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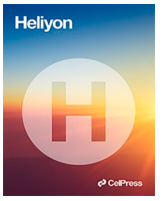

Research article

Sour orange (*Citrus aurantium*) seed, a rich source of protein isolate and hydrolysate – A thorough investigation

Marzieh Rownaghi, Mehrdad Niakousari *

Department of Food Science & Technology, College of Agriculture, Shiraz University, Shiraz, Iran

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ABSTRACT

Sour orange (*Citrus aurantium*) seeds are typically discarded by juice processors as waste. This study aimed to extract protein isolates, produce hydrolysates from de-oiled sour orange seeds (SOS), and characterize their physicochemical properties. Previous studies have described methods to obtain protein isolates and hydrolysates from agricultural residues. However, there is limited data on the SOS. This research characterized protein isolates and hydrolysates from SOS, emphasizing yield, purity, and amino acid composition. Protein isolates were extracted using borate saline buffer, saline, and distilled water. Enzymatically hydrolysis was conducted using Protamex® (a commercial protease) at concentrations ranging from 0.2 to 5 g enzyme/100g protein isolate. Differential scanning calorimetry, electrophoresis, and FT-IR spectroscopy were utilized to characterize the isolates and hydrolysates. Data showed that using 5 % saline resulted in protein extraction with a yield and purity of 30 and 86 %, respectively. DSC analysis revealed that the denaturation temperature of the protein isolate was 68 °C, while the hydrolysates exhibited structural instability, as indicated by a decrease in enthalpy change compared to the isolate. The protein isolate had a 76° contact angle. The amino acid profile showed a significant presence of glutamic acid (130.530 mg/g) and arginine (70.210 mg/g). Electrophoresis analysis exhibited four major bands of the protein. The bands' intensity decreased, and new bands appeared after hydrolysis. The enzyme hydrolysis was confirmed using the O-phthaldialdehyde method and FTIR. Findings revealed that based on the free amine group quantity, the hydrolysate obtained using 5 g enzyme/100g protein isolate was 14.220 ± 0.299 μmol/mg protein. The study concluded that sour orange seeds are a good source of protein, with protein isolates and hydrolysates exhibiting desirable characteristics. More research needs to be conducted to acquire further information about their functional properties and potential applications.

1. Introduction

Citrus aurantium, popularly known as bitter (sour) orange, is a hybrid of *citrus maxima* (pomelo) and *citrus reticulata* [1]. Each whole fruit bears at least 10–15 seeds, with an average weight of 0.2 g when dried. *Citrus aurantium* belongs to the Rutaceae family and comprises numerous fruits such as oranges, grapefruits, and lemons. They grow in tropical and subtropical regions of China, the European Union, and Brazil [2]. Citrus fruits, especially sweet and sour oranges, are globally recognized for their bioactive components and versatility in culinary [3]. Even though sweet oranges are typically consumed fresh, sour oranges are mostly used in salad dressing,

* Corresponding author. Department of Food Science and Technology, College of Agriculture, Shiraz University, Shiraz, Iran.
E-mail addresses: Marzieh.rownaghi@gmail.com (M. Rownaghi), niakosar@shirazu.ac.ir (M. Niakousari).

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sherbet, and cooking [4].

Citrus fruits are processed to produce juice, jam, or marmalade. Wastes produced during processing, such as peels, seeds, and pomaces, might have a significant potential for value-added products and good sources of edible oil, essential oil, pectin, and protein [3].

The byproduct of citrus seeds contains valuable compounds such as oil, protein fiber, limonoids, and flavonoids. Hesperidin, neohesperidin, naringin, and narirutin are the most abundant flavonoids found in *Citrus aurantium* seeds [5]. Bitter orange seeds are identified to contain phenolic acids categorized as hydroxybenzoic acids, notably vanillic acid at a concentration of 3.3 μg/g DW, hydroxycinnamic acids including caffeic acid at 5 μg/g DW, *trans*-ferulic acid at 3 μg/g DW, and *p*-coumaric acid at 15 μg/g DW [6]. However, the exploitation of citrus seed proteins remains largely underdeveloped [3]. Various citrus seeds are reported to have between 4 and 21 % protein (dry weight basis) and contain 16-18 different types of amino acids. The largest concentrations of these are in glutamic acid, arginine, aspartic acid, and methionine [3]. Compared to other citrus seeds, the sour orange seed comprises two distinct components: the outer shell and the inner core. Preliminary examinations revealed that the inner core exhibited a higher protein content than the outer shell [7].

