Fabrication and characterization of gels with optimum stiffness and syneresis from *Lathyrus sativa* protein isolate

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ABSTRACT

Meat consumption is inconsistently associated with the development of coronary heart disease, stroke, and diabetes mellitus, limiting quantitative recommendations for consumption levels. Plant protein foods can serve as a complete and well-balanced source of amino acids for meeting human physiological requirements without causing these diseases. Protein gelation is important to obtain desirable sensory properties and textural structures in foods. Gelation phenomenon requires a driving force to unfold the native protein structure, followed by an aggregation retaining a certain degree of order in the matrix formed by the association between protein strands. Protein gelation has been traditionally achieved by heating, but some physical and chemical processes form protein gels in an analogous way to heat-induction. The characteristics of each gel are different and dependent upon factors like protein concentration, ionic strength and/or enzyme level. Response surface methodology was employed to evaluate the influence of 3 independent variables including protein concentration, TG, and $CaCl_2$ at 3 variation levels on the stiffness (N/m) and syneresis (%). The experiments were designed based on Central composite Face Center Design to obtain maximum stiffness with minimum syneresis for the gels. The results indicated that *Lathyrus sativa* Protein Isolate (LSPI) could be used as a substitute for meat protein or as a nutritious and functional additive that plays an important role, similar to previous experience with soy protein. According to the results, the optimum condition for producing LSPI gels had significant influence on stiffness and syneresis. Applying desirability function method, optimum operating conditions were found to be protein content of 10.39%, $CaCl_2$ concentration of 0.60 M, and TGase: 41.73U.g^{-1}. At this optimum point, stiffness and synersis were found to be 184.33(N/m) and 0.33 (%), respectively.

INTRODUCTION

Meat consumption is inconsistently associated with development of coronary heart disease, stroke, and diabetes, limiting quantitative recommendations for consumption levels[1]. Plant protein foods contribute approximately 65% of the per capita supply of protein on a worldwide basis. These sources of protein are discussed in relation to their amino acid content, human amino acid requirements, and dietary protein quality. These can serve as a complete and well-balanced source of amino acids for meeting human physiological requirements[2].

Protein gelation is important to obtain desirable sensory properties and textural

structures in foods. Gelation phenomenon requires a driving force to unfold the native protein structure, followed by an aggregation retaining a certain degree of order in the matrix formed by the association between protein strands. Protein gelation has been traditionally achieved by heating, but some physical and chemical processes form protein gels in an analogous way to heat-induction[3]. The characteristics of each gel are different and dependent upon factors like protein concentration, ionic strength and/or enzyme level. Calcium ions interact with proteins and are also the most commonly used source of cations in food systems. Their gelation mechanism induces changes in the cross-link of the gel and alters the mechanical properties of gel[4]. Electrostatic repulsion extensively opposes protein–protein interactions preventing gel formation. When the solution is cooled to room temperature and mixed with salt, the electrostatic charges are shielded and a gel is formed[5]. Protein isolate cold-set gelation showing differences in the degree of aggregation of preheated proteins at various calcium chloride concentrations. Modifications in protein and/or calcium concentration modify gel characteristics[6]. Mulvihill & Kinsella[7] reported that β-lactoglobulin gel strength increased almost linearly as sodium chloride concentration increased, calcium chloride increasing gel strength at lower concentrations (20 mM) than sodium chloride (100 mM).

Enzyme-induced gelation refers to cross-linking between protein chains resulting in the formation of a gel structure. The most widely used enzyme to form food gels is transglutaminase (TGase), a protein-glutamine γ-glutamyltransferase (EC 2.3.2.13) enzyme capable of catalysing acyl transfer reactions, introducing covalent cross-links between proteins[8]. When TGase acts on protein molecules, ε-(γ-glutamyl) lysine crosslinks are formed[9]. The commercial use of TGase in the food industry started with the manufacturing of surimi in Japan. Addition of TGase increases the elasticity and firmness of surimi gel, and can minimize wastage by stabilising fluctuations in raw material quality[10]. Nowadays TGase is not only used in the Japanese fish industry: it is exerted world-wide for the preparation of meat, dairy, bakery, soy products, pasta etc. to improve the texture and to modify the properties of prepared foods in general[9]. Wan et al.[11] reported that addition of some salt resulted in increased gel strength of uncooked and cooked surimi gels and in a synergistic effect to the TGase.

