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The effect of limited proteolysis on canola protein gelation

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Abstract

The objective of this study was to improve canola protein gelation properties through limited enzymatic hydrolysis with trypsin, ficin and bromelin either alone or in combination with the cross-linking enzyme transglutaminase (TG). Proteolysis alone was found to reduce gel strength to below that of the control. This was accompanied by a decrease in molecular weight, observable through SDS-PAGE analysis. Micrographs corroborated the rheological data and showed that protease application brought about a loss of structure. In contrast, limited proteolysis was shown to be a suitable pretreatment to cross-linking with TG, a frequently used cross-linking enzyme. Previous research suggests and this study corroborates that opening the protein structure prior to TG treatment, can enhance its effectiveness. Gels, treated with both a protease and TG, were significantly stronger than those treated with TG alone. It was concluded that while limited proteolysis does not improve canola protein gelation on its own, it can significantly improve gelation properties when combined with TG.

Keywords: Gelation; Canola; Proteolysis; Cross-linking

1. Introduction

Enzymatic hydrolysis has been shown to improve the functional properties of soy, sunflower, peanut (Beuchat, 1977) and rapeseed protein isolates (Hartnett & Satterlee, 1990; Kim, Park, & Rhee, 1990; Mahajan & Dua, 1998). Pour-el and Swenson (1976) performed an extensive experiment comparing the effectiveness of many proteases in improving soy protein gelation properties. In their analysis, they discovered ficin and bromelin to be the most effective at improving the properties of irreversible soy protein gels (Pour-el & Swenson, 1976). Trypsin on the other hand was found to be the least effective, consistently producing gels too weak to support their own weight (Pour-el & Swenson, 1976). When applied to commercial soy protein isolate the microbial protease alcalase was shown to yield a three fold improvement in thermal aggregation, a prerequisite for gelation (Kim et al., 1990). Hartnett and Satterlee (1990) showed that there is an optimal degree of pepsin hydrolysis at which the strongest soy protein gels are formed. This signifies that, while limited proteolysis can improve gelation, excessive proteolysis is detrimental. In the case of papain and bacterial protease, however, even short incubation times resulted in poor heat set gels and increasing the reaction time resulted in a complete loss of gelling ability (Hartnett & Satterlee, 1990). This effect was attributed to the different hydrolytic specificities of these enzymes (Hartnett & Satterlee, 1990).

Limited treatment with proteases leads to some protein hydrolysis and consequently partial unfolding of the protein structure. Unfolding of the native protein exposes buried hydrophobic groups (Kang et al., 1994) and other interactive groups, which are then free to interact with neighboring polypeptides. Since hydrophobic interactions play a major role in the gelation of canola proteins (Léger & Arntfield, 1993) it is hypothesized that limited proteolysis has the potential to improve canola protein gelation.

Additionally, limited proteolysis can be used as a pretreatment to cross-linking with TG. TG has been shown to effectively improve the gel forming ability of many proteins, including rapeseed (Hyun & Kang, 1999). It is widely

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used in the food industry for the purpose of cross-linking proteins (Aguilera & Rademacher, 2004). A prerequisite for cross-linking with TG is the availability of lysine and glutamine residues, which through the action of TG become covalently bound forming either inter- or intra molecular ε-(γ-Glu)-Lys cross-links (De Jong & Koppelman, 2002; Nielsen, 1995). Kang et al. (1994) showed that the amount of surface lysine and glutamine residues were correlated with the amount of cross-links formed. A variety of methods can be applied to destabilize a protein, thus increasing the exposure of its lysine and glutamine residues (De Jong & Koppelman, 2002). EDTA (De Jong & Koppelman, 2002), heat treatment (Kang et al., 1994) and reduction with DTT (Aboumahmoud & Savello, 1990) as well as cysteine and sulfite have been used to partially unfold proteins prior to TG treatment (De Jong & Koppelman, 2002). Many vegetable proteins are globular (Nielsen, 1995). It is therefore expected, that any treatment which opens the structure and makes the buried reactive groups more available, would improve the TG cross-linking in vegetable proteins (Nielsen, 1995). It was hypothesized, that enzymatic hydrolysis would also increase the availability of these groups and could thus be a useful pretreatment to cross-linking with transglutaminase.