Protein molecules are found as filaments or globules in their natural state [8]. Fibrous proteins, known for their insolubility, are the predominant structural elements in animal tissues. These proteins commonly exhibit parallel configurations, which are strengthened by establishing hydrogen bonds among their molecular structures [8]. Globular proteins are found to be soluble in water, acid/base, and salts and form complex spherical structures by creating mainly hydrogen, ionic, hydrophobic, and disulfide bonds and perform a wide range of functions in living systems [8]. The protein separation and isolation process usually includes protein extraction and precipitation, and at each stage, some factors are said to affect the purity and yield of protein. Such factors affect protein solubility and precipitation [9]. Since proteins are not considered the main part of the grain, they are often linked to other components of the cell wall [9], or due to the seed's grinding process, their solubility might be reduced, resulting in lower yield [10]. Hence, selecting an optimum extraction technique as well as operating conditions and choosing the most suitable solvent, can significantly impact protein functionality and productivity [11].

Nowadays, food industries prefer biological modification strategies over chemical ones. Studies have shown that hydrolysis of protein usually leads to the synthesis of peptides and hydrolyzed proteins, which can improve bioavailability and stability [12]. Bioactive peptides have been implicated to have antioxidant, anticancer, immune booster, and antimicrobial properties. These bioactive peptides have unique qualities for their use in the food, pharmaceutical, and cosmetic industries [13]. Protease enzymes used for this purpose are produced by microorganisms, animals, and plants. They are most commonly applied in food biotechnology and in the production of bioactive peptides to reduce allergies and indigestibility [14]. Therefore, selecting an appropriate method for protein extraction and ultimately optimizing the effective parameters of enzymatic hydrolysis is critical and crucial for improving functional properties [13].

In the present study, the sour orange seeds (SOS) were characterized, and high-purity proteins and protein hydrolysates were obtained by optimizing combined extraction/purification techniques.

2. Materials and methods

2.1. Materials

Unprocessed wet seeds were provided by a juice processing plant (Lemondis Plant, Mahram Group Co., Shiraz, Iran). Boric acid, sodium borate, sodium chloride, Sodium hydroxide, hydrochloric acid, L-homoserine, O-phthalaldehyde, 2-mercaptoethanol, citrate buffer, buffer maleate, phosphate buffer, sodium dodecyl sulfate, sodium tetrahydroborate, methanol, β-mercaptoethanol were all product of Merck (Germany) and/or Kimia-Mavad (Iran) and were all of standard analytical grade. Protamex® (a blend of microbial endo-proteases from Novozyme, 1.5 AU-N/g) was purchased from Novo Nord Darou (Tehran, Iran).

2.2. Methods

2.2.1. Protein extraction and isolation

Protein isolate was obtained from SOS according to the method adopted by Ref. [15]. The wet seeds were air-dried utilizing natural ventilation at ambient room temperature to about 4 % moisture content. To optimize the protein preparation, the inner core of the dried seed was separated from the outer shell. The seeds were milled (Blend-X Classic BLP607WH, Havant, England), passed through a sieve mesh size 20 (ASTM-E:11, Damavand, Iran), and mixed with N-hexane at 1:8 (powder: hexane) and de-oiled in a Soxhlet. The crude protein was extracted from de-oiled seeds powder using three extracting solvents: borate saline buffer (BSB), 1 and 5 % brine solution, and distilled water (control). BSB buffer (1 M boric acid, 0.025 M sodium borate, and 0.075 M sodium chloride solution, pH = 8.45) was used in 1:10 and 1:5 (powder: solvent). Brine solutions of 1 and 5 % and distilled water were used in ratios of 1:10 (powder: solvent). The extraction was performed on a magnetic stirrer (Alfa, D-500) for 1 h at 25 °C. For all extraction solvents, the suspension was centrifuged at 2600×*g* for 20 min, and the supernatant containing crude protein was separated and acidified by 1 M HCl until reaching a pH = 3. To precipitate and obtain the protein isolate, the resulting suspension was centrifuged at 2600×*g* for 25 min at 4 °C. The protein isolate was washed with deionized water, adjusted to pH = 7, freeze-dried, and stored at 4 °C in dark vials for further analysis [15].

2.2.2. Enzymatic hydrolysis of protein isolate using Protamex®

The protein isolate obtained from the method adopted by Ref. [15]. It was mixed with distilled water at 1:10 while the pH was adjusted to 8 using 0.5 N NaOH solution. The Protamex® was added to the prepared protein isolate solution at a concentration of 0.2, 1, and 5 g enzyme/100g of protein isolate. The resulting suspension was incubated for 330 min at 50 °C. Finally, the enzymatic reaction was stopped by heating the solution at 85 °C for 10 min. This was followed by centrifuging at 8000×*g* for 15 min. The supernatant containing protein hydrolysate was stored in a freezer at −20 °C. The mentioned supernatant and precipitated solid's dry matter content were evaluated [16].

2.2.3. Characterizations of the protein isolate and hydrolysates

2.2.3.1. pH, dry matter, protein content (Kjeldahl method), and yield. pH, dry matter, protein content, and yield for all solvents were examined as described by Ref. [17] with slight modification. The evaluation of the above parameters was carried out for the sediments and supernatant. The correction factor for protein content in the Kjeldahl test was 5.5 [18].

