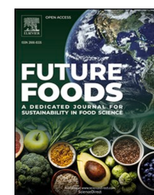
Future Foods 9 (2024) 100310

Contents lists available at ScienceDirect

Future Foods

journal homepage: www.elsevier.com/locate/fufo



Heat-induced nanoparticles from pumpkin leaf protein for potential application as β-carotene carriers

Milica N. Perović [a], Zorica D. Knežević Jugović [b], Mirjana G. Antov [a,*]

[a] University of Novi Sad, Faculty of Technology, Blvd. Cara Lazara 1, Novi Sad, Serbia
[b] University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade, Serbia

ARTICLE INFO

Keywords:
Leaf protein
Enzyme-assisted extraction
Secondary structure
β-Carotene
Surface hydrophobicity
Quenching constant

ABSTRACT

Nanoparticles prepared by heat treatment of protein from pumpkin leaves were evaluated as potential carriers of β-carotene. White protein fraction was recovered from green juice produced by pressing the leaves, with the step of enzyme-assisted extraction (green protocol) or without it (conventional protocol). Heat treatment of white protein fractions from conventional and green protocols at 90 °C and pH 9.3 during 20 min induced formation of nanoparticles with peak diameter 18 nm and 21 nm, respectively. Due to heating, portion of β-sheets in nanoparticles from both native protein fractions decreased by approximately 15 %, associated with an increase in surface hydrophobicity-to-area ratio. Quenching constant of β-carotene for nanoparticles was increased nearly 100 times by heating the white protein fraction recovered in green protocol. Native white protein fraction from conventional protocol and corresponding nanoparticles exhibited high ability to bind β-carotene, with quenching constant 3×10^5 L/mol and 3.3×10^5 L/mol, respectively. White protein fraction from pumpkin leaves appeared to be a suitable substrate for the fabrication of nanoparticles by heat treatment, with potential application as β-carotene nanocarriers in food matrices.

Introduction

Food proteins are remarkable molecules - besides being valuable nutrients, they affect physicochemical characteristics of food matrices. Consequently, proteins greatly contribute to functional properties and food quality, and therefore are considered the most valuable functional ingredients in food products (Kinsella and Melachouris, 1976). Faced with fast raise in human population at global level, efforts of both food industry and research are lately accelerated to find new protein sources. Proteins from these new sources should not negatively impact product quality and safety, and at the same time, their production should take into account the environmental issue (Aiking, 2011; Henchion et al., 2017).

In addition, along with the changes in socio-economic milieu and risen awareness of food-health relation, a considerable attention has been focused on the design of foods and food ingredients with enhanced nutritional and functional properties (Henchion et al., 2017). However, direct incorporation of certain types of bioactive components into food products is challenging because of their low solubility, poor stability, and low oral bioavailability. The suitability of proteins for the fabrication of different nanostructures able to encapsulate wide spectrum of bioactive components for their incorporation in food matrix is well known. Protein nanocarriers can improve solubility, stability and bioavailability of such compounds thus improving nutritional and functional properties of foods (Mohammadian et al., 2020). There are numerous methods for the fabrication of nanocarrier structures for their application in food systems (McClements, 2014). Among them, heat treatment is simple and inexpensive, and induces unfolding of protein molecule. This results in promoted aggregation of the unfolded molecules through exposed hydrophobic amino acid residues (Sponton et al., 2017). It was shown that heat-induced protein nanoparticles were able to bind linoleic acid (Perović and Antov, 2022; Sponton et al., 2020), which could be beneficial for both its improved incorporation in food matrices and protection from oxidation.

Protein from various sources can be used for the preparation of nanocarriers, yet preference is given to plant proteins due to their availability and low toxicity as well as renewability of plant-derived protein sources. In addition, production of plant proteins is more sustainable and cost-effective in comparison to animal proteins (Aiking, 2011). Even more, protein produced from waste plant materials such as leaves are of high interest to the food industry due to its abundancy and good functional properties (van de Velde et al., 2011).

