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The evaluation of proteases as coagulants for soy protein dispersions

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Abstract

The ability of different proteases to induce the gelation of soy protein isolate dispersions (5.33% w/w) was studied. The coagulation time and gel firmness were determined using dynamic viscoelastic measurements. Among the six protease tested, papain was the most effective coagulant in terms of gel strength and coagulation speed; the second was alcalase. Degree of hydrolysis (DH), pH and viscosity profiles of soy proteins were tested during the coagulation with different proteases. The result suggested that strong interactions, other than electrostatic interaction, existed between peptides in papain and alcalase induced coagulation. Thermal and pH stability tests indicated that papain was more stable than alcalase in the temperature (60–90 °C) and pH (5.8–7.0) ranges studied. Higher papain dosages within the range of 5–13.3 U/ml resulted in firmer soy protein gels, but concentrations higher than 13.3 U/ml produced weaker gels. With the addition of 0.625 mM cysteine, the soy protein coagulation ability of papain was improved. A soy protein gel formed with 0.025% papain in the presence of 0.625 mM cysteine had about the same strength as that induced by 0.133% papain without cysteine.

Keywords: Soy protein; Protease; Coagulant; Gel formation

1. Introduction

Soy protein is a popular food ingredient used throughout the world for its nutritional and functional properties, especially after the FDA in the United States allowed a soy health claim in 1999 (Fukushima, 2001). This claim has sparked the development of new soy foods. Tofu is one of the most important traditional soy foods in the eastern world, but it has a limited shelf life. Soy powder that could be rapidly coagulated into tofu after rehydratation with hot water could be a potential new product, since convenience has become an important component for food service (Pszczola, 2000). However, one of the difficulties in making instant tofu is that more than 30 min is needed with traditional coagulants, such as $CaCl_2$ or glucono-delta-lactone (GDL), to coagulate or gel soy milk. Good temperature control during the gel forming process is also required.

Proteases have been found to coagulate soy protein, with early reports appearing in 1970 s and 1980s. Utaka and Fukazawa (1976) reported that soy protein dispersion could be gelated by ficin. Fuke and Matsuoka (1980), Fuke, Sekiguchi, and Matsuoka (1985) found that bromelain could gelate both soy protein dispersions and soy milk. Murata et al. (1987) reported that some commercial microbial proteases could also gelate soy protein dispersions. More attention has been paid to this phenomenon and enzymatic modification of functional properties (Feng & Xiong, 2003; Kim & Park, 1990) and bioactivities (Joo, Sang, Lee, Lee, & Oh, 2004; Wang et al., 1995) of soy proteins have been reported. Because coagulation of soy proteins occurred in many cases during the preparation of soy protein digests, the process was made difficult and resulted in low yields. Limited coagulation of soy proteins has also been considered a way of producing soy protein gels with novel texture and functional properties (Inouye, Nagai, & Takita, 2002; Nagai & Inouye, 2004). Recently, coagulation mechanism of some microbial proteases on soy protein was investigated; reports have suggested that

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the coagulation was due to a hydrophobic interaction among the peptides produced by proteases (Aoyama et al., 2000; Nagai & Inouye, 2004).

The objective of this research was to survey the gelation ability of various protease systems under conditions that would be encountered during instant soy protein coagulation. The soy protein coagulating ability of several commercial protease systems was evaluated via dynamic viscoelastic measurements. Thermal and pH stabilities of selected protease systems were compared, and the coagulation parameters of the most effective protease on soy protein were determined.

2. Materials and methods

2.1. Materials

Defatted soy flour was purchased from Sanjiang Soy Company (Heilongjiang, China). The enzyme alcalase, flavourzyme, neutrase, and Protemax were from Novo Nordisk (Tianjin, China). Papain was purchased from Xipu Biotech. Ltd. (Panzhihua, China), and bromelin was purchased from Sigma (St. Louis, MO, USA). Other chemical reagents were of analytical grade.