2.2.3.2. Amino acid profile. The amino acid profile was determined using the high-performance liquid chromatography (HPLC) method as adopted by Ref. [10] with brief modifications. Briefly, protein isolate was digested with 6 N HCl, and then the solution obtained was diluted at a ratio of 1:20 with 125 mM borate. Afterward, 50 μL of L-homoserine and 800 μl of methanol were added to 200 μL of the above solution while mixing thoroughly for 20 s. To derivatize the sample, 100 μL of 125 mM borate solution and 50 μL of a solution containing (0.025 g phthalaldehyde + 250 μL of borate + 2250 μL of methanol + 25 μL of 2-mercaptoethanol) was added to 250 μL of the sample. This was followed by adding 25 μL of 0.75 M HCl solution after 2 min. The amino acid profile was determined by an Agilent LC-MS mass spectrometer (G1313). The HPLC separation of derivatized amino acids utilized two mobile phases: A (30 mmol/L potassium dihydrogen phosphate buffer with 0.4 % tetrahydrofuran and pH 7.0) and B (50 % acetonitrile with water). The flow rate was constant at 1 mL/min with linear gradient changes from 100 % A to 52 % A/48 % B, then to 40 % A/60 % B, and finally back to 100 % A over 35 min, including a 10 min equilibration period before the next sample injection. The calibration curve was drawn by injecting each amino acid standard in different quantities [10].

2.2.3.3. Protein isolate isoelectric point. To determine the isoelectric point of protein, 0.5 g/L protein isolate together with various buffers, including citrate, maleate, and phosphate, respectively, in the range of pH = 1 to pH = 6, were prepared. To adjust the pH in the above range, dilute solutions of HCl and NaOH (0.1 M) were applied. To eliminate scattered particles, the samples were homogenized. Then, the zeta potential was measured at each pH using a dynamic light scattering device (DLS, SZ-100, Horiba Japan). The region where the zeta potential is zero is defined as the protein's isoelectric point [19].

2.2.3.4. Thermal characteristics. Differential scanning calorimetry (DSC) was used to estimate the net heat energy, as well as the onset (T_{onset}) and maximum temperatures (T_{max}) for endothermic transitions of protein isolate and its hydrolysates with varying degrees of hydrolysis. The thermal properties were determined with a DSC system (NETZSCH DSC 214 Polyma) based on the description given by Ref. [10].

2.2.3.5. Hydrophobicity measurement. Contact angle measurement was conducted as described by Ref. [20] with some modifications. After pelletizing the protein isolate and placing it in the measuring device (V-Tech Contact Angle Meter, VT984), 20 μL of distilled water was dropped on the sample's surface. An image was taken, and the contact angle was evaluated using Image-J (1.52v) software.

2.2.3.6. Predicting the extent of hydrolysis using FTIR. FTIR analysis was applied to examine the protein isolate and its hydrolysates. The FTIR device (Tensor II, Bruker Germany) was set to wavenumbers 400 to 4000 cm^{-1} and 25 °C temperature [21].

2.2.3.7. Measurement of free amine group (OPA method, 1983). The hydrolysis usually results in a change in the free amine group. This may be evaluated by the *o*-phthaldialdehyde (OPA) method. To achieve this, 200 μL of the protein isolate sample and its hydrolysates were mixed with 2 μL of fresh *o*-phthaldialdehyde solution. The fresh OPA solution was prepared by mixing 2.5 mL of sodium dodecyl sulfate solution 20 % (w/w), 25 mL of 100 mM sodium tetra-hydroborate, and 40 mg of *o*-phthaldialdehyde in 1 mL of methanol, and 100 μL of β-mercaptoethanol. The final volume reached to 50 mL. The mixture was incubated for 2 min at 25°C, and the absorption was read at a wavelength of 340 nm in a spectrophotometer (Benchtop double beam UV-VIS spectrophotometer, Model-526). For quantification of peptide content, L-leucine was used as a standard [22].

2.2.3.8. L-leucine standard curve preparation. 0.4–1 mg/mL of L-leucine solution were prepared. Each prepared concentration was then mixed with 2 ml of OPA solution. After 2 min, the absorbance of the samples at a wavelength of 340 nm was read in a spectrophotometer compared to the control sample [22].

2.2.3.9. Electrophoretic analysis. Protein isolate and its hydrolysates solutions(with a 3 % concentration) were analyzed by SDS-PAGE as described by Ref. [23]. The Protein isolate and its hydrolysates solutions were combined with a loading buffer that included β-mercapto ethanol to achieve a final 2 mg/mL concentration. Subsequently, the samples underwent a 5-min heating process in boiling water and were subjected to analysis via SDS-PAGE using 4 % stacking gels and 7.5 % resolving gels, utilizing a Mini Protean Tetra

Table 1
Proximate analysis, pH, and yield of protein isolates.

