J. Product. & Dev., 27(2): 263- 279 (2022)

SDS-PAGE ELECTROPHORESIS AND SOLUBILITY CHARACTERISTICS OF CASEIN – *Chlorella Vulgaris* PROTEIN ISOLATE CO-PRECIPITATE MIXTURES

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ABSTRACT

Protein isolate was obtained from microalgae Chlorella vulgaris, the protein percent in the isolated protein was 56%. A solution (3.5% protein) of Chlorella vulgaris protein isolate (CPI) was blended with fresh skim milk (pH 6.6) at volume ratios of 5:95 , 10:90 and 15:85 (vol:vol) Chlorella protein solution to fresh skim milk and the protein mixtures were co-precipitated at pH4 using 2N-Hcl. The obtained three Casein – Chlorella protein co-precipitate mixtures were separated electrophoretically using SDS – PAGE. The molecular weights of most separated mixtures had intermediate values between molecular weight of cow's milk casein and chlorella protein.

Solubilities of the obtained three mixtures were higher than that of the cow's milk casein at pH6, ranged from 80 to 87.1% comparing with cow's milk casein (60%).

***Conclusively,** these results suggested that, casein – chlorella protein mixtures may find their uses in acidic foods.*

Key words: SDS-page electrophoresis, solubility characteristics, casein, *Chlorella Vulgaris*, protein isolate, co-precipitate mixtures

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INTRODUCTION

Proteins of animal origin, such as milk proteins (casein and whey proteins) have a good functional properties, but are also expensive and are not available in sufficient quantities (Hofi, 2011; Alu′datt *et al.*, 2013).

The production of functional food grade casein in the 1960′s coincided with the development of processed food products that required functional proteins (Fox and Kelly, 2004). Also, proteins from traditional crops such as soy, wheat, rice, corn, may become limited in time (Kinsella and Shitly,1978), therefore dairy companies are seeking untraditional sources of protein ingredients, the developments of supplementary sources of food proteins, *e.g*. microalgae protein is the main goal for several research workers (Radkova *et al.*, 2019, Geada *et al*, 2021). Microalgae are a good source of proteins, for example Chlorella vulgaris microalgae contain 51 – 58% protein on dry basis, (Becker, 2007). This attracted the attention of scientists as an alternative food protein source (Safi *et al.*, 2014).

On the other hand, emphasis is now being placed on designing new blends of dairy and vegetable protein. These blends which combine the low cost of vegetable proteins and good functional properties of dairy proteins (Hinderink *et al.*, 2021).

The successful adoption of new proteins will depend upon, the availability, cost, nutritive value and safety, also, the functional properties of proteins should be fully described (Culbertson, 2005). The Functionality of proteins can be largely divided in four areas, solubility, is important in milk beverages ; emulsification is important in processed cheese and foaming is vital for ice cream and gelation is important in fermented milk, cheese and meat products (Poure-El,1981 ; Culbertson, 2005; Kinsella,1982).

Therefore, the aim of this study was to prepare protein isolate from *Chlorella vulgaris* microalgae mixing with skim milk protein to produce co-precipitate mixtures of casein and chlorella protein. The electrophoretic and functional properties of the obtained chlorella protein isolate, casein, and casein – chlorella protein co-precipitate mixtures were then examined.

MATERIALS AND METHODS

MATERIALS:

Microalgae biomass: *Chlorella vulgaris* biomass in freeze-dried form was obtained from the Algae Biotechnology Unit, National Research Center, Dokki, Cairo, Egypt.

Bulk fresh cow′s skim milk (pH6.6) was used for preparation acid casein and co-precipitate mixtures of cow′s milk Casein and Chlorella protein isolate. All chemicals were analytical grade.

