studied the solubility of sugar beet (*B. vulgaris* L.) leaves RuBisCO and reported a typical U-shape solubility curve at a pH range of 2–9. Similar results were reported by Lamsal et al. (2007) for alfalfa (*M. sativa* L.) RuBisCO, which was least soluble at pH levels of 3–5 and highly soluble at pH 6 and above. Similarly, moringa leaf RuBisCO has significantly lower solubility at pH 4, measuring at ~9%, compared to a much higher solubility of ~58% at pH 10 (Rawdkuen, 2020). Similar solubility trends were also reported by Martin et al. (2014) and Martin et al. (2019) for duckweed (*L. minor* L.) and spinach (*S. oleracea* L.) RuBisCO isolates. These observations align with the common trend which suggests that solubility of proteins decreases at a pH close to their Ip and increases in alkaline environments due to reduced electrostatic interactions promoting aggregation and precipitation at acidic pH levels. This phenomenon occurs due to a reduction in electrostatic repulsion between protein molecules.

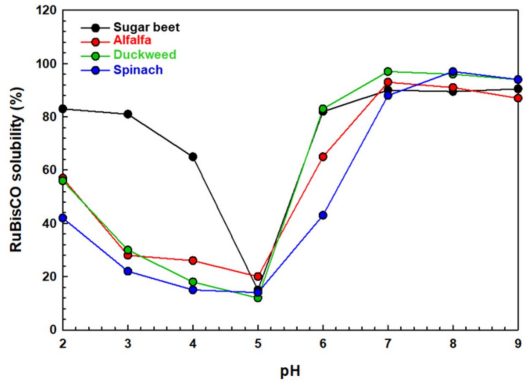

FIGURE 2
Extracted RuBisCO protein solubility of 10 g kg^{-1} protein dispersions as a function of pH at 25°C from sugar beet (*B. vulgaris* L.), alfalfa (*M. sativa* L.), duckweed (L. minor L.), and spinach (*S. oleracea* L.). Data adapted from Kobbi et al. (2017), Lamsal et al. (2007), Martin et al. (2019), and Martin et al. (2014).

5.2 Thermal properties

Thermal gravimetric analysis (TGA) stands as a widely utilized thermal analytical method in food research, holding significant

importance in ensuring food quality within the industry (Yilmaz et al., 2019). Among the food macronutrients, proteins are the primary focus of thermal analysis. Research has extensively explored the thermal properties of proteins and investigated how various environmental factors induce conformational changes in food proteins (Turgeon and Rioux, 2011). Researchers have extensively examined the thermal denaturation of tissue proteins and food enzymes (Sun-Waterhouse et al., 2014). TGA serves as a valuable tool in comprehending the behavior of proteins under different thermal conditions, contributing to the understanding and maintenance of food quality and processing standards. However, very limited information has been reported in the past on the thermal properties of RuBisCO. Previous studies showed that the denaturation temperature (T_d) of isolated RuBisCO from spinach (*S. oleracea* L.) and alfalfa (*M. sativa* L.) ranged between 64.9 and 67.5°C (Béghin et al., 1993; Libouga et al., 1996; Martin et al., 2014), which is lower than the reported T_d of other plant proteins (Table 1).

6 Functional properties of RuBisCO

6.1 Gelling properties

Protein denaturation leading to network formation is a fundamental step initiating gelation, a critical functional property in food science. This process significantly influences the texture and structure of food products (Klost et al., 2020). The gel-forming ability of RuBisCO has been investigated, and the best results were obtained using RuBisCO extracted from alfalfa (*M. sativa* L.). These positive outcomes were achieved after subjecting the alfalfa-derived RuBisCO to 80°C under a highly alkaline environment (pH 12) for 30 min. Under these controlled conditions, the protein likely underwent denaturation and subsequent network formation took place, resulting in the creation of a gel-like structure with desirable textural and functional properties (Barbeau and Kinsella, 1988). Gels produced using spinach (*S. oleracea* L.) leaf RuBisCO have also shown promising results. These spinach (*S. oleracea* L.) RuBisCO gels showed enhanced resistance and stability compared to soy (*G. max* L. Merr.) protein gels. This highlights the potential advantages of RuBisCO proteins for specific food applications where gel stability is crucial (Martin et al., 2014). Moreover, the response of RuBisCO gels as a function of pH has also been studied and results showed that at lower pH levels, RuBisCO gels were more brittle and prone to break. This pH-dependent behavior can be crucial in tailoring the textural properties of RuBisCO gels for specific food or industrial applications (Libouga et al., 1996; Di Stefano et al., 2018).

TABLE 1 Reported denaturation temperature (T_d) of isolated RuBisCO from spinach (*S. oleracea* L.), alfalfa (*M. sativa* L.), and other plant proteins.

Protein	T_d (°C)	References
Spinach (*S. oleracea* L.) RuBisCO	64.9	Martin et al. (2014)
Alfalfa (*M. sativa* L.) RuBisCO	66.5–67.5	Libouga et al. (1996) and Béghin et al. (1993)
Duckweed (*L. minor* L.) RuBisCO	70.0	Tan et al. (2022)
Pea (*L. oleraceus Lam.*) protein	82.0–84.0	Martin et al. (2014) and Munialo et al. (2014)
Soybean (*G. max* L.) protein β-conglycinin glycinin	70.0–73.3 90.0–93.3	Kitabatake et al. (1990), Renkema et al. (2002), and Martin et al. (2014)

6.2 Emulsifying properties

The ability to produce and sustain emulsions within a variety of food systems is one of the key surface-active properties of food proteins (Zhou et al., 2021). Several factors influence the emulsifying capacity of a protein, including hydrophobicity (Yan et al., 2021), molecular flexibility (Cui et al., 2020), and purification process (Zhang et al., 2020).

