Analysis of Products, Mechanisms of Reaction, and Some Functional Properties of Soy Protein Hydrolysates

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ABSTRACT: Soybean protein isolates were hydrolyzed with papain, bromelain, cucurbita, hieronymin, and pomiferin. The highest hydrolysis rate for cucurbita and the lowest for papain was detected at 720 min. Gel filtration, reversed-phase liquid chromatography, and electrophoresis showed that the action of each protease was different. Pomiferin acted on the native structure of β-conglycinin and glycinin, generating a large number of small hydrolysis products with hydrophilic and hydrophobic characteristics. The hydrolysates obtained with cucurbita showed residual structures that were almost intact and very similar to the substrate and contained a low number of small peptides. The hydrolyzates obtained with papain, bromelain, and hieronymin had hydrolysis products with structures similar to the partially hydrolyzed native isolate. The molecular masses of these products were similar to or lower than the controls. Polypeptides of low molecular mass were also detected. The prevalence of one-by-one and zipper mechanisms was suggested for cucurbita and pomiferin, respectively. For the other proteases both mechanisms were likely to coexist. The solubility of hydrolysates and their ability to form and stabilize foams correlated well with the structural properties and with the suggested mechanisms of hydrolysis. The best properties were presented by the hydrolysates prepared with cucurbita. Foaming ability for pomiferin hydrolysates was as high as that for unhydrolyzed soy isolate, but the foams were extremely unstable.

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The globulins β-conglycinin (7S) and glycinin (11S), the major components of soybean protein isolates, have specific functional properties that can be modified by physical, chemical, and enzymatic treatments (1). Enzymatic hydrolysis of 7S and 11S fractions as well as commercial soybean protein isolates has been studied by several methods (2). The purpose of these studies has been to increase the solubility of protein isolates both from animal and plant origin through enzymatic hydrolysis (3,4). In some cases it has been possible to correlate the degree of hydrolysis with changes in solubility (2). An increase in emulsifying properties was also observed after papain hydrolysis of soybean protein isolates (5).

Commercial proteases, such as trypsin, alcalase and papain, have been used in the majority of studies reported in the literature; different results have been obtained with respect to the improvement of the functional properties of the modified proteins (6,7). These enzymes preferentially hydrolyze β-conglycinin over glycinin (2). Studies performed with 7S and 11S fractions hydrolyzed with trypsin allowed identification of the end products as well as the reaction mechanisms (8,9). Zipper and one-by-one mechanisms seemed to participate in glycinin hydrolysis, where a stable intermediate was identified. This intermediate product, named glycinin T, had a lower molecular weight than the substrate (8). In the case of β-conglycinin, it was possible to identify five stable reaction products; two originated from the hydrolysis of α and α′ subunits and three from the β subunit (9).

The compact structure of soybean storage proteins makes it difficult for proteases to act. The use of novel enzymes would allow products to be obtained with different structural and functional properties. For this reason the main aim of this work was to study and compare the action on soybean protein isolates of three new vegetable proteases and two commercial ones. Analyses of the hydrolysis products, possible reaction mechanisms, and solubility of the hydrolysates and their ability to form and stabilize foams were carried out.

MATERIALS AND METHODS

Isolate preparation. The protein isolate was prepared from defatted soybean flour (Santista Alimentos, S.A., Porto Alegre, Brazil), containing 42.0% w/w protein, by extracting the protein with alkaline water (1:10 wt/vol flour/water ratio) for 2 h at room temperature. The pH of the suspension was periodically adjusted to 8 with 2 N NaOH, and then it was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was retained and brought to pH 4.5 with 2 N HCl. In this condition an isoelectric precipitate was obtained that was separated by centrifugation at 5,000 × *g* for 5 min at 4°C. Native isolates were prepared by suspending the isoelectric precipitate in water (5% wt/vol of proteins), and the pH was adjusted to 8.0 with 1 N NaOH before freeze-drying.

Proteases. In the present study the following enzymes were used: Papain (EC 3.4.22.2) (Sigma Chemical Co., St. Louis, MO), a cystine protease from type III papaya latex; bromelain

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(EC 3.4.22.4) (Sigma Chemical Co.), a cystine protease from pineapple; cucurbita (Cuc), a serine protease purified from *Cucurbita ficifolia* at the Laboratorio de Bioquímica, Universidad Católica de Valparaíso, Chile, according to Curotto *et al.* (10); hieronymin (Hier) a cystine protease extracted from *Bromelia hieronymi* Mez at the Laboratorio de Botánica, Universidad Nacional de La Plata, Argentina (11); and pomiferin (Pom) a serine protease extracted from *Maclura pomifera* (Raf.) Schneid. ("osage orange") at the same laboratory as hieronymin (12).

Preparation of protein hydrolyzates. Solutions or dispersions of soy protein isolates (30 mg/mL) in pH 8.0, 0.01 M phosphate buffer were mixed with enzyme solution (0.4 mg/mL in the same buffer) in a ratio of 4:1 in a stirred thermostatted bath at 40°C. The reaction was stopped at predetermined times by the addition of 19% wt/vol trichloroacetic acid (TCA). One Anson Unit (AU) is the amount of enzyme that digests hemoglobin under the same conditions at an initial rate such that the amount of product per minute, not precipitated by 0.3 N TCA, is equivalent to the color produced by 1 meq of tyrosine.