2.2. Preparation of soy protein isolates (SPI) and preheated SPI dispersions

Defatted soy flour was extracted with 10 times its weight of deionized water (adjusted to pH 8.0 with 2 M NaOH) for 2 h at room temperature. The mixture was centrifuged at 3000*g* for 20 min in order to remove the insoluble material. The pH of the supernatant was adjusted to 4.5 with 2 M HCl, and the insoluble fraction was collected by centrifugation (3000*g*, 10 min). The precipitate was washed with deionized water and then adjusted to pH 7.0 with 2 M NaOH, which yielded soy protein isolate (SPI) dispersion. SPI dispersions (8% w/w) were preheated at 90 °C for 30 min and then cooled with ice-water to yield preheated SPI dispersions that were used for all coagulation experiments.

2.3. Assay for protease activity

A spectrophotometric neutral protease assay was used (Godfrey & Reichelt, 1983). One neutral protease unit is the amount of enzyme that will liberate 1 μmol of tyrosine/min under the assay conditions (37 °C, pH 7.0).

2.4. Coagulability of soy protein dispersions with proteases

Preheated SPI dispersion (2 ml) was mixed with 1 ml freshly prepared protease solution and the mixture was then placed on the sample plate of a rheometer (AR1000 Rheometer, TA instrument, Dover, DE, USA). Final concentration and unit activity of proteases in the protease–SPI mixture used are shown in Table 1. The sample was then subjected to time ramp oscillation at 40 °C, 1 Hz frequency and 1.0 Pa stress. The gap between the two plates was set to 500 μm. The changes of storage modules (G'), loss modules (G'') and loss angle tangent ($\tan\delta = G''/G'$) were recorded over a period of 40 min.

Table 1
Final composition of SPI–protease mixture

Mixture	Protease used		SPI (%, w/w)
	Weight fraction (%, w/w)	Unit (U/ml)	
SPI–alcalase	0.074	10.0	5.33
SPI–brominase	0.163	10.1	5.33
SPI–flavourzyme	0.411	10.2	5.33
SPI–neutrase	0.245	10.2	5.33
SPI–papain	0.10	10.2	5.33
SPI–protamax	0.08	10.1	5.33

2.5. pH changes during the enzyme reaction

Preheated SPI dispersion(10 ml) was mixed with 5 ml freshly prepared protease solution in a beaker and then incubated at 40 °C in a water bath. Final concentration of protease was equal to that shown in Table 1. The pH of the mixture during incubation was measured with a pH meter.

2.6. Degree of hydrolysis test during the enzyme reaction

Preheated SPI solution was mixed with papain, alcalase or neutrase at the concentration listed in Table 1. Each mixture was transferred to test a set of test tubes with a total volume of the mixture being 1 ml. Test tubes were incubated in a water bath set at 40 °C as noted. After each defined reaction time, one test tube was taken out from the water bath and the enzyme reaction was stopped by the addition of 5 ml 1% SDS (85 °C) to the tube and holding the tube at 85 °C for 15 min with occasional shaking. The measurement of the degree of hydrolysis (DH) followed the method established by Adler-Nissen (1979). With his method, the content of free amino groups, expressed as leucine equivalents, was assayed after the reaction with trinitrobenesulphonic acid (Sigma, St. Louis, MO, USA).

2.7. Dynamic viscoelastic profile and pH profile of SPI coagulated by glucono-delta-lactone (GDL)

Dynamic viscoelastic and pH profiles of the SPI–GDL gels were measured using the same method as that used for testing coagulability of protease except that the temperature was set at 50 °C.

2.8. Preparation of soy protein solution with different DH and measurement of its viscosity

Preheated soy protein solution (5.33%) was adjusted to pH 8.0 with 1 M NaOH solution and then hydrolyzed with papain, alcalase or neutrase to obtain soy peptide solutions

with different DH's by the pH-stat method (Adler-Nissen, 1986). Protease was added gradually to ensure that at each desired DH, the pH of soy protein solution did not change within 5 min, 1 M NaOH solution was used for pH control. After a certain DH was reached, 5 ml of the solution was taken for viscosity measurement. Viscosity was tested using a rheometer (AR1000 Rheometer, TA instrument, Dover, DE, USA) at 25 °C, cone geometry (4° cone angle, 4 cm diameter) was used, and viscosity at shear rate range of 0.1–10 S^{-1} (log mode) was recorded.