Compared with other commercial proteins, such as bovine serum albumin and soy (*G. max* L. Merr.) protein, RuBisCO has lower emulsifying activity (Anoop et al., 2023), but it is higher than egg albumin (Nieuwland et al., 2021). Notably, RuBisCO extracted from dried alfalfa (*M. sativa* L.) has high emulsifying properties (capacity 158-219m^2g^{-1} protein, stability 17–49 min), particularly at higher pH levels *viz.*, ≥7 (Hojilla-Evangelista et al., 2017). In alkaline pH conditions, oil–water interfaces expand as a result of protein unfolding (Zhang et al., 2021). Additionally, heat treatment before emulsification can enhance emulsion strength and stability (Sarkar et al., 2016). Heating alfalfa (*M. sativa* L.) RuBisCO before emulsification results in improved emulsifying activity, as reported by Wang and Kinsella (1976), Lamsal et al. (2007), and Hojilla-Evangelista et al. (2017). Furthermore, ultra-centrifuged alfalfa (*M. sativa* L.) RuBisCO concentrates exhibited notably superior emulsion capacity than acid-precipitated concentrates, likely due to a higher content of native protein forms in the ultra-centrifuged samples (Lamsal et al., 2007).

6.3 Foaming properties

RuBisCO possesses excellent foaming capacities, primarily due to its structural composition, which includes both hydrophilic and hydrophobic regions and moieties. For instance, duckweed (*L. minor* L.) RuBisCO had a superior foaming capacity (194%) compared to egg whites foaming capacity (122%) as shown in Figure 3. This increased foaming capacity suggests advantages of RuBisCO applications where foam capacity is a critical factor, such as in various culinary and food preparation processes (Muller et al., 2023). Van de Velde et al. (2011) found a highly desirable foaming capacity of RuBisCO than soy and whey protein isolates at pH 4.5 and 7.0. This relatively low foaming capacity observed in native soy (*G. max* L. Merr.) protein isolates could be linked to their complex tertiary and quaternary protein structures. These structures make it challenging for soy (*G. max* L. Merr.) proteins to efficiently stabilize air bubbles and create a stable foam. Soy (*G. max* L. Merr.) proteins have a relatively globular tertiary structure, which means they are already folded into a compact shape. This structure reduces the ability of soy (*G. max* L. Merr.) proteins to interact effectively with air-water interfaces and form stable foams (Shao et al., 2016). Additionally, soy (*G. max* L. Merr.) proteins tend to form larger aggregates in their native state due to their quaternary structure. These

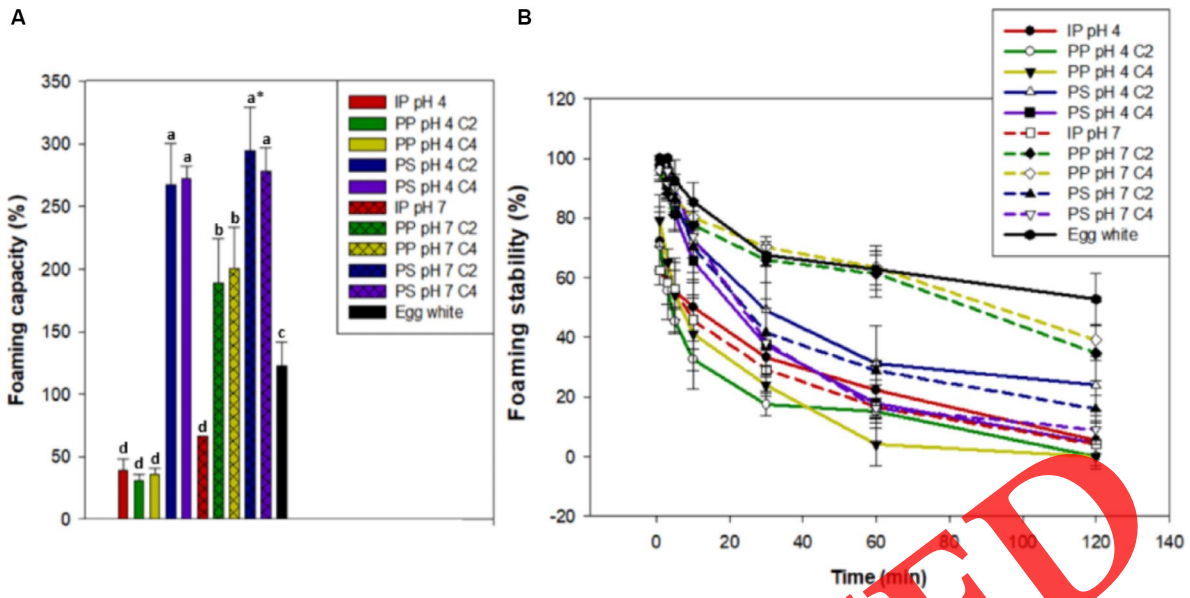

FIGURE 3
Foaming properties (**(A)** foaming capacity and **(B)** foam stability) of duckweed (*L. minor* L.) RuBisCO solution (1.5%, w/w) at pH 4 and 7 of the initial leaf powder (IP), purification RuBisCO pellet (PP), and purification supernatant (PS) obtained from an initial powder concentration of 2 and 4% (C2 and C4, respectively) compared to egg white (11% protein w/w). Data adapted from Muller et al. (2023).

larger aggregates are less suitable for generating and stabilizing small air bubbles in a foam (Han et al., 2023). On the other hand, Lupin (*Lupinus* spp.) protein's remarkable foaming capability in acidic solutions distinguishes it from soy (*G. max* L. Merr.) protein and makes it particularly well-suited for use in food products like yogurts or salad dressings that have acidic pH conditions (Shrestha et al., 2021). Contrary to these conventional legume proteins, isolated RuBisCO from alfalfa (*M. sativa* L.; Nissen et al., 2021), vegetable wastes (Famuwagun et al., 2020a; Sedlar et al., 2021), and mulberry (*Morus atropurpurea* Roxb.) leaves (Sun et al., 2015) have shown excellent foaming capacities in both acidic and alkaline conditions, showing the potential for application of RuBisCO as protein source in plant-based coffee whiteners, ingredient in cocktails and other associated products.

7 Nutritional, functional, and organoleptic properties of RuBisCO

RuBisCO is a non-allergic protein with excellent nutritional value due to its good digestibility and essential amino acid composition (Sánchez and Vázquez, 2017; Di Stefano et al., 2018). This protein provides all essential amino acids, including required calories (Mariotti and Gardner, 2019). The functional properties of RuBisCO have been linked with its bioactive peptides. Various functional properties such as memory-enhancing, appetite-stimulating, and antioxidative have been linked to the RuBisCO peptides (Di Stefano et al., 2018).