Gel filtration chromatography. Sephacryl S300 HR (Amersham Pharmacia, Uppsala, Sweden) was used with 0.01 M sodium phosphate buffer pH 8. Absorbance was measured at 280 nm and protein contents of the collected fractions were determined as described by Lowry (13). Protein standards used were: tyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovoalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.7 kDa). The following calibration curve was obtained:

$$K_{av} = 1.804 - 0.297 \log MW \quad [1]$$

(where K_{av} is the fraction of the stationary gel volume that is available for diffusion of a given solute species and MW is molecular weight).

Reversed-phase high-performance liquid chromatography (RP-HPLC). Samples obtained at distinct hydrolysis times, frozen at −80°C, and thawed just before use were analyzed by RP-HPLC with a Partisil-5 (type C18, 250 × 4.6 mm i.d.) column from Whatman (Whatman Inc., Clifton, NJ). The column was maintained at 35°C; the flow rate was 1.45 mL/min; the sample size was 50 µL, and the quantity of protein was determined by the method of Lowry *et al.* (13). A 90-min gradient of 20–40% acetonitrile in water containing 0.1% trifluoracetic acid was used. The protein was eluted with 20% acetonitrile for the first 10 min and 45% acetonitrile for 20 min following the gradient, yielding a 150-min chromatogram. All samples were analyzed at least in duplicate. Proteins were detected at 230 nm with a photodiode ultraviolet (UV) detector (Waters model 996; Millipore Corp., Milford, MA).

Surface hydrophobicity (H_o). Values of H_o were determined by the hydrophobicity fluorescence probe 1-anilino-8-naphthalene-sulfonate (ANS) according to the method described by Hayakawa and Nakai (14). Fluorescence intensity (FI) was measured at 365 nm (excitation) and 484 nm (emission) using a PerkinElmer 2000 (PerkinElmer Corp., Norwalk, CT). The initial slope of the FI vs. protein concentration plot (calculated by linear regression analysis) was used as an index of protein hydrophobicity (H_o).

Electrophoresis. All gels were run in mini-slabs (Mini Protean II Model; Bio-Rad Laboratories, Hercules, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (15) as modified by Petruccelli and Añón (16). Nondenaturing polyacrylamide gel electrophoresis (Native-PAGE) was done in 4–7.5% (wt/vol) acrylamide linear gradient gels with the same buffer system used in SDS-PAGE but without SDS.

Determination of free amine groups. Free amine residues from the hydrolyzed proteins were determined by the trinitrobenzenesulfonic acid (TNBS) reaction (17). Measurements at 340 nm were performed in triplicate, using 1.5 mM L-leucine as reference.

Determination of solubility. Solubility determinations were performed on soy protein isolates and hydrolysates obtained by 12-h bromelain digestion. In both cases pH was adjusted to 2.5 and samples were lyophilized. Solubility determinations were made in a pH region of 3.6 to 8.0 (ionic strength 0.1–0.2). Native isolates and hydrolysates were dissolved for 1 h at room temperature to reach a concentration of 1% wt/vol. Samples were centrifuged at 10,000 × *g* for 10 min at 4°C. Protein was measured by the method of Lowry *et al.* (13). Solubility was expressed as grams of soluble protein/100 grams of sample. Determinations were conducted in duplicate.

Foaming properties. All assays were performed as previously described (18). N_2 gas was sparged at a flow rate of 1.70 mL/s through 6 mL of 0.5 mg/mL of sample (native and hydrolysates) in 0.1 M phosphate buffer, pH 8.0. Bubbling was continued until a fixed volume of foam (295 mL) was reached, or after a maximum elapsed bubbling time of 1.5 min. Determinations were performed in duplicate. The maximal volume of liquid incorporated to the foam (V_{max}, mL), the liquid volume in the foam at the end of the bubbling (V_f, mL), and the rate of liquid incorporation to the foam (v_i, mL/min) were determined. The times for half-drainage of the liquid that was incorporated into the foam at the end of the bubbling period ($t_{1/2}$, s) were also measured.

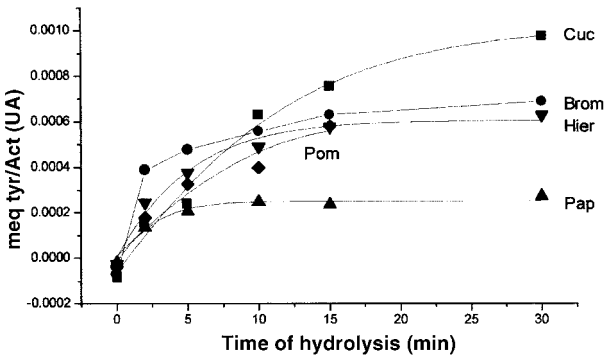

FIG. 1. Soluble product–time plots for proteases: bromelain (Brom: ●), cucurbita (Cuc: ■), papain (Pap: ■), hieronymin (Hier: ▼), and pomiferin (Pom: ◆). Abbreviation: meq tyr/Act (UA), meq tyrosine/enzyme activity, expressed in Anson units.