Grass pea (*Lathyrus sativus* L., Leguminosae) is an important crop for green feed and grain for both animal and human consumptions that grows in the South Asia and Ethiopia, with less intense production in China, Mediterranean region and Europe[12]. *L. sativus* has considerable potential as a forage crop in semi-arid regions. It has large amount of energy and protein (25.6–28.4%) and the predominant amino acids of *L. sativus* protein isolate (LSPI) are aspartic acid, glutamic acid, arginine, leucine, and lysine (one of two substrates of TGase for the formation of ε-(γ-glutamyl) lysine covalent bonds). However, LSPI is poor in terms of sulphurous amino acids (eg. methionine, cysteine, and tryptophan) is limited[13].

There is no published information about the gelation of LSPI. The objectives of this study were, therefore, to assess, using response surface methodology (RSM), the stiffness and syneresis of LSPI gel samples produced by variables concentration of TGase and $CaCl_2$. Finally, the appropriateness of the models to predict optimum gelation conditions was evaluated.

MATERIALS AND METHODS

Materials

L. sativus seeds were purchased from a local market in Mashhad, Iran. Probind TX Transglutaminase was obtained from BDF

Natural Ingredients, S.L., Girona, Spain. All other chemicals used in experiments were of analytical grade.

Preparation of LSPI

L. sativus seeds were cleaned manually to remove all foreign matter. *L. sativus* seed flour was defatted using hexane (1:5 w/v) by stirring for 60 min prior to being milled and sieved using a mesh 18 sifter. The defatted flour was dried in the ventilator overnight at 20°C. LSPI was prepared from defatted *L.sativus* seed flour, as described by[14]. In brief, defatted flour stirred at 30°C for 1 h at pH 9.9 with flour to deionized water ratio 1:10 (w/v). After centrifugation at 8000g for 15 min, supernatants were pooled and the pH of the soluble proteins was adjusted to the isoelectric point (pH 4.5) for1 h. After centrifugation at 8000g for 15 min, the precipitate was washed with deionized water adjusted to pH 4.5, then dried by freeze dryer after resolved at pH 7.0. The protein content was 917.5 g kg^{-1} determined by the Kjeldhal method and considering 6.25 as the conversion rate of nitrogen to crude protein.

Sample preparation

Protein isolate dispersions (8-12% w/v) of pH 7.0 were first prepared by heating in test tubes immersed in a ben marry bath at 90°C for 15 min and were occasionally agitated by hand to facilitate protein solubilization. The dispersions were then poured into plates and were followed by the addition of TGase (10-50 U/g) and $CaCl_2$ (0-0.60 M) at 48°C. The samples were then left in an incubator at 48°C for 2 h and then left in the refrigerator at 4°C for 2 h.

Stiffness determination by penetration

Cylindrical gels having uniform geometry (25mm height x 25mm diameter) were subjected to penetration testing at room temperature using a texture analyser (TA.XT PLUS; Canners Ltd., Ontario, Canada) equipped with a 10 N force transducer. The plunger velocity was set to 1 mm s^{-1}. The samples for Stiffness determination were prepared beforehand using circular molds to obtain gelled disks of diameter 25 mm and height of 25 mm. For the penetration test, a 4 mm diameter probe having a flat end was allowed to penetrate 50% of the sample length, and the first peak was used as a penetration force. Force/deformation data were monitored for four gels of each treatment, and the averaged initial slope of the force/deformation curves was taken as an indicator of gel stiffness.

Syneresis

For syneresis measurements, gel samples were moved in conical centrifugation tubes. The gels were centrifuged at 1000 ×g for 20 min. The separated serum was removed with a pasteur pipette, and syneresis (%) was calculated as the mass of released serum related to the total mass of the gel before centrifugation. Three gel samples were analyzed for each treatment.