2.9. Residual activity of protease (alcalase and papain) after various pre-heat-treatments

Four sets of 1% protease solutions were incubated in test tubes in a water bath at 60, 70, 80 and 90 °C, respectively. Tubes were removed from the water bath and cooled in an ice bath after 1 or 2 min intervals. Residual protease activity was measured according to the assay reported by Godfrey and Reichelt (1983).

2.10. Relative activity of protease at different pHs

Enzyme activity of alcalase and papain was measured with the same assay except that the pH of the enzyme solution and substrate buffer were both adjusted to 5.8, 6.1, 6.4, 6.7 or 7.0.

2.11. Influence of papain concentrations and the addition of cysteine on the gelation properties of SPI dispersions

A preheated SPI dispersion (2 ml) was mixed with 1 ml freshly prepared protease solution, and it was immediately placed on the sample plate of the rheometer. The sample was then subjected to a temperature ramp with an applied oscillation stress at 1 Hz frequency and 1.0 Pa stress. The temperature ramp was programmed from 40 to 85 °C at 20 °C/min and then decreased back to 50 °C at 4 °C/min. This temperature profile was used to simulate an "instant gelation" of a SPI dispersion. The gap between the two plates was set to 500 μm. The storage modules (G'), loss modules (G'') and loss angle tangent ($\tan\delta$) were recorded. The influence of papain concentration used on the gelation properties of preheated SPI dispersion with addition of 0.0625 mM cysteine before preheating was measured using the same method.

3. Results and discussion

3.1. Soy protein coagulation ability of different proteases

In order to compare the coagulation ability of different proteases on soy proteins, six different proteases at concentrations providing the same activity (10 U/ml) (Table 1) were used individually to coagulate soy protein isolate (SPI) dispersions (5.33%, w/w) at 40 °C. The development of the gel with different proteases was evaluated by measuring their elastic modulus (G') profiles as shown in Fig. 1. It was found that by the addition of protease, G' increased in each of the six SPI–protease systems. By comparing the G' value after 40 min, it was clear that papain produced the firmest soy protein gel among the six proteases, followed by alcalase, bromelin, flavorzyme, protamax, and neutrase. In terms of coagulation speed, the rank of the six tested proteases was similar to that of gel strength. Papain was also the fastest. Bromelin–SPI system coagulated faster than that of alcalase at the beginning, but after 16 min, the alcalase–SPI gel was stronger. It took about 8 min for alcalase–SPI system to start coagulation.

Murata et al. (1987) also compared the coagulation of soy milk with various commercial proteases. The formation of soy milk gel was judged by a change in appearance. This method was similar to a visual method used to test milk coagulation (Arima, Iwasaki, & Tamura, 1967). The water binding ability of these soy milk gels was also compared. The results of the study showed no marked differences among the enzymes tested with respect to their coagulation and water binding abilities. In this study, there was a significant difference in the development of G' among the gels formed with six proteases examined. However, the final $\tan\delta$ (G''/G') values of all the six protease–SPI systems were similar after 40 min of hydrolysis (Table 2). This suggests that the nature of gels which were produced by the six proteases was similar but the strength of peptide interactions was different.

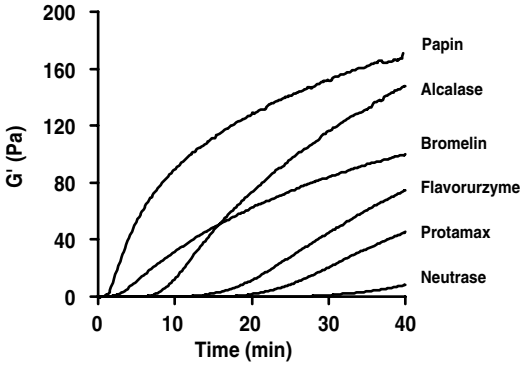

Fig. 1. The development of the elastic modulus G' during the coagulation of SPI dispersions with six proteases at 40 °C.

