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Effects of Ultra-high Pressure Homogenization on the Emulsifying Properties of Whey Protein Isolates under Various pH

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Abstract The effect of ultra-high pressure homogenization on the emulsifying properties of whey protein was investigated in a model emulsion made with whey protein isolate and soya oil under various pH. The emulsifying properties, the average diameter of the oil droplets (d_{vs}), and the protein load, were measured for each emulsion produced at different homogenization pressures (50 to 200 MPa) and pH values (4.6 to 8.0). According to the results of variance analysis and response surface, the pH had more influence on oil droplet size and protein load than homogenization pressure. The model equations, which were obtained by response surface analysis, show that pH and homogenization pressure had the major effect on oil droplet size and protein load. Higher homogenization pressure decreased the average droplet size and the protein load. Homogenization at high pressure, as opposed to low pressure, causes no overprocessing, but the effect was pH-dependent. The average diameter of the oil droplets increased slightly by decreasing the pH from 8.0 to 6.5 and then increased dramatically toward the isoelectric point of whey protein (i.e., at pH 4.6). Moreover associated droplets were found at acidic pH and their size was increased at high temperature.

Keywords: high pressure homogenizer, emulsion, whey protein, emulsifying property, protein adsorption

Introduction

Homogenizer is one of the most important devices in the food and dairy industry. It is more effective in reducing droplets size in emulsion than other equipments, such as high-speed blender, ultrasonicater, and colloid mills (1,2). Homogenization is realized by dynamic pressure operations in homogenizing valve. The non-homogenized liquid is forced through a narrow gap between the valve seat and the homogenizing valve under high pressure and low velocity. When the liquid leaves the gap, there is a rapid increase in velocity and an instantaneous drop in pressure. Some phenomena due to this extreme condition, such as shear, turbulence, and cavitations, have been proposed to explain the mechanisms of homogenization (3-5).

High pressure homogenizer can produce smaller oil droplets, improve stability, enhance the functional properties, and modify the protein membrane composition in emulsion system (6-8). Because of this effectiveness, the high pressure homogenizer has been developed to manufacture higher commercial value products and to extend shelf life. In recent, Kheadr *et al.* (9) observed that high pressure homogenization of milk could reduce total bacterial counts and improve its microbiological quality. These results proposed the possibility that high pressure homogenization treatment could be used as cold pasteurization of milk. Moreover, the application is extended to the chemical, pharmaceutical, and biotechnological processing to reduce the polymer molecular weight, disrupt cells, and produce small particles (10).

The most important factor in determining homogenization efficiency is the energy density, which varies depending on the homogenization pressure and time scale of the process. The properties of the final product are therefore modified by the homogenization pressure. Recent technological breakthroughs improve that high pressure homogenization can be carried out at pressure of up to 200 MPa. Such high pressure may enhance emulsifying properties as well as open possibilities for new applications (5,10). However, no attempt has been made to find the effect of such high pressure homogenization on emulsions. Because homogenizers in the food processing lines are actually used at 2.5-50 MPa, most studies were only performed within this pressure range. In order to extend their applications, it is necessary to understand the effect of higher pressure exceeding 50 MPa. Therefore, the objective of this study was to investigate the effect of homogenization at high pressure (50-200 MPa) and pH on the emulsifying properties of whey protein isolate (WPI) in a model emulsion. We used a central composite experimental design and response surface methodology. The surface response methodology can be used to evaluate individual parameters or their combined influence.

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Materials and Methods

Materials Whey protein isolate (WPI, 95% protein content) and soya oil were purchased from Davisco Food International Inc. (Le Sueur, MN, USA) and local store, respectively, and used without further purification treatment. Urea was purchased from BDH Inc. (Toronto, ONT, Canada). Ethylenediamine tetraacetic acid (EDTA) and β-mercaptoethanol were obtained from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. These chemical reagents were of analytical grade.