METHODS:

Chlorella Vulgaris protein isolation

C. Vulgaris biomass powder was subjected to two pretreatments before alkaline protein extraction. The first, the dried cell biomass was mixed with organic solvent hexane at ratio of 1:4 (w/v) Chlorella powder: hexane. The solvent cell powder mixture was stirred for 30 min, centrifuged at 3000xg for 15 min, and hexane phase was discarded. Hexane extraction was repeated three times as described above. The extracted cells were left at room temp. to evaporated the residual hexane. The second pretreatment was as follows:

100 g of hexane extracted cells powder was mixed with 250 mL of distilled water and the pH of the cell suspension was adjusted to 12 using 2N – NaOH solution. Aliquots of the alkaline cell suspension (25 g) were mixed with 30 g sand (acid washed and neutralized). The cells – sand mixture was manually ground using a mortar and pestle for 5 min. Then, the collected ground cells, their volume were restored to 500 mL of distilled water, and left under gravity for 2 h. Then the cells were removed from the sand by decanding and the adhered cells with sand was collected by washing the sand with distilled water. The protein was isolated from previous cells by alkaline solution. The pH of cells was readjusted to pH 12, the suspension resultant was the stirred for 2 h at 40℃ while maintaing the pH at 12. The extraction mixtures centrifuged at 6000xg for 30 min.

The alkaline supernatant was collected and it′s pH was adjusted to pH 4 with 2N-HCl solution to precipitate chlorella protein isolate (CPI). The collected isolated protein paste was weighted and it′s protein content was determined.

The protein isolate yield was calculated as follows:

$$\%\ \textit{Protein yield} = \frac{\textit{Amount of protein in the isolated paste}}{\textit{Amount of protein in corresponding biomass}} \times 100$$

The resultant protein paste was freeze-dried and it′s smell and colour were organoleptically examined and compared with the original chlorella cell powder.

***Preparation of acid casein*:**

It was obtained by adjusting the pH of the skim milk to pH4.6.

The three co-precipitated protein mixtures and acid casein were designated as: Mix-5%, Mix-10% and Mix-15% and Cn for Casein – Chlorella protein co-precipitate mixtures contain 5, 10 and 15% chlorella protein, and acid casein, respectively.

Preparation of cow's milk Casein – Chlorella vulgaris protein isolate co-precipitate mixtures:

The methods used in production of cow′s milk Casein – Chlorella protein co-precipitate mixtures based on the previous method used by **Fayed and Morshed (1990)** with some modifications. A solution (3.5%) of chlorella protein isolate (CPI) was blended with fresh skim milk (pH6.6) at volume ratios of 5:95, 10:90 and 15:85, chlorella protein solution: fresh skim milk. The protein mixtures were co-precipitated at pH 4 (using 2N-Hcl).

The resultant protein isolates were freeze-dried. Gross chemical composition of all protein precipitates were determined and it′s smell and colour were organoleptically examined and compared with the individual used proteins, chlorella protein and casein.

Chemical analysis:

The chemical analysis of *Chlorella vulgaris* biomass, protein isolates of Chlorella, Casein and Casein – Chlorella protein co-precipitate mixtures were performed according to the international standard methods (ISO). Moisture content was determined (ISO 6496:1999) crude ash determined (ISO 5984:2002), crude protein determined (ISO 5983 – 1: 2002), crude fat determined the method described in (Official Journal of European Union (EN), 2009), L54/37, volume 52, and crude fiber was determined the methods described in (Official Journal of the European (EN), 2009, L54/40, volume 52).

The carbohydrate content was determined according to **Agarwal *et al.* (2015).** Uronic acid content was determined according to Method of **Ahmed and Labavitch (1982).**

The chlorophyll content was determined according method of **Yu *et al.* (2017).**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS – PAGE.

SDS – PAGE method was used to characterize the chlorella protein isolate (CPI), acid casein (Cn) and protein mixtures, Mix-5, Mix-10 and Mix- 15. Electrophoresis performed depending on method used by **Robert *et al* (1983)** using 3.5% stacking gel (w/v) and 12% polyacrylamide gel (w/v). The gel was run at 240 v (constant voltage) for 5 h. The molecular weight of proteins was evaluated using protein molecular weight marker 250 – 11000 KDa.

Gels were scanned with Syn- Gene system and image captured analysis was performed using vision Capt. Software [Fille version: 4.0102 – Serial No 17292'' 14518" `Sme′ mpcs.].

Determination of protein solubility:

Protein solubility of (CPI), Cn, and Mix-5, Mix-10 and Mix-15 was determined using method of Aoki *et al* (1980) with some modifications as follows; sample of freeze-dried protein (1 g) were dispersed in 50 mL of distilled water.

The protein dispersion was stirred for 30 min while adjusting the pH to selected pH values in the range of 2 – 11 with a 0.1M – NaOH or 0.1M – Hcl.