7.1 Amino acid composition

The amino acid composition is essential in determining the overall nutritional quality of proteins. A protein containing balanced levels of nine essential amino acids is considered a complete protein (Pérez-Vila et al., 2023). RuBisCO obtained from spinach (*S. oleracea* L.) provides more than enough essential amino acids, as outlined in the WHO/FAO/UNU Report (FAO, 2013). The literature reported the essential amino acid composition of spinach (*S. oleracea* L.) RuBisCO seems to be comparable or even superior to that of animal proteins, especially beef and egg proteins (Figure 4). A larger subunit (LSU) of RuBisCO is highly conserved and because of this Rubisco protein isolated from different plants may have a similar nutritional profile (Liu et al., 2017; Pearce and Brunke, 2023). However, like other proteins, the nutritional value of RuBisCO is also affected by antinutritional compounds such as phenolics, phytic, and oxalic acids present in the plant tissues (Pérez-Vila et al., 2022).

7.2 Functional peptides derived from RuBisCO

During digestion, RuBisCO proteins can undergo proteolytic cleavage, resulting in the formation of functional or bioactive peptides. With both positive and negative impacts on health, these functional peptides have usually short chain amino acid sequences that can interact with various other proteins in the human body. Some RuBisCO-derived peptides demonstrated antihypertensive antioxidant, anti-cancer, anti-allergy, and anti-atherosclerotic properties, including appetite-regulating and memory consolidation effects (Yang et al., 2003; Kaneko, 2021; Kaneko et al., 2022; Zarandi-Miandoab et al., 2023; Shu et al., 2024). Notably, two peptides originating from the RuBisCO LSU, Rubiscoin-5 (amino acid sequence: YPLDL) and Rubiscolin-6 (amino acid sequence: YPLDLF), were δ-opioid receptor binding peptides of spinach (*S. oleracea* L.) RuBisCO (Yang et al., 2001). These rubiscolins are selective δ-opioid receptor agonists that have antinociceptive effects upon oral administration (Karasawa et al.,

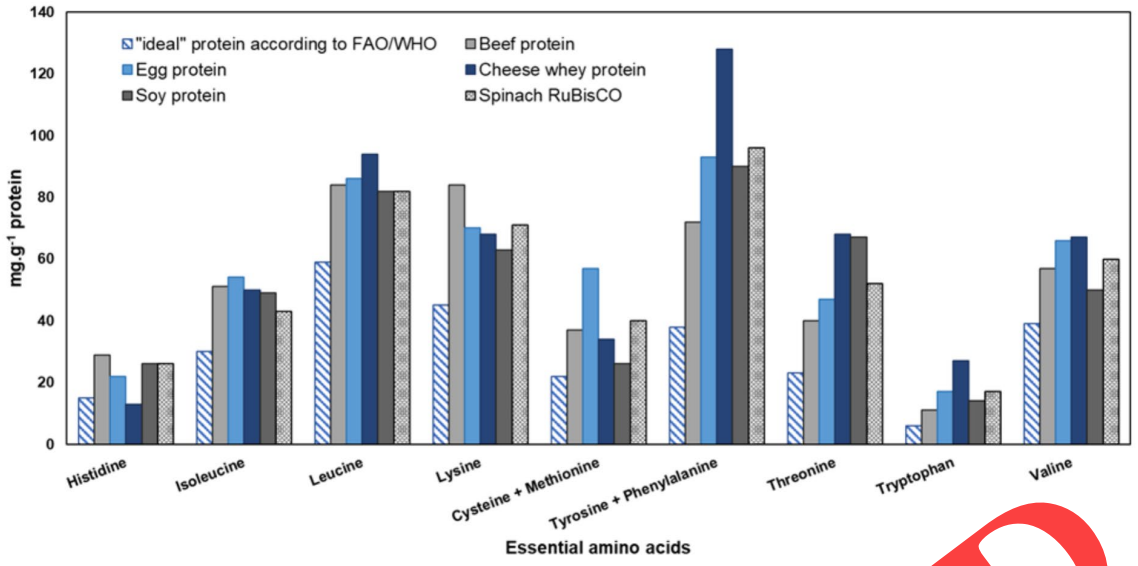

FIGURE 4
Essential amino acids composition of spinach (*S. oleracea* L.) RuBisCO and other commercial animal and plant proteins in comparison with the "ideal" protein according to FAO/WHO. Data adapted from FAO (2013) and Pearce and Brunke (2023).

2021). Further, 3 nmol/mouse intracerebroventricular or 100 mg/kg after oral administration of rubiscolin-6 has been linked with improved memory consolidation in mice (Yang et al., 2003). Although, no significant effects of rubiscolin-5 were observed on mice memory consolidation (Yang et al., 2003). Rubiscolin-6 is also found to induce an antidepressant-like effect through the activation of the δ-opioid receptor in mice (Mitsumoto et al., 2019). Similarly, it may play a role in reducing anxiety (Karasawa et al., 2023). In mice, intraperitoneally administration of rubiscolin-6 showed the orexigenic effect that may apply to treat anorexia and cachexia, conditions linked with severe loss of food intake and appetite (Ataka et al., 2022). Spinach-origin rubiscolin-contains naturally occurring opioid peptides, which are selective δ-opioid receptor agonists with antinociceptive effects. The YHIEPV peptide derived from the pepsin-pancreatin digestion of Rubisco was found effective in improving neural leptin responsiveness and limiting dietary-related weight gain in mice (Kaneko et al., 2022).

7.3 Digestibility

Excellent digestibility is linked with protein quality. Animal proteins, like those found in meat and milk, are highly susceptible to enzymatic hydrolysis, with over 95% digestion efficiency. Conversely, unprocessed protein-containing plant products often exhibit lower digestibility rates (ranging from 50 to 80%) due to challenges in disintegrating cellular walls and the presence of anti-nutritional factors. However, the digestibility of plant protein isolates increases after removing the cell wall and other components (Kaur et al., 2022). Higher bio-accessibility has been reported to the amaranth (*Amaranthus tricolor* L.), chia seeds (*Salvia hispanica* L.), broad beans (*Vicia faba* L.), and alfalfa leaf protein concentrate in the stomach and small intestine (Ramírez-Rodrigues et al., 2022). RuBisCO, interestingly, has been observed to undergo rapid degradation by digestive enzymes, often breaking down quickly within seconds (Tanambell et al., 2024). In the intestinal phase, RuBisCO is hydrolyzed to a high extent, mainly into the smaller peptides or amino acids. RuBisCO digestibility was shown to be independent of processing history and purity (Tanambell et al., 2024). Non-proteinaceous components such as phenolic molecules bind to RuBisCO and decrease its degradation, thereby reducing its nutritional value (Pedone et al., 1995; McNabb et al., 1998; Molan et al., 2000; Bunglavan and Dutta, 2013).