* Corresponding author.
E-mail address: mantov@uns.ac.rs (M.G. Antov).

https://doi.org/10.1016/j.fufo.2024.100310
Received 31 August 2023; Received in revised form 29 December 2023; Accepted 2 February 2024
Available online 2 February 2024
2666-8335/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The aim of this study was to evaluate the potential of white protein fraction isolated from pumpkin leaves for the fabrication of nanoparticles. Suitability of protein nanoparticles as nanocarriers for β-carotene was studied. β-Carotene is a precursor of vitamin A but also beneficially affects human health acting as anticancer agent and antioxidant, in the prevention of cardiovascular diseases etc. (Krinsky and Johnson, 2005). In the food industry, β-carotene is commonly used as dietary supplement and as colorant. However, due to its poor water-solubility, high susceptibility to degradation, etc., its incorporation into food matrix is challenging (Qian et al., 2012). Various techniques were studied to stabilize β-carotene molecule (Qian et al., 2012; Saiz-Abajo et al., 2013), some of them with proteins as carriers (Deng et al., 2016). In this study, protein was isolated from pumpkin leaves by two protocols – one was conventional based on pressing of raw material; the other was considered as the green one because it included a step of recovery of white protein in additional amounts through the enzyme-assisted extraction of fibrous pulp. Nanoparticles were induced by heat treatment and their characteristics regarding size, surface hydrophobicity and secondary structure were analyzed. The ability of protein nanoparticles to bind β-carotene was evaluated by fluorescent spectroscopy.

Materials and methods

Material

Green leaves were collected from fields (JS&O, d.o.o. Novo Miloševo, Serbia) during harvest of hull-less pumpkin (*Cucurbita pepo* L.) during the late summer of 2022. Undamaged leaves were washed, dried with paper tissue and stored at −18 °C in the freezer till processed and analyzed. Dry matter (DM) content in green leaves determined by oven drying at 60 °C until constant mass was 20.46 ± 0.66 %.

β-Carotene (synthetic, purity ≥95 %), sodium azide (purity ≥99.5 %) and Bovine Serum Albumin (BSA, purity ≥96 %) were purchased from Sigma–Aldrich. All other chemicals used were of analytical grade.

Conventional protocol for the preparation of white protein fraction

Green leaves were pressed in cold press juicer (Angel Juicer, model 8500, Angel Juicer Co. LTD, South Korea) to produce two streams – green juice and fibrous pulp. After the addition of sodium metabisulfite (0.5 %) green juice was heated at 55 °C for 30 min in water bath with shaking for the thermal coagulation of green protein fraction. After being cooled to 4 °C suspension was centrifuged in Sorvall RC 5B (15,000 × *g*, 20 min, 4 °C) to remove precipitated green proteins. Then white protein fraction was separated from supernatant (brown juice) by isoelectric precipitation at pH 4.5 under constant stirring. Precipitate was separated by centrifugation (15,000 × *g*, 20 min, 4 °C) and collected protein was reconstituted in distilled water along with pH adjustment to 7. Protein solutions were subjected to freeze drying at −40 °C for 24 h at laboratory lyophilization equipment (Martin Crist Alpha LSC 2–4, Osterode, Germany) to deliver white protein fraction from conventional protocol (CP). Conventional extraction was run in three repetitions.

Enzyme preparation

Enzyme complex (NS22119, Novozymes' Bioethanol Kit), a commercial cocktail containing cellulase, arabinanase, β-glucanase, hemicellulase, pectinase and xylanase was applied for the hydrolysis of cell wall polysaccharides. Cellulolytic activity of was assayed according to Ghose (1987) and one FPU was defined as the amount of enzyme that released 2 mg of reducing sugars from 50 mg of Whatman No.1 filter paper in 1 h under the standard conditions of hydrolysis (temperature 50 °C, pH 4.8). Cellulolytic activity of Enzyme complex was determined to be 30 FPU/mL.

Enzyme-assisted protocol for the preparation of white protein fraction

Pumpkin leaves were pressed and white protein fraction from green juice was recovered as described in Section 2.2. Fibrous pulp was suspended in 0.25 mmol/L acetate buffer (pH 5.5) to solid:liquid = 1:5 (m/v) and extraction was carried out with the addition of 7.5 FPU/g_{DM} during 30 min, at 45 °C. Then suspension was cooled to 4 °C and centrifuged at 10,000 × *g* for 20 min, and sodium metabisulfite was added to supernatant (0.5 %). White protein fraction was recovered from supernatant following equal steps applied in the processing of the green juice in conventional protocol (see Section 2.2). White protein fraction recovered from the fibrous pulp by enzyme-assisted extraction was pulled together with the white protein fraction recovered from the green juice by the conventional protocol, and freeze dried at −40 °C for 24 h to deliver white protein fraction from green protocol (GP). Green extraction was run in triplicate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CP and GP were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 12 % TruPAGE Precast Gels (Sigma Aldrich Co., St. Louis, MO, USA) under reducing conditions with discontinuous buffer system. Electrophoresis was performed for 45 min at constant voltage 180 V and 100 mA. Gels were fixed and stained with Coomassie Blue R250 for 1 hour and de-stained in distilled water. Protein markers kit 7 kDa – 240 kDa (SERVA Electrophoresis, Heidelberg, Germany) was used.