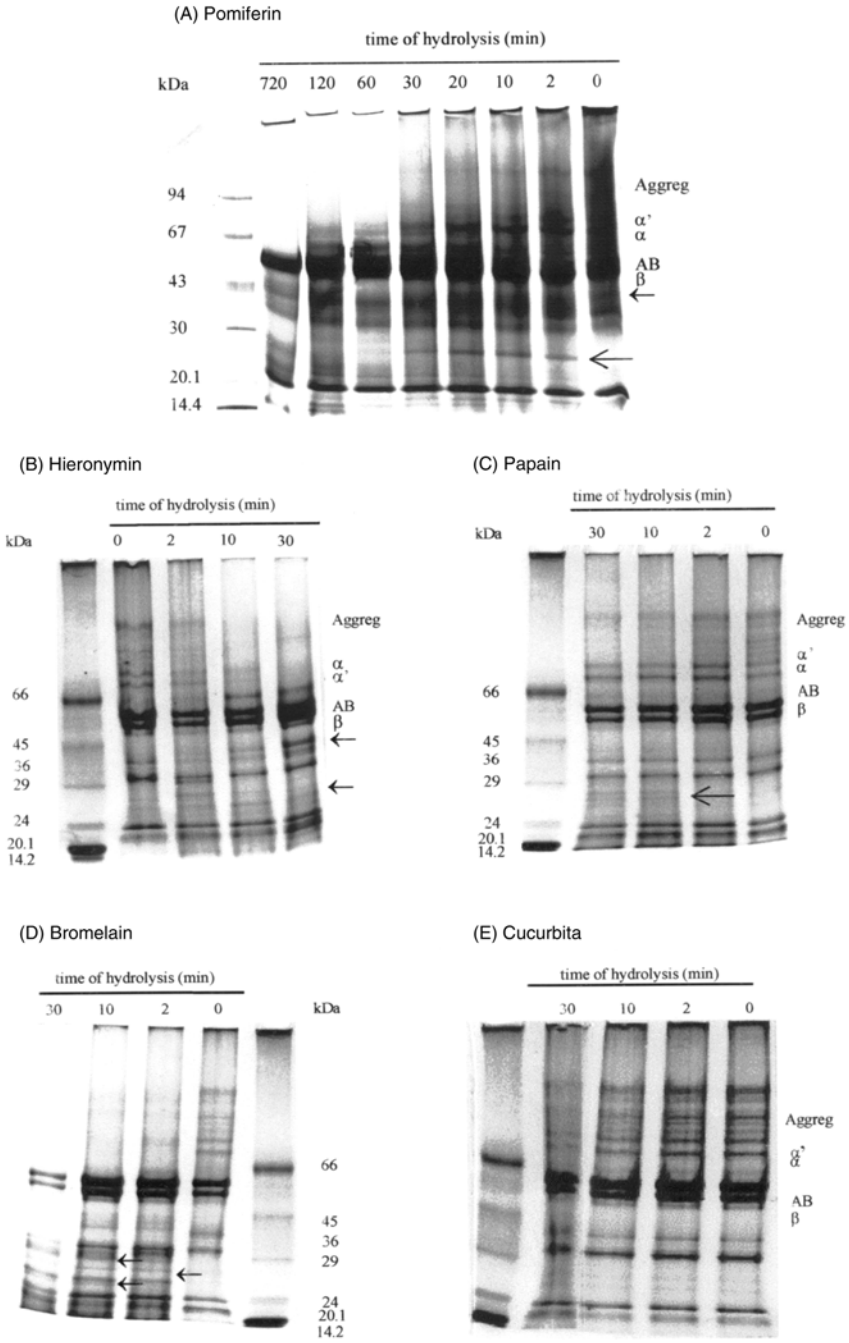

FIG. 2. Denaturing electrophoresis [sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] of the hydrolysis products of the trichloroacetic acid (19% wt/vol)-insoluble fractions obtained at different reaction times. The enzymes used were (A) pomiferin, (B) hieronymin, (C) papain, (D) bromelain, and (E) cucurbita. Arrows indicate hydrolysis products.

Statistical analysis. Data obtained were statistically evaluated by variance analysis (ANOVA) and means were compared employing the Tukey test with $P < 0.05$ (19). Both analyses were carried out using the SYSTAT statistical software (20).

RESULTS AND DISCUSSION

Characterization of hydrolysis products. Hydrolysis reactions carried out under the described conditions yielded a soluble fraction constituted by low molecular weight peptides and a TCA-insoluble fraction containing peptides and proteins of higher molecular mass. Kinetic studies and protein characterization were then performed on these two fractions.

Figure 1 shows the soluble product/time plots obtained for the five proteases. A standardization was made in terms of units of enzyme activity. In all cases, the more rapid hydrolysis was observed at the beginning of the reaction, reaching a plateau between 10 and 30 min. The highest initial rate of hydrolysis cor-