7.4 Allergenicity

With the growing demand for proteins for human consumption, it is noteworthy that all commercially available proteins are universally acknowledged as major allergens, a recognition held in both Europe and the United States. This serves as a cornerstone for understanding the allergenic potential and implications associated with protein sources. For instance, in soybean alone, there are approximately 15 allergenic proteins, which pose significant dietary restrictions for individuals with allergies (Pi et al., 2021). Similarly, wheat, which contains over 100 main proteins, is known to be allergenic, primarily due to gluten (Menezes et al., 2024). Among other plant-based protein sources, lupin is listed as an allergen in the EU and Australia (Villa et al., 2020), and per Food and Drug Administration (FDA) recommendations, individuals allergic to peanuts (*Arachis hypogaea* L.) may also show sensitivity to lupin (*Lupinus* spp.), which can be severe and life-threatening (Grossman, 2022).

In contrast to other plant-based proteins, RuBisCO protein is classified as non-allergenic (Goodman and Leach, 2004; Ahrens et al., 2014). Thus, it can be used as based line control for testing allergenic experiments (Goodman et al., 2008; Nayak et al., 2013; Karasawa et al., 2023). In dogs, a large sub-unit of RuBisCO was responsible for the immunoglobulin E-mediated green grass allergy (Mason et al., 2023). There is only one reported case where a 23-year-old woman experienced an allergic reaction to spinach (*S. oleracea* L.) consumption. Biochemical analysis linked symptoms of asphyxia and

angioedema with an allergic reaction to RuBisCO, which affected her lips and tongue (Foti et al., 2012).

7.5 Antinutritional components

Commonly occurring phytochemicals in leaves such as phytates, oxalates, tannins, saponins, alkaloids, and cyanogenic glycosides are generally considered as antinutrients as they can impair gastrointestinal functions and metabolic performance leading to reduced digestibility and bioavailability of RuBisCO. Therefore, these phytochemicals must be removed during the extraction of RuBisCO (Di Stefano et al., 2018). Heat treatment is the most effective method to reduce antinutrients factors in green leafy vegetables. Cooking and blanching remove antinutrients by breaking the plant cell wall and leaching soluble compounds. However, this practice can also leach out RuBisCO. According to a study on anti-nutrient reduction on amaranth (*Amaranthus tricolor* L.), bathua (*Chenopodium album* L.), fenugreek (*Trigonella foenum-grecum* L.), and spinach (*S. oleracea* L.) leaves, blanching the leaves for 10 to 15 min significantly reduced the amount of phytic acid (Yadav and Sehgal, 2003).

7.6 Flavor

RuBisCO being a leaf protein has undesirable grassy flavors, which makes it difficult as an alternative to meat protein in human food (Ducrocq et al., 2020). In plants, compounds like aldehydes, ketones, and alcohols are the primary volatile organic compounds responsible for the "green" and "grassy" flavor notes of plant proteins (Ebert et al., 2022). However, it is noteworthy that the purification of RuBisCO can help to make it odorless, including colorless (Smit et al., 2015).

8 Extraction of RuBisCO

In 1773, the French biochemist Hilaire M. Rouelle isolated proteins from green leaves, even though the understanding of proteins was limited during that era (Takanishi, 1989). The initial juice extraction from leaves was performed using a mortar and pestle, which he referred to as green juice. He achieved this by heating the solution until it was too hot to keep a finger in it for an extended period. The green coagulum was separated by filtration. The brown juice that remained was then further heated, producing a light coagulum. While the extraction techniques have evolved significantly since then, the fundamental principles remain the same: extracting foliage juice, eliminating the green components, and ultimately refining and concentrating the white portion. The pictorial representation of typical RuBisCO extraction methods is presented in Figure 5. Also, various RuBisCO extraction methods (lab/pilot scale) reported in the literature are presented in Table 2.

8.1 Green juice extraction through pressing: an essential step in protein isolation

RuBisCO is a globular protein found within leaf cells. Foliar protein extraction starts with breaking down the cellular walls to release juices (Di Stefano et al., 2018). To maximize protein extraction from leaf tissues, it is essential to achieve a high degree of cell defragmentation (Streatfield, 2007). Screw pressing is a highly effective approach for extracting green juices, both in laboratory and pilot-scale operations (Lamsal et al., 2003). In this process, foliar fluids are extracted, and the fibrous pulp is expelled at the end of the screw. An appropriate screw press allows to extract up to 70% of the total green juice from the biomass. By incorporating water during the extraction, proteins trapped in the fibrous pulp are washed out, leading to a higher protein recovery (Tenorio et al., 2016).

Recent studies have demonstrated that the homogenization of cauliflower (*Brassica oleracea* L.) leaf biomass via ultrasonication resulted in more thorough cellular disintegration enhancing protein recovery (Pérez-Vila et al., 2022). Protein recovery can also be increased using alkaline solutions instead of water during the juice extraction process. This is attributed to the increased protein solubility and more efficient chloroplast disruption in an alkaline solution, leading to greater protein recovery (Kumar et al., 2021). Fresh or frozen leaves are the most commonly used raw materials, although dried leaf material is employed in some studies. In these cases, protein solubility and thus yield increases in alkaline solutions, i.e., at pH 10 (Anoop et al., 2023).