Then, the volumes were adjusted to 100 mL with distilled water. The protein dispersions were centrifuged at 6000xg for 30 min. The N content of the clear supernatant was determined by the Kjeldal Method (ISO: 5983-1(2002).

$$\% \text{ Nitrogen solubility} = \frac{\text{N in total supernatant}}{\text{N in 1g protein}} \times 100$$

RESULTS AND DISCUSSION

1-Gross chemical composition of C. vulgaris powder.

The gross chemical composition of *C. vulgaris* biomass used in our study is shown in Table (1). It could be seen that crude protein (%) of chlorella biomass represented the highest component in chlorella powder. It′s content was 35.5%. This make *Chlorella vulgaris* a good source for unconventional protein.

Also, Chlorella biomass contain significant amounts of carbohydrate (including charged carbohydrate uronic acid), 23.01% for carbohydrates and 4.1% for uronic acid. Also, chlorella cells contained 1510 mg of chlorophyll / 100 gram of cells and this in agreement of green colour of chlorella. The gross composition for *Chlorella vulgaris* obtained in the present study was in agreement with other studies **(Ursu *et al.*,2014 ; Tohamy *et al.*,2018).**

Table (1). Gross chemical composition of *Chlorella vulgaris* powder

Components	**%**
Moisture	11.51
Crude protein	35.5
Fat	1.98
Ash	24.7
Carbohydrate	23.01
Fiber	3.3
Uronic acid	4.1
Chlorophyll, mg %	1510

2-*Effect of extraction procedure on protein isolation yield*

Chlorella protein isolated yield obtained from the used extraction technique was 26%. This obtained yield, still low in respect to the crude protein in the original biomass (35.5% protein). From these results, we concluded that, the alkaline solution (NaOH – pH 12) was critical factor in protein extraction from *C. vulgaris*. On other hand, the pretreatment of cells by grinding with sand had no signification effect on protein extraction. These results are in agreement with results obtained by Safi *et al,* (2014) who

reported that, the maximum yield of protein isolated from *C. vulgaris* was 33.2% by using alkaline extraction solution of NaOH.

The major problem hampered protein extraction from *C. vulgaris* is the cell wall. This wall is rigid and robust, composed of cellulosic microfibrillar structure **(D′Hondt *et al,* 2017; Safi *et al*, 2014).**

Also, it could be observed that the colour and smell of protein isolate past characterized with light green colour and clean smell comparing with the original chlorella biomass which had dark-green colour and fishy smell. **Rackis *et al*.(1979**) observed that the flavor scores of hexane /ethanol extracted soybean flakes are significantly higher than those not extracted.

***Gross chemical composition of protein isolate powder*.**

Table (2) shows the gross chemical composition of protein isolate obtained from *C. vulgaris* biomass. The protein percent in isolated protein was 56%, while lipid and fiber (%) were very low and this may be due to hexane extraction and separation process during alkaline extraction. On the other hand, protein isolate still have high amounts of carbohydrates (29.1%) comparing with the original cells. **Teuling *et al* (2017)** found that, the final purified protein isolates from four unicellular microalgae contained 62 – 77% protein and 9 – 24 % carbohydrates.

Table (2) Gross chemical composition of *C. vulgaris* protein isolate powder.

Component	**(%)**
Moisture	8.1
Crude protein	56
Lipids	0.22
Fiber	0.077
Ash	6.51
Carbohydrate	29.10
Uronic acid	3.9
Chlorophyll, mg %	1860

***Casein – Chlorella protein co-precipitate mixtures*:**

Mixtures of casein and chlorella protein were obtained by mixing a solution (3.5%) of chlorella protein isolate and fresh skim milk (pH6.6), and solubilizing both under alkaline conditions and co-precipitated the protein

mixtures at pH 4. Three casein / chlorella protein co-precipitate mixtures containing 5 , 10 and 15%of chlorella protein were obtained.

The gross chemical composition of the obtained protein mixtures comparing with casein and chlorella protein are shown in Table (3). The protein content of the three obtained protein mixtures ranged from 51.77 to 58.99% comparing with chlorella protein isolate (56%) and casein (59.24%).

The high protein content of the mixtures suggested the use of these mixtures as a new unconventional protein source for food industry.

Table (3) Gross chemical composition of Casein , Casein/Chlorella protein mixture and *Chlorella vulgaris* protein isolate powder.

