Functional Properties of Proteolytic Enzyme Modified Soy Protein Isolate

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The effects of proteolytic enzyme modification of soy protein isolate (SPI) on its molecular and functional properties were evaluated by treating a commerical SPI, Ardex F, with Alcalase, α-chymotrypsin, trypsin, Liquozyme, and rennet. Trypsin effectively decreased the molecular size of SPI followed by Alcalase and α-chymotrypsin. Hydrolytic breakdown occurred more extensively in the α′, α, and β subunits of 7S globulins than in the acidic and basic polypeptides of 11S globulins. Partial hydrolysis of SPI contributed to improving its solubility at pH 7.0 and 4.5, emulsifying capacity, and ability to undergo thermal aggregation. The extent of contribution was dependent upon the enzymes used, duration of proteolytic treatment, and functional properties sought after.

Soy proteins, due to good nutritional value and abundant availability as byproducts of oil processing, have received considerable attention for the replacement of expensive animal proteins in conventional foods (Kinsella, 1979). Nevertheless, their less than desirable functional properties have limited their expanded utilization in food applications (Kinsella, 1979; Morr, 1979). Moreover, functionalities of commerically available soy proteins are further lowered as a result of denaturation during the prior defatting process. Improvement of their functional properties, therefore, is highly desirable so that they may successfully perform specific functions in food applications.

To improve functionalities of low-functional proteins, chemical modification has been extensively studied. Among the various chemical modification techniques, acylation with acid anhydrides appears to be most widely studied with proteins from many plant sources (Franzen and Kinsella, 1976; Choi et al. 1982, 1983; Shukla, 1982; Johnson and Brekke, 1983; Kim and Kinsella, 1987). Although succinylated proteins were functionally superior to unmodified proteins, incorporation of chemically modified proteins in foods will have to clear regulatory safety guidelines. Furthermore, chemically modified proteins are of nutritional concern because succinylated bonds were not hydrolyzed by gastric and pancreatic proteases (Shukla, 1982). In contrast, enzyme-modified proteins are not likely to be a safety concern, and thus enzymatic modification may be a viable alternative to chemical modification.

Enzymatic modification is usually performed through limited proteolysis (Richardson, 1977). Puski (1975) investigated the functionalities of soy protein isolates (SPIs) that were hydrolyzed with *Aspergillus oryzae* protease at varying ratios of enzyme to substrate. Adler-Nissen and Olsen (1979) reported improved emulsifying capacity, solubility, and foaming capacity of SPIs after Alcalase and Neutrase treatment. Functional changes in some specific fractions such as 7S (Lehnhardt and Orthoefer, 1982) or 11S globulins (Mohri and Matsushita, 1984) rather than as ordinary SPIs were also reported after their hydrolysis with pepsin and bromelain, respectively.

The present study was conducted to obtain further information on the effects of proteolytic modification on SPI functionalities. Alterations in SPI oligomeric structures during the modification were also studied. With a view to provide information more readily applicable to practical need, both a commerical SPI and several commerical proteases were used instead of laboratory-purified SPIs and proteases, respectively.

MATERIALS AND METHODS

Materials. A commerical SPI, Ardex F, was purchased from Archer Daniels Midland Co. (Decatur, IL). Alcalase 0.6 L (microbial protease) and Liquozyme 120 L (crude microbial α-amylase) were obtained from Novo Laboratories, Inc. (Wilton, CT). Trypsin (T2395) and α-chymotrypsin (C4129) were purchased from Sigma Chemical Co. (St. Louis, MO). Double-strength microbial rennet (Emporase EL-400) was from Dairyland Food Laboratories, Inc. (Waukesha, WI). All chemicals used were reagent grade.

Preparation of Enzyme-Modified SPIs. SPIs were dispersed in distilled water with a Hobart Kitchen-Aid mixer (Hobart Corp., Troy, OH) equipped with a water bath to prepare a 20% (w/v) solid concentration except for Liquozyme treatment; above 15%, the protein suspension became gel-like under the proteolytic conditions used (see Table I). The pH of the suspension was adjusted with 6 N NaOH to the optimum pH for hydrolytic reaction of each enzyme. When the suspension reached optimum temperature, an enzyme (2% of SPIs, w/w) was added to the suspension. Prior to addition, enzyme powder was dissolved in a small amount of distilled water. After incubation with each enzyme for 5, 10, and 30 min, respectively, the suspensions were heated to inactivate enzymes. Preliminary studies showed no noticeable proteolytic activities after heat treatment done as in Table I. The hydrolysates were neutralized to pH 7.0, to which hydrogen peroxide (50% strength) was added to 2% levels (w/w) as an antimicrobial as well as a bleaching agent, freeze-dried, ground, and placed in sealed glass bottles for storage at 5 °C until analyzed. Since heat treatment was applied to denature enzymes used, heat-treated controls were also prepared in the same manner as the hydrolysates except no enzyme was added and SPI suspensions were heated at 87 °C for 5 min.