Preparation of protein nanoparticles

Freeze-dried white protein fractions were reconstituted in 50 mmol/L NaCl and pH was adjusted to 9 with 1 mol/L NaOH. Sodium azide (0.2 g/L) was added to prevent microbial growth. Mixtures were stirred for 8 h and kept overnight at 4 °C to achieve complete hydration. Afterwards, dispersions were centrifuged at 9000 × *g* for 10 min in order to eliminate insoluble materials. Concentration of protein in supernatants was determined with Bradford method (Bradford, 1976) with BSA as standard.

For the preparation of protein nanoparticles, supernatants were diluted with 50 mmol/L NaCl to final concentration 4 g/L. The pH value was adjusted 9.3 with 0.5 mol/L NaOH. Prepared solutions were heated in a water bath at 90 °C for 20 min, and then immediately cooled in cold water (Sponton et al., 2017). After the heat treatment no insoluble materials were observed in any of the samples. Samples from three repeated experiments were kept at 4 °C until further analysis.

Fourier-transform infrared spectroscopy and elements of secondary structure

Fourier-transform infrared (FTIR) spectra of native protein and nanoparticles (each in two samples) measured by a Bruker ALPHA FTIR Spectrometer (Bruker, Billerica, MA, USA) with a Platinum ATR that holds a diamond ATR module. The spectra were recorded in reflective mode from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, and resulting spectra were calculated from two repetitions. The relative contents of secondary structures (α-helix, β-sheet, β-turns, random coils) were calculated. All original protein spectra were baseline corrected between 1700 and 1600 cm^{-1} and then fitted with Gaussian band profiles by using Origin software (OriginLab Corporation, Northampton, MA, USA). The number of bands and positions were taken from their second derivative spectra.

Characterization of protein nanoparticles

Size of nanoparticles

The particle size distribution, based on volume, was measured by

dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) in three repetitions. Refractive indexes used for the solvent and the protein aggregates were 1.33 and 1.50 respectively. The Z-average of nanoparticles were determined as well under these assays.

Parameters of surface hydrophobicity of nanoparticles

Surface hydrophobicity (*SH*) was analyzed by measuring the amount of bromophenol blue (BPB) bound by protein and calculated as described earlier (Chelh et al., 2006). Additional parameters required to estimate nanoparticles surface hydrophobicity were calculated based on the mean diameter, taking into account that the particles were spherical in shape.

Fluorescence quenching and Stern–Volmer quenching constant

The interaction between native proteins and nanoparticles with β-carotene was measured by protein intrinsic fluorescence spectroscopy using spectrofluorometer Jasco FP-8550 (Jasco, USA) according to Deng et al. (2016) with slight modifications. Steady-state intrinsic emission fluorescence of CP and GP, and nanoparticles at constant protein concentration (0.5 mg/mL) was recorded from 300 to 450 nm, with an excitation wavelength of 280 nm. The *β*-carotene stock solution (2.5 mmol/L) was prepared in absolute ethanol, and then added dropwise to protein solution till reaching required concentration in the range from 0 to 30 μmol/L. The highest resulting concentration of absolute ethanol was 1.2 % (v/v) and had no appreciable effect on native proteins and nanoparticles structures. After being vortexed for 20 s to ensure complete nanocomplex formation, the mixtures were immediately subjected to the fluorescence determination. Control was run with β-carotene to check autofluorescence effect of ligand (Bohin et al., 2014). Fluorescence signal (calculated as the mean from two repetitions) was corrected for the inner filter effects (IFE) according to the Eq. (1) (Lakowicz, 2006):

$$F_{corr} = F_1 \times 10^{(A_{ex}+A_{em})/2} \quad (1)$$

where F_{corr} is the absorbance IFE-corrected fluorescence intensity, F_1 is the measured (uncorrected) fluorescence intensity, A_{ex} and A_{em} are the absorbances at the fluorescence excitation and fluorescence emission wavelengths, respectively.