	Extracting solvents				
	Distilled water	BSB	BSB	NaCl 1 %	NaCl 5 %
Ratio[a]	(1:10)	(1:5)	(1:10)	(1:10)	(1:10)
Initial pH°	4.950 ± 0.028[b]	8.213 ± 0.017[a]	8.260 ± 0.016[a]	4.910 ± 0.021[b]	4.750 ± 0.093[c]
AKS[¥] pH	9.916 ± 0.012[a]	7.817 ± 0.049[e]	8.213 ± 0.020[d]	9.803 ± 0.012[b]	9.133 ± 0.023[c]
AKS[¥] **dry matter (%)**	42.930 ± 0.100[b]	27.130 ± 0.567[e]	27.765 ± 0.263[d]	45.286 ± 0.398[a]	38.206 ± 0.758[c]
AKP[b] **dry matter (%)**	53.866 ± 0.560[d]	72.870 ± 0.567[a]	72.235 ± 0.165[b]	51.88 ± 0.385[e]	66.386 ± 0.754[c]
AKS[¥] **protein content (%)**[c]	29.167 ± 2.082[c]	23.246 ± 0.872[d]	17.057 ± 0.816[e]	35.666 ± 1.027[b]	45.100 ± 1.444[a]
Protein yield (%)	16.286 ± 0.825[b]	2.150 ± 0.291[d]	1.245 ± 0.163[e]	15.166 ± 0.221[c]	30.020 ± 0.424[a]

Different letters within a row represent significant differences at $p < 0.05$.
[¥]AKS= Alkaline supernatant contained crude protein.
[a] Ratio = Ratio of Seed powder to extracting solvent; [O]Initial pH = pH measured after adding seed powder to the solvent.
[b] AKP= Alkaline pellet.
[c] Based on (Kjeldahl).

system (Bio-Rad Laboratories, USA). Each well was loaded with 10 μL of the samples. Following analysis, the gels were stained with Coomassie Brilliant Blue R-250. The marker in this experiment was a ladder with a molecular range between 10 and 180 kDa (Sigma Aldrich) [23].

2.3. Statistical analysis

Analyses were carried out using analysis of variance (ANOVA). Results were expressed as the standard error of the mean of standard deviation (Mean ± STD). Statistical significant difference ($p < 0.05$) was determined using Duncan's multiple range test in a completely randomized design using SAS version 9.4.

3. Results and discussion

3.1. pH, percentage of dry matter, protein content, and yield

It is essential to observe the pH values during various stages of the extraction process. These values are summarized in Table 1. The initial pH of distilled water and brine solutions was about 5 before adding SOS powder. The original pH of the BSB buffer was 8. The latter remained unchanged on adding SOS powder (BSB buffer capability). When extraction was carried out using distilled water and brine solution, the pH was raised to 11 by adding NaOH solution. The pH of the supernatant containing crude protein decreased to about 9 after centrifugation. This drop in pH may be partially attributed to the effect of the pellet formed in the extraction flask.

Referring to data in Table 1 regarding the dry matter and crude protein content in the supernatant, it may be concluded that in suspensions at higher pH values, the solubility of the crude protein was greater. This is probably due to the negative charge of the protein, hence the increased attraction of protein-water [24]. The yield of protein isolate was significantly increased ($p \leq 0.05$) when saline water was applied as the extracting solvent. Interestingly, this was the case for the purity of protein isolate, too. Based on the Kjeldahl method, the highest purity of about 85 % was accomplished when saline water at 5 % concentration was used in the extraction process (Table 1). This value in the case of extraction by BSB (at a ratio of 1:5) was about 78 %. Alkaline extraction is the most widely used method for producing protein isolate due to its ease of use and low cost [25].

Our results were consistent with those reported by other researchers [26]. noted that the alkaline extraction method for soy proteins showed a recovery rate of around 40 % when pH = 10 was utilized to solubilize them [26]. Similarly [27], findings indicated that the ability to extract proteins from the canola meal improved as the pH level of the extraction solution was elevated. The extraction efficiency was approximately 27 % at pH = 10 and rose to 58 % at pH = 12 [27]. Furthermore, some publications compare the ultrasound- or enzyme-assisted processes with conventional alkaline techniques [28,29]. In an attempt [28], established that the ultrasonic treatment enhanced protein extraction yield, solubility, and functional characteristics when using camelina flixweed seeds as the raw material [28]. This improvement in yield might be attributed to the disruption of seed texture induced by ultrasound treatment. The disruption likely facilitated the release of proteins from the seed matrix, leading to increased solubility [28]. [21] revealed that the utilization of ultrasound combined with alkaline extraction led to an increase of 11 % in the extraction yield of pea protein compared to the conventional alkaline method [21]. A small study on protein extraction from citrus fruits indicated that applying microwave and enzyme-assisted processes gave rise to a higher extraction yield than the conventional method [30]. It is crucial to recognize that excessive alkaline conditions in the protein extraction process may result in alterations in the function and digestibility of proteins. Such alterations encompass denaturation, breakdown of proteins, formation of cross-links, and depletion of crucial amino acids [25]. In order to maintain the quality of proteins, it is essential to establish a delicate balance between improving yield and preserving functionality and digestibility [31].