Degree of Hydrolysis (DH). This was estimated by measuring the nitrogen content soluble in 10% trichloroacetic acid (TCA) as discussed by Yamashita et al. (1970) and Edwards

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Table I. Conditions for the Hydrolysis of Soy Protein Isolate with Proteases and Their Inactivation

enzyme	reaction pH	reaction T, °C	inactivation T, °C	inactivation time, min
Alcalase	8.0	50	87	5
α-chymotrypsin	8.0	37	87	5
trypsin	8.0	37	87	5
Liquozyme	7.0	68	98	10
rennet	6.8	31	71	2

and Shipe (1978). A 1-g SPI was suspended in 100 mL of water. A 1-mL aliquot of the suspension was assayed for nitrogen by the micro-Kjeldahl method (AOAC, 1975). A 10-mL aliquot of the aqueous suspension of enzyme-modified SPIs (1% (w/v)) was mixed with 10 mL of 20% TCA and then centrifuged at 12100*g* for 15 min. The soluble nitrogen in the supernatant was assayed by the micro-Kjeldahl method. The percent DH was expressed as follows:

$$\% \text{ DH} = \frac{10\% \text{ TCA-soluble N}}{\text{total N}} \times 100 \quad (1)$$

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE). The protein hydrolysates were analyzed by SDS-PAGE following the general procedure of Weber and Osborn (1969) using 7.5% acrylamide gels. Briefly, SPIs (2 mg/mL) were made up in 0.01 M phosphate buffer at pH 7.0 with 1% SDS and 1% β-mercaptoethanol and then incubated at 37 °C for 2 h. A 50-μL sample in sucrose and bromophenol blue was loaded on top of the gel and electrophoresed at 8.0 mA/gel for about 8 h. The gels were stained overnight in 0.25% Coomassie brilliant blue R-250, 10% acetic acid, and 50% methanol and destained in 7.5% acetic acid with 5% methanol. Molecular weights of polypeptides were estimated by using four marker proteins including bovine serum albumin (67K), ovalbumin (43K), chymotrypsin A (25K), and ribonuclease A (14K).

Nitrogen Solubility. This was estimated by the method of Franzen and Kinsella (1976) with a slight modification. SPIs were dissolved in 0.1 N NaOH (1% (w/v)), and 6.0-mL aliquots were pipetted into 15-mL centrifuge tubes. The pH of the aliquots was then adjusted to pH 12.0 with 1 N HCl or 1 N NaOH, and the tubes were centrifuged at 4000*g* for 20 min. The protein in the supernatant was measured by the biuret method (Gornall et al., 1949) and assumed to have 100% nitrogen solubility. Similarly, hydrolysates were dissolved in 0.1 N NaOH, and the pH of the aliquots (6 mL) was adjusted to 4.5 and 7.0, respectively. After shaking at 300 rpm at 25 °C for 1 h, the suspension was centrifuged at 4000*g* for 20 min. The protein in the supernatant was determined by the biuret method. The percent solubilities of enzyme-modified SPIs were obtained by dividing the protein content of the supernatant at pH 4.5 and 7.0, respectively, with that at pH 12.0.

Emulsifying Capacity. The method of Swift and Sulzbacher (1963) was modified slightly. One liter of soybean oil (Wesson) was colored with 0.1 g of Oil-Red-O biological stain. The colored oil (20 mL) was added to a 1% suspension of SPIs and enzyme-modified SPIs (25 mL, pH 7.0) and dispersed at low speed with a T-line mixer (Rheostat 50) for 1 min, and the speed was then increased to full power. After 30 s, more oil was added at a rate of about 14 mL/min. The volume of oil consumed until the inversion of the initially formed emulsion, which was accompanied by sudden changes in oil color and viscosity, was recorded and expressed as emulsifying capacity.