The quenching kinetics of intrinsic fluorescence intensity of native proteins and heat-induced nanoparticles by *β*-carotene molecule was described by the Stern–Volmer equation as follows:

$$\frac{F_0}{F} = 1 + K_{SV} \times C_{BC} \quad (2)$$

where F_0 and F were corrected fluorescence intensities in the absence and the presence of β-carotene, respectively; C_{BC} was the concentration of β-carotene in the dispersions; K_{SV} was the Stern-Volmer quenching constant. K_{SV} was calculated from the Stern–Volmer plot with F_0/F against C_{BC} for native proteins and nanoparticles.

Statistical analysis

Statistical analysis was carried out using Statistica (TIBCO Softver Inc.) to compare differences between results by the analysis of variance (One-way ANOVA), followed by Tukey's HSD test. Results of statistical analysis were assumed to be significant when $p < 0.05$. Each experiment was carried out at least in triplicate (unless otherwise stated) and the results were presented herein as mean value ± standard deviation.

Results and discussion

Yield of white protein fraction from conventional and green protocol was 0.61 ± 0.08 % and 2.68 ± 0.09 % (based on pumpkin leaves dry matter), respectively. More than four times increased yield was achieved by the addition the step of enzymatic extraction of fibrous pulp in recovery protocol.

Secondary structure of white protein fractions from pumpkin leaves and heat-induced nanoparticles

FTIR spectra of white protein fractions recovered from pumpkin leaves with or without the assistance of enzymes (Fig. S1) differed a bit at positions assigned to different secondary structures of plant proteins (Shevkani et al., 2019); this reflected the differences in portions of elements of secondary structure between them (Table 1). In general, portion of β-sheets was dominant in both analyzed fractions, with GP having slightly higher value than CP. On the other side, higher percentage of α-helices was present in CP than in GP, while percentages of other elements of protein secondary structure were almost equal. These results indicated differences in composition of CP and GP in terms of proteins that were extracted by two applied protocols. It is known that different proteins had different percentages and ratios of α-helices and β-sheets in their secondary structures (Yu, 2005). It was found that albumin is a dominant protein fraction in leaf proteins, followed by glutelin (Rawiwan et al., 2023; Sun et al., 2017), although their content varies among different plant species. Protein patterns of CP and GP analyzed by SDS PAGE (Fig. 2S) were similar with slight differences. Bands at 14 and 55 kDa corresponding to the small and large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), respectively, (Fiorentini and Galoppini, 1983) were present in both white protein fractions but slightly more intense in GP. Band at 23 kDa, which can be attributed to leaf globulins (Rawiwan et al., 2023), was more distinctive in CP. On the other side, there were more protein bands between 70 and 100 kDa in GP than in CP; those proteins were found to be characteristic for albumin and globulin leaf proteins (Rawiwan et al., 2023). These findings suggested that ratios of different proteins recovered from pumpkin leaves by protocols with and without the assistance of enzymes were different. Consequently, differences in portions of elements of secondary structure in CP and GP were recorded by FTIR analysis.

Heat treatment induced changes reflected on FTIR spectra of nanoparticles in comparison to corresponding fractions (Fig. S1). Applied treatment decreased content of β-sheets and increased content of random coils in both protein fractions (Table 1). Portion of α-helices was increased and that of β-turns almost unchanged. These results are in an agreement with the fact that heat treatment differently affects different elements of secondary structure in protein molecule with β-sheet structure more prone to denaturation (Wang et al., 2014). As the consequence of differences in portions of secondary structures in native fractions, nanoparticles with different proportion of elements of secondary structures were obtained (Table 1). So, resulting nanoparticles

Table 1
Percentages of the elements of secondary structures of native white protein fraction isolated with or without the assistance of enzyme from pumpkin leaves and corresponding heat-induced nanoparticles*.

	α-Helix (%)	β-Sheet (%)	Random coil (%)	β-Turn (%)
CP				
Native protein	25.35 ±0.50^{b}	49.83 ±0.23^{a}	8.05±0.07^{b}	16.88 ±0.58^{a}
Nanoparticles	27.95 ±0.06^{a}	34.97 ±0.03^{b}	19.81±0.26^{a}	17.25 ±0.35^{a}
GP				
Native protein	19.09 ±0.13^{b}	52.97 ±0.18^{a}	9.25±0.35^{b}	18.27 ±0.39^{a}
Nanoparticles	21.04 ±0.06^{a}	37.83 ±0.24^{b}	23.87±0.18^{a}	17.24 ±0.35^{a}

Different letters represent the differences in means of the samples, according to Tukey's HSD test at $p < 0.05$ level; FTIR analysis was run in two repetitions of each protein/nanoparticles.