Table 2
Amino acid composition in sour orange seeds.

Amino acid	mg/g Protein isolate	Amino acid requirements for adults (mg/g protein) [$$]
Aspartic acid	50.743 ± 0.162	
Glutamic acid	130.587 ± 0.057	
Glycine	20.949 ± 0.055	
Arginine	70.201 ± 0.051	
Alanine	20.390 ± 0.147	
Serine	20.336 ± 0.021	
Histidine	10.202 ± 0.239	16
Threonine	10.937 ± 0.055	9
Tyrosine	10.767 ± 0.180	19
Methionine	7.179 ± 0.211	17
Valine	30.778 ± 0.087	13
Phenylalanine	30.081 ± 0.070	19
Isoleucine	20.480 ± 0.068	13
Leucine	40.518 ± 0.021	19
Lysine	10.755 ± 0.049	16
Tryptophan	5.414 ± 0.016	11.3

[$] WHO/FAO; Mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day).

Table 3
Thermal characteristics of protein isolate and its hydrolysates.

Sample	T_{onset} (°C)	T_{max} (°C)	ΔH[a] (J/g)
Sour orange protein isolate	51.167 ± 0.850^{c}	68.183 ± 0.686^{a}	12.020 ± 1.138^{a}
Protein hydrolysate obtained by 0.2 g enzyme/100g protein isolate	57.877 ± 0.854^{b}	66.973 ± 0.132^{a}	4.393 ± 0.318^{b}
Protein hydrolysate obtained by 1 g enzyme/100g protein isolate	59.567 ± 0.618^{a}	63.857 ± 0.292^{b}	3.263 ± 0.292^{b}
Protein hydrolysate obtained by 5 g enzyme/100g protein isolate	27.100 ± 0.342^{d}	31.833 ± 0.850^{c}	0.337 ± 0.143^{c}

Different letters within a column represent significant differences at $p < 0.05$.
[a] ΔH = denaturation enthalpy change.

3.2. Amino acid profile composition

Table 2 shows the presence of essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and non-essential amino acids (arginine, tyrosine, aspartic acid, glutamic acid, glycine, alanine, and serine) in the protein isolate. The total amount of essential and non-essential amino acids was approximately 166.344 and 323.973 mg/g Protein isolate, respectively. Glutamic acid (130.587 ± 0.057 mg/g Protein isolate) and tryptophan (5.414 ± 0.016 mg/g Protein isolate) were the highest and lowest amino acids in the protein isolate. This may give a good clue about the importance of proteins extracted from sour orange seeds. Results indicated that based on FAO/WHO nutritional recommendations, the amounts of histidine, methionine, and lysine in the SOS protein isolate were lower than the recommended levels. Amino acids operate as the nitrogenous backbone for essential substances like hormones and serve as the construction blocks of proteins. An organism on its own cannot produce the essential amino acids; hence, they must be exogenously provided through diets. An insufficient intake of essential amino acids can lead to the development of various medical conditions and diseases [30]. reported the amino acid profiles of lemon, orange, and grapefruit seeds. They realized that asparagine, cysteine, hydroxyproline, and tryptophan were missing from their profiles, whereas glutamic acid, leucine, and glycine were amongst the highest group of amino acids [30]. Another study revealed that the primary amino acids found in the Citrus lemon peel were serine, glycine, aspartic acid, proline, and alanine [32]. On the other hand, the primary amino acids found in the peel of *Citrus maxima* were proline, aspartic acid, serine, glycine, and alanine [32]. [33] conducted an interesting study on the variation of amino acids in the lemon seeds as they germinated. They realized that the germination process intensifies the amino acid content of seeds. This result may be explained by the fact that reserved proteins are hydrolyzed to produce free amino acids, which are subsequently utilized to create new fragments [33]. Parameters such as genetics, ecological parameters, variances in cultivar, growth stage, preparation process, and seasonal collection of the plant may explain the variation in the amino acid content of the seeds [32].

3.3. The isoelectric point of protein isolate

The isoelectric point of the protein refers to the point where the surface charges of the particle are neutral. Therefore, at isoelectric pH, the zeta potential is zero. In the present study, utilizing different buffers at different pH ranges and measuring zeta potential showed that pH = 2.9, the value of zeta potential is zero. Thus, this pH was considered the desired point for the precipitation of SOS protein isolate, which was almost consistent with the research conducted on citrus seeds [30]. The presence of glutamic acid (with an isoelectric point of 3.08) as the protein's dominant amino acid can justify this isoelectric point value [34]. [30] study identified proteins of grapefruit, lemon, and orange seeds obtained from defatted press cakes undergone various pre-treatment procedures

Table 4
Effect of enzymatic (Protamex®) treatment on protein isolate.