Thermal Aggregation. This was determined according to the method of Kramer and Kwee (1977) with slight modifications. Suspensions of SPIs and enzyme-modified SPIs (2%, pH 6.8) were agitated on a magnetic stirrer for 15 min, and two aliquots (1.0 mL each) were assayed for protein content by the biuret method. Another two aliquots (10 mL each) were placed in screw cap test tubes and heated in a boiling-water bath for 20 min. After rapid cooling to ~22 °C and centrifugation at 2000*g* for 20 min, the protein content of the supernatant was assayed. Thermal aggregation of enzyme-modified SPIs was represented by the difference in protein content of the supernatant caused by heating:

$$\% \text{ thermal aggregation} = [(\text{absorbance before heating} - \text{absorbance after heating})/\text{absorbance before heating}] \times 100 \quad (2)$$

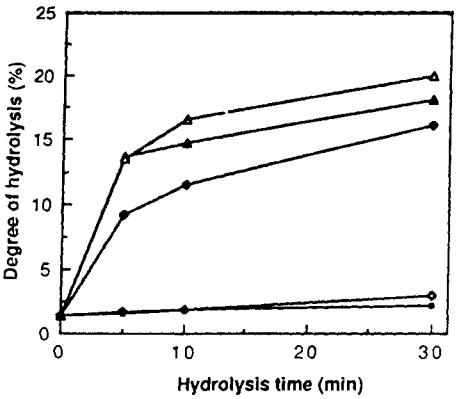

Figure 1. Time course of proteolytic degradation of Ardex F by five different proteases at 2:100 (w/w) enzyme/substrate: (▲) Alcalase; (◆) α-chymotrypsin; (△) trypsin; (◇) Liquozyme; (■) rennet. Protein concentrations were at 20% except for Liquozyme treatment at 15%.

Statistical Analysis. All data represent a mean value of triplicate analyses on each hydrolysate from three incubation times. Significant differences among treatments were determined by Duncan's multiple range test ($p < 0.05$).

RESULTS AND DISCUSSION

Extent of Enzymatic Modification. The initial DH values of untreated and heat-treated controls were ca. 1.4 and 1.3%, respectively. Since the difference in DH between untreated and heat-treated controls is indicative of the effect from the heat treatment, no significant difference ($p < 0.05$) in DH values between the two controls suggested that heat treatment for the inactivation of the enzymes (Table I) would not complicate the evaluation of DH for enzyme-modified SPIs.

The hydrolysis of SPIs with Alcalase, α-chymotrypsin, and trypsin proceeded at a rapid rate during the initial 5 min and then slowed down thereafter (Figure 1). DH values varied from ca. 2 to 20% after 30 min of incubation, depending upon the enzymes used. Trypsin gave the highest DH values and was more effective for proteolysis ($p < 0.05$) than α-chymotrypsin. This may be accounted for partly by SPI compositional features; 7S and 11S globulins, major components of soy proteins, contain a larger number of basic amino acids than aromatic ones (Wolf, 1972) and thus may contain more cleavage sites for trypsin to act on. Liquozyme showed very little increase in DH values even after 30 min of incubation. These results were not surprising because the proteolytic activity of Liquozyme (crude α-amylase) is due to contaminated protease. SPI, unlike milk proteins, was not easily degraded by rennet.

Alterations in Molecular Structures. Subunit degradation occurring during the proteolytic enzyme treatment was investigated by SDS-PAGE (Figure 2). Untreated control displayed five major bands, P80, P76, P50, P34, and P18, and three minor bands, P100, P38, and P28, which were labeled from their molecular masses in kilodaltons (kDa). The first three major bands, P80, P76, and P50, were identified as the α′, α, and β subunits, respectively, of β-conglycinin (7S globulin). These molecular masses are higher than those reported by Thanh

