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Opposite Contributions of Glycinin- and β-Conglycinin-Derived Peptides to the Aggregation Behavior of Soy Protein Isolate Hydrolysates

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Abstract The aggregation behavior as a function of pH was studied for hydrolysates obtained by hydrolysis of soy protein isolate (SPI) and glycinin- and β-conglycinin-rich protein fractions with subtilisin Carlsberg. The substrates were hydrolyzed up to degrees of hydrolysis (DH) of 2.2% and 6.5%. Compared with nonhydrolyzed SPI, a decrease in solubility was observed for the hydrolysates of SPI [0.8% (w/v) protein, I=0.03 M] around neutral pH. At pH 8.0, glycinin hydrolysates had a much lower solubility (~43% and 60%, respectively, for DH 2.2% and 6.5%) than SPI and β-conglycinin-derived hydrolysates, which were almost completely soluble. Peptides that aggregated were all larger than 5 kDa, and as estimated by size-exclusion chromatography their composition was almost independent of the aggregation pH. The solubility of hydrolysates of SPIs with a varying glycinin and β-conglycinin composition showed that glycinin-derived peptides are the driving force for the lower solubility of SPI hydrolysates. The solubility of SPI hydrolysates at pH 8.0 was shown not to be the sum of that of glycinin and β-conglycinin hydrolysates. Assuming that the separate hydrolysis of glycinin and β-conglycinin did not differ from that in the mixture (SPI), this indicates that β-conglycinin-derived peptides have the ability to inhibit glycinin-derived peptide aggregation.

Keywords Aggregation · Solubility · Proteolysis · Soy protein isolate · Glycinin · β-Conglycinin · Subtilisin Carlsberg

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Introduction

Soy protein aggregation followed by the formation of a space-filling gel network plays a major role in the structure formation of various soy-based foods, such as meat analogs, tofu, or fermented soy milk.[1] In addition to these existing food structures, new structures would open possibilities to expand or to improve the use of soy proteins in foods. A possible way to obtain such new structures may be via the modification of the aggregation properties of proteins. Such modifications may be induced physically, e.g., via heat treatment, or chemically, e.g., by using proteolytic enzymes.[2]

The improvement of soy protein aggregation and the subsequent change in soy protein gelation by pretreatment with proteases has not yet received much attention in the literature, although hydrolysis can be a means of expanding the gelling properties of whey proteins[3,4] and soy proteins.[5,6]

The probable reason for this lack of attention is the fact that hydrolysis is assumed to be unfavorable for the gelling properties of proteins, because hydrolysis increases the number of charged groups and reduces the molecular mass, both of which generally hamper gelation.[7] On the other hand, during hydrolysis, the folded structure of the protein is altered, resulting in the exposure of buried hydrophobic groups, which might induce aggregate formation.

Interestingly, upon hydrolysis of proteins, aggregates may be formed,[8,9] indicating that peptides are formed with an aggregation behavior different from that of the nonhydrolyzed protein. Under specific conditions, these aggregates can form gel networks, which have improved properties compared with the nonhydrolyzed protein.[4,5,10,11] In contrast to the formation of aggregates, peptides can also prevent aggregation. Peptides derived from milk or soy proteins were shown to have the ability to inhibit blood platelet aggregation.[12,13]

In a previous study,[6] it was shown that hydrolysis of soy protein isolates (SPI) results in the formation of peptides that aggregate at a pH value higher than the pH at which the nonhydrolyzed proteins aggregate. Understanding the characteristics and origin of these aggregating peptides will enable optimization of the hydrolysis process with respect to the yield in peptides having aggregating properties, which can be used as functional ingredients in foods.

The two main proteins present in soy are glycinin and β-conglycinin, which represent ~42% and ~34%, respectively, of all the proteins present in soybeans.[14] Glycinin is composed of an acidic (acidic p*I*; ~35 kDa) and a basic polypeptide (basic p*I*; ~20 kDa) connected by one disulfide bridge[15] and mainly exists as a hexamer (11S; pH 7.6, I=0.5 M). Glycinin can dissociate into its 7S and 3S form upon decreasing the ionic strength or changing the pH.[16] β-Conglycinin exists as a trimer with a molecular mass of 150–200 kDa, and contains three major subunits: α′ (72 kDa), α (68 kDa), and β (52 kDa).[15] Glycinin and β-conglycinin have different gelling properties: upon heating, glycinin is able to form stronger gels than β-conglycinin.[17] In heat-induced gels made of mixtures of glycinin and β-conglycinin, the gel hardness of the mixed preparations was intermediate between gels of glycinin and β-conglycinin.[18,19] Analogous to this, it can be hypothesized that peptides originating from glycinin or β-conglycinin may also have a different contribution to the aggregation behavior of hydrolysates made from SPI. Therefore, the main purpose of the present study was to elucidate the individual contributions of glycinin- and β-conglycinin-derived peptides to the aggregation behavior of SPI hydrolysates.