Enzyme/protein isolate	0.2 g/100g	1 g/100g	5 g/100g
SDPH[€] (%)	65.653 ± 0.065[c]	70.243 ± 0.0328[a]	68.150 ± 0.057[b]
Pellet dry matter (%)	34.183 ± 0.063[a]	29.206 ± 0.217[c]	32.003 ± 0.139[b]

Different letters within a row represent significant differences at $p < 0.05$.
[€]SDPH= Supernatant dry matter containing protein hydrolysates.

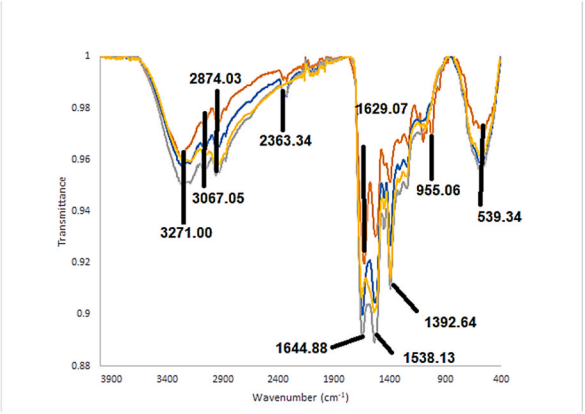

Fig. 1. FT-IR spectrum of protein isolate and hydrolysates of sour orange seeds
Protein isolate as control (no added enzyme)
Hydrolyzed proteins obtained by using a concentration of 0.2g enzyme/100g protein isolate
Hydrolyzed proteins obtained by using a concentration of 1g enzyme/100g protein isolate
Hydrolyzed proteins obtained by using a concentration of 5g enzyme/100g protein isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(microwave roasted, cold pressed, solvent extracted, and enzyme-treated methods). They realized that the extraction method did not affect the isoelectric point of proteins; hence, they reported a pH of 4 as the isoelectric point of citrus seeds proteins [30].

3.4. Thermal characteristics and hydrophobicity of protein isolate

Storage, heat treatment, and chemical alterations, including microbial hydrolysis, acidification, dehydration, and mechanical processing, significantly impact proteins' structural integrity and functionality. These treatments often result in denaturation, posing challenges in using proteins as food additives, particularly in terms of resolubilization post-extraction and drying. Understanding food proteins' conformation and denaturation characteristics is crucial for predicting and monitoring their thermal behavior in food systems [35]. In Table 3, the analysis of the onset temperature denaturation (T_{onset}), the maximum temperature (T_{max}), and enthalpy change (ΔH) of protein isolate and its hydrolysates were summarized. T_{max} and ΔH values for protein isolate were 68.183 ± 0.686 °C and 12.020 ± 1.138 J/g, respectively. ΔH values for protein hydrolysate obtained by 0.2g enzyme/100g protein isolate was 4.393 ± 0.318 J/g, while this value for protein hydrolysate obtained by 5g enzyme/100g protein isolate was 0.337 ± 0.143 J/g. T_{max} values for protein hydrolysate obtained by 0.2 and 5g enzyme/100g protein isolate were 66.973 ± 0.132 and 31.833 ± 0.850 °C, respectively. It is clear that the application of the enzyme as well as its increasing concentration in the reacting media, improves the degree of hydrolysis, the maximum temperature (T_{max}) and enthalpy change(ΔH) decreased. This shows the increase in structural instability of the protein, leading to denaturation at lower energy levels [30]. Our findings were consistent with those reported by Ref. [36]. They expressed that the hydrolysis of chickpea protein using Alcalase and Flavourzyme decreased the denaturation enthalpy, which may be an additional marker for the decline in thermal stability [36]. Another study revealed that the method of extracting lemon seeds using cold pressing and solvents affected the temperature at which denaturation occurred in the proteins of citrus seeds. This indicates that using different solvents for extraction may induce protein changes, leading to denaturation at lower temperatures. However, it is important to note that this conclusion did not apply to orange seeds exposed to microwaves or grapefruit seeds treated with enzymes before oil extraction [30]. In general, the denaturation temperature illustrates the extent of changes in the protein structure based on breaking hydrogen bonds, and a higher denaturation temperature indicates the presence of heat-sensitive proteins in the denatured sample [30].

The hydrophobicity of the protein isolate was measured by calculating the contact angle. This angle was measured as 76.026 ± 4.133° for the SOS protein isolate. No data on the contact angle of citrus protein isolates was previously reported in the literature. However, there is a research reporting a contact angle of approximately 64.4 for soy protein isolate [20]. [37] study investigated the

contact angle of milk protein isolate and realized that the most significant contact angle was recorded at pH = 7.4, at which it reduced from 118 to 106°, while the lowest contact angle was reported at pH = 8.4, where it declined from 107 to 90°. Consequently, results revealed that milk protein isolate powder had a hydrophobic surface with contact angles larger than 90° and that the pH of the water droplets had no significant effect on milk protein powder wettability [37]. Increased surface hydrophobicity could potentially influence the functional characteristics of a protein, particularly in applications such as foam and emulsion, where this attribute plays a crucial role in specific food product functionalities [38]. Additionally, powder wettability is important in dispersion and is a precursor to dissolution. The degree of hydrophobicity is directly related to the contact angle value, and the droplet formed related to the higher contact angle on the protein's surface structured a more rounded shape [39].