In addition, the incorporation of other chemicals into the juicing process has been found effective in increasing protein recovery. For example, $Na_2S_2O_5$ is a preservative and antioxidant (E-number E223) compound and it effectively slows down browning reactions during the protein extraction process (Nynäs, 2018). Similarly, detergents

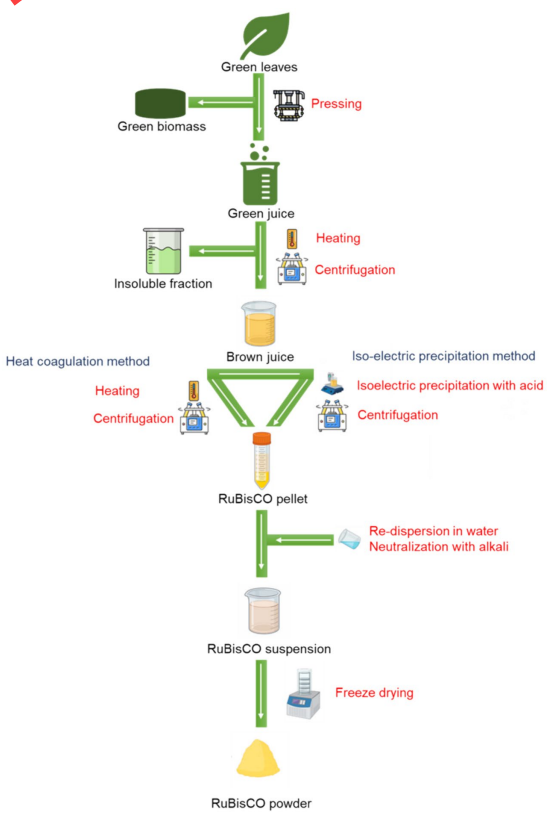

FIGURE 5
Pictorial representation of typical RuBisCO extraction method.

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TABLE 2 Some RuBisCO extraction methods (lab/pilot scale) reported in the literature.

Plant biomass	Green juice extraction	Removal of large particles	Removal of a green fraction	Concentration and purification	The final product (white or green protein concentrate)	References
Dried alfalfa (*M. sativa* L.)	Solubilization at pH 10, 50°C	Centrifugation (10,000×*g*, 20 min)	• Thermal coagulation at 50°C for 2 h • Centrifugation (10,000×*g*, 20 min)	• Isoelectric precipitation at pH 3.5 • Resolubilization and neutralization • Dialysis (3.5 kDa)	White protein	Hojilla-Evangelista et al. (2017)
Fresh alfalfa (*M. sativa* L.)	Hammer mill and multi-cone press	Centrifugation (3,066×*g*)	• Thermal coagulation at 55°C • Centrifugation (3,066×*g*)	• Isoelectric precipitation at pH 3.5 • Resolubilization and neutralization • Ultrafiltration	White protein	Lamsal et al. (2007)
	Chopping and pressing	Filtration	• Thermal coagulation at 55°C using tubular heat exchanger • Centrifugation	• Ultrafiltration (10 kDa cut-off) • Two rounds of intermittent diafiltration were conducted with a transmembrane pressure set at 250 kPa, with inlet and outlet pressures maintained at 300 and 200 kPa, respectively.	White protein	Firdaous et al. (2017)
	Screw pressing	Centrifugation (10,000×*g*)	• Thermal coagulation at 60°C for 20 s • Centrifugation (10,000×*g*)	• Heat coagulation at 80°C • Centrifugation • Washing at pH 4.5	White protein	Edwards et al. (1975)
	Hammer mill and screw press	Centrifugation	• Flocculant • Centrifugation	• Isoelectric precipitation at pH 4.0 • Centrifugation	White protein	Fiorentini and Galoppini (1983)
	Screw press	Filtration	Ultrafiltration (15 kDa)	• Heat coagulation using steam injection (95°C for 30 s) • Centrifugation	White protein	Koschuh et al. (2004)
	Screw pressing	Centrifugation (2,500×*g*, 15 min)	–	• Isoelectric precipitation at pH 3.5 • Centrifugation (2,500×*g*, 15 min)	Green protein	Hansen et al. (2022)
	• Milling, pressing, drying and granulation of green juice • Ammonious extraction from dried green juice at pH 10	Centrifugation (10,000×*g*, 20 min)	• Isoelectric precipitation at pH 3.0 • Centrifugation (10,000×*g*, 20 min)	• Microfiltration • Washing	White protein	Kobbi et al. (2017)

(Continued)

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TABLE 2 (Continued)

Plant biomass	Green juice extraction	Removal of large particles	Removal of a green fraction	Concentration and purification	The final product (white or green protein concentrate)	References
Rye grass (*Lolium perenne* L.)	Screw press	Centrifugation (15,000×*g*, 30 min)	• Thermal coagulation at 50°C for 30 min • Centrifugation (15,000×*g*, 30 min)	• Isoelectric precipitation at pH 3.5 • Centrifugation (15,000×*g*, 30 min)	White protein	Pérez-Vila et al. (2022)
Spinach (*S. oleracea* L.)		Centrifugation (15,000×*g*, 35 min)	• Thermal coagulation at 50°C for 30 min • Centrifugation (15,000×*g*, 35 min)	• Clear juice pH adjusted to 8 using solid Tris-base and 4 M NaOH • Filtration (0.45 μm) • Ion exchange Chromatographic purification using Q-sepharose FF column • Diafiltration (3.0 kDa cut off)	White protein	Martin et al. (2014)
Sugar beet (*B. vulgaris* L.)		Centrifugation (17,000×*g*, 45 min)	• Addition of $Na_2S_2O_5$ (2 g kg^{-1}) and $CaCl_2.2H_2O$ (200 mmol L^{-1}) • Thermal coagulation at 50°C for 15 min • Centrifugation (17,000×*g*, 45 min)	• Ultrafiltration (100 kDa cut off) • Diafiltration against $Na_2S_2O_5$ (2 g L^{-1}) to remove small peptides, polyphenols, and inorganic compounds • Further diafiltration against demineralized water to remove salts	White protein	Martin et al. (2019)
Tropical aquatic plants *viz.*, *Typha latifolia* L., *Hygrophila spinosa* Schumach, *Ottelia alismoides* L. Pers. and *Polygonum barbatum* L.	Pulping and pressing	Centrifugation	• Thermal coagulation at 80°C • Centrifugation		Green protein	Dewanji et al. (1997)
Dried duckweed (*L. minor* L.)	Rehydration at various concentrations *viz.*, 2–6% w/w	–	• Thermal coagulation at various temperatures *viz.*, 50–80°C and various pH levels *viz.*, 9–11 for 2 h • Centrifugation (12,000×*g*, 20 min)	• Washing and centrifugation at 12,000×*g*, 20 min	White protein	Muller et al. (2023)
Unicellular organisms *viz.*, *Arthrospira (spirulina) maxima*, *Nannochloropsis gaditana*, *Tetraselmis impellucida*, and *Scenedesmus dimorphus*	• Rehydration in K_3PO_4 buffer pH 11 • Bead milling	Centrifugation (70,000×*g*, 30 min)	Dialysis (12–14 kDa cut off)	• Ion-exchange chromatography • Dialysis (12–14 kDa cut off)	White protein	Teuling et al. (2017)