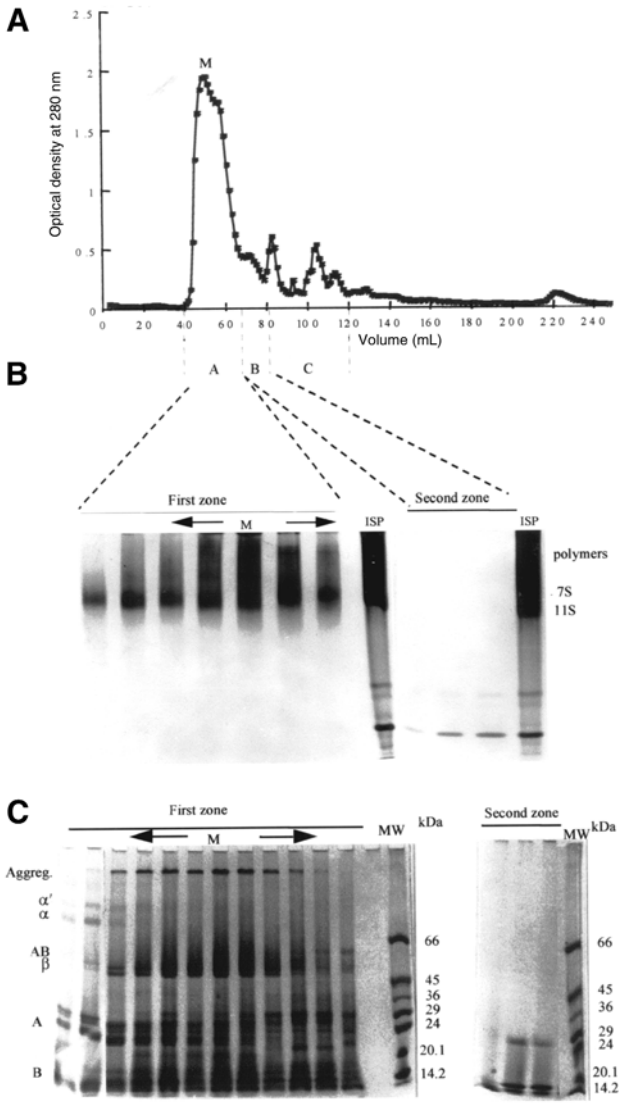

FIG. 3. Nonhydrolyzed soy protein isolate. (A) Gel filtration chromatograph and electrophoresis of the eluted fractions, (B) native PAGE, and (C) SDS-PAGE. Abbreviations: M, maximum peak of the first zone of the chromatogram; ISP, soy protein isolate. For other abbreviations see Figure 2.

responded to bromelain and the lowest to pomiferin and hieronymin.

The degree of hydrolysis for some proteases was determined with the TNBS method at the end of the reaction (720 min), being 47, 40, 28, and 22% for pomiferin, papain, cucurbita, and bromelain respectively.

Owing to the complexity of soybean isolate, protease might hydrolyze exposed areas at the beginning, without affecting the three-dimensional structure of 7S and 11S storage protein. For this reason the TCA-insoluble fractions of the hydrolysis products were characterized by denaturing electrophoresis (Fig. 2). The TCA-insoluble fraction at t_0 showed a typical electrophoretic pattern of soy protein isolates. After 10 min of hydrolysis, several differences in the electrophoretic patterns of the hydrolysates can be observed, as regards the nature of the protein species hydrolyzed by the enzymes and the products they generated. An evident effect of bromelain, hieronymin, and pomiferin on high-molecular-mass aggregates and on α′, α, and β-conglycinin subunits was observed (Fig. 2B, C, and D). New protein species (molecular masses lower than that of the AB-11S, indicated in Fig. 2 with arrows) as end products of the enzymatic activity were also detected. In the case of pomiferin the appearance of intermediate molecular mass products was observed after 2 min of hydrolysis; some of them disappeared after 30 min of reaction (Fig. 2A). In the case of papain (Fig. 2C) a similar behavior is observed at longer hydrolysis times (720 min of hydrolysis; results not shown). As regards cucurbita, no significant differences were observed in comparison to the substrate without hydrolysis (Fig. 2E).

These results suggest that the proteolytic activity proceeds initially on the 7S fraction and later on the 11S fraction, where the 7S fraction is hydrolyzed to a greater extent than the 11S fraction, and the polypeptide B is not attacked. The last result agrees with the localization of the basic 11S polypeptide in the interior of the glycinin molecule.

It has been shown that proteolytic cleavage can modify the state of association/dissociation of soybean storage protein during glycinin biosynthesis (21). To analyze the possible modifications of the soybean protein structures by the enzymatic ac-

TABLE 1
Molecular Masses and Their Compositions from Peaks in the Gel Filtration Chromatograms of the Control Isolate and of the Hydrolyzed Isolates

	Gel filtration chromatogram			
	Zone A	Zone B	Zone C	
Enzyme	(1,500–205 kDa)	(205–30 kDa)	(30–0.5 kDa)	SDS-PAGE[a]
Nonhydrolytic treatment	917.0	40.9	4.3	Aggregates, α′, α, and β-7S, AB, A and B-11S. 26.9, 17.1, and 16.1 kDa
Bromelain	1,112.0 411.0	39.0	5.0	Aggregates, α′, α, and β-7S, AB, A and B-11S. 26.9, 17.1, 16.1, 20.0 kDa
Hieronymin	682.4 469.7 366.1	105.0 44.1	4.7	Aggregates, α′, α, and β-7S, AB, A, and B-11S. 26.9, 17.1, and 16.1 kDa
Pomiferin	479.0	44.7	4.8	α′, α, 20–14 kDa, absence of aggregates. 26.9, 17.1, and 16.1 kDa
Cucurbita	910.2	58.8	5.5	Aggregates, α′, α, and β-7S, AB, A and B-11S. 26.9, 17.1, and 16.1 kDa
Papain	435.3	36.1	4.9	Absence of aggregates