Emulsion preparation The high pressure homogenizer used in this study was EmulsiFlex-C50 (Avestin Inc., Ottawa, ONT, Canada). WPI was dissolved in deionized water at 0.5%(w/w) concentration and stirred for 1 hr at room temperature. After dissolving WPI, the pH was adjusted with 0.1 M HCl and 0.1 M NaOH (pH 4.6-8.0), and this protein solution was used as the aqueous phase. Premixed emulsions were prepared by mixing 90%(w/w) of protein solution with 10%(w/w) soya oil using Ultra-turrax (model T25; IKA Labortechnik, Staufen, Germany). Emulsions were produced at selected homogenization pressures (50-200 MPa) for a first pass and then at 3 MPa for a second pass. The second pass can separate weakly flocculated droplets formed after first pass, but has no effect on the reduction of droplet size. The average diameter of the oil droplets and protein load were immediately measured on the fresh emulsions.

Droplet size measurement The emulsions were diluted 500 times with a dissociating buffer (pH 7.0) containing 8 M urea, 50 mM EDTA and 10 mM β-mercaptoethanol to disperse the aggregated/flocculated oil droplets formed during emulsification (11). This diluted solution was then stirred for 5 min. Emulsion globule size was estimated by photon correlation spectroscopy (model 370; Pacific Scientific, Hiac/Royce Instruments Division, CA, USA).

Protein load To measure the concentration of whey proteins adsorbed on oil droplets, the method of Zahar and Smith (12) was used with modification. Fresh emulsion was centrifuged at 40,000×g for 60 min to separate the serum and cream phrases. The separated cream layer was removed with a spatula, and stored at 4°C until protein and oil content were determined. Protein and oil content in the cream phases were measured by the Kjeldahl (13) and the Mojonnier method (14), respectively. Protein load was calculated as protein quantity (mg) adsorbed per m^2 of interface.

Optical microscopy measure The state of oil droplets formed under various conditions was observed by using optical microscope (Leitz Laborlux S; Leica Mikroskople & System, Wetzlar, Germany) was used. No pigments were used, and emulsion was only diluted with deionized water. All samples were observed at a magnification of 100×.

Experimental design A central composite design was used to study the effects of independent variables. The fundamental variables selected in this study were the pH of protein solutions before emulsion formation and the homogenization pressure used to form emulsions. Based on the capacity of high pressure homogenizer and preliminary experiments, the ranges of each variable were 50-200 MPa of homogenization pressure and pH 4.6-8.0. These independent variables were distributed using a central composite design as described in Table 1.

Statistical analysis The results of variance analysis and the response surface equations were obtained using the general linear models (GLM) and response surface analysis by least squares regression (RSREG) procedures, respectively, of the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) program.

Result and Discussion

Response surface analysis Average droplet diameters and protein loads obtained at different pH values and homogenization pressures are shown in Table 1. The results of the variance analysis are shown in Table 2.

Table 1. Experimental design and responses for the average diameter of the oil droplets and protein load

Independent variables		Dependent variables	
Pressure (MPa)	pH	Droplet diameter (d_{vs}) (nm)	Protein load (mg/m^2)
72	4.6	638.2	2.64
72	7.4	448.7	1.63
178	4.6	506.5	2.39
178	7.4	362.1	1.25
125	6.0	429.8	1.68
125	6.0	425.5	1.62
125	6.0	426.4	1.61
125	6.0	423.4	1.63
125	6.0	421.1	1.63
50	6.0	500.4	1.70
200	6.0	319.3	1.58
125	4.6[1)]	589.8	2.46
125	8.0	410.6	1.29

[1)]The original pH was initially 4.0, but in order to obtain better analysis in whole pH range, the pH in the 12th emulsion was modified to 4.6.

Table 2. Variance analysis for the average diameter of the oil droplets and the protein load as a function of pH and homogenization pressure

	Average diameter			Protein load		
	SS[1)]	F value	% Explained	SS	F value	% Explained
Pressure (X_1)	37,459.54	85.09***	41.64	0.181	0.045***	7.48
pH (X_2)	51,955.23	157.36***	57.75	2.232	0.744***	92.19
Pressure × pH	507.51	47.46**	0.56	0.005	0.005	0.21
Error	42.78		0.05	0.003		0.12
Total	89,965.06			2.421		

[1)]Sum of square; **significant at 0.05 level; ***significant at 0.001 level.