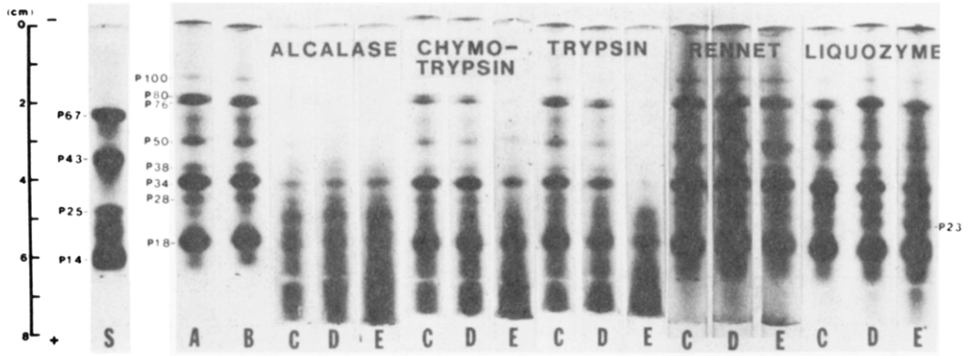

Figure 2. Time course for enzymatic proteolysis of Ardex F as shown on 7.5% SDS-PAGE gels: S, molecular weight standards; A, untreated control; B, heat-treated control; C, D, and E, gels for Ardex F modified for 5, 10, and 30 min, respectively, with each protease labeled. For standards: P67, bovine serum albumin; P43, ovalbumin; P25, chymotrypsinogen A; P14, ribonuclease A. For Ardex F: P100, unknown; P80, P76, and P50, α', α, and β subunits of 7S globulins, respectively; P38 and P34, acidic polypeptides of 11S globulins; P18, basic polypeptide of 11S globulins; P28, unidentified. Amount of proteins applied: 100 μg/sample.

and Shibasaki (1977) but were in good agreement with the values for the α', α, and β subunits given by Beachy et al. (1981) and Lillford and Wright (1981). The last two major bands, P34 and P18, were identified as acidic and basic polypeptides, respectively, of glycinin (11S globulin) according to Badley et al. (1975) and Mori et al. (1981). The minor band P38 would be a fraction of acidic polypeptides according to Mori et al. (1981), who resolved acidic polypeptides into two bands having molecular masses of 38 and 34.8 kDa, with the latter a major band. The other two minor bands, P100 and P28, were not known and thus left unidentified in this study.

Whereas the untreated and heat-treated controls were of almost identical pattern, considerable differences in gel patterns between controls and Alcalase-modified SPIs were noticed; bands for the α', α, and β subunits (P80, P76, and P50) and three minor bands (P100, P38, and P28) were no longer observed in the hydrolysates. The decrease in the band intensities of both acidic (P34) and basic polypeptides (P18) became apparent, and an extra minor band (P23) appeared with prolonged proteolytic treatment. Since equal amounts of hydrolysates were loaded throughout SDS-PAGE, darker intensity of basic polypeptide bands implied that they may be more resistant to enyzmatic breakdown than acidic polypeptides. With α-chymotrypsin treatment, the band intensities of the 7S globulin subunits (P80, P76, and P50) and three minor bands (P100, P38, and P28) gradually decreased and became unnoticeable after 30 min of incubation. Whereas only a slight decrease was noticed in the band intensity of the acidic polypeptides (P34), the basic polypeptides (P18) seemed almost unaffected. This might be due to a difference in compactness of structures between the two polypeptides; i.e., basic polypeptides are more hydrophobic (Badley et al., 1975; Peng et al., 1982) and thus may be more compact, making them less accessible to enzyme attack. SDS-PAGE patterns of trypsin hydrolysates were similar to those of α-chymotrypsin hydrolysates except for greater proteolytic degradation.

While a considerable degradation in 7S globulin subunits are caused by proteases that showed higher DH values, other proteases with low DH values, i.e., Liquozyme and rennet, revealed almost unaffected protein bands, implying less pronounced degradation in their structures. Neverthesless, the appearance of an extra band (P23) suggested some changes must have taken place by Liquozyme treatment.

Nitrogen Solubility. Solubility characteristics of protein are among the most important functional properties since many functional performances of proteins depend upon their capacity to go into solution initially. The differences in solubilities between untreated and heat-treated controls at pH 4.5 (4.4 vs 5.7%) as well as 7.0 (80.4 vs 81.5%) were not significant ($p > 0.05$). Consequently, changes induced in nitrogen solubility of modified SPIs would be due mainly to proteolytic treatment.