3.5. The effectiveness of Protamex® on citrus protein isolate formation

Supernatant dry matter containing protein hydrolysates and pellet dry matter are summarized in Table 4. The amount of dry matter in the supernatant containing hydrolysates proteins was at its highest and lowest level when using enzyme in 1 and 0.2 g enzyme/100g protein isolate, respectively. A datasheet provided by the manufacturer (Novonesis Co., Denmark) indicates that presence of 10g protein/100g of Protamex® enzyme with an activity of about 1.5 AU-N/g [40]. Proteases are enzymes that bind specifically or non-specifically to their target protein and perform hydrolysis processing. In general, one of the factors affecting enzyme activity is the easy attachment of the enzyme to the substrate. It is anticipated that the attachment of the enzyme to the protein isolate powder can facilitate the hydrolysis process better than its attachment to the seed powder [41].

3.6. FT-IR spectroscopy of protein isolate and hydrolysates of sour orange seeds

FTIR diagram is used to investigate functional groups and secondary structure of protein. The FTIR spectra of SOS protein isolate and its hydrolysates are shown in Fig. 1. The FTIR spectra of enzymatic hydrolysates with varying degrees of hydrolysis did not show any new infrared absorption bands and exhibited similarities to the protein isolate spectrum, although with differing intensities. This observation indicated that distinct structural modifications occurred while retaining the same functional groups, thereby preserving the secondary structure of the proteins without causing damage. As shown in Fig. 1, the isolated protein and its hydrolysates samples showed a broad peak in the 3500-3000 cm^{-1} range. However, the intensity of the transmittance peak of protein isolate (no added enzyme) was higher than in hydrolysates samples. In general, the characteristics of the absorption peak in the range of amide A (3500-3000 cm^{-1}) were due to the stretching vibrations of the N–H and O–H functional group, related to the hydrogen bonds of the polypeptide [34]. Hydrogen bond forces in protein molecules are necessary to maintain the stability of secondary structures. Therefore, the change in the state of the hydrolysate proteins compared to the non-hydrolysate sample can be attributed to the change in the secondary structure of the isolated protein under the influence of the enzyme [42].

The region 3000-2800 cm^{-1}, correlated to the C–H group's stretching vibrations, represents the proteins' hydrophobic region. However, the C–H bands for all hydrolysates were shifted from 2930 (protein isolate) to 2960 cm^{-1} (Fig. 1), which might be associated with the exposure of buried hydrophobic patches with increased aliphatic side chains following the unfolding of proteins during enzymatic hydrolysis [43]. The range of absorption in the wavelength 1600-1700 cm^{-1}, which is usually used to estimate the secondary structure of the protein and is related to amide I; So that 1610-1640 cm^{-1} for β-sheet structures, 1640-1650 cm^{-1} range for random-coil, α-helix, and β-turn respectively occupy 1650-1658 cm^{-1} and 1660-1695 cm^{-1} [44]. As shown in Fig. 1, the 1629.1, 1645.31, 1650.74, and 1650.87 cm^{-1} peaks for protein isolate and hydrolysates protein obtained by 0.2, 1, and 5g enzyme/100g protein isolate, respectively, indicated that the secondary structure of protein isolated and its hydrolysates were affected by the enzyme concentration [42]. Moreover, the FT-IR spectra (Fig. 1) indicated a reduction in the intensity of the transmittance peak at 1536 cm^{-1} in the hydrolysate samples compared to the protein isolate. This decrease might be attributed to the formation of primary amines due to hydrolysis [45]. These outcomes were consistent with previously published for protein isolate and its hydrolysates [42,46,47].

3.7. Free amine group (OPA method)

The amount of free amine groups in the hydrolysate samples was calculated after exposing 100g of the isolated protein to 0.2, 1, and 5g Protamex®. The control sample was the protein isolate with no added enzyme. The lowest free amine concentration was 4.192 ± 0.322 μmol/mg protein, corresponding to the control sample. The addition of 0.2g enzyme to the hydrolyzing system increased the free amine to 7.266 ± 0.283 μmol/mg protein. This relatively low increase compared to the control system might be attributed to the insufficient catalytic sites when using this low enzyme concentration. Increasing the enzyme to 1g and 5g enhanced the hydrolysis and increased the amount of free amine group to 9.636 ± 0.339 and 14.220 ± 0.299 μmol/mg protein, respectively. It might be worth mentioning that this increasing trend in hydrolysis and the amount of free amine group cannot be generalized as no other enzyme concentration was used in this study. Similarly [48], stated that in the hydrolysis of yak whey protein, the effect was more significant during the first 2 h of the process [49]. also revealed that the degree of hydrolysis increased rapidly during the first 2 h of the alkaline protease substrate reaction and then rose slowly. These observations are probably attributed to the accumulation of enzyme, hence leading to inhibition of the active sites of the catalytic protein [13]. Moreover, the application of the Michaelis-Menten equation may justify these findings. When the enzyme concentration was low enough to allow for complete dissolution, the rate and degree of protein hydrolysis increased rapidly with increasing enzyme concentration. This might result in an increase in the maximum rate of hydrolysis. However, once the enzyme concentration reaches its saturation point, the rate of enzymatic reaction will be stabilized [50].