The effects of limited proteolysis, alone and in combination with TG, on the strength of heat induced canola protein gels was evaluated. Rheological measurements performed with a texture analyzer, SDS-PAGE and microscopy were used to evaluate the resulting networks.

2. Materials and methods

2.1. Canola protein isolate

A commercial canola protein isolate was obtained from BMW canola. The isolate contained 87% protein as determined by Kjeldahl (Chung, 2003) using a N to protein conversion factor of 5.7 (Uruakpa, 2004). The pH of a 10% solution was 6.2 ± 0.1.

2.2. Enzyme hydrolysis

Commercial canola protein isolate was treated with trypsin (T-1426, Sigma, St. Louis, USA), bromelin (B-4882, Sigma, St. Louis, USA) and ficin (F-4165, Sigma, St. Louis, USA), all of which were obtained from Sigma (St. Louis, USA). The enzymes were applied in concentrations ranging from 0 to 20 mg/g CPI. Canola protein isolate was mixed with 0.1 M NaCl (Fisher Scientific, Ottawa, Canada) to obtain a 10% (w/v) dispersion. To allow for complete suspension, the samples were vortexed frequently within a 2 h period. The enzymes were also dissolved in 0.1 M NaCl. An appropriate aliquot of the enzyme solution was then added to the canola protein isolate solution. After briefly vortexing, the mixture was placed into a 40 °C water bath (Magni Whirl Constant Temperature Bath, Blue M Electric Company, Blue Island, USA) for 2 min. An 8 mL aliquot of the treated sample was placed into a small (diameter = 41 mm) round aluminum box (LeeValley, Winnipeg, Canada) and covered with a lid to prevent evaporation. To terminate enzyme hydrolysis and initiate gel formation, the suspension was heated to 95 °C for 15 min (Stabil-therm constant temperature cabinet, Blue M Electric Company, Blue Island, USA). Gels were then cooled to room temperature prior to rheological analysis. The time to transfer the solution into the aluminum dish and place it into the oven was exactly 2 min resulting in a total treatment time of 4 min.

2.3. Enzymatic hydrolysis and cross-linking combined

The proteases ficin (F-4165, Sigma, St. Louis, USA) and trypsin (T-1426, Sigma, St. Louis, USA) were applied at three low levels ranging from 2×10^{-2} mg/g CPI to 2×10^{-14} mg/g CPI. The samples were vortexed and then kept at room temperature for 2 min prior to TG treatment. The proteases were not inactivated prior to TG application.

A bacterial Ca^{+2} independent TG was used. The Activia TI transglutaminase enzyme (Ajinomoto, Paramus, USA) was also dissolved in 0.1 M NaCl at a rate of 1 g (100 Units) in 10 mL. An appropriate aliquot (0.25 mL) of the enzyme solution was then added to 10 mL of canola protein isolate solution to produce a TG concentration of 2.5 Units/g protein isolate. After briefly vortexing the mixture, it was placed into a preheated water bath (Magni Whirl Constant Temperature Bath) for the desired treatment time. An 8 mL aliquot of the treated sample was placed into a small (diameter = 41 mm) round aluminum box (LeeValley, Winnipeg, Canada) and covered with a lid to prevent evaporation. To terminate enzyme hydrolysis and initiate gel formation the suspension was heated to 95 °C for 15 min (Stabil-therm constant temperature cabinet, Blue M Electric Company, Blue Island, USA). Gels were then cooled to room temperature prior to rheological analysis. The time to transfer the solution into the aluminum dish and place it into the oven was exactly 10 min.

2.4. Rheology

To evaluate the effect of enzyme treatment on the texture of canola protein gels a uniaxial compression test was performed with a TA-TX2i Texture Analyzer (Stable Micro Systems Ltd., Godalming, England). The method of O'Kane, Happe, Vareijken, Gruppen, and Van Boekel (2004) was adapted to suit the requirements of this experiment. A spherical plunger (diameter = 12 mm) was utilized. Samples were compressed to 50% at a rate of 0.1 mm/s. The trigger point was 0.01 N. The resulting data was interpreted using Texture Expert Version 1.22 analysis software (Stable Micro Systems Ltd., Godalming, England). The program produces a force–displacement curve. Two parameters, the elasticity and the hardness of the material were determined.