Table 2
The SPI gel properties after 40 min, following the addition of protease [a,b,c]

Mixture	G' (Pa)	G'' (Pa)	δ (°)	pH
SPI–alcalase	148.0 ± 7.2	33.8 ± 3.4	12.9 ± 0.6	6.27 ± 0.13
SPI–brominase	99.3 ± 5.0	22.5 ± 1.6	12.7 ± 0.3	6.25 ± 0.09
SPI–flavourzyme	74.7 ± 3.5	15.7 ± 1.1	11.9 ± 0.3	6.25 ± 0.07
SPI–neutrase	8.1 ± 1.1	1.7 ± 0.3	12.0 ± 0.6	6.32 ± 0.11
SPI–papain	171.3 ± 6.7	39.6 ± 1.5	13.0 ± 0.1	6.32 ± 0.06
SPI–protamax	44.6 ± 5.0	8.6 ± 0.6	10.8 ± 0.5	6.32 ± 0.07

[a] The composition of each mixture was listed in Table 1.
[b] The reaction was done at 40 °C.
[c] The results were mean values from 3 measurements plus standard deviation.

One possible reason for the difference in coagulation speed could be the different stability of proteases when soy protein was used as a substrate. In order to address this possibility, the development of DH profiles of the soy proteins during coagulation with the strong coagulation enzymes, papain and alcalase, and the poor coagulation enzyme, neutrase, were examined (see Fig. 2). The gel strength of papain and alcalase induced gels after 40 min were about 20 times higher than that of neutrase, but the DH of soy protein dispersions with any of the 3 enzymes was similar, but slightly higher for the one with neutrase. This suggested that soy protein was digested to a similar extent by different proteases. The difference in hydrolysis pattern and peptides interaction, however, afforded different coagulation speed and strength.

The enzymatic hydrolysis of protein dispersions resulted in a decrease of pH (Whitaker, 1972), and the decrease of pH may influence the coagulation of SPI, as demonstrated by Kaoru, Yoh, and Etsushiro (1995). The final pH values after 40 min of protease–SPI incubation are shown in Table 2. The pH was reduced from 7.0 to 6.25–6.32, which also suggested that all six proteases had hydrolyzed soy protein to a similar degree. Glucono-delta-lactone (GDL) is a commonly used acid coagulant of SPI solutions, the mechanism of GDL coagulation is that GDL gradually lowers the pH of soymilk upon the formation of gluconic acid when the dispersion is heated. This promoted electrostatic interactions among soy protein molecules as their pI was approached. Fig. 3 shows the development of the elastic modulus G' during the coagulation of SPI dispersion with GDL (0.4%) at 50 °C. According to the G' profile, no gel formation was detected at pH 6.25. Only when the pH was reduced to about 5.8 the SPI dispersion began to coagulate. To obtain a similar G' value as that in the papain–SPI system, the pH should be lowered to 5.42 (Fig. 3). This suggests that the drop of pH during enzyme reactions was not a factor in soy protein coagulation with the addition of the proteases.

The viscosity change profiles of soy protein solution during hydrolysis with papain, alcalase or neutrase were tested, while the electrostatic interactions among soy peptides were blocked by holding the pH at 8.0. The viscosity

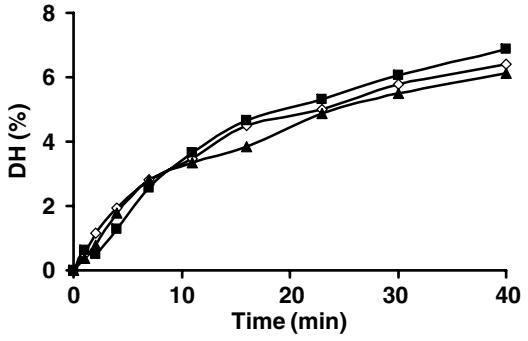

Fig. 2. Hydrolysis degree changing profiles of soy protein during coagulation with papain (◇), alcalase (▲) or neutrase (■) at 40 °C.