According to the measured responses, pH has higher sum of square values than homogenization pressure. pH and homogenization pressure occupy 57.75 and 41.64% of the total effect on the change in average diameter respectively. This result shows that droplet size is relatively more dependent on pH than homogenization pressure, but homogenization pressure plays also an important role. However homogenization pressure have only 7.48% of the total effects on the variation of protein load, whereas the effect of pH are 92.19% indicating that pH is major factor on the change in protein load.

The estimated regression coefficients for each dependent variables obtained from response surface analysis are shown in Table 3. The best explanatory model equation for average droplet diameter is as follows:

$$Y = 2199.5 - 0.01527X_1 - 471.0X_2 - 2.01 \times 10^{-7}X_1^2 + 2.25\ X_2^2 + 0.001518X_1X_2$$

where, Y is the average droplet diameter, and X_1 and X_2 are homogenization pressure and pH, respectively. The coefficient determination is 0.9989, and this model equation also is very significant at level above 0.0001. The model equation for protein load is:

$$Y = 9.497 - 4.98 \times 10^{-5}X_1 - 2.063X_2 + 3.607 \times 10^{-10}X_1^2 - 0.1313X_2^2 - 4.717 \times 10^{-6}X_1X_2$$

The coefficient determination of this model equation is 0.9962, and is also significant ($p<0.0001$). These equations can adequately describe the effect of homogenization pressure and pH on droplet size and protein load.

The model equations must be validated in order to confirm whether they can give the same values for droplet size and protein load within desired conditions. Experimental values of 4 emulsions, which have produced different droplet sizes and protein load, were compared with values calculated from model equations. These results are shown in Table 4. The calculated values are consistent with experimental values, except for the emulsion formed at pH 8.0 and 200 MPa. These emulsions were obtained by using the extreme values for each variable. Since model-related errors may be higher at the end values of the variable ranges, we must exercise caution in interpreting these 'end-value' results.

Table 3. Coefficients values of the model[1)] equations estimated by surface response analysis

	Coefficients	
	Average diameter	Protein load
Constant	2,199.5***	9.497***
Linear		
Pressure (P)	-0.01527**	-4.98×10^{-5}**
pH	-471.0***	-2.063***
Quadratic		
P^2	-2.01×10^{-7}	3.607×10^{-10}
pH^2	32.25***	0.1313***
Interaction		
P × pH	0.001518*	4.717×10^{-6}*
R^2	0.9899	0.9962
Probability	<0.0001	<0.0001

[1)]Response model is $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{12}X_1X_2$ where, Y: dependent variables average diameter and protein load), X_1, and X_2: homogenization pressure, and pH of protein solution, respectively. β_0, β_1, β_2, β_{11}, β_{22}, and β_{12}: regression coefficients in model equations.
*, **, *** Significant at 0.10, 0.05, 0.001 level, respectively.

Average droplets diameter Figure 1, the response surface for the average droplet diameter, clearly shows the change in average droplet diameter at different pH values and homogenization pressures. The average droplet diameter remains unchanged between pH 6.2 and 8.0, but a sharp increase is observed below pH 6.2. The droplet size reaches the highest value at acidic pH, near the isoelectric point of whey proteins. For example, in emulsions formed at 125 MPa, the droplet size is changed from 410 nm at pH 8.0 to 425 nm at pH 6.0, but is close to 600 nm at pH 4.6. This pH effect is substantiated by optical microscopy measurements. The microscopy analysis of emulsions formed at different pH values and homogenization pressures are shown in Fig. 2. Since the microscopic images give useful information to understand the changes in emulsion

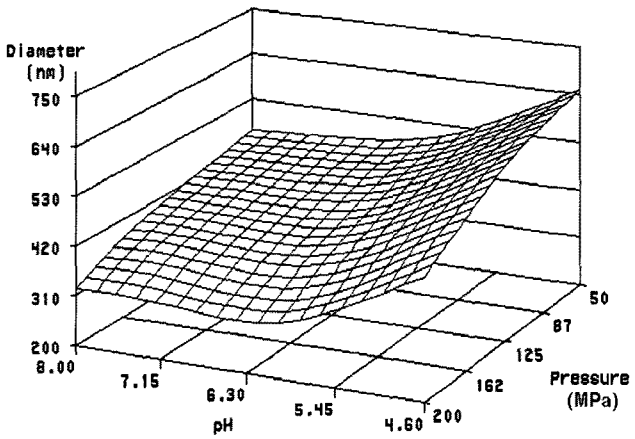

Fig. 1. Response surface for the average diameter of the oil droplets as a function of homogenization pressure and pH. Other emulsion conditions are as following; whey protein=0.5%, soy oil=10%, temp=room temperature.