To calculate elasticity:

Elasticity is stress divided by strain (Lardner & Archer, 1994). To calculate stress, the force was divided by the projected surface area of the ball probe (34.56 cm^2). The maximum displacement considered in the calculations (1 mm) was divided by the original sample thickness (8 mm) to obtain the strain. The line of the force displacement graph was consistently linear up to a displacement of 1 mm, above this value linearity was lost. Consequently calculations were restricted to the first mm of displacement.

To calculate hardness:

The peak force achieved at any point during the test was used as an indicator of gel hardness. This was either the rupture point or the force at maximum displacement (4 mm).

2.5. Electrophoresis

To evaluate subunits in the protein, the method of Aluko and McIntosh (2001) was followed with minor modification. Samples were dissolved (5% w/v) in 1 M Tris–HCl (T-1503, Sigma, St. Louis, USA) buffer at pH 8 containing 10% SDS (L-3771, Sigma, St. Louis, USA) 5% 2-mercaptoethanol (M-7154, Sigma, St. Louis, USA) and 0.01% Pyronin Y (P-6653, Sigma, St. Louis, USA). Samples were boiled for 10 min and then vortexed (Vortx Genie 2, Scientific Industries Inc., Bohemia, USA) to promote dissolution. The sample was then centrifuged at 14,000*g* (Biofuge A, Canlab, West Germany) to settle out any remaining particulate matter and 4 μl of the supernatant were applied to a 4–20% gradient gel (161-1159, Bio-Rad, Hercules, USA). The standard (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad, Hercules, USA) was prepared as per manufacturers instructions and 5 μl were loaded into one slot of each gel. Gels were run for 2 h and 20 min at 10 amp per gel (Mini-ProteanR 3 Cell, Bio-Rad, Hercules, USA). A staining solution consisting of 0.08% (w/v) Comassie Brilliant Blue G-250 (B-1131, Sigma, St. Louis, USA), 10% (w/v) ammonium sulfate (ACS 093, BDH Inc., Toronto, Canada) and 2.5% (w/v) phosphoric acid (Fisher Scientific, Ottawa, Canada) was used to visualize the protein bands. Twenty percent of ammonium sulfate (ACS 093, BDH Inc., Toronto, Canada) was used to destain the gels. The gels were scanned to provide a permanent record of the results (Epson Perfection Scanner, Epson, Toronto, Canada).

2.6. Microscopy

To examine the protein gel microstructure, the method of Uruakpa (2004) was followed with minor modifications. Freeze-dried samples were fractured by hand and prepared for microscopy by mounting on studs with carbon paint. The samples were then sputter coated with gold/palladium (60/40) under a 2×10^{-1} Torr vacuum. Micrographs were obtained through the use of a Cambridge Instruments stereoscan 120 scanning electron microscope (Cambrigde, UK). The acceleration voltage was 30 kV. Micrographs at magnifications of 50×, 200× and 1000× were obtained for each sample.

2.7. Experimental design

2.7.1. Phase 1 – proteolysis

The three proteolytic enzymes trypsin, ficin and bromelin were each applied at five levels ranging from 2×10^{-6} to 20 mg/g. The exact treatment levels were: 2×10^{-6} mg/g, 2×10^{-4} mg/g, 2×10^{-2} mg/g, 2 mg/g, 20 mg/g. All other parameters such as treatment temperature, treatment time and CPI concentration were held constant at 40 °C, 4 min and 10% respectively. Each treatment was applied in duplicate.

2.7.2. Phase 2 – proteolysis and cross-linking combined

Treatment with 2×10^{-2} mg/g 2×10^{-6} mg/g, 2×10^{-10} mg/g and 2×10^{-14} mg/g was carried out in duplicate. The samples were then treated with TG under the following conditions: TG = 2.5 units, treatment time = 60 min, treatment temperature = 40 °C. The CPI concentration was kept constant at 10%.