[a]SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

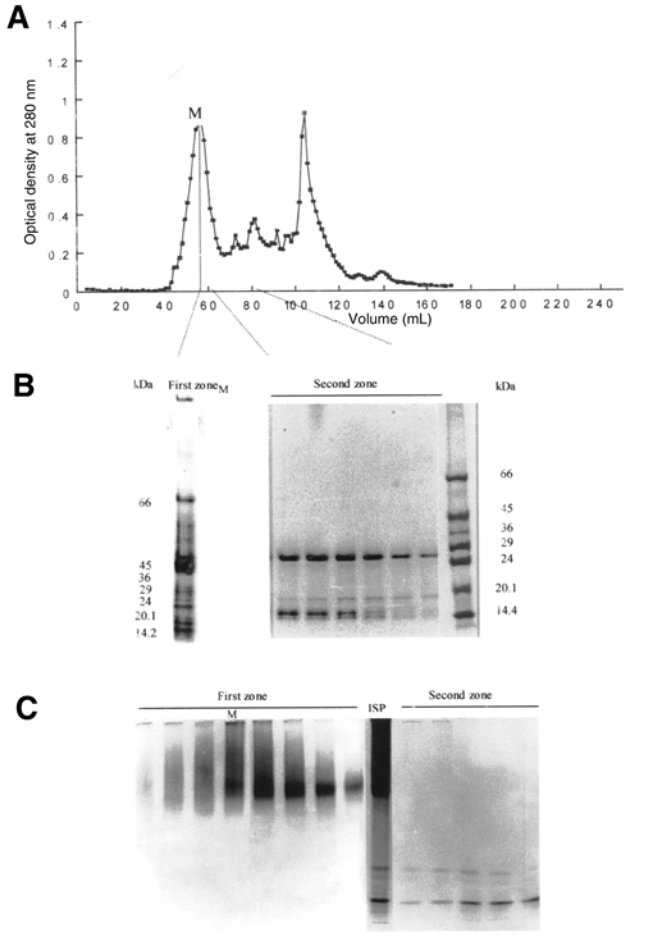

FIG. 4. ISP hydrolyzed for 30 min with Pom (A). Gel filtration chromatogram, (B) SDS-PAGE and (C) native PAGE. For abbreviations see Figures 1 and 2.

tion, the hydrolysates obtained were also characterized by molecular exclusion chromatography. Profiles were divided into three zones: A, B, and C. Zone A comprised molecular masses ranging from 1,500 to 205 kDa; zone B, from 205 to 30 kDa; and zone C, from 30 to 0.5 kDa. Results showed that the 7S and 11S fractions of the nonhydrolyzed soybean protein isolates eluted as one peak (Fig. 3A, Table 1). The molecular mass maximum was higher than that corresponding to the trimeric and hexameric forms of β-conglycinin and glycinin, respectively (180–210 for 7S and 350 for 11S), suggesting the existence of forms of higher degrees of association. In these conditions the glycinin and β-conglycinin could not be resolved. The electrophoretic profiles of zone A showed that the protein eluting at the beginning was enriched in the 7S fraction while at longer times of elution the 11S became dominant (Fig. 3B and C). In zone B, a small peak was present, and the SDS-PAGE pattern of its fractions showed three polypeptides (Fig. 3A and C, Table 1) that copurify with the 7S globulin (data not shown). These results agree with those obtained by Cole and Cousin (22).

The results of the first chromatogram peak for 30 min of hydrolysis with pomiferin (Fig. 4, Table 1) or papain (Table 1) revealed important molecular mass differences compared with

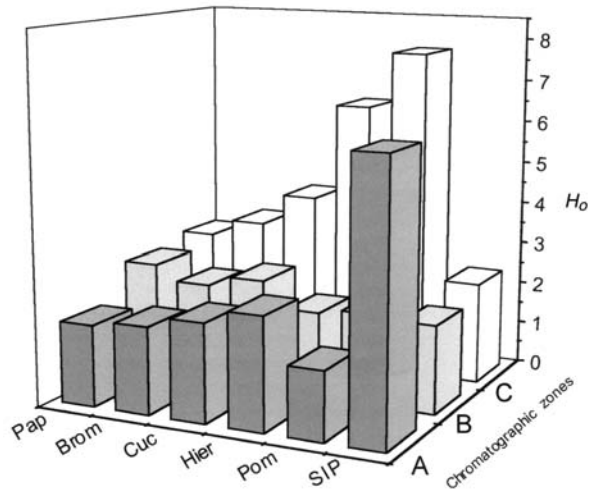

FIG. 5. Surface hydrophobicity (H_o) (14) of the gel filtration chromatogram fractions of soy protein and 30 min enzymatic hydrolysates, zones A–C, obtained from the control (nonhydrolyzed soy protein isolate) and from the hydrolysis products after 30 min of reaction. For abbreviations see Figures 1 and 3.

those of the other proteases. In both cases an important molecular mass decrease to values like those of the trimeric and hexameric forms of β-conglycinin and glycinin was observed, coinciding with the decrease in the amount of high molecular weight aggregates detected by electrophoresis (Fig. 4B and C, Table 1). These results suggest that the forms of higher degree of association present in soybean isolates are very sensitive to pomiferin and papain. The shift of the maximum in the first zone toward smaller molecular masses and the 280 nm absorbance increase in the low molecular mass zones of the chromatograms (Fig. 4A) are all evidence of the presence of peptides as hydrolysis products.

The chromatographic gel filtration profiles of the isolates hydrolyzed for 30 min with bromelain and hieronymin (Table 1) were similar to that of the control (nonhydrolyzed soy protein isolate), although in the first zone the hydrolysates present a broad peak with two and three molecular mass maxima, respectively. For the hieronymin hydrolysates the appearance of a new peak in zone B was also detected. In the case of cucurbita the chromatographic profile was similar to that of the nonhydrolyzed soy protein isolate (Table 1). These results suggest that bromelain and hieronymin have little effect on destabilization of these aggregates, and cucurbita has almost no effect.

The electrophoretic patterns of the hydrolyzed samples of the first peak of the gel filtration chromatograms were enzyme specific (Table 1). Low molecular mass hydrolysis products were retained in the structure of the protein fractions. These products were released only after the protein structure was denatured with SDS. This behavior is consistent with that observed by Kamata *et al.* (9) and Shutov *et al.* (8) when hydrolyzing purified glycinin and β-conglycinin with trypsin.