Materials and methods

Materials and chemicals Defatted soybean meal was used, after being prepared from Hyland soybeans (non-GMO) supplied by Fa. L.I. Frank (Twello, The Netherlands) as described previously.[6] Subtilisin Carlsberg from *Bacillus licheniformis* [10 units/mg solid: one unit releases color, equivalent to 1.0 μmol (181 μg) of tyrosine per minute from casein at pH 7.5 at 37°C as measured using the Folin-Ciocalteu reagent] was obtained from Sigma Chemical Co. (St. Louis, MO, USA; article no. P-5380). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Sigma.

Analysis of the protein content The nitrogen content of various samples was determined in duplicate by the Dumas method using an NA2100 Nitrogen and Protein Analyzer (CE Instruments, Milan, Italy) according to the instructions of the manufacturer. Methionine was used as a standard. The nitrogen correction factor for purified glycinin and β-conglycinin is 5.60, based on their average amino acid compositions as found in Swiss-Prot [http://www.expasy.org; primary accession numbers used were as follows: P04776 (glycinin 1; A1a and B2 subunits), P04405 (glycinin 2; A2 and B1a subunits), P11828 (glycinin 3; A1b and B1b subunits), P02858 (glycinin 4: A5, A4 and B3 subunits), P04347 (glycinin 5; A3 and B4 subunits), P11827(β-conglycinin, α′-chain), P13916 (β-conglycinin, α-chain), and P25974 (β-conglycinin, β-chain)].

Because β-conglycinin is a glycoprotein and is reported to contain ~4.5% carbohydrates,[14,20] a nitrogen factor of 5.87 is appropriate for this protein. During proteolysis, only the polypeptide chain is cleaved, and all the sample preparations are standardized on the same polypeptide content. For β-conglycinin, this means that a protein factor of 5.60 is used instead of 5.87. In this work, we regard SPI as containing only glycinin and β-conglycinin; therefore, a nitrogen factor of 5.60 is used for SPI as well.

Preparation of soy protein isolates From the defatted soybean meal, an SPI was prepared by precipitation of the proteins at pH 4.8 using 1 M HCl as described previously.[6] Densitometric analysis of the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel showed that at least 90% of the protein bands present in the SPI originate from glycinin or β-conglycinin [Figure 4 (1)]. This SPI is further denoted as SPI 64/36, in which 64/36 is the ratio of glycinin/β-conglycinin in the preparation (Table 1), as determined by high-performance size-exclusion chromatography (HP-SEC). In the preparation of the SPI, the clear solution at pH 8.0, obtained just after centrifugation and just before the pH decrease to pH 4.8, is denoted as pH 8.0 protein extract.

In addition to the SPI 64/36, SPIs were also prepared by precipitation at other pH intervals, which resulted in SPIs with different glycinin/β-conglycinin ratios by making use of the difference in the pH-dependent solubility of the two proteins.[21] Two sequential precipitations were performed, both starting with the pH 8.0 protein extract from soybean meal.

The first sequential precipitation resulted in three SPIs precipitated in the pH intervals 8.0–6.2, 6.2–5.0, and 5.0–4.8. First, a precipitate was prepared by decreasing the pH to 6.2. The pH of the supernatant obtained at pH 6.2 was

Table 1 Glycinin and β-conglycinin content of glycinin, SPIs, and β-conglycinin; the pH interval at which they were obtained, h_{tot}; and the protein content of the freeze-dried preparations

Sample	Precipitation pH interval	Glycinin (%)[a]	β-Conglycinin (%)[a]	h_{tot}[b] (meqv/g)	Protein content (%)[c]
Glycinin	6.2–8.0	92	8	8.85	90.4 (±3.7)
SPI 92/8	6.2–8.0	92	8	8.85	85.9 (±0.6)
SPI 79/21	5.6–8.0	79	21	8.81	81.3 (±2.9)
SPI 64/36[d]	4.8–8.0	64	36	8.77	83.3 (±1.6)
SPI 49/51	5.0–6.2	49	51	8.73	76.2 (±1.7)
SPI 35/65	4.8–5.6	35	65	8.70	74.2 (±1.0)
SPI 22/78	4.8–5.0	22	78	8.66	78.1 (±3.9)
β-Conglycinin	4.8–5.0	12	88	8.63	80.3 (±4.4)

[a] As determined by HP-SEC.
[b] h_{tot}: Total number of peptide bonds in the protein substrate (meqv/g protein).[22]
[c] The protein content is expressed as (w/w) % and measured in duplicate.
[d] SPI 64/36 is same as used previously and can be considered as a reference SPI.[6]

subsequently decreased to pH 5.0 to precipitate another part of the proteins. Next, the pH of the supernatant obtained at pH 5.0 was set to 4.8 followed by centrifugation. This first sequential precipitation resulted in three SPIs: SPI 92/8, SPI 49/51, and SPI 22/78.