2.8. Statistical analysis

Results were analyzed with Microsoft Excel and SAS 8. Statistical parameters determined include average, standard deviation and difference tests (LSD, Bonferroni *t* test and Duncan's multiple range test).

3. Results

3.1. Proteolysis

Despite the apparent potential of limited proteolysis to improve soy protein gelation, no beneficial effects on canola protein gelation were observed. Applied as a pretreatment to heat induced gelation, limited amounts of protease did not alter gel strength. Increasing amounts of enzyme however reduced gel elasticity and hardness. The following data demonstrate the detrimental effect of all three of these enzymes on gel strength. The apparent decrease in gel strength can be explained by the decrease in molecular weight caused by these enzymes. SDS-PAGE analysis clearly demonstrated that protease treatment leads to the loss of high molecular weigh bands and an increase in the number of low molecular weigh constituents. As an example, the effect of trypsin on the subunit composition is shown in Fig. 1. Micrographs corroborated the rheological data and showed that protease application was accompanied by loss of structure. This is visualized in Fig. 2, which shows the effect trypsin on the microstructure of CPI gels.

3.1.1. Trypsin

The detrimental effect of trypsin on canola protein gelation is illustrated in Table 1. Both elasticity and hardness

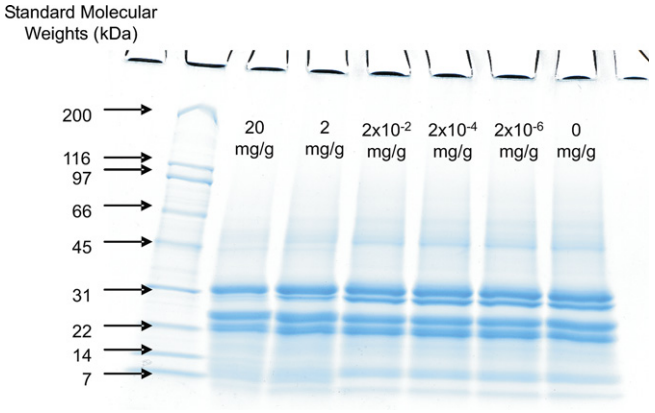

Fig. 1. SDS-PAGE composition of canola protein isolate treated with varying levels of trypsin. CPI %, treatment time and temperature were kept constant at 10%, 60 min and 40 °C respectively.

decreased with increasing amounts of trypsin. Duncan's multiple range test shows that treatments ranging from 2×10^{-6} to 2×10^{-2} mg/g did not significantly differ from the untreated sample, in terms of both elasticity and hardness. Treatment with 2 mg/g trypsin, however, significantly reduced gel strength. No meaningful hardness measurement could be obtained at a treatment level of 20 mg/g, as the gel collapsed under moderate pressure, and this treatment level is therefore not included in Table 1. Preliminary experiments included treatments in the range of 40–60 mg/g trypsin which resulted in a complete loss of the gel forming ability (data not included).

Fig. 1 clearly shows the loss of high molecular weight polypeptides brought about by trypsin. Peptides at or above 45 kDa and the polypeptide at approximately 30 kDa, grew fainter with increasing amounts of trypsin. As the amount of trypsin increased more low molecular weight (22 kDa and below) bands appeared on the SDS-PAGE gel. These changes became particularly evident at the 2 mg/g treatment level, the same point at which the gel strength was significantly reduced compared to the control.

The micrographs in Fig. 2 visualize the loss of structure induced by trypsin. Treatment with 2×10^{-6} mg/g and 2×10^{-4} mg/g trypsin showed no significant differences in microstructure. A significant loss in structure was observed at treatment levels of 2×10^{-2} mg/g and above. At 2×10^{-2} mg/g a loss of cell walls resulted in very large elongated cells. These elongated cells are clearly visible at a magnification of 50× and 200×. As the trypsin concentration increased further the cell structure was lost and the material appeared flakier. At levels of 20 mg/g the cell walls appeared extremely rough, as is best observed at a magnification level of 1000×. This loss of cell walls and structure helps explain the reduction in gel strength observed during rheological measurements.