To find out whether the hydrolysis of soy proteins carried out with our proteases modifies protein surface characteristics, the surface hydrophobicities of the chromatographic gel filtration fractions obtained from the control and from the hydroly-

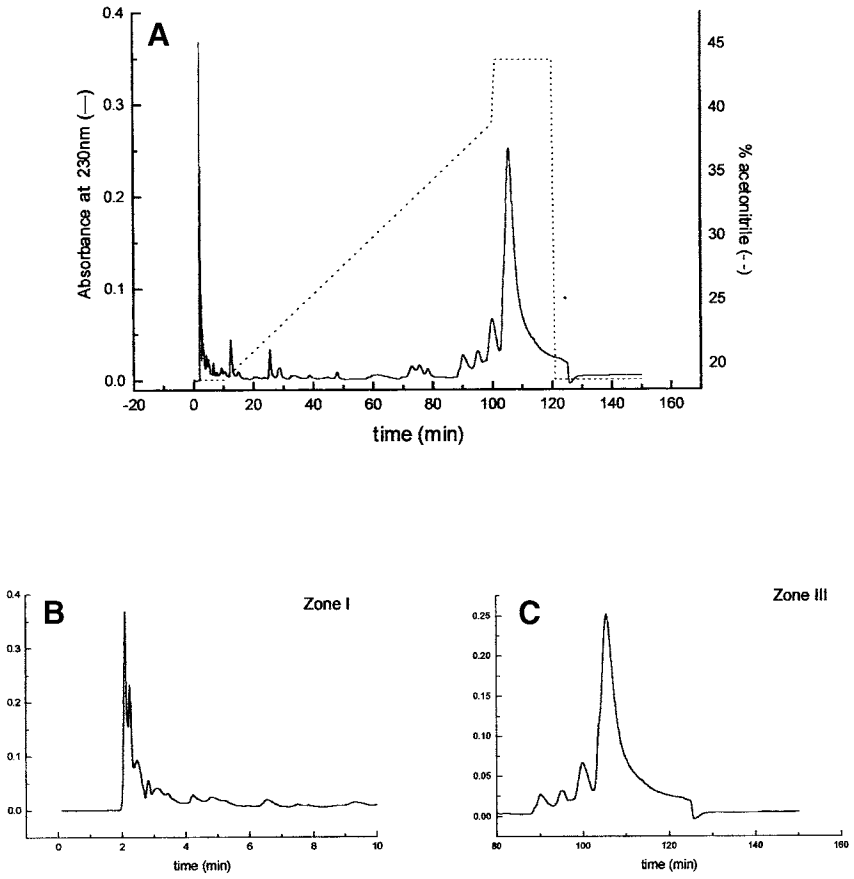

FIG. 6. Reversed-phase high-performance liquid chromatogram (RP-HPLC) corresponding to (A) the soy protein isolate, with enlarged (B) first and (C) third zones. (.....) Elution gradient (% acetonitrile in water) used.

sis products after 30 min of reaction were determined (Fig. 5). In the control protein isolate the high molecular mass proteins (zone A, Fig. 3) were much more hydrophobic than in the different hydrolyzed isolates of the same zone. Both in the control and hydrolyzed isolates, the polypeptides that elute in zone B showed similar hydrophobicities, whereas those products of low molecular mass that eluted in zone C had different hydrophobicities. The most hydrophobic products were generated by the action of pomiferin and hieronymin, followed by those released by cucurbita, bromelain, and papain.

These results for 30 min of hydrolysis time indicate that the native structure of the substrate was modified in such a way that the high molecular mass aggregates (zone A) became hydrolyzed, resulting in stable structures of lower hydrophobicity. Likewise, hydrolysis generates products of lower molecular mass and markedly different hydrophobicities, depending on the enzyme used.

Hydrolysates were analyzed by RP-HPLC so as to study the possible changes in the relation between molecular mass and hydrophobicity of the products obtained at longer reaction time. The results obtained are shown in Figure 6. Three zones can be identified in the chromatograms of control isolate: I, II, and III, corresponding to 0–10, 10–80, and 80–150 min retention times, respectively. The control soy protein isolate shows several peaks in zone I, which corresponds to 5% of the total area of the chromatogram. Zone II shows a large number of small peaks, while zone III presents four peaks representing 85% of the total area. These results are comparable to those obtained by Wolf *et al.* (23) for an acid precipitate of soy proteins.

Figure 7 shows the results obtained from a comparative study carried out on the hydrolyzed soy proteins obtained with the five enzymes by RP-HPLC (zones I and III). For each enzyme, an increase in the intensity of the peaks of the zone I was observed with respect to the nonhydrolyzed isolate (Figs. 6 and 7A). The area of this zone increased from 5 to 70% of the total. The largest increases (in the amounts of soy protein hydrolyzed) in the area of zone I, as well as the most noticeable changes in the RP-HPLC profile, were associated with pomiferin, cucurbita, and papain. These results are in accord with the changes observed by gel filtration chromatography for pomiferin and papain, but they are quite surprising for cucurbita where almost no variation was detected. By contrast, hieronymin affected the profiles less than the others did. In the hydrolyzed isolates zone III represents about a quarter of the total area, depending on the enzyme used (Fig. 7B). In this zone the area of peaks of sam-

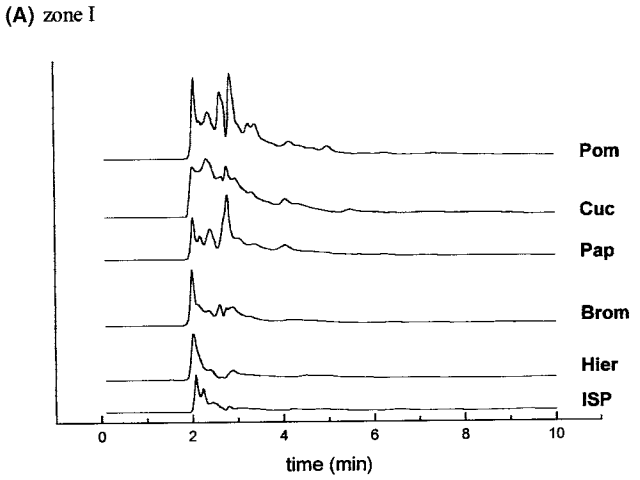

FIG. 7. Enlarged first (A) and third (B) zones of RP-HPLC corresponding to an ISP and the same isolate hydrolyzed during 720 min with Pom, Brom, Cuc, Hier, and Pap. For abbreviations see Figures 1,3, and 6.