Components	**(%)**				
	Casein	**Mix-5 %**	**Mix-10 %**	**Mix-15 %**	**Chlorella protein isolate**
Moisture	13.9	10.13	13.5	12.4	8.1
Crude protein	59.24	58.99	53.86	51.77	56.0
Lipids	7.95	7.45	6.85	4.97	0.22
Fiber	0.0	0.099	0.099	0.076	0.077
Ash	5.17	5.96	5.0	3.71	6.51
Carbohydrate	13.74	17.37	20.69	27.08	29.10
Uronic acid	3.0	5.1	4.7	4.2	3.9
Chlorophyll (mg%)	--	1170	1320	1590	1860

The organoleptic properties of isolated proteins revealed that green-colour, which observed with chlorella protein isolate became lighter in the mixtures and no-off-flavour was observed in the co-precipitate mixtures. Also, the curds of the protein – mixtures became more finer as the proportion of chlorella protein in the mixtures increased. These results, may be again suggest that the obtained casein – chlorella protein co-precipitate mixtures may have new functional properties and this may serve to expand the use of chlorella protein in dairy and food industry.

Electrophoretic properties of Casein – Chlorella vulgaris protein co-precipitate mixtures:

Table (4) Fig.(1) are show the molecular weight of protein fractions separated by SDS – PAGE. When the molecular weight of separated casein,

Table (4) Protein fractions obtained by SDS – PAGE technique for casein, chlorella protein isolate, and casein – chlorella protein mixtures.

Fraction No.	Casein		Casein/Chlorella protein mixtures						Chlorella protein Isolate	
			Mix-5		Mix-10		Mix-15			
	Mw	%	Mw	%	Mw	%	Mw	%	Mw	%
1	329.22	19.116	333.47	18.796	342.15	20.112	342.15	19.475	346.57	21.262
2	150.80	11.594	210	7.009	182.33	10.804	184.68	12.643	191.94	10.409
3	93.58	8.683	108.95	16.496	116.19	11.018	121.28	9.669	141.11	13.728
4	63.85	10.818	70.14	7.657	65.59	19.207	68.28	14.618	72.04	12.111
5	48	6.797	48	9.074	37.88	8.763	39.92	9.091	38.89	11.925
6	26.36	21.793	31.47	8.360	27.31	13.738	27.31	15.979	27.80	12.088
7	10.84	21.199	24.27	16.526	11.32	16.358	11.16	18.525	11.00	18.477

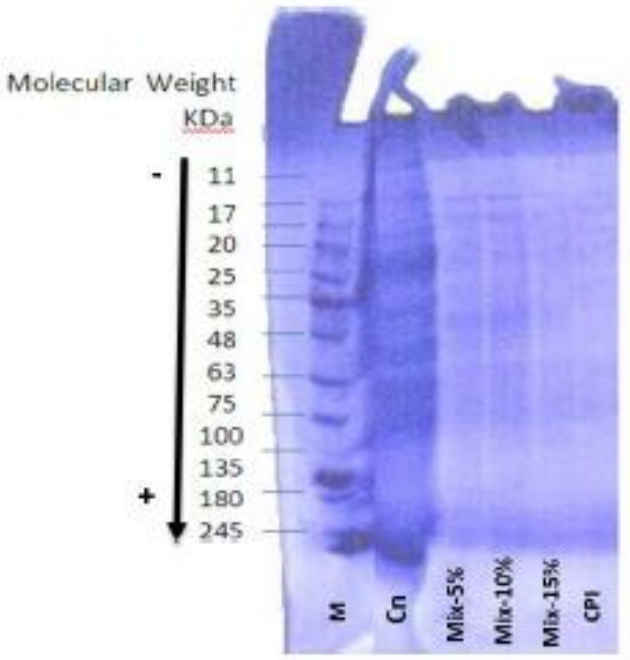

Figure (1) SDS-polyacrylamide gel electrophoresis of casein (Cn), chlorella protein isolate (CPI), casein / chlorella protein co-precipitate mixtures , Mix-5, Mix-10 , Mix-15 and protein molecular weight marker (M) .

chlorella protein isolate and casein – chlorella protein mixtures were investigated. It could be seen that, the first four fractions for all separated proteins bands characterized with high molecular weight, ranged from 36.85 to 346.57 KDa. By comparing these fractions for chlorella protein with corresponding fractions of those of casein , chlorella protein fractions characterized by higher molecular weight than of casein (Table 4).