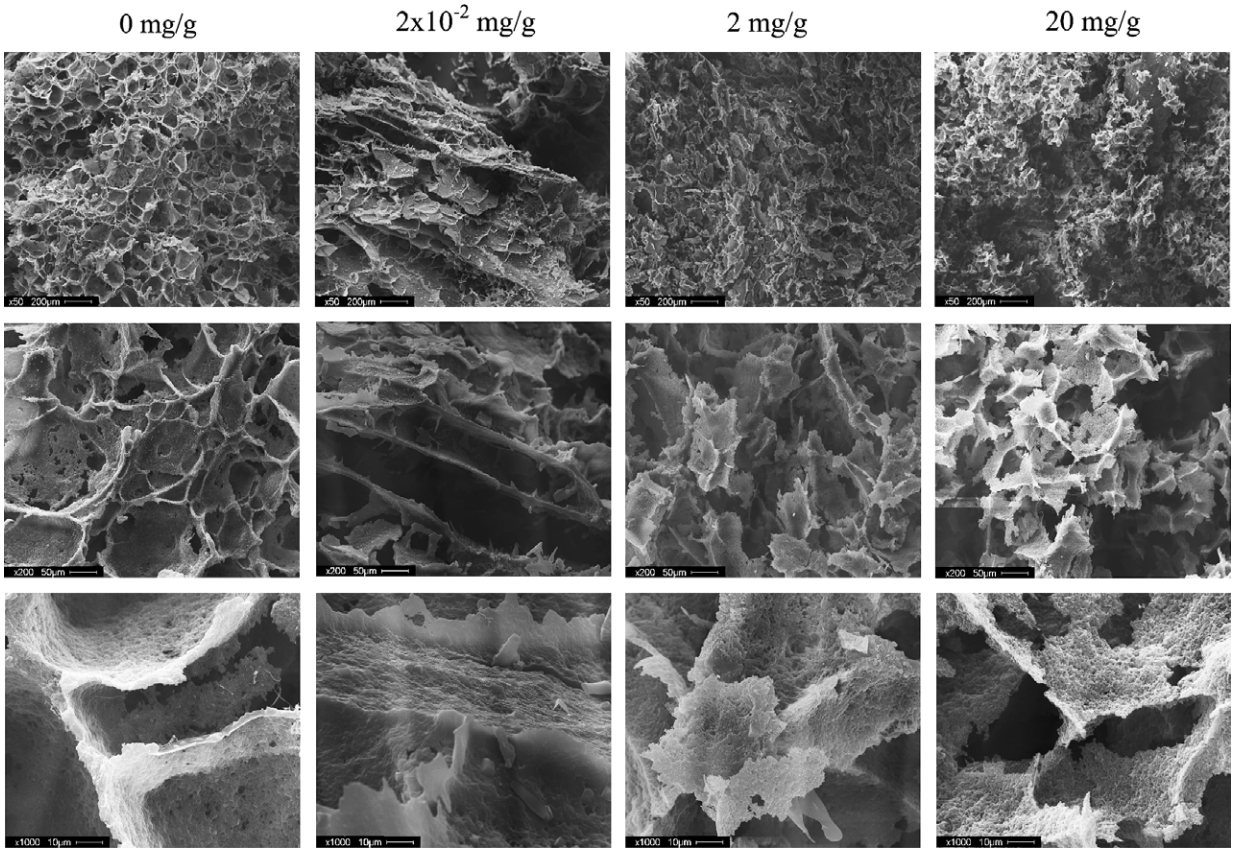

Fig. 2. The effect of trypsin treatment on the microstructure of heat set canola protein gels. CPI %, treatment time and temperature were kept constant at 10%, 60 min and 40 °C respectively. Magnifications were as follows: top row 50×; middle row 200× and bottom row 1000×.