Table 4. Comparison between the calculated results obtained from the model equations and the experimental results

Emulsion condition		Droplet diameter (d_{vs}) (nm)		Protein load (mg/m^2)	
pH	Pressure (MPa)	Calculated value	Experimental value	Calculated value	Experimental value
6.0	50	498.7	500.4±2.4	1.72	1.70±0.07
6.0	178	361.1	370.8±9.4	1.55	1.59±0.04
4.6	125	580.3	589.8±9.7	2.48	2.46±0.09
8.0	200	352.6	368.5±10.6	1.24	1.20±0.05

properties, it has been used in many studies on emulsion system (15). In emulsion at pH 7.4 and 72 MPa (A in Fig. 2), all small-size oil droplets are clearly separated, even though some aggregated droplets are shown. In contrast, large oil droplets are found in emulsions formed at pH 4.6 and at same pressure (72 MPa) (B in Fig. 2). Moreover, there are more flocculated and/or aggregated oil droplets than at pH 7.4, although some oil droplets still remained small. Such flocculation between droplets at pH 4.6 would coalesce and increase the size of oil droplets. This phenomenon is enhanced when emulsion is formed at 50°C (C in Fig. 2).

pH-induced changes in droplet size can also be explained by the variation in electrostatic charge of adsorbed whey proteins at different pH values. First, at pH far from the isoelectric point, the increase in the net charge within the adsorbed protein could form an electrical layer in vicinity of droplets and thus occurrs the repulsive force between droplets. The formation of repulsive forces in adsorbed proteins prevents droplets from approaching and flocculating. Near the isoelectric point, however, the adsorbed proteins do not have any net charges, and thus the electrostatic repulsive forces between droplets are essentially negligible. This fact permits droplets to flocculate and aggregate. Moreover, the changes in electrostatic charge of protein near isoelectric point lead protein to the formation of complex and the reduction of solubility. This means that the hydrophobic groups buried in inside of protein have a more difficulty to expose, and thus they need more time to adsorb at interface than protein molecules at other pH. According to Mohan and Narsimhan (16), in sodium caseinate-stabilized emulsions at different pH values, the coalescence rate is dependent on the change in interdroplet electrostatic repulsive forces. The electrostatic repulsive forces between droplets were highest at pH 7 and lowest at pH 5 (i.e., near the pI of sodium caseinate), and the coalescence rate was therefore increased as the pH varied from 7.0 to 5.0. Demetriades *et al.* (17) also reported an increase in droplet size near the isoelectric point of whey proteins. In their results, the droplet distributions at pH 3 and 7 were similar, whereas the considerable aggregation of droplets and the formation of a larger bimodal distribution were induced at pH 5.

From the results of Fig. 1, the average diameter of the oil droplets generally decreases with higher pressure. The average droplet size in emulsions formed at pH 8.0 decreases from 470 to 320 nm, as the homogenization pressure increases from 50 to 200 MPa. Increasing pressure generally leads to a further decrease in emulsified droplet size (2,18,19), and some authors reported that an increase in homogenization pressure above the optimal value could increase droplet size (i.e., 'overprocessing') (20,21). However, this phenomenon has not been observed in the present results. The pressure range in our study is much higher (50-200 MPa) than the other studies. Such high pressure may have other effects on adsorbed whey proteins. Moreover, high pressure has been used to produce fat substitutes, which made from protein aggregates. During this production, the high pressure has an effect for fragmentation of the protein aggregate to microparticule form (5). So, it could potentially have affected the break-up of protein aggregates formed near pH 4.6. The smaller protein aggregates which are characterized by different emulsion stabilizing properties (i.e., reduced conformational flexibility) could then be easily adsorbed at the oil interface. At other pH values, homogenization at higher pressure can also affect the surface properties of whey protein and lead to a continuous decrease in droplet size.