Also, the first four fractions were represented 50.22% and 57.51% of total separated proteins of casein and chlorella proteins, respectively. Our results were in agreement with results obtained by **Andreeva *et al* (2021),** who showed molecular weight distribution for the first separated fractions of

C. vulgaris ranged between 67 – 227 KDa. Teuling *et al* (2017) reported that the obtained protein fractions of microalgae higher then 250 KDa expected to be protein aggregation complex.

Also, **Garcia *et al* (2018)** reported that protein extracted from most microalgae were often covalently bound to carbohydrates, lipid, and pigments forming complex with protein. This is clear in Table (3), which shows the gross chemical composition of chlorella protein isolate, were high content of carbohydrates and chlorophyll were associated with chlorella protein.

Also, Fig.(1) and Table (4) showed that, the three fractions separated later, characterized with bands having low molecular weights, of 48, 26.36 and 10.84 KDa for casein and 38.98, 27.80 and 11 KDa for chlorella protein. These low molecular weight fractions represented 49.77% and 42.49% of total separated protein for casein and chlorella protein, respectively. The low molecular weight fractions may represent the protein fractions of chlorella and casein. **Ursu *et al*. (2014)** showed that the majority of chlorella proteins were separated in the apparent molecular weight range between 12 – 75 KDa.

Also, the low molecular weight observed with casein were in the range of traditional casein fractions (19 – 26 KDa) [Fox,1989].

When casein – chlorella protein mixtures examined [Fig. (1) and Table (4)], we found that these mixtures followed the same trends observed with high and low molecular weights fractions which mentioned above for casein and chlorella protein fractions, but the most mixtures had intermediate values between casein and chlorella proteins.

Nitrogen solubility:

The nitrogen solubility at different pHs of casein, chlorella protein mixtures are shown in Fig.(2). and Table (5). At the acidic side of the solubility curve (pH 2 – 5), the lowest solubility were observed at pH(3 – 4) for chlorella protein and at pH 5 for casein, whereas the lowest solubility of protein mixtures were observed at pH (4 – 5). These pHs may be the nearest points of isoelectric-pH for algae protein and casein.