Table 1
The effect of protease treatment on the elasticity and hardness of heat set canola protein gels

Protease (mg/g)	Trypsin		Ficin		Bromelin	
	Elasticity (N/mm^2)[a]	Hardness[a] (N)	Elasticity[a] (N/mm^2)	Hardness[a] (N)	Elasticity[a] (N/mm^2)	Hardness[a] (N)
0	0.0137 ± 0.0025A	1.57 ± 0.22A	0.0136 ± 0.0025A	1.57 ± 0.22A	0.0137 ± 0.0024A	1.57 ± 0.22A
2×10^{-6}	0.0115 ± 0.0013A	1.31 ± 0.28A	0.0113 ± 0.0003AB	1.20 ± 0.05AB	0.0110 ± 0.0000AB	1.31 ± 0.28A
2×10^{-4}	0.0104 ± 0.0013AB	1.28 ± 0.26A	0.0115 ± 0.0013AB	1.31 ± 0.14AB	0.0117 ± 0.0005AB	1.28 ± 0.26A
2×10^{-2}	0.0104 ± 0.0005ABC	1.38 ± 0.07A	0.0089 ± 0.0005BC	0.99 ± 0.14B	0.0100 ± 0.0001AB	1.38 ± 0.07A
2	0.0078 ± 0.0003BC	0.76 ± 0.11B	0.0065 ± 0.0014CD	na[b]	0.0087 ± 0.0006B	0.76 ± 0.11B
20	0.0055 ± 0.001C	na[b]	0.0033 ± 0.0004D	na[b]	0.0032 ± 0.0001C	na[b]

CPI concentration, treatment time and temperature were kept constant at 10%, 60 min and 40 °C respectively.
[a] Means with the same letter are not significantly different ($p < 0.05$) (Duncan's multiple range test); samples were at minimum run in duplicate.
[b] Not available.

3.1.2. Ficin

Like trypsin, ficin had a negative impact on canola protein gelation. Table 1 illustrates the reduction in gel strength induced by ficin treatment. Both elasticity and hardness were reduced by ficin. Duncan's multiple range test shows that treatments of 2×10^{-6} and 2×10^{-4} mg/g were not significantly different from the untreated sample in terms of both elasticity and hardness, while treatment levels greater than 2×10^{-4} mg/g showed a significant reduction in gel strength. No meaningful hardness measurements could be obtained at 2 and 20 mg/g, as these gels collapsed under moderate pressure. These treatment levels are therefore not included in Table 1.

The reduction in gel strength was again associated with a reduction in molecular weight. This became particularly visible at the 2 mg/g treatment level. Peptides at or above 45 kDa grew fainter at 2 mg/g ficin and almost completely disappeared at a treatment level of 20 mg/g. At 20 mg/g ficin, the polypeptides between 22 and 30 kDa were also partially lost. The loss of high molecular weight bands was accompanied by an increase in low molecular weight material. A treatment level of 2×10^{-2} mg/g, despite producing a gel of significantly reduced strength, showed no differences in the molecular weight distribution of the polypeptides (data not included).

As the ficin concentration increased, the cell structure was reduced and the material appeared flakier. Treatment with 2×10^{-6} mg/g and 2×10^{-4} mg/g ficin showed no significant differences in microstructure. A significant loss in structure was observed at treatment levels of 2×10^{-2} mg/g and above. This supports the results obtained during rheological analysis and coincides with the point at which MW reduction became apparent in SDS-PAGE analysis. Additionally, the control produced fairly flat and smooth edges along the fracture plane. Gels treated with 2 or 20 mg/g ficin produced jagged edges at the break. This further indicates that the cell walls contain many structurally weak points.

3.1.3. Bromelin

Bromelin, like trypsin and ficin, proved to be detrimental to canola protein gelation. Table 1 illustrates that both elasticity and hardness decreased with increasing amounts of bromelin. Treatment of canola protein with up to 2×10^{-2} mg/g was not found to significantly reduce strength. At 2 mg/g and 20 mg/g both hardness and elasticity were significantly reduced, compared to the control. No meaningful hardness measurement could be obtained at a treatment level of 20 mg/g bromelin, as the gel collapsed under moderate pressure and this treatment level is therefore not included in Table 1.

Changes in the molecular weight distribution become apparent at 2 mg/g, the same level at which gel strength was significantly reduced compared to the control. At 2 mg/g, bromelin bands at and above 20 kDa grew thinner and fainter, indicating hydrolysis of these polypeptides by bromelin. At the same time, low molecular weight (less than 20 kDa) material accumulated. At 20 mg/g this trend was even more pronounced.