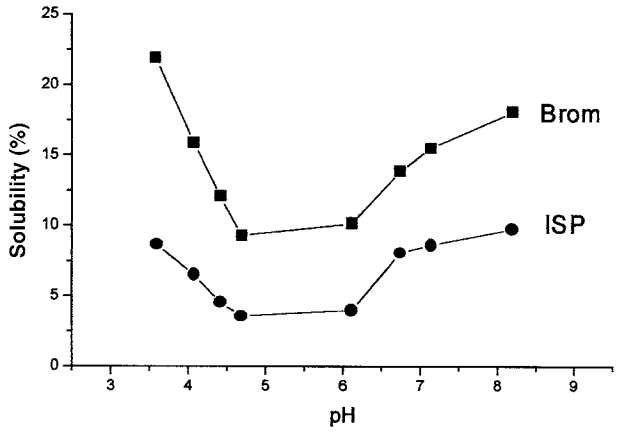

FIG. 8. Solubility percentage as a function of pH. ISP (●); hydrolysate after 12 h of reaction with Brom (■). For abbreviations see Figures 1 and 3.

ples hydrolyzed with pomiferin, bromelain, and hieronymin decreased, whereas for the hydrolysates obtained with cucurbita and bromelain, the peak area also decreased while shifting toward longer retention times, suggesting an increase in hydrophobicity in the protein components.

Reaction mechanisms. The characterization of the hydrolysates by RP-HPLC, molecular exclusion chromatography, and electrophoresis allowed us to conclude that the hydrolysis products resembled the structure of the partially hydrolyzed native isolate, which contained hydrolysis products in its structure as well as soluble hydrophilic and hydrophobic peptides of smaller molecular mass. The presence of either one product or the other allowed us to assign the mechanism of reaction for each protease. The Linderstrom-Lang model (24) considers equilibrium between the native and denatured forms of the protein, the latter being the substrate in the hydrolysis reaction. The model also describes two different pure mechanisms: zipper and one-by-one, which are defined by the rate of production of both intermediate and final hydrolysis products. The first proposed mechanism (zipper) proceeds through a fast step in which the structure the protein is destabilized, exposing peptide bonds that result in various intermediate products. The second mechanism (one-by-one) proposes a first slow step in which molecules are hydrolyzed one-by-one to yield the final products, and consequently no intermediate products can be detected. However, in most cases protein hydrolysis proceeds under a combination of both mechanisms (25). In the case of complex reaction mixtures where the products of hydrolysis are used as substrates (protein hydrolysis), inhibition by end-products should be included in the model.

The gel filtration profiles (Table 1) and the electrophoretic patterns obtained at short reaction times (Fig. 2E) for the cucurbita hydrolysates showed a residual protein structure that was almost intact and very similar to the substrate. However, at longer reaction times the RP-HPLC profiles (Fig. 7A and B) showed the presence of an important amount of hydrophilic hydrolysis products of very-low-molecular mass, suggesting the prevalence of the one-by-one mechanism. In the case of pomiferin, a visible decrease in the molecular mass of the different species and the appearance of hydrophobic hydrolysis products of low molecular mass were detected at short reaction times by gel filtration chromatography and electrophoresis (Table 1; Figs. 2A, 4, and 6). Meanwhile, a decrease in products of intermediate size (Fig. 2A) and the production of a significant amount of hydrophilic peptides (Fig. 7B) were observed at reaction times exceeding 30 min, suggesting that the zipper mechanism would be operating. For the other proteases, both mechanisms are likely to coexist with a slight prevalence of the one-by-one for hieronymin and papain, and of the zipper for bromelain.

Solubility, foaming capacity, and stability. The structural properties and hydrolysis mechanisms discussed earlier can be analyzed in relationship with both physicochemical and functional properties, such as solubility and foaming properties of the reaction products.

Solubility was measured in 0.01 M phosphate buffer (pH 8) of the protein isolate and of the hydrolysates. The buffer was selected to identify the components that may be participating