Experimental design and statistical analysis

Response surface methodology (RSM) was employed for determining the effect of three independent variables (LSPI concentration, x_1; TGase, x_2, and $CaCl_2$, x_3) at three variation levels on the stiffness (N/m) and syneresis (%). RSM is an efficient strategy for optimizing a multivariable process, due to its practical use in their optimization. The technique provides mathematical and statistical procedures to study relationships between one or more responses (dependent variables) and a number of factors (independent variables)[15]. A central composite rotatable design (CCRD) was employed to create the experimental design using RSM statistical package (Design Expert version 6.01, Statease Inc., Minneapolis, USA). Experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to

extraneous factors. The model proposed for the response is given below:

$$Y= \beta_0+ \beta_1 x_1+ \beta_2 x_2+ \beta_3 x_3+ \beta_1 \beta_1 x_1^2+ \beta_2 \beta_2 x_2^2+ \beta_3 \beta_3 x_3^2 + \beta_1 \beta_2 x_1 x_2+ \beta_1 \beta_3 x_1x_3+ \beta_2 \beta_3 x_2x_3+ \varepsilon$$

The coefficients of polynomial model were represented by β_0 (constant term), β_1, β_2 and β_3 (linear effects), $\beta_1\beta_1$, $\beta_2\beta_2$ and $\beta_3\beta_3$ (quadratic effects), and $\beta_1\beta_2$, $\beta_1\beta_3$ and $\beta_2\beta_3$ (interaction effects).The significance of each coefficient was determined using the *p*-value. The adequacy of the models was verified using model analysis, lack-of fit test, coefficient of determination (R^2) and adjusted-R^2 analysis. Responses were monitored and results compared with model predictions. Numerical optimization technique of the Design-Expert software was used for simultaneous optimization of the multiple responses. The desired goals for each variable and response were chosen. The aim of the optimization was to minimize the syneresis while stiffness was maximized.

RESULTS AND DISCUSSION

In order to establish the optimized gelation of LSPI, RSM was used in this study. Lack of fit test and R^2 are usually employed to verify a model adequacy. The significance of the lack of fit test shows that the points are not completely distributed around the model; as a result, the model could not be applied to predict the values of the independent variables. Therefore, the in the significance of the lack of fit test implies that the model has been able to fit the data properly. It is necessary that a model has a $R^2>0.80$ to be capable of fitting the data adequately.

The empirical data of syneresis, and stiffness of gel were analyzed by fitting polynomial quadratic equations using multiple regressions through in RSM. The adequacy of the polynomial quadratic equation was determined according to the R^2. The R^2 values of syneresis and stiffness of gel were equal to 0.90 and 0.89, respectively.

The model fitness was determined by the lack of fit test ($p>0.05$) which shows the model suitability for predicting the variations. This test revealed that the model was accurate enough to predict the responses. The linear lacks of fit mean of squares were also insignificant, indicating the quadratic model was adequate and other models did not affect the responses. Statistical analysis of the fitted models demonstrated that the quadratic model was the most significant one for all of the responses.

The effect of the independent variables on stiffness

The *p*-value was utilized as a useful parameter to evaluate the significance of every coefficient. The lower values of this parameter indicate the more significance of the corresponding coefficient. When the *p*-value was lower than 0.05, it can be concluded that model terms are significant. The influence of various gelation conditions on LSPI gel stiffness has been reported by the coefficient of the second order polynomials (Data not shown). In order to aid visualization, the response surfaces for stiffness are presented in Figure 1.