such as Tween 80, an amphipathic compound can accelerate the disruption of cell membranes by increasing the release of membrane-bound proteins (Gomes et al., 2020). Proteolytic enzymes have also been effectively used for increasing protein extraction, but it is important to note that only degraded peptides of varying sizes could be obtained. Additionally, buffer solutions of reducing agents and sucrose have been found effective in protein extractability from the solutions. However, this also increases the extraction costs and the end product quality may be affected (Sari et al., 2015).

8.2 Strategies for green protein separation

The juice extracted from leaves is not solely composed of soluble proteins; it also includes chlorophyll, proteins associated with chlorophyll, membrane fragments, and other undesirable substances, all of which have the potential to influence the quality end product (Ayele et al., 2021a). Eliminating the green juice enhances the functional properties of the white fraction and reduces the green color as well as any grassy smells and tastes (Hansen et al., 2023). Protein precipitation in green leaf tissues occurs at varying temperatures and facilitates fractionation. For example, in the green fraction, protein aggregation occurs at temperatures between 50°C and 65°C, while the soluble white proteins precipitate at 80–82°C (Nynäs, 2018). One proposed method for fractionating the proteins is sequential thermal treatment an intermediate separation step. For instance, heating at 60°C for 20 s through steam injection is sufficient to coagulate the green fraction, while extraction at milder temperature may require prolonged treatment duration, i.e., 50°C for 30 min (Phillips and Williams, 2011). Various temperatures and complete processes have been employed for protein extraction from different plant sources. Another approach to removing the green fraction involves the use of flocculants, which induce larger particles to settle. The proteins that aggregate can be readily separated through either centrifugation or filtration, resulting in a clear brown supernatant (Fiorentini and Galoppini, 1983; Barros et al., 2015).

8.3 Purification and concentration techniques for leaf-based white proteins

The elimination of the green fraction from leaf juice yields a brown juice containing white proteins. Further purification of brown juice may be required depending on the intended use of the final product, as several substances in brown juice can affect the nutritional and functional properties of a protein. Purification methods include salt fractionation, chromatography, and membrane filtration, which have traditionally been seen as costly but are becoming more affordable and efficient (Tenorio et al., 2016). Iso-electric precipitation is another method where proteins precipitate at pH close to their respective Ip, typically within pH 3.5–4.5, allowing the separation of proteins from soluble compounds (Hadidi et al., 2020). The co-precipitated substances from the precipitate are removed using a pH-adjusted solution and then re-dissolved in neutral or slightly alkaline water (Soo et al., 2021). A dialysis step might be needed for further purification and exclusion of salts and other small molecules from the concentrate (Barashkova and Rogozhin, 2020). High protein concentration can also be achieved by thermal denaturation of the white fraction at 95–100°C, simultaneously providing pasteurization for extended shelf life (Nynäs et al., 2021).

To achieve high yields and streamline the process, one option is to produce total leaf protein concentrates that contain both green and white protein fractions (Balfany et al., 2023). This is achieved by fully precipitating the green juice, which can be done through heating at 80°C, acidification using hydrochloric acid, or fermentation. Fermentation involves the application of natural microorganisms or inoculated lactic bacteria to reduce the solution pH to 3.5 and to accelerate protein precipitation (Liese et al., 2023). Another option is to precipitate unfractionated proteins through freezing and subsequent thawing of the green juice, resulting in a curd primarily composed of chloroplasts. Ultrafiltration of the green juice provides yet another method for obtaining a concentrated protein solution (Santamaría-Fernández and Lübeck, 2020).

8.4 Limitations related to RuBisCO production

To achieve high yields and streamline the process, one option is to produce total leaf protein concentrates. Commercial production of RuBisCO necessitates the utilization of techniques capable of handling substantial quantities of plant material while adhering to food safety standards. Various feasibility studies have identified promising approaches, warranting further research into their practicality for extracting proteins from green leaves. Assessing the existing techniques for extracting protein from green leaves is imperative for any business endeavor. The primary obstacles associated with RuBisCO extraction (refer to Figure 6) are outlined below.

In contrast to extracting proteins from animal-derived sources, where the protein is readily accessible, proteins in leaves are encapsulated within tough cell walls reinforced with cellulose (Hadidi et al., 2022). Maceration of green raw material is commonly achieved by mechanical pressing (twin-screw press), in which the interlocking screws compress the plant tissues against a screen, facilitating the collection of juice (Ayele et al., 2021b). Other extraction methods, such as sugarcane rolls, hammer mills, or shredders can also be used, although the twin-screw presses are the most commonly used technique for leaf tissues of crops such as sugar beet (*B. vulgaris* L.), spinach (*S. oleracea* L.), and alfalfa (*M. sativa* L.) (Anoop et al., 2023). During the extraction, various lysis chemicals may be added to enhance product yield (Sari et al., 2015). Since RuBisCO is located inside chloroplasts, rupturing cell walls and then chloroplast membranes to release the protein can be challenging. Therefore, lysis enzymes such as cellulase, hemicellulose, or pectinase are usually added to break down the cell walls and enhance protein yields (Guo et al., 2024).