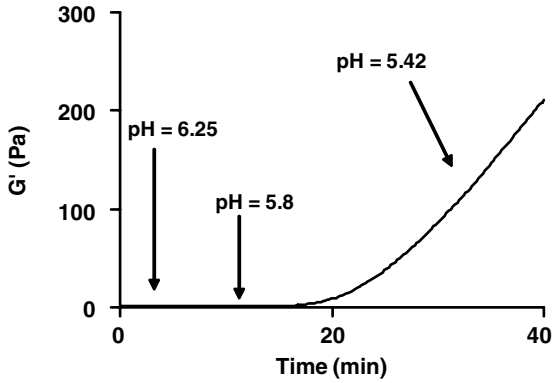

Fig. 3. The development of the elastic modulus G' during the coagulation of SPI dispersion with the breakdown of the acidulant glucono-delta-lactone (0.4%) at 50 °C.

of neutrase–SPI system decreased with increasing DH as given in Table 3. When the DH was 5%, the viscosity decreased to 0.0052 Pa S, was only 5.6% of the viscosity of untreated SPI solution. This suggested that electrostatic interactions were responsible for the slight coagulation of neutrase–SPI system, although it is not a key factor for strong coagulation. For alcalase–SPI and papain–SPI systems, the viscosity increased with hydrolysis, since electrostatic interactions were blocked, the increase in viscosity reflected the existence of other types of interactions between soy peptides, such as hydrophobic interaction or the formation of intermolecular disulphide bonds.

3.2. Thermal and pH stability of alcalase and papain

Rapid soy milk coagulation and a firm gel set are prerequisites for the choice of a protease as an instant soy milk coagulant. According to the coagulation ability results (Fig. 1), papain and alcalase were chosen as potential instant SPI coagulants. In addition to stronger coagulation ability, the protease should also be thermal and pH stable to be used an instant coagulant. Thermal stability is needed

Table 3
The viscosity change of soy protein solution during protease digestion[a,b]

DH, %[c,d]	Viscosity at shear rate 10 S^{-1} (Pa S)[e]		
	Neutrase	Alcalase	Papain
0	0.092 ± 0.006	0.092 ± 0.006	0.092 ± 0.006
1	0.014 ± 0.001	0.061 ± 0.001	0.091 ± 0.005
2	0.008 ± 0.001	0.132 ± 0.012	0.346 ± 0.021
3	0.007 ± 0.001	0.413 ± 0.021	0.408 ± 0.033
4	0.006 ± 0.001	0.612 ± 0.036	0.889 ± 0.037
5	0.005 ± 0.001	0.782 ± 0.029	1.093 ± 0.046

[a] Soy protein concentration was 8%.
[b] Temperature for viscosity test was set at 25 °C.
[c] pH was controlled at 8.0 by addition of 1 M NaOH, DH was calculated by pH-stat equation.
[d] Enzyme was gradually added for slow hydrolyzing and better control of DH, viscosity was tested right after certain DH was reached.
[e] The results were mean values from 2 measurements plus standard deviation.

for the retention of residual activity after rehydration of the SPI powder with hot water, at about 95 °C. The pH stability is needed since the pH decreased during coagulation (Table 2).