Generally, the forces involved in the maintenance of the native protein structure in solution are also influence on network forming during protein gelation[16]. The changes of gel stiffness with protein content and $CaCl_2$ concentration at a constant TGase concentration are depicted in Figure 1. It is evident that with increasing $CaCl_2$ concentration, gel stiffness increased, but the increase of protein content had a falling effect on this property. At less protein content, $CaCl_2$ did not have a considerable effect on gel stiffness when compared to more protein content. Electrostatic interactions had high sensitivity to ionic environment and can be manipulated by adding of minerals. The addition of $CaCl_2$ was beneficial to the gel textural properties of proteins and resulted in a more compact

gel network. The addition of $CaCl_2$ could improve the gel strength of the gel system. It is believed that $CaCl_2$ could improve gelation by promoting the unfolding of proteins and changing the aggregation and conformation of proteins via hydrophobic interactions and Ca bridges[17]. Increased salt concentrations increased the ionic, hydrogen, and hydrophobic bonds, which in turn could improve the gel strength and its textural properties[18]. For example, carboxyl groups can act as binding site for Ca^{2+}, and thus induce a determinant influence on gel forming ability. Therefore, this increasing effect is likely due to the involvement of Ca^{2+} ions in electrostatic interactions and as a result, reinforcement of the protein network structure. Additionally, based on the DLVO theory, by adding salt, the distance distribution of repulsive potential changes; therefore, the energy barrier decreases, leading to particles to be aggregated. An increase in salt concentration can lead to a fast drop of the repulsive potential with distance in comparison to the influences at less salt concentrations[19]. The influences of the van der Waals forces and electric double layer mentioned by the DLVO theory are to allow the aggregates to get near enough for interaction via inter-molecular forces like covalent, hydrophobic, and hydrogen bonds[20]. Mao et al.[21] reported that when the Ca^{2+} concentration increased, the stiffness of mixed gellan gel increased up to an optimum concentration (10 mM), but the magnitude of this parameter decreased with further added Ca^{2+}. Lee et al.[22] also reported that when the salt concentration increased, the gel strength of myofibrillar protein gels containing different levels of red bean protein isolate was improved. In another study, Mulvihill[7] found that increasing concentrations up to 10 mM $CaCl_2$, resulted in a considerable increase in hardness and decrease in cohesiveness of 10% protein β-lactoglobulin gels. The magnitude of stiffness increased with the amount of protein as a result of increased number of bonds.

Although calcium ion concentration has a considerable influence on gel texture, conflicting results of different researchers demonstrate its impact mainly depends on other factors than total amount of calcium present[23]. For instance, transglutaminase enzyme is one of the most important factors which can change gelation process. The main purpose of TGase utilization in food formulation is to improve their physical and textural properties like stiffness which has been reviewed in previous studies[24]. The changes occurred in gel stiffness with increasing protein content and TGase concentration at a constant $CaCl_2$ concentration is given in Figure 1. An increase in TGase content in all protein concentrations was accompanied by an increase in gel stiffness (Figure 1) which may be related to producing covalent bonds between molecular chains, and consequently improve the textural properties of gels[25]. In conclusion, the highest stiffness of LSPI gel was realized to be at a maximum calcium ion and TGase concentration and protein content. But we believe that we can with the appropriate salt concentration improve the strength of LSPI gels mediated by TGase, however, the combination of proteins and TGase was effective to improve the textural properties in low-salt systems.

The effect of the independent variables on syneresis

Syneresis is defined as loss of water during aging of gels and showed the instability of gel network. LPSI gels with more protein concentrations demonstrated a less syneresis compared to those having lower concentrations. With increasing protein concentration, the space available for holding water decreases and as a result, syneresis increases[27].