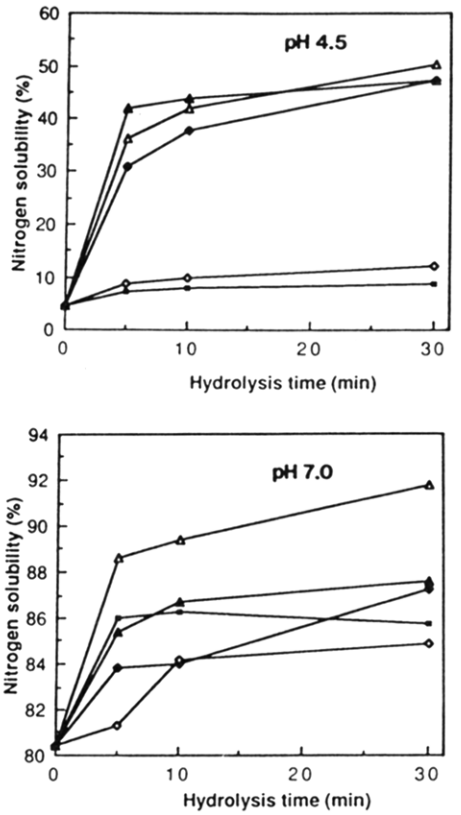

Figure 3. Nitrogen solubilities at pH 4.5 and 7.0 in water of Ardex F treated with (▲) Alcalase, (◆) α-chymotrypsin, (△), trypsin, (◇) Liquozyme, and (■) rennet.

At pH 4.5, Alcalase increased the solubility most efficiently ($p < 0.05$) during the initial 5 min of incubation, reaching ca. a ten fold rise (Figure 3). After that, only a slight increase was observed. On the other hand, trypsin and α-chymotrypsin continued to increase the solubility, reaching about the same value as achieved by Alcalase after 30 min ($p > 0.05$). A slight increase in solubility, about threefold and twofold, resulted from Liquozyme and rennet treatment, respectively, after 30 min of incubation. Solubility was improved at neutral pH, but not as much as at pH 4.5.

It is interesting to note that the solubility at pH 4.5 of enzyme-modified SPIs was highly correlated with their

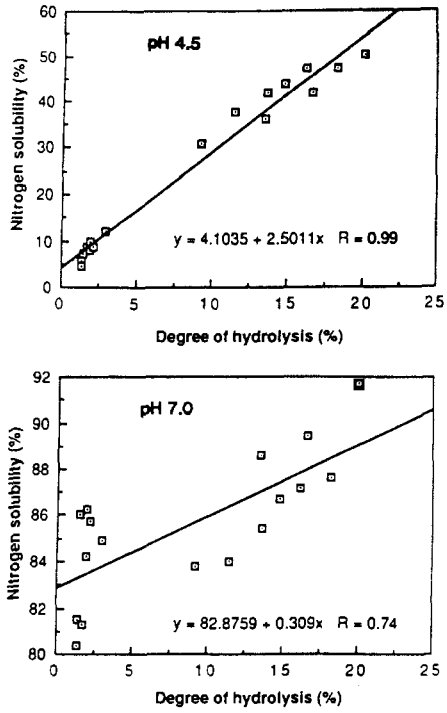

Figure 4. Correlation of nitrogen solubilities at pH 4.5 and 7.0 of Ardex F hydrolysates and their degree of enzymatic hydrolysis.

DH (r = 0.99). At pH 7.0, they were less strongly correlated (r = 0.74). Correlations between solubility and DH at both pH values are presented in Figure 4. For the high correlation between solubility at pH 4.5 of modified SPIs and DH, the hydrolytic breakdown of SPI oligomeric structures, especially 7S globulins, would be responsible. The isoelectric points of 7S subunits are reportedly at pH 5.18, 4.90, and 5.65–6.00 for the α', α, and β subunits, respectively (Thanh and Shibasaki, 1977). Their proteolytic degradation (as shown in Figure 2) would, therefore, have changed their charge properties, leading to the observed increase in the solubility. The increased solubility of enzyme-modified SPIs in the acidic pH range would help their utilization in acid foods such as mayonnaise, salad dressings, acidic beverages, etc., and therefore proteolytic modification of SPIs with Alcalase or trypsin could represent a significant source of acid-soluble proteins.

Emulsifying Capacity. Interactions of proteins and lipids are commonly found in many food systems, and thus the ability of proteins to form stable emulsions is important. The emulsifying capacity of the heat-treated control (136 mL) was not significantly ($p > 0.05$) different from that of the untreated control (146 mL).