The thermal stabilities of papain and alcalase were measured over a temperature range of 60–90 °C (Figs. 4 and 5). Both papain and alcalase were stable at 60 and 70 °C. When incubated at 80 °C, the enzyme activity of alcalase decreased rapidly to 20% residual activity after 6 min, but, papain activity was retained at a 60% level after 6 min at 90 °C. As to pH stability, papain also showed a higher stability than alcalase within the pH range of 5.8–7.0 (Table 4). This corresponds to the range of pH in a SPI–protease mixture during enzymatic coagulation. As pH dropped from 7.0 to 6.1, relative activity of papain rose gradually. There was then a small decrease when the pH was reduced to 5.8, but the activity was still higher than that at pH 7.0. For alcalase, a decline in the activity was observed on reducing the pH from 7.0 to 5.8. In conclusion, papain was more thermally and pH stable than alcalase, and thus may be considered to be more suitable as an instant coagulant for soy protein dispersions.

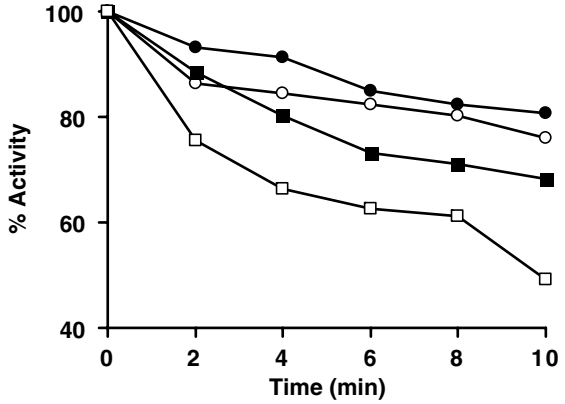

Fig. 4. Residual activity profiles of papain after heating at 60 (●), 70 (○), 80 (■) and 90 °C (□).

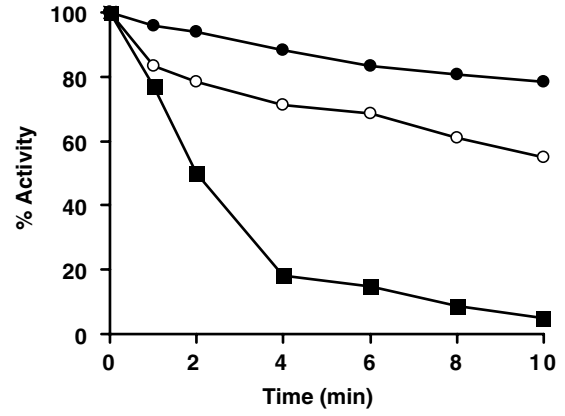

Fig. 5. Residual activity profiles of alcalase after heating at 60 (●), 70 (○), and 80 °C (■).

Table 4
Relative activity of papain and alcalase at different pH's

	Relative activity (%)[a,b,c]				
	pH 7.0	pH 6.7	pH 6.4	pH 6.1	pH 5.8
Papain	100	109.3 ± 1.3	114.5 ± 2.1	119.6 ± 0.9	110.2 ± 1.9
Alcalase	100	92.7 ± 1.5	78.1 ± 0.8	64.8 ± 1.2	51.7 ± 2.6

[a] Protease activity at pH 7.0 was considered to be 100%.
[b] Relative activity = protease activity at certain pH/protease activity at pH 7.0.
[c] The results were mean values from 2 measurements plus standard deviation.

3.3. Influence of papain usage level on gelation properties of SPI

The instant coagulation properties of papain on soy powder during rehydration with hot water were tested by recording the development of storage modulus (G'). The temperature profile during soy powder rehydration with hot water was simulated by the temperature control system of the rheometer. After loading the SPI dispersion with papain on the rheometer, the temperature was raised from 40 to 85 °C, which corresponds to the temperature increase of a soy powder with addition of hot water, the highest rate of temperature change provided by rheometer (20 °C /min) was used. The temperature reduction stage was programmed from 85 to 50 °C at 4 °C/min. With this temperature profile, the coagulation properties of SPI with papain at different usage levels, from 5 to 20 U/ml was measured (Fig. 6). It can be seen that initial coagulation time (the point at which G' start to increase) was shortened by increasing papain concentration. It took 80 s with 5 U/ml of papain, but with 20 U/ml of papain, coagulation of the SPI dispersion occurred immediately after mixing. It was also found that when papain usage levels were within the range of 5–13.3 U/ml, the G' values of papain–SPI mixtures gradually increased as a function of time, and the final value of G' was higher with higher levels of papain. When the concentration of papain was