Upon disrupting the leaf material, diphenols and quinones can be formed due to the activation of polyphenol oxidase. The resulting quinones can undergo additional reactions, either self-reacting to generate brown pigments or interacting with proteins (Selvarajan et al., 2018). These brown compounds not only diminish consumer acceptance of the final product but impair protein functional quality. While PVPP (polyvinylpolypyrrolidone) is often employed to mitigate browning in laboratory-scale purifications (Xu and Diosady, 2002), this method is unsuitable for large-scale applications. Alternatively, antioxidants such as ascorbic acid or metabisulfite can effectively

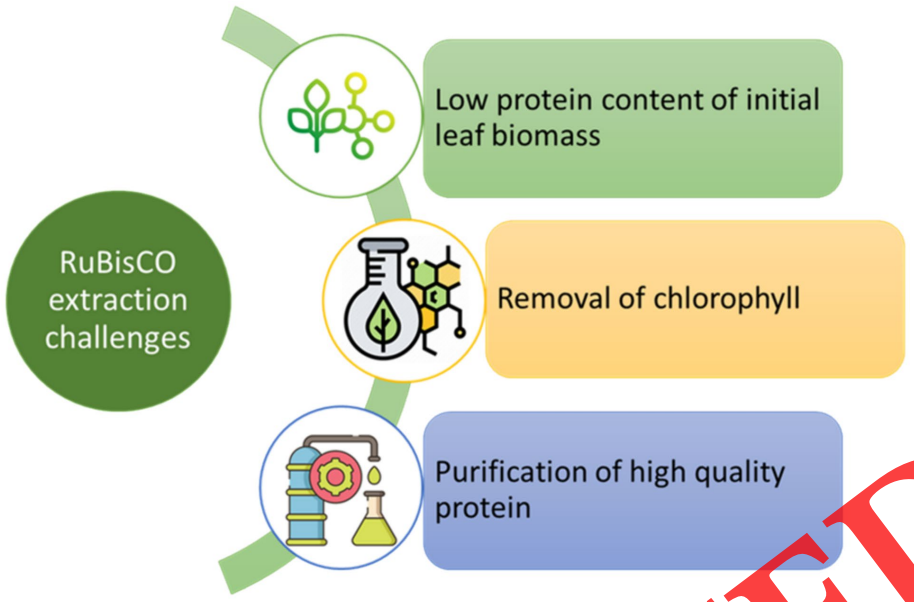

FIGURE 6
Technical challenges associated with RuBisCO extraction.

inhibit polyphenol oxidase activity, thereby reducing the occurrence of browning.

The RuBisCO extraction and separation from leaf tissues present a significant challenge in food protein production. Key goals include the removal of green chlorophyll to yield a colorless compound, the elimination of small molecules linked to bitter or 'vegetal' tastes, and the concentration of the material into a practical form (Kapel et al., 2006). Some biorefineries have adopted a straightforward approach by precipitating all proteins from the juice, yielding protein concentrates, which can be used as animal feed (Santamaría-Fernández and Lübeck, 2020). Nevertheless, when taking consumers into account, the product's attractiveness is compromised by the green color, undesirable taste, and the presence of minor components. Green juice comprises soluble proteins, primarily RuBisCO, as well as insoluble proteins, including significant cell debris such as cell walls and broken organelles. While higher centrifugation rates are viable on a laboratory scale, alternative methods become necessary during pilot or commercial production (Udenigwe et al., 2017).

Maintaining the native structure of the RuBisCO molecule during the entire process of extraction is typically crucial for ensuring the quality of the end product. Methods employed must ensure that RuBisCO remains undenatured. One straightforward approach involves heating the juice to 50–55°C for 20–30 min (Tenorio et al., 2016). This process induces the aggregation of chlorophyll and associated proteins, collectively termed as 'green protein'. Significantly, the temperature range employed is below the threshold for denaturing RuBisCO. The precipitated compounds can then be separated through centrifugation or decanting.

In specific cases, eliminating green proteins initially is considered satisfactory, and the resulting supernatant can be freeze-dried to produce white protein concentrate, as observed in studies by Tamayo Tenorio et al. (2017b). However, multiple methods are commonly utilized for increasing the purity of white proteins in the supernatant and segregating it from additional smaller molecules that could impact taste, color, aroma, or functionality (Tamayo Tenorio et al., 2017a). Isoelectric precipitation (Ip) is a commonly used method in large-scale RuBisCO extractions, where pH is reduced to ~4.5, approaching the Ip, and precipitated RuBisCO is subsequently recovered via centrifugation (Kobbi et al., 2017). High temperature (80°C) treatment is also used for purification of the white fraction containing RuBisCO, however, RuBisCO denaturation and gelation occur upon the subsequent cooling of the white fraction, as reported by Koschuh et al. (2004). Typically, treatments are customized to maintain the minimal stress on protein extraction and purification steps to attain a purified protein product with desired quality traits *viz.*, colour, flavour, and physicochemical properties. Such treatments include membrane filtration, where end products are purified and concentrated by eliminating undesirable components (Mohammad et al., 2012). In microfiltration, fibrous material, chloroplast, and bacterial cells can be separated from protein by varying the pore size. Nowadays, many researchers regularly use centrifugal filters to separate desired proteins from other impurities in solutions. A membrane with a cut-off lower than that of the protein allows the separation of water and small molecules from the sample. Diafiltration, aimed at preserving the protein fraction, is sometimes combined with the aforementioned filtration method to effectively remove small molecular weight contaminants (Martin et al., 2019). Nieuwland et al. (2021) adopted this method to produce a concentrated duckweed (*L. minor* L.) RuBisCO. They commenced the process with microfiltration (0.45 μm) to eliminate microbes and insoluble green fraction, followed by ultrafiltration (100 kDa cut-off) to concentrate RuBisCO.

The incorporation of washing steps is a common practice aimed at enhancing the quality of the product by removing impurities, such as phenols. These impurities tend to interact with the protein fraction (Wang et al., 2003). Membrane fouling, besides the associated impurities in the end product, can reduce the efficiency of the filtration process in RuBisCO extraction and purification (Zhang et al., 2015). Moreover, proteins may bind to the fouling layer of non-permeating material, leading to a reduction in achievable yields (Hoffmann et al.,

2019). Certain purification techniques integrate continuous flow membrane systems where the fouling layer is consistently washed away, and the permeate volume is replenished with buffers. As a result of this process, protein flux is enhanced while concentration effects are mitigated. Nevertheless, these techniques may be challenged by impurities such as phenolic compounds, lipids, and carotenoids that persist in the protein fraction, primarily when permeating it (Zhang et al., 2015). Amendments, such as temperature increments have been found effective for improving membrane flux and membrane specificity (Zhao et al., 2017). Further, optimizing other extraction parameters such as pH and electric conductivity can increase the yield of soluble proteins and their functional properties, as demonstrated in studies by Lamsal et al. (2007) and Nissen et al. (2021).