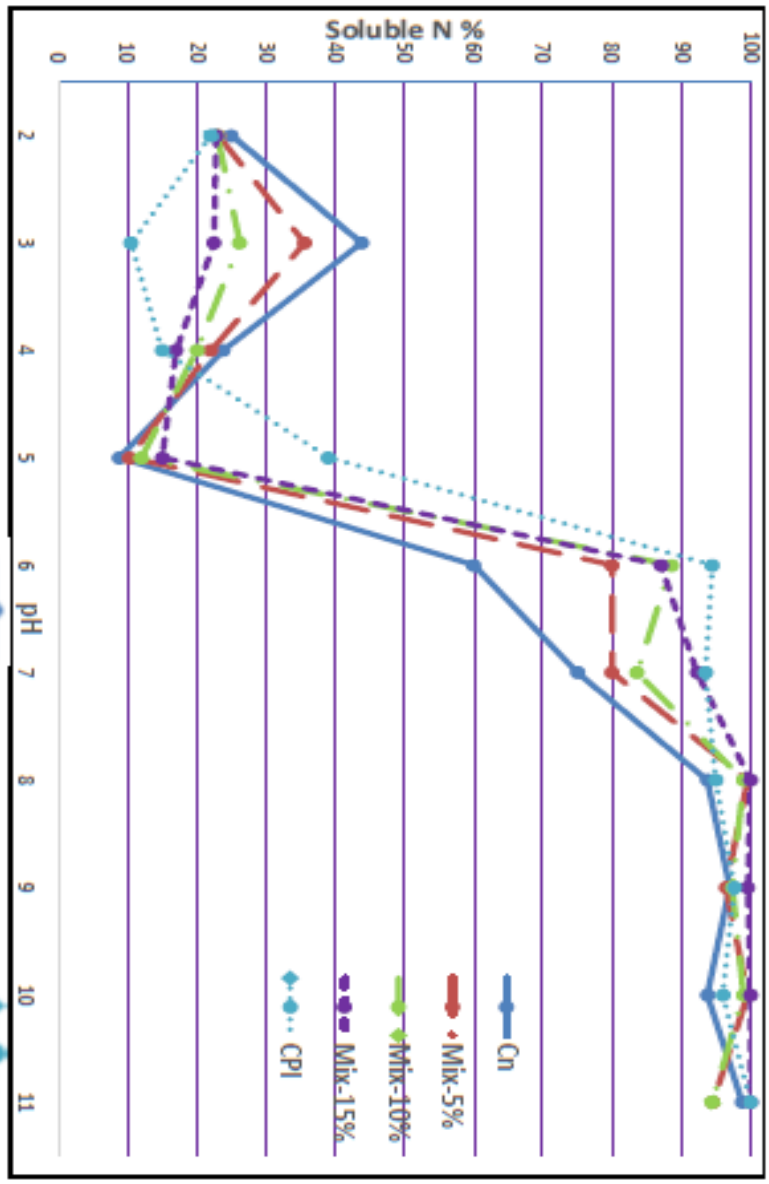

Figure. (2). Solubility as function of pH of Casein (Cn), Chlorella protein isolated (CPI) and Casein / Chlorella protein co-precipitate(5 , 10 and 15%) mixtures.

Table (5). Solubility as function of pH of casein , chlorella protein isolate and casein / chlorella protein co-precipitate mixtures

pH	Casein	Mix-5 %	Mix-10 %	Mix-15 %	Chlorella protein isolate
2	25	23.1	23	22.8	22
3	43.8	35.6	26.2	22.5	10.5
4	23.8	22	20	17	15
5	8.8	10.3	12	15	39
6	60	80	88.6	87.1	94.5
7	75	80	83.6	92.3	93.5
8	93.8	99.6	99	100	95
9	97	96.5	97.4	99.6	97.7
10	93.8	99.6	99	100	96
11	98.8	94.6	94.5	100	100

Same trends were observed by **Ursu *et al*. (2014)** for chlorella protein at pH (3 – 4). At pH 6 (Fig. 2), Most of chlorella proteins was soluble (93.7%) comparing with casein (60%). The mixtures showed solubility higher than that of casein at pH 6.

Thus, the solubility of casein at pH 6 was improved by mixing chlorella protein with casein, the solubility of protein mixtures ranged from 80 to 87.1% comparing with that of casein (60%). At alkaline pH(7 – 11), the solubility of all studied proteins showed high solubility ranged from 75% to 100%. The solubility of protein mixtures was in agreement with solubility result obtained for casein – plant protein mixtures obtained by **Fayed and Morshed (1990) ; Fayed (1997).**

From our solubility results it can be concluded that Casein – Chlorella protein mixtures at pH 6 had higher solubility than that of Casein at the same pH . These results suggest that , Casein – Chlorella protein mixtures may find their uses in acidic foods.

Conclusively, these results suggested that, casein – chlorella protein mixtures may find their uses in acidic foods.

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دراسة التفريد الكهربي و الذوبان لمخاليط مترسبة معاً من الكيزين ومعزول بروتين طحلب *Chlorella vulgaris*

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تم عزل بروتين من طحلب *Chlorella vulgaris* و كانت نسبة البروتين في المعزول ٥٦% و استخدم البروتين المعزول في انتاج ثلاثة مخاليط من الكيزين و بروتينات الطحلب تحتوي على ٥ ، ١٠ ، ١٥ % من بروتين الطحلب عن طريق خلط محلول به ٣.٥ % من بروتين الطحلب مع اللبن الفرز الطازج (pH 6.6) بنسب حجم ٥:٩٥، ١٠:٩٠, ١٥:٨٥ ثم تم ترسيب مخلوط البروتينات معاً على pH4. ثم أجرى تفريد البروتينات المتحصل عليها باستخدام جهاز التفريد الكهربي للبروتين -SDS PAGE و وجد ان اقسام البروتينات المفصولة كان لمعظمها أوزان جزيئية تقع بين تلك التي للكيزين و بروتين الطحلب .
و عند تقدير الاذابة لتلك المخاليط على pH 6 وجد أن لها إذابة تفوقت على تلك التي للكيزين حيث تراوحت الاذابة بين ٨٠- ٨٧.١ % في حين كانت إذابة الكيزين ٦٠% و **التوصية**:من هذه النتائج تؤكد صلاحية المخاليط للدخول في صناعة منتجات الأغذية و الألبان و خاصة الأغذية الحامضية .