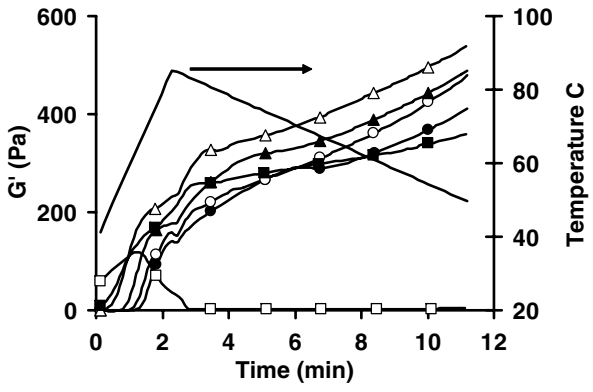

Fig. 6. Instant coagulation profiles of papain–SPI mixture with different Papain usage levels. 5 U/ml (●), 6.7 U/ml (○), 10 U/ml (▲), 13.3 U/ml (△), 16.7 U/ml (■), and 20 U/ml (□).

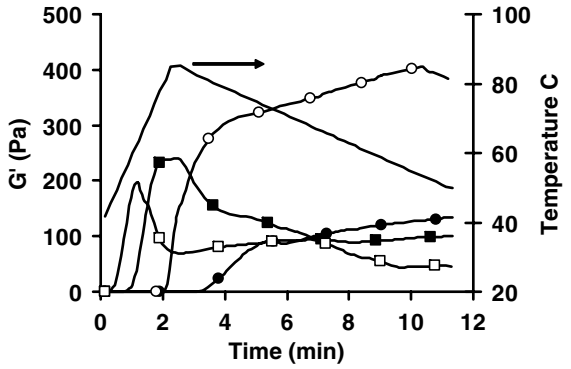

Fig. 7. Instant coagulation profiles of papain–SPI mixture with different papain usage level at the presence of 0.625 mM Cys, 1 U/ml (●), 2.5 U/ml (○), 5 U/ml (■), and 10 U/ml (□).

raised to 16.7 U/ml, G' values of the papain–SPI system were still higher than that with lower papain usage levels during the early stage, but later the G' values descended and the final G' value was less than that with 13.3 U/ml papain. With a papain concentration of 20 U/ml, G' values initially increased but then a sharp decline followed after 1 min, and no gel was formed at the end. These results indicated that the DH and speed affect the final SPI gel strength.

Papain is an –SH protease. Oxidative reagents can inhibit its activity and a reducing reagent can enhance it. Machiko and Setsuro (1984) reported that the addition of cysteine reduced coagulation time with –SH proteases. The influence of cysteine (Cys) (0.625 mM) with different levels of papain on instant coagulation properties of soy milk were measured (Fig. 7). It was found that 5 U/ml papain would over hydrolyze SPI dispersions and a decrease in the G' resulted after 2 min. With the addition of 0.625 mM Cys, the G' values of the gel formed with 2.5 U/ml papain was about the same as that by 13.3 U/ml papain without Cys. This means that the addition of Cys increased the enzyme activity of papain by about 5 times; a suitable papain usage level for coagulation was thus decreased to 1/5 of the original concentration.

4. Conclusions

Among the six commercial proteases, papain and alcalase were the most effective SPI dispersion coagulants. The difference in coagulating ability among proteases was due to their different hydrolysis pattern on soy protein which resulted in different interaction between soy peptides. Since papain had a higher thermal and pH stability than alcalase, it was more suitable as an instant coagulant for soy protein. Higher papain usage levels than 13.3 U/ml would not form stronger gels compared to lower papain concentrations. The presence of a reducing regent lowered the concentration of papain necessary for a soy protein gel of equivalent strength produced with higher concentrations of papain alone.

Acknowledgements

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