* Condition of heat treatment: 90 °C, 20 min, pH 9.3.

fabricated from CP finally had higher portion of α-helices and lower portion of β-sheets and random coils in comparison to those fabricated from GP.

Characterization of heat-induced nanoparticles from pumpkin leaf white protein fractions

Nanoparticle size parameters, such as diameter, area to volume ratio (A/V) as well as surface hydrophobicity to area ratio (*SH*/A), are crucial for their possible application in food industry. Heating of native protein solutions induced formation of nanoparticles with monomodal particles size distribution (Fig. 3S). Diameter (peak) of nanoparticles prepared from both CP and GP were shifted towards higher values in comparison to native proteins (Table 2). This can be attributed to heat-induced aggregation of protein molecules due to hydrophobic interaction between hydrophobic side residues of amino acids. Actually, during heat treatment a partial unfolding of protein occurs, dominantly due to denaturation of β-sheet structure (Wang et al., 2014). So, previously buried hydrophobic regions in protein structure become exposed and prone to form hydrophobic interactions leading to aggregation (Sponton et al., 2015). Mean diameter (Z-average) of nanoparticles prepared from CP during heat treatment increased while that of nanoparticles from GP was not significantly changed in comparison to corresponding native proteins. This resulted in larger CP-nanoparticles in comparison to GP-nanoparticles, with relatively lower and higher A/V ratio, respectively. Heat treatment at applied conditions also greatly increased surface hydrophobicity in both protein samples (Table 2), which was in agreement with literature (Wang et al., 2014). When surface hydrophobicity was expressed per area, heating of white protein fractions resulted in nanoparticles from GP having higher value than those from CP.

Evaluation of interaction between nanoparticles from white protein fractions and β-carotene via fluorescence quenching kinetics

Surface hydrophobicity of protein and nanoparticles plays an important role in interactions with various hydrophobic bioactive compounds. β-Carotene is compound with high hydrophobic nature that makes it readily for binding to the hydrophobic regions on the surface of the proteins (Deng et al., 2016). Intrinsic fluorescence quenching technique was used for monitoring the binding of β-carotene to white protein fractions and heat-induced nanoparticles, and results of the quenching constant (calculated from fluorescent spectra, Fig. S4) was presented in Table 2. Ability of native protein fractions to bind β-carotene considerably differed, with CP having K_{SV} almost five hundred times higher than GP despite higher surface hydrophobicity-per-area of the later one. Heat treatment under applied conditions increased quenching constant of β-carotene for both CP- and GP-nanoparticles in comparison to corresponding native proteins. This increase was more prominent for nanoparticles fabricated from white protein recovered by green protocol, while K_{SV} of CP was just slightly affected. The interaction between different hydrophobic bioactive molecules (including β-carotene) and proteins usually occurs by intermolecular hydrophobic interactions. Yet, values of *SH*/A were not consistent with results of quenching constant of β-carotene for nanoparticles. K_{SV} for β-carotene and heat-induced nanoparticles from CP and GP were higher and in the range, respectively, of those reported for soy protein isolate (Deng et al., 2016). However, quenching constant of β-carotene with β-lactoglobulin was considerably higher, 1.31×10^7 L/mol (Allahdad et al., 2019). Such a high ability of β-lactoglobulin to bind hydrophobic ligands resulted from the presence of hydrophobic cavity in its structure; even more, docking studies revealed that binding of β-carotene to this hydrophobic cavity resulted in conformational changes of protein (Allahdad et al., 2019). In comparison to β-lactoglobulin, lower K_{sv} in this study might suggests that complexation of CP, GP and nanoparticles with β-carotene may occur only on the hydrophobic clusters on the surface as it was suggested previously (Deng et al., 2016). In addition, it was found that in some cases van der Waals forces and hydrogen bonding can be involved in the interactions between hydrophobic bioactive molecules and proteins (Livney, 2010). So, differences in binding ability of proteins and nanoparticles in this study might be the result of different mechanisms of interaction with β-carotene.