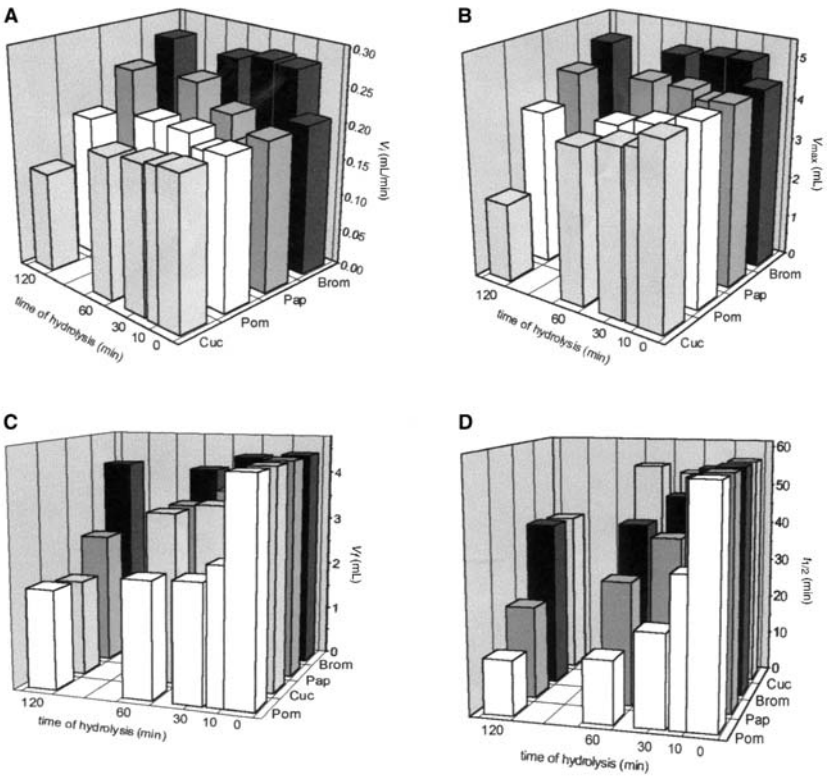

FIG. 9. Foaming properties of the nonhydrolyzed ISP and hydrolysates obtained with Pom, Pap, Cuc, and Brom. The foaming parameters were determined at 0 (native isolate), 10, 30, 60, and 120 min of hydrolysis. (A) Rate of liquid incorporation to the foam, v_i; (B) volume of liquid incorporated to the foam, V_{max}; (C) volume of liquid at the end of bubbling, V_f; (D) time for half-drainage of the liquid that was incorporated into the foam at the end of the bubbling, $t_{1/2}$, in min.

as the soluble fraction. The native isolate showed high solubility (89.75 ± 1.90%). No significant differences were observed ($P < 0,05$) in the solubility of the hydrolysates obtained with pomiferin, hieronymin, and cucurbita in comparison with the control at different reaction times (10, 30, 60, and 120 min of hydrolysis).

Solubility was also analyzed as a function of pH, both on the substrate and on the hydrolysate after 12 h of reaction with bromelain. The solubility of the soy isolate was very low in all cases (Fig. 8), probably due to its storage at low pH. During this time, proteins may have been denatured and became aggregated. The lowest solubility for the substrate was found at pH 4.5, corresponding to the isoelectric pH. The hydrolysate obtained with bromelain showed a higher solubility than the substrate over the pH range studied (Fig. 8), reaching a minimum at pH 4.5. These results reflected an enzymatic modification of the proteins of the isolate, suggesting that the increase in solubility may be due to the presence of small peptides and a partially hydrolyzed structure as components of the hydrolysis products.

Foaming properties of native isolate and hydrolysates obtained with pomiferin, bromelain, papain, and cucurbita at different reaction times are shown in Figure 9. The native isolate, which was not hydrolyzed, showed good ability to form and stabilize foams (Figs. 9A, B, C, D; time 0). Samples hydrolyzed with bromelain produced foams with spherical bubbles, similar to the control. The initial rate for the formation of foams increased slightly after 10 min and remained constant until 120 min of reaction. The time for half-drainage decreased after 30 min of hydrolysis, whereas the maximal volume of liquid incorporated into the foam did not vary. On this basis, it could be assessed that the hydrolysates obtained with bromelain have good foaming capacity, although the foams they produced are unstable and of low density in comparison to the control (time 0).

The hydrolysates obtained with papain had an initial rate of foaming that increased with the reaction time. The maximal volume of incorporated liquid remained constant, whereas the final volume of bubbling and the time for half-drainage decreased significantly. These results indicated that as the degree of hydrolysis increased, the foaming capacity of the isolate was gradually lost, in terms of foam formation and stabilizing capacity.

Foams obtained with the hydrolysates prepared with cucurbita contained spherical bubbles similar to those of the control although they were slightly bigger. The initial rate of foam formation remained constant up to 60 min but decreased significantly after long times (120 min). At intermediate times (60 min) the foam properties were similar to the control (Fig. 9A, B, D), but decreased as foaming progressed.

Finally, the results obtained with protein hydrolysates prepared with pomiferin indicated that the initial rate of foam formation was similar to the control (time 0) regardless of time. Small bubbles that increased their diameter initially composed those foams. This process negatively affected foam stability. The drastic drops in time for half-drainage of liquid with further hydrolysis confirmed this observation. In conclusion, the foaming ability for hydrolysates was as high as that for the unhydrolyzed isolate, but the foams had an extremely low stability.

The behavior described for each hydrolysate correlated well with the structural properties and with the mechanisms of hydrolysis that were previously discussed. Hydrolysates prepared with pomiferin showed a large number of small peptides with hydrophobic and hydrophilic properties, together with a low proportion of native structure similar to the substrate. For this reason, a zipper mechanism was ascribed to this reaction. Molecules would be free to migrate and absorb at the interface. However, they would have a reduced ability to interact, since smaller peptides migrate to the interface at the same rate at which they are released. Those observations are reflected in the foaming ability and their low stability.

Hydrolysates prepared with cucurbita showed a similar structure to the control, with an almost intact residual structure and a low number of small peptides, suggesting a one-by-one mechanism for this reaction. In addition, the presence of such intact residual structure of the hydrolysates obtained with cucurbita provides scientific grounds for the interpretation of the similarities found with the control. Working with bromelain and papain, we observed a combination of zipper and one-by-one mechanisms that lead to products with certain properties and correlated well with their ability to form and to stabilize foams.

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