9 Potential commercial usage

RuBisCO emerges as a potential vegan protein ingredient in the current market of plant-based processed foods due to lesser environmental impact, good PDCAAS, favorable sensory and physical properties, as well as potential bioactivities that contribute to human health, all without triggering allergenicity concerns. Figure 7 highlights the primary advantages of incorporating RuBisCO as a food supplement.

In contrast to the environmental impacts associated with the production of many other proteins, particularly those of animal origin, RuBisCO distinguishes itself as a premier protein option, particularly in terms of adherence to circular economy principles and sustainability. Its widespread presence in all photosynthetic plants, bacteria, and fungi, attributable to its role in oxygen reaction and catalytic inefficiency, facilitates its easy isolation from green vegetable waste generated by various industries. Although commonly isolated from sources *viz.*, alfalfa (*M. sativa* L.) and sugar beet (*B. vulgaris* L.) leaves, RuBisCO can be extracted from various crops, including those yielding common industrial waste such as carrot or tomato leaves. This characteristic makes it an exceptionally eco-friendly vegan protein source for food and feed purposes. Furthermore, macro and microalgae present additional promising sources of RuBisCO due to

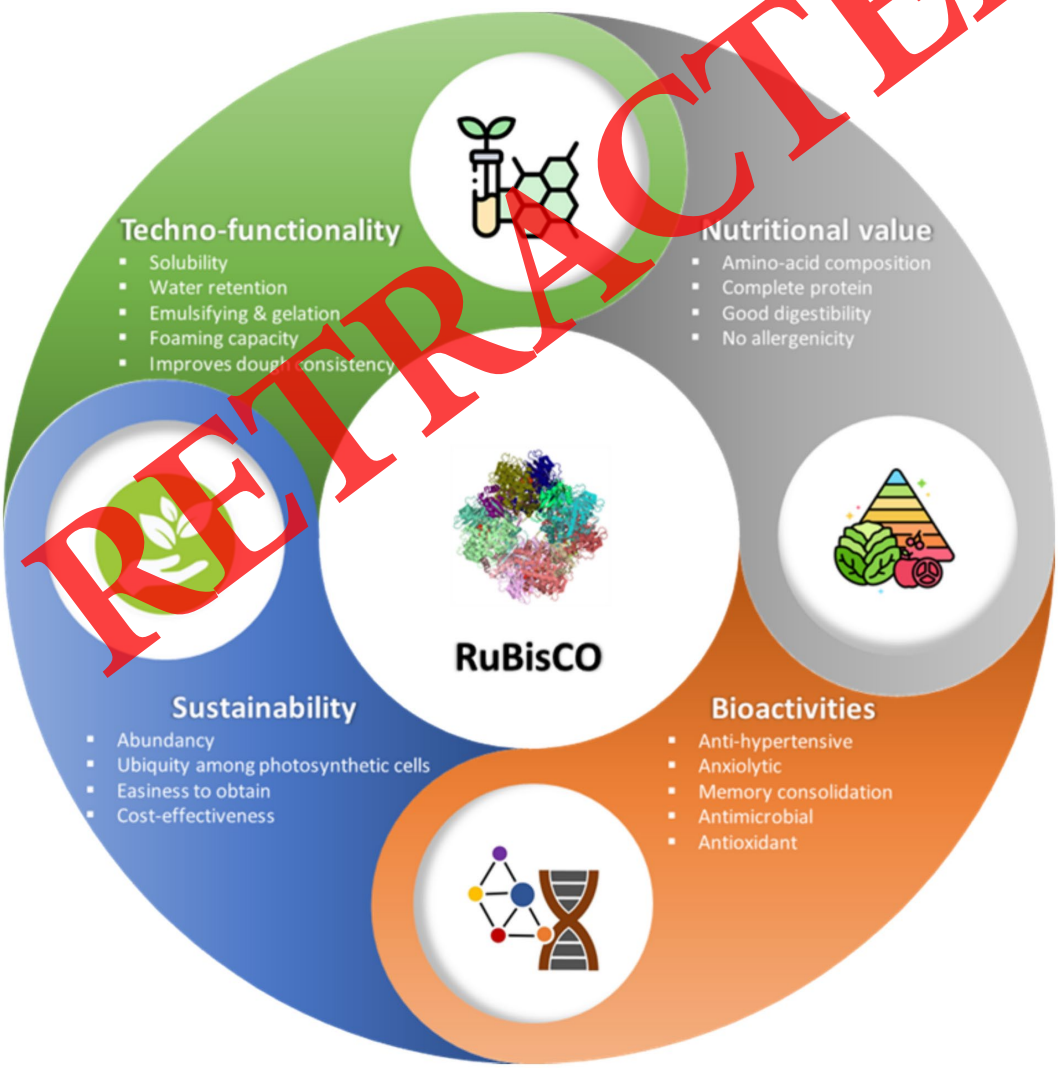

FIGURE 7
Benefits of utilizing RuBisCO as a food ingredient.

their rapid growth and ability to produce substantial biomass in short timeframes.

10 Conclusion

Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) a carboxylase enzyme is a widely distributed plant protein stored in green tissues of plants, algae, cyanobacteria, various photosynthetic bacteria, and even some non-photosynthetic alga and non-photosynthetic chemoautotrophic bacteria. RuBisCO is a globular protein with a molecular weight of 550 kDa. RuBisCO possesses significant potential in meeting the growing global demand for vegan proteins and can be a promising solution for addressing malnutrition due to its wide availability, nutritional composition, and good techno-functional properties. However, it is currently underutilized in the food industry due to the lack of scalable extraction methodology and the inferior quality of RuBisCO end product mainly due to its greenish appearance and undesirable grassy off-flavor. Therefore, more effective techniques for its decoloration and purification are needed without compromising its digestibility and bioavailability. Additionally, numerous studies reported the presence of bioactive peptides in RuBisCO. However, further studies are warranted to validate the health benefits and risks associated with these bioactive peptides. Altogether, RuBisCO due to its unique physicochemical and functional properties can be a sustainable source of protein in the future for vegetarian diets.

Author contributions

MN: Writing – original draft, Visualization, Data curation, Conceptualization. DK: Writing – original draft, Conceptualization. NU: Writing – review & editing. KU: Writing – review & editing. MA: Writing – review & editing, Supervision, Conceptualization.

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