Table 2
Size parameters, surface hydrophobicity (*SH*) and β-carotene quenching constant (K_{SV}) of white protein fractions from pumpkin leaves and corresponding heat-induced nanoparticles.

	Peak (nm)	Z-average (nm)	*SH* (μg BPB/mg)	A/V* (1/nm)	*SH*/A** (μg BPB/nm^2)	K_{SV} (L/mol)
CP						
native protein	7 ± 0.81^b	207 ± 19.79^b	14.6 ± 0.58^b	0.11	2.7×10^{-5}	3.0×10^5
nanoparticles	18 ± 0.69^a	272 ± 12.02^a	40.8 ± 0.39^a	0.08	4.4×10^{-5}	3.3×10^5
GP						
native protein	13 ± 1.06^b	62 ± 5.36^a	19.1 ± 0.28^b	0.38	3.9×10^{-4}	6.2×10^2
nanoparticles	21 ± 0.08^a	55 ± 1.88^a	58.0 ± 0.40^a	0.43	1.5×10^{-3}	6.0×10^4

Different letters represent the differences in means of the samples, according to Tukey's HSD test at $p < 0.05$ level; measurement were run in three repetitions for each sample; A/V and *SH*/A were calculated from mean values;.
* area/volume ratio – A/V;.
** surface hydrophobicity/area of nanoparticles – *SH*/A.

Conclusions

White protein fractions recovered from pumpkin leaves by conventional and green protocol appeared to be good substrates for the fabrication of nanoparticles by heat treatment. Prepared nanoparticles showed high ability for binding β-carotene. This indicated their high potential for possible application in food matrices as nanocarriers for β-carotene.

Ethical statement - Studies in humans and animals

All authors confirm that presented work doesn't involve usage of human subjects or human data, or research on animals.

CRediT authorship contribution statement

Milica N. Perović: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Zorica D. Knežević Jugović:** Conceptualization, Resources, Supervision, Project administration. **Mirjana G. Antov:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition, Investigation, Methodology, Supervision.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgement

The financial support by the Science Fund, Republic of Serbia,

Project MultiPromis, Grant No.7751519 is greatly acknowledged.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2024.100310.

References

Aiking, H., 2011. Future protein supply. Trends Food Sci. Technol. 22 (2–3), 112–120. https://doi.org/10.1016/j.tifs.2010.04.005.

Allahdad, Z., Varidi, M., Zadmard, R., Saboury, A.A., Haertlé, T., 2019. Binding of β-carotene to whey proteins: multi-spectroscopic techniques and docking studies. Food Chem. 277, 96–106. https://doi.org/10.1016/j.foodchem.2018.10.057.

Bohin, M.C., Vincken, J.-P., Westphal, A.P., Tripp, A.M., Dekker, P., van der Hijden, H.T. W.M., Gruppeb, H., 2014. Interaction of flavan-3-ol derivatives and different caseins is determined by more than proline content and number of proline repeats. Food Chem. 158, 408–416. https://doi.org/10.1016/j.foodchem.2014.02.145.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Chelh, I., Gatellier, P., Santé-Lhoutellier, V., 2006. Technical note: a simplified procedure for myofibril hydrophobicity determination. Meat Sci. 74 (4), 681–683. https://doi.org/10.1016/j.meatsci.2006.05.019.

Deng, X.X., Zhang, N., Tang, C.H., 2016. Soy protein isolate as a nanocarrier for enhanced water dispersibility, stability and bioaccessibility of β-carotene. J. Sci. Food Agric. 97 (7), 2230–2237. https://doi.org/10.1002/jsfa.8033.

Fiorentini, R., Galoppini, C., 1983. The proteins from leaves. Plant Foods Hum. Nutr. 32, 335–350. https://doi.org/10.1007/BF01091193.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59 (2), 257–268. https://doi.org/10.1351/pac198759020257.

Henchion, M., Hayes, M., Mullen, A., Fenelon, M., Tiwari, B., 2017. Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. Foods 6 (7), 53. https://doi.org/10.3390/foods6070053.

Kinsella, J.E., Melachouris, N., 1976. Functional properties of proteins in foods: a survey. CRC Crit. Rev. Food Sci. Nutr. 7 (3), 219–280. https://doi.org/10.1080/10408397609527208.

Krinsky, N.I., Johnson, E.J., 2005. Carotenoid actions and their relation to health and disease. Mol. Aspects Med. 26, 459–516. https://doi.org/10.1016/j.mam.2005.10.001.