Bromelin, like trypsin and ficin caused a loss of structure. While treatment with 2×10^{-6} mg/g to 2×10^{-2} mg/g produced no significant differences in microstructure, a significant loss in structure was observed at treatment levels of 2 and 20 mg/ml. This supports the results indicating a significant loss of elasticity at 2 mg/g and above. It does however not corroborate the results indicating that the gel hardness is affected at a treatment level as low as 2×10^{-2} mg/g. At levels of 2 and 20 mg/g the cell walls again appeared very rough.

3.2. Proteolysis and cross-linking combined

Limited proteolysis was expected to hydrolyze some peptide bonds, thereby opening the protein structure and increasing the availability of the active groups lysine and glutamine, making it a better substrate for TG. Results indicate that limited proteolysis with trypsin or ficin can indeed improve the strength of TG treated heat induced CPI gels.

The results obtained from treating CPI with trypsin, prior to treatment with TG, produced significantly stronger heat induced gels than pretreatment with TG alone. As shown in Table 2, gel hardness was improved significantly compared to the control at a treatment level of 2×10^{-14} mg/g trypsin. Treatment levels of 2×10^{-10} to 2×10^{-2} mg/g also improved the hardness compared to the control, with greater improvements at lower trypsin

Table 2
The effect of a combined trypsin and TG treatment on the properties of heat set canola protein gels

Protease (mg/g)	Trypsin		Ficin	
	Elasticity (N/mm^2)[a]	Hardness[a] (N)	Elasticity[a] (N/mm^2)	Hardness[a] (N)
0	0.0300 ± 0.0014A	2.541 ± 0.194A	0.0300 ± 0.0014A	2.541 ± 0.194A
2×10^{-14}	0.0335 ± 0.0031A	3.074 ± 0.195A	0.0335 ± 0.0036A	3.085 ± 0.087A
2×10^{-10}	0.0312 ± 0.0008A	2.936 ± 0.124AB	0.0311 ± 0.0006A	2.871 ± 0.054AB
2×10^{-6}	0.0311 ± 0.0012A	2.829 ± 0.152AB	0.0300 ± 0.0021A	2.849 ± 0.187AB
2×10^{-2}	0.0314 ± 0.0010A	2.827 ± 0.007AB	0.0306 ± 0.0014A	2.650 ± 0.163B

CPI concentration and protease treatment conditions were kept constant at 10% CPI and 4 min of treatment time at 40 °C. Treatment conditions for the subsequent TG treatment were kept constant at 2.5 units TG/g CPI and 60 min treatment time at 40 °C.

[a] Means with the same letter are not significantly different ($p < 0.05$) (Duncan's multiple range test); samples were at minimum run in duplicate.

levels, but the improvements were not statistically significant. Likewise limited proteolysis seemed to improve the elasticity of TG treated CPI gels, with greater improvements at lower levels of trypsin. However these differences were not statistically significant either.

Treatment with ficin mirrored the results obtained with trypsin. The results obtained from treating CPI with ficin, prior to treatment with TG, produced stronger heat induced gels than pretreatment with TG alone. Table 2 shows that the hardness of TG treated CPI gels was improved significantly through treatment with 2×10^{-14} mg/g ficin. Treatment levels of 2×10^{-10} to 2×10^{-2} mg/g also improved the hardness compared to the control, with greater improvements at lower treatment levels, but the improvements were not statistically significant. Likewise limited proteolysis induced statistically insignificant improvements in elasticity of TG treated CPI gels, with greater improvements at lower levels of ficin.

SDS-PAGE revealed no significant differences in the polypeptide chains of the protease treated CPI (data not included). This could indicate that either, the proteolysis treatment did not alter the proteins ability to interact with TG or that the isolates ability to act as a substrate was improved, but that the increase in cross-linking was not large enough to show up on SDS-PAGE.

In contrast to the rheological data, no significant differences in the microstructure were induced through the application of limited proteolysis prior to TG treatment (data not included). In all cases highly structured, well formed networks were visible. Cell walls were generally smooth and fairly thick. In conclusion, the beneficial effect of proteolysis as a co-treatment to TG was not reflected in the microstructure of the gels.