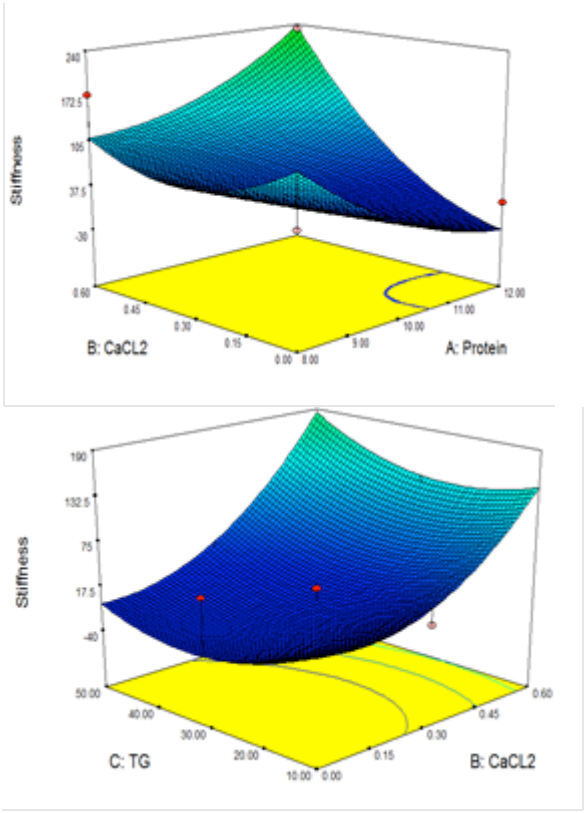

Figure 1. The effect of $CaCl_2$, Protein and TGase concentration on stiffness.

The degree of syneresis is related to the aggregation of the three-dimensional gel network structure and density[28]. In the presence of $CaCl_2$, the carboxyl groups on various molecules may act as binding site for Ca^{2+}. The intermolecular ionic interaction between carboxyl groups and Ca^{2+} ions leads to an increase in shrinkage of the network structure[28]. Therefore, it is expectable that the added $CaCl_2$ can lead to the increasing degree of syneresis.

Recently, the use of TGase has proved to be an appropriate substitute for altering the technological characteristics of raw products[26]. As it can be seen in Figure 2, increasing TGase concentration diminished gel syneresis. This effect can be attributed to the effect of TGase on α-amine groups of lysine residues connected to proteins which resulted in more water holding capacity[9].

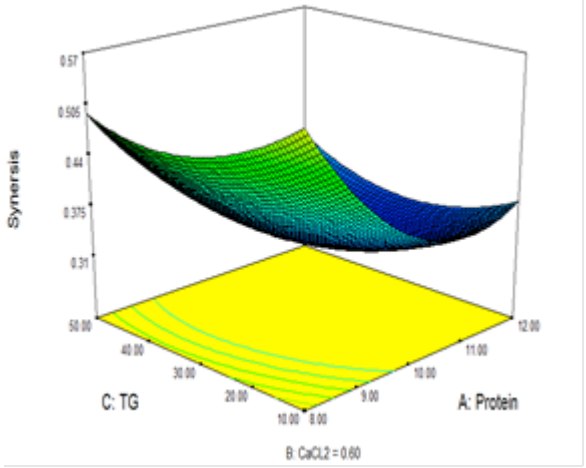

Figure 2. The effect of the independent variables on syneresis.

Optimization and verification of the models

Optimization of gelation procedure was designed to obtain maximum stiffness with minimum syneresis for the gels. The appropriateness of the models to predict optimum gelation condition was evaluated (Table 1).

Table 1. Optimum gelation condition

	Goal	Lower limit	Upper limit	Optimum
Protein	Is in range	8	12	10.39
$CaCL_2$	Is in range	0	0.60	0.60
TGase	Is in range	10	50	41.73
Stiffness	Maximize	5.08	484.05	184.33
Syneresis	Minimize	0.31	0.68	0.32
Desirability				0.61

CONCLUSION

These results, suggest that using as a substitute for meat protein or as a nutritious and functional additive, *Lathyrus sativa* isolate protein could play an important role, similar to what is done with soy protein. The result of this research revealed that the optimum condition for producing LSPI gels had significant influence on stiffness (N/m) and syneresis (%). With increasing $CaCl_2$ concentration, gel stiffness improved, but the increase of protein content had a falling effect on this feature. With increasing TGase concentration gel syneresis decreased. According to these results, it was found that the optimum condition for producing LSPI

gels is protein content of 10.39%, $CaCl_2$ concentration of 0.60 and TGase content of 41.73. At this condition, stiffness and syneresis were 184.33 and 0.32, respectively.

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