Protein load The change in the amount of whey proteins adsorbed at the oil droplets interface as a function of pH and homogenization pressure is shown in Fig. 3. The protein load increases progressively as the pH approaches 4.6. At 125 MPa, the protein load is 1.29 mg/m^2 at pH 8.0 and 1.63 mg/m^2 at pH 6.0. Lowering the pH to 4.6 causes the protein concentration to increase to 2.48 mg/m^2. Such pH-induced changes in protein load are almost the same at other homogenization pressures. Our results on pH-induced changes are in agreement with previous studies (22,23).

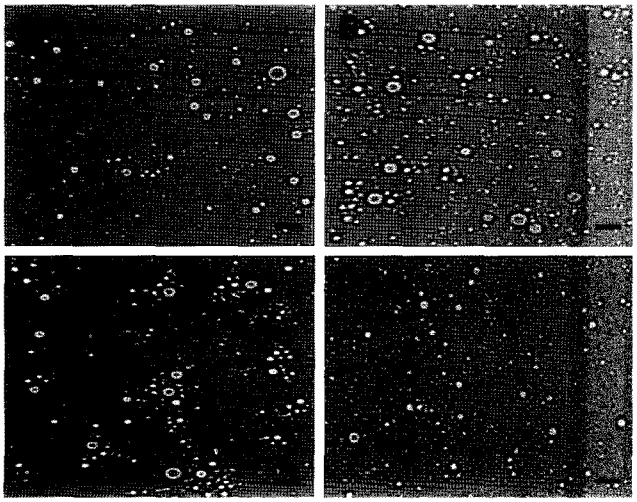

Fig. 2. Microscopy images of emulsions formed at different homogenization pressures and pH values. (A) 72 MPa, pH 7.4, 20°C; (B) 72 MPa, pH 4.6, 20°C; (C) 72 MPa, pH 4.6, 50°C; (D) 178 MPa, pH 4.6. Scale bar=5 mm.

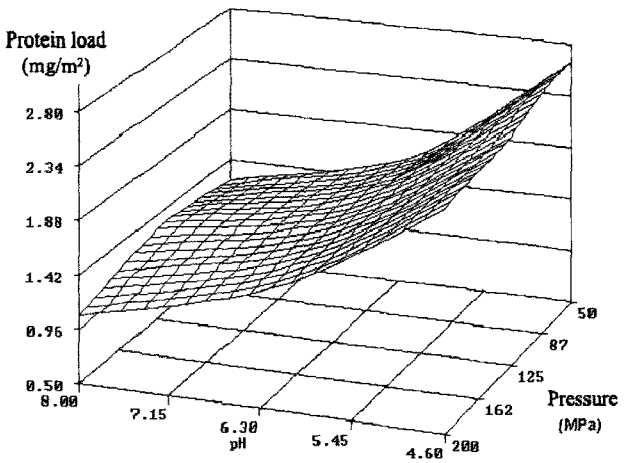

Fig. 3. Response surface for the protein load as function of homogenization pressure and pH. Other emulsion conditions are as following: whey protein=0.5%, soy oil=10%, temp=room temperature.

The adsorption behavior of whey proteins can be explained by pH-induced structural changes in the 2 major whey proteins, β-lactoglobulin (β-Lg). β-Lg forms a stable dimer at pH values between 6.7 and 5.2, but between pH 5.2 and 3.5, it associates into octamers (24). Therefore, the migration of the protein molecules to the interfaces may be accomplished by octamers form at acidic pH; hence, contrary to other pH values more proteins could be adsorbed on interfaces. Apart from the structural changes, the increase in adsorbed whey proteins at acidic pH is also explained by changes in the net charge. Near the isoelectric point, the electrostatic repulsion between proteins diminishes, which could facilitate the formation of interactions between protein molecules and of protein aggregates. This phenomenon may cause further adsorption of protein molecules than other pH. Waniska and Kinsella (25) showed that the rate of adsorption and packing of β-Lg at the interfacial film was maximal near the isoelectric point. Shon *et al.* (26) recently reported that emulsion activity (EA), which is based on the protein's ability to adsorb, spread and stabilized the oil/water interface, was depend on pH. Lower EA was found at pH 4.5 than other pH (3.0, 7.0, and 9.0) and they indicated that it may due to increased protein-protein interaction.