Enzymatic modification, except rennet treatment, increased the emulsifying capacity of SPIs (Figure 5). Trypsin and Alcalase steadily increased emulsifying capacity, reaching almost a twofold increase (about 250 mL) after 30-min incubation. α-Chymotrypsin and Liquozyme increased the emulsifying capacity of SPIs gradually but were not as effective as either trypsin or Alcalase. No significant improvement in emulsifying capacity resulted from rennet treatment. In light of the quaternary structures of 7S and 11S globulins with high molecular masses, approximately 141–170 and 320–363 kDa, respectively (Kinsella, 1979), the increase in number of peptide molecules as well as exposed hydrophobic amino acid residues induced by proteolytic disintegration of subunits would contribute to the increased emulsion formation.

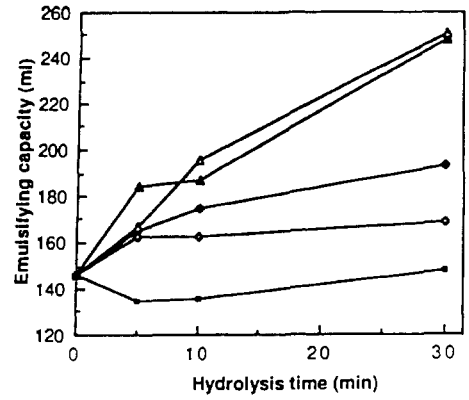

Figure 5. Emulsifying capacities of Ardex F as a function of proteolytic treatment: (▲) Alcalase; (◆) α-chymotrypsin; (△) trypsin; (◇) Liquozyme; (■) rennet.

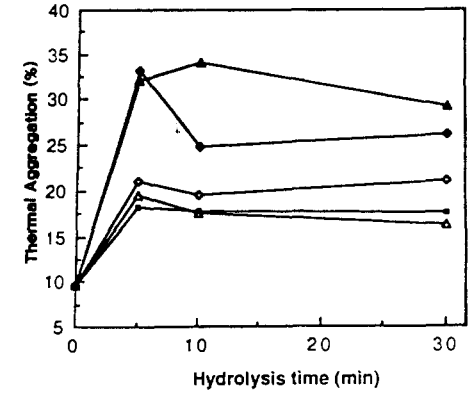

Figure 6. Thermal aggregation of Ardex F as a function of proteolytic treatment: (▲) Alcalase; (◆) α-chymotrypsin; (△) trypsin; (◇) Liquozyme; (■) rennet.

The improved emulsifying capacity of SPIs through limited hydrolysis with Alcalase and Neutrase was also reported by Adler-Nissen and Olsen (1979). Puski (1975) reported similar results with microbial proteolytic enzyme treatment on SPIs.

Thermal Aggregation. Interactions between protein molecules, when induced by heating, may give rise to the formation of protein aggregates. When such heat-induced aggregation takes place at a protein concentration high enough to entrap water in the three-dimensional matrices of the aggregates, gelation or coagulation will result. The aggregation of protein molecules is, therefore, regarded to be an absolute prerequisite for gel formation (Hermansson, 1986). The ability of modified SPIs to undergo thermal aggregation was evaluated in the present study. Among the functional properties tested, thermal aggregation was the only functional property that showed a significant difference ($p < 0.05$) between untreated (9.7%) and heat-treated controls (16.1%). Heating SPI suspensions above 60 °C was reported to induce dissociation and, in turn, unfolding of subunit polypeptides followed by the irreversible transformation of sol to progel (Circle et al., 1964; Catsimpoolas and Meyer, 1970). Therefore, the initial heating of SPI suspensions at 87 °C for 5 min for the heat denaturation of enzymes used might have caused increased thermal aggregation in the heat-treated control. However, the reason for this was not further investigated in the current study.

Enzyme treatment was effective in increasing thermal aggregation of SPI (Figure 6). In accordance with its high DH (Figure 1), Alcalase increased thermal aggregation most effectively, reaching about threefold improvement, which was followed by α-chymotrypsin and Liquozyme.