Lakowicz, J.R., 2006. Instrumentation for fluorescence spectroscopy. Principles of Fluorescence Spectroscopy. Springer US, Boston, MA, pp. 27–61.

Livney, Y.D., 2010. Milk proteins as vehicles for bioactives. Curr. Opin. Colloid Interface Sci. 15 (1–2), 73–83. https://doi.org/10.1016/j.cocis.2009.11.002.

McClements, D.J., 2014. Nanoparticle- and Microparticle-Based Delivery Systems: Encapsulation, Protection and Release of Active Components. CRC Press.

Mohammadian, M., Waly, M.I., Moghadam, M., Emam-Djomeh, Z., Salami, M., Moosavi-Movahedi, A.A., 2020. Nanostructured food proteins as efficient systems for the encapsulation of bioactive compounds. Food Sci. Hum. Wellness 9 (3), 199–213. https://doi.org/10.1016/j.fshw.2020.04.009.

Perović, M.N., Antov, M.G., 2022. The influence of enzymatic pretreatment of chickpea on properties of protein nanoparticles prepared by heat treatment. LWT 163, 113545. https://doi.org/10.1016/j.lwt.2022.113545.

Rawiwan, P., Xiang, J., Quek, S.Y., 2023. Optimisation of protein extraction from selenium-enriched Brassicaceae leaves. Fut. Foods 8, 100273. https://doi.org/10.1016/j.fufo.2023.100273.

Qian, C., Decker, E.A., Xiao, H., McClements, D.J., 2012. Physical and chemical stability of β-carotene-enriched nanoemulsions: influence of pH, ionic strength, temperature, and emulsifier type. Food Chem. 132, 1221–1229. https://doi.org/10.1016/j.foodchem.2011.11.091.

Saiz-Abajo, M.-J., Gonzalez-Ferrero, C., Moreno-Ruiz, A., Romo-Hualde, A., Gonzalez-Navarro, C.J., 2013. Thermal protection of β-carotene in re-assembled casein micelles during different processing technologies applied in food industry. Food Chem. 138, 1581–1587. https://doi.org/10.1016/j.foodchem.2012.11.016.

Shevkani, K., Singh, N., Chen, Y., Kaur, A., Yu, L., 2019. Pulse proteins: secondary structure, functionality and applications. J. Food Sci. Technol. 56 (6), 2787–2798. https://doi.org/10.1007/s13197-019-03723-8.

Sponton, O.E., Perez, A.A., Carrara, C.R., Santiago, L.G., 2015. Linoleic acid binding properties of ovalbumin nanoparticles. Colloids Surf., B 128, 219–226. https://doi.org/10.1016/j.colsurfb.2015.01.037.

Sponton, O.E., Perez, A.A., Ramel, J.V., Santiago, L.G., 2017. Protein nanovehicles produced from egg white. Part 1: effect of pH and heat treatment time on particle size and binding capacity. Food Hydrocoll. 73, 67–73. https://doi.org/10.1016/j.foodhyd.2017.06.033.

Sponton, O.E., Perez, A.A., Stechina, M.F., Santiago, L.G., 2020. Production of protein nanovehicles by heat treatment of industrial egg white in a batch reactor. J. Food Eng. 268 https://doi.org/10.1016/j.jfoodeng.2019.109740.

Sun, C., Wu, W., Ma, Y., Min, T., Lai, F., Wu, H., 2017. Physicochemical, functional properties, and antioxidant activities of protein fractions obtained from mulberry (*morus atropurpurea* roxb.) leaf. Int. J. Food Propert. 20, S3311–S3325. https://doi.org/10.1080/10942912.2016.1238389.

van de Velde, F., Alting, A., Pouvreau, L., 2011. From waste product to food ingredient : the extraction of abundant plant protein RuBisCo. New Food 14, 10–13.

Yu, P., 2005. Protein secondary structures (a-helix and b-sheet) at a cellular level and protein fractions in relation to rumen degradation behaviours of protein: a new approach. Brit. J. Nutr. 94, 655–665. https://doi.org/10.1079/BJN20051532.

Wang, Z., Li, Y., Jiang, L., Qi, B., Zhou, L., 2014. Relationship between secondary structure and surface hydrophobicity of soybean protein isolate subjected to heat treatment. J. Chem., 475389 https://doi.org/10.1155/2014/475389, 2014.