Although homogenization pressures have less effect on protein load than pH, whey protein adsorption is dependent on pressure changes (Fig. 3). Protein load is reduced as homogenization pressures are increased. For example, when emulsions are formed at pH 7.0, the protein load is about 1.37 mg/m^2 at 50 MPa, whereas it decreases to 1.25 mg/m^2 at 200 MPa.

The increase in homogenization pressure reduces the size of oil droplets (larger surface area), and hence total amount of adsorbed proteins is therefore increased to stabilize emulsion (Fig. 4). These results show effectively the change in protein amount by the increase in area of interface. However, it is found that the protein load diminished in present result (Fig. 3) in spite of the increase in surface of oil droplets. These results therefore show that the change in protein load is not totally related with the protein amount adsorbed at interface due to the increase of interfacial surface. We may propose that another mechanism is needed to stabilize the increased interface except protein adsorption. This is likely to determine that the spreading or unfolding of the already adsorbed proteins cover the some part of large interfaces resulting from high pressure.

However, many studies reported the increase in the interfacial protein adsorption at higher pressures. Cano-Ruiz and Richer (27) reported that protein load on the milk fat globule membrane increased with homogenization pressures. They obtained a protein load of 9.79 and 11.88 mg/m^2 when milk was homogenized at pressures of 60 and 90 MPa, respectively. McCrae (28) also showed that about 2-5% of the total protein load was increased after homogenization by the adsorption of serum protein on the fat surface. Such increase in protein load is due to adsorption of large casein micelle at the interface. From this difference, it is also shown that protein load may be dependent on the type and properties of protein. In our results, it is found that different protein load is produced at same droplet size. For example, the droplet size is approximately 470 nm in emulsions formed at pH 8.0 and

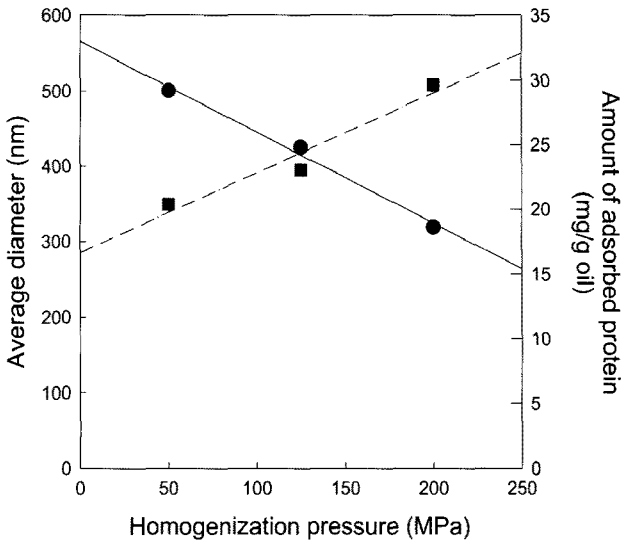

Fig. 4. Average diameter (●) and total amount of adsorbed protein (■) as a function of homogenization pressure. All emulsions were prepared at pH 6.0.

50 MPa as well as at pH 4.6 and 200 MPa, but the latter condition (2.35 mg/m^2) leads to a greater increase in protein adsorption than the former (1.29 mg/m^2). It is shown that all changes in protein load are not dependent on the increase in interfacial surface of oil droplets, this is, the modification of protein properties induced by emulsion conditions could produce the different amount of protein adsorption at same droplet size. Tornberg (20) showed that the type of protein adsorbed has a large influence on the proteins adsorption on fat globules.

When the effect of pressure on protein load is compared at different pH, it is found that high pressures are more effective in emulsion prepared at pH 4.6 to decrease protein load than in that at other pH. First, the effect of high pressure at this pH is the dissociation of flocculated droplets. In Fig. 2, emulsion treated by 178 MPa (D) has less flocculation of droplets than that by 72 MPa (B). High pressures (>50 MPa) can be used to form protein microparticles, to disrupt microorganism, and to modify polymers (5,10,29). Because of their high ability, large changes in protein load at pH 4.6 could be occurred. It is possible that high pressures could modify the properties of protein aggregates formed near isoelectric point.

Acknowledgments

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