It was unexpected that trypsin, which also achieved high DH, showed the least improvement in thermal aggregation. This suggests that the thermal aggregation of enzyme-modified SPIs was also influenced by factors other than the decrease in molecular size. It is not clear from the current study as to what these other possible factors would be. However, it should be noted that 11S globulins are largely responsible for thermal aggregation (Catsimpoolas et al., 1970; Wolf and Tamura, 1969) and, especially, that the basic polypeptide precipitates almost quantitatively upon heating (Badley et al., 1975; Peng et al., 1982), whereas 7S globulins prevent their thermal aggregation through the formation of a soluble complex (German et al., 1982; Damodaran and Kinsella, 1982). Therefore, under the circumstances that structural alteration also takes place in 11S globulins through proteolytic treatment, the mere absence of 7S globulins may not contribute much to increasing thermal aggregation of SPIs. This is further supported by the observation that thermal aggregation was higher with Liquozyme and rennet treatment, which showed almost no alteration in 11S globulins, than with trypsin treatment, where a slight decrease in the band intensities of 11S globulins was apparent (Figure 2). However, the strength of such inference is reduced by the observation that the band intensities of 11S globulins were lighter in Alcalase than in trypsin treatment. Nevertheless, the proteolytic degradation of SPIs positively contributed to improving thermal aggregation.

Proteolytic enzyme modification is an effective way to improve SPI functionality. Proteolytic enzyme treatment increased solubility, emulsifying capacity, and thermal aggregation of SPIs, with the extent of improvement dependent upon proteases, duration of proteolytic treatment, and functional properties sought after. The proteolytic modification of SPIs could be performed well at high concentrations (20% protein suspensions), which is of practical importance. Among the enzymes tested, trypsin was the most effective in proteolytic performance, followed by Alcalase and α-chymotrypsin. More extensive enzymatic breakdown was observed with 7S subunits as compared to 11S globulins, supporting that the former may have more hydrophilic surface than the latter (Lewis and Chen, 1979) and that the latter may possess more of a compact structure than the former (Wolf, 1972). In accordance with DH, nitrogen solubility was considerably increased. Emulsifying capacity was also significantly improved by trypsin, Alcalase, and α-chymotrypsin treatment. Alcalase was most effective in improving the ability of SPIs to undergo thermal aggregation. All these modified functional properties of SPIs are expected to affect quality attributes of food products containing them. Currently, the functional performance of enzyme-modified SPI in food systems is under evaluation in our laboratory.

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Effect of Gibberellic Acid and 2-(3,4-Dichlorophenoxy)triethylamine on Nootkatone in Grapefruit Peel Oil and Total Peel Oil Content

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The nootkatone content in grapefruit peel oil extracted from flavedo and the peel oil content of fruit receiving preharvest treatment with 20 or 50 ppm gibberellic acid (GA) and/or 50, 125, or 250 ppm 2-(3,4-dichlorophenoxy)triethylamine (DCPTA) were determined. Treatment by GA reduced the rate of increase in nootkatone concentration observed in control fruit with maturation, and the effect was dose-dependent. When DCPTA was used alone as the growth regulator, nootkatone content increased significantly. When 50 ppm GA followed DCPTA treatment at the three levels used above, the effect of GA predominated and nootkatone content was significantly lower than that found in untreated fruit. Treatment by GA generally increased peel oil concentration.

Traditionally, to export grapefruit from Florida to locations such as Japan with a quarantine against fruit flies, it has been necessary to subject fruit to fumigation or to adverse physical conditions to kill any Caribbean fruit fly eggs or larvae present. Treatment of grapefruit with the growth regulator gibberellic acid (GA) to sustain resistance to Caribbean fruit flies until late in the season might make it possible to ship GA-treated fruit early in the season (when fruit demand and price are high) without postharvest disinfection treatments. This is the period when the fruit is most susceptible to peel damage from the cold storage treatment currently used to kill any fruit fly eggs or larvae present (Ismail et al., 1986). Use of GA or other growth regulators could affect the content or composition of grapefruit peel oil, e.g., the nootkatone content, and thereby influence the infestation of the fruit by Caribbean fruit fly larvae (Greany et al., 1983).

Coggins and co-workers (1969) attempted to establish a maturity or senescence index for California navel oranges and to determine the effect of GA treatment on fruit senescence (Lewis et al., 1967). They steam-distilled the essential oil of GA-treated vs untreated fruit and analyzed volatile flavor components by gas chromatography (GC). The concentration of many of the oil components was not affected by GA treatment, but octanol, linalool, and geranial levels increased, while valencene content decreased after GA treatment. Since changes in valencene concentration were concomitant with biochemical and physiological changes associated with senescence, it was thought possible to use valencene content as an indicator of the

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