4. Discussion

4.1. Proteolysis

In summary, all three proteases, reduced the strength of gels produced. The reduction in hardness and elasticity was generally associated with a reduction in the molecular weight of the constituent polypeptides. It is therefore hypothesized that this reduction impairs the ability of the protein to form a good network.

These results are contrary to the findings of Pour-el and Swenson (1976) and Hartnett and Satterlee (1990) who found that limited hydrolysis can improve the gelation properties of soy and Sanchez and Burgos (1995) who found limited proteolysis to promote gelation in sunflower proteins. Although these authors signified success with certain enzymes, other enzymes destroyed the gel forming ability even when applied at low levels.

Pour-el and Swenson (1976) found ficin and bromelin to be most effective in improving soy protein gels. Trypsin on the other hand was found to be least effective, consistently producing gels too weak to support their own weight (Pour-el & Swenson, 1976). Hartnett and Satterlee (1990) showed that limited pepsin hydrolysis can yield stronger soy protein gels. In the case of papain and bacterial protease however even short incubation times resulted in poor heat set gels and increasing the reaction time resulted in a complete loss of gelling ability (Hartnett & Satterlee, 1990). Puski (1975) found that even limited enzyme treatment with a protease preparation from *Aspergillus oryzae* significantly reduced the viscosity and prevented gel formation of soy protein solutions. Regenstein, Grunden, and Baker (1978) likewise determined that proteolysis with either ficin or bromelin reduced the coagulum strength of egg albumin. This indicates that successful improvement of gel forming ability strongly depends on enzyme specificity and the substrate protein. This can explain why bromelin and ficin, which were reported to improve soy gelation, did not provide the desired improvement of canola protein gelation. Trypsin on the other hand consistently performed poorly (Kim et al., 1990; Pour-el & Swenson, 1976), which was corroborated by this study.

4.2. Proteolysis and cross-linking combined

Combining TG treatment with exceedingly low levels of either trypsin or ficin improved the rheological properties of the CPI gels. Lower levels of proteases produced better gels, with only the lowest application level tested (2×10^{-14} mg/g) having produced a significant improvement. This improvement in gel strength could however not conclusively be linked to an increase in cross-linking via SDS-PAGE analysis or an improvement in microstructure via microscopy. These results corroborate the findings that

partial denaturation can enhance the effects of TG. EDTA was found to destabilize α-lactalbumin and made it a good substrate for TG (De Jong & Koppelman, 2002). Heat treatment was found to increase the amount of surface active groups and to maximize ε-(γ-Glu)-Lys cross-linking of glycinin (Kang et al., 1994). The reducing agent dithiotreitol (DTT) is also commonly used to improve glutamine and lysine accessibility. Aboumahmoud and Savello (1990) determined that DTT was required for cross-linking of whey, via TG treatment, to take place. Nonaka et al. (1989) found DTT treatment to improve the ability of TG to react with bovine serum albumin, human serum albumin and con albumin. For these proteins a structural change, induced through the reduction of disulfide bonds, was required for them to act as a substrate for TG (Nonaka et al., 1989). Other food-grade reductants, such as cysteine and sulfite, have also been shown to enhance the ability of β-lactoglobulin to form cross-links during TG treatment (De Jong & Koppelman, 2002). These reductants were however found not to be as effective as DTT (De Jong & Koppelman, 2002). This study indicates that limited proteolysis can provide yet another suitable method for improving the action of TG.

5. Conclusions

Limited proteolysis was found to be an effective pretreatment for cross-linking with TG. Treatment with both a protease and TG prior to heat set gelation yielded harder gels than TG treatment alone. Treatment with proteases alone, however, negatively impacted canola protein gelation. At low levels of treatment no significant differences in elasticity or hardness were observed. With increasing amounts of protease gel strength deteriorated. It is concluded that trypsin, ficin and bromelin alone are not suitable for the improvement of canola protein isolate gelation. A review of the available literature, however, indicates that success is highly enzyme specific. Before dismissing limited proteolysis as a method for improving canola protein gelation, the effects of other proteases should therefore be investigated.

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