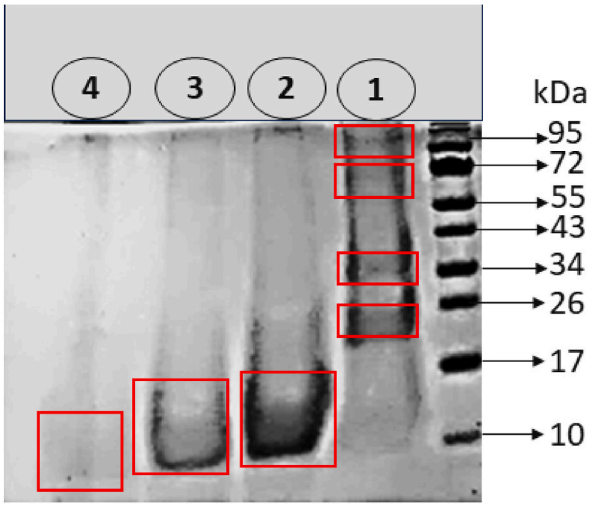

Fig. 2. SDS-PAGE analysis of protein isolate and its hydrolysates 1: Protein isolate; 2: Protein hydrolysate with 0.2g enzyme/100g protein isolate; 3: Protein hydrolysate with 1g enzyme/100g protein isolate; 4: Protein hydrolysate with 5 g enzyme/100g protein isolate.

3.8. Electrophoretic characterization

Fig. 2 illustrates the SDS-PAGE electrophoresis bands of protein isolate and its hydrolysates samples. Electrophoresis analysis showed that the protein isolate comprises two major bands at around 26 and 40 kDa. Moreover, two bands at around 72 and 95 kDa were observed, although they were not dominant. Enzymatic hydrolysis influenced the molecular weight distribution of SOS protein isolate, as new bands appeared following the hydrolysis. Fig. 2 shows that bands around 10 kDa were formed in the hydrolysate obtained using 0.2g enzyme/100g protein isolate, which almost disappeared with increasing enzyme concentration. The highest decrease in molecular weight was observed in hydrolysate produced with 5g enzyme/100g protein isolate, which seems to be lower than 10 kDa. This observation was in accordance with the data presented in Ref. [3]. Similarly [51], reported molecular weights of 19, 27, 33, and 50 kDa for the proteins derived from palm kernels [52]. also estimated the molecular weight of defatted *Bunium persicum Bioss*. Press cake protein ranged from 25 to 72 kDa, and in the hydrolyzed samples, a weak band at approximately 10 kDa was observed at 80 min, specifically for proteins hydrolyzed by pancreatin. Conversely, no band was observed in other samples, indicating a significant reduction in molecular weight [52]. The results of this study provided further validation of the findings regarding the degree of hydrolysis.

4. Conclusion

In this study, sour orange seeds (SOS), a waste from the citrus processing plant, were effectively utilized to produce added-value products such as crude protein, protein isolate, and hydrolysates. Examination of various extracting solvents and pH revealed valuable insights into optimizing protein yield and content. A 5 % NaCl solution proved to be particularly effective for preparing protein isolates. Additionally, the seeds contained significant concentrations of essential amino acids, including glutamic acid, aspartic acid, and arginine. Furthermore, the study provided detailed information on protein isolates' conformation and denaturation characteristics as well as their hydrolysates. The impact of enzyme concentration on protein hydrolysis was studied. Higher enzyme concentrations led to increased hydrolysis, confirmed by electrophoretic characterization. FTIR spectroscopy analysis demonstrated a significant alteration in the secondary structure composition following hydrolysis by different concentrations of enzymes. This research highlights the potential for valorizing waste products such as sour orange seeds to generate added value for the citrus industry. By producing protein isolate and hydrolysates from these seeds, both the environmental concerns associated with waste disposal as well as the growing demand for sustainable protein sources are addressed. However, the study neither investigated a wider range of Protamex® concentrations nor other proteases. Potential variations in seed composition were also noted, and further research is needed to explore scalability and economic feasibility. In summary, this study contributes to our understanding of the potential of sour orange seeds as a valuable resource in the citrus industry, highlighting avenues for future research and development to maximize their utilization and benefits.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Marzieh Rownaghi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mehrdad Niakousari:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32503.

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