The second sequential precipitation resulted in two SPIs precipitated in the pH intervals 8.0–5.6 and 5.6–4.8. First, a precipitate was prepared at pH 5.6. The pH of the supernatant obtained at pH 5.6 was decreased to pH 4.8 followed by centrifugation. This second sequential precipitation resulted in two SPIs: SPI 74/21 and SPI 35/65. The compositions of the five SPIs obtained can be found in Table 1.

In the two sequential precipitations, the pH was decreased by addition of 1 M HCl. All suspensions were stirred for at least 1 h before centrifugation (15 min, 12,000×*g*, 20°C). All the pellets, obtained at different pH values (6.2, 5.6, 5.0, and 4.8), were washed once with Millipore water (v/v; ratio precipitate/water ~1:9) followed by suspension in water and adjustment of the pH to 8.0 before they were freeze dried.

Purification of glycinin Glycinin was purified in a similar way as the SPI 92/8 preparation with the difference that the precipitate obtained at pH 6.2 was directly suspended in water at pH 8.0 overnight at 4°C. After centrifugation (30 min, 12,000×*g*, 20°C), the supernatant was dialyzed against distilled water. The pH of the dialyzed protein dispersion was adjusted to pH 8.0 and the clear solution obtained was freeze dried. The freeze-dried product was denoted "glycinin" (Table 1).

Purification of β-conglycinin To obtain purified β-conglycinin, the supernatant from the glycinin purification (pH 6.2) was adjusted to pH 5.0, stirred for 1 h at ambient temperature, and centrifuged (30 min, 18,000×*g*, 20°C). The pellet was discarded and the pH of the supernatant was adjusted to 4.8 to induce precipitation of β-conglycinin. The suspension was stirred for 1 h, followed by centrifugation (30 min, 12,000×*g*, 20°C). The precipitate was suspended overnight at 4°C in 35 mM sodium phosphate buffer (pH 8.0). After centrifugation (30 min, 18,000×*g*, 20°C), the supernatant was applied onto a Superdex 200 column (60× 10 cm, Amersham, Uppsala, Sweden) and eluted with a 35 mM sodium phosphate buffer (pH 8.0) at a flow rate of 40 mL/min at ambient temperature. The eluate was monitored by UV detection at 220 nm and 200 mL fractions were collected. Fractions containing β-conglycinin, as analyzed by SDS-PAGE (molecular mass bands >43 kDa), were pooled and dialyzed against distilled water. The pH of the dialyzed protein dispersion was adjusted to pH 8.0 and the clear solution obtained was freeze-dried. The freeze-dried product was denoted "β-conglycinin" (Table 1).

Hydrolysis of soy protein isolates, glycinin, and β-conglycinin The SPIs, glycinin, and β-conglycinin were suspended in Millipore water at a concentration of 1.5–2% (w/w) protein and stirred overnight at 4°C. The pH was adjusted to 8.0, if necessary. SPI solutions were centrifuged (18,000×*g*, 20 min, 20°C), while the glycinin and β-conglycinin solutions were filtered using a 0.45 μm filter (Schleicher & Schuell GmbH, Dassel, Germany). The protein content of the supernatant and filtrates was determined using the Dumas method. Solutions were diluted to 1% (w/w) protein using Millipore water followed by heating at 95°C for 30 min. The heated solutions all had a clear to slightly opaque appearance. Hydrolysates with degrees of hydrolysis (DH) of 2.2% and 6.5% were prepared from the heated SPI, glycinin, and β-conglycinin solutions by hydrolysis at pH 8.0 at 40°C, using subtilisin

Carlsberg. The pH and DH were controlled using the pH-stat method by using a 719S Titrino (Metrohm ion analysis, Herisau, Switzerland).[22] The h_{tot} used for the calculation of the DH was calculated based on the amino acid composition of the various preparations, assuming that only glycinin and β-conglycinin were present (Table 1). The enzyme concentrations used were ~3 or 13 units/g protein for the DH values of 2.2% and 6.5%, respectively. The enzyme was dissolved in Millipore water and directly added to the solutions. The molarity of the NaOH solution used to maintain the pH at pH 8.0 varied from 0.1 to 0.3 M. When the desired DH was reached, the enzymatic hydrolysis was stopped by addition of a 100 mM phenylmethylsulfonyl fluoride stock solution in 2-propanol to a final concentration of 1 mM. The pH-stat experiment was stopped after the pH remained stable at pH 8.0. Hydrolysates were freshly prepared before each experiment.

Gel electrophoresis The protein composition of SPIs, glycinin, and β-conglycinin and their hydrolysates was examined using SDS-PAGE under reducing conditions (10 mM 2-mercaptoethanol). A 10–20% Tris–HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) was used on a Mini-protean II System (Bio-Rad Laboratories) according to the instructions of the manufacturer. The gel was calibrated with marker proteins with molecular masses ranging from 14.4 to 94 kDa (Amersham Biosciences, Roosendaal, The Netherlands) and was stained with Coomassie brilliant blue.

Solubility of the soy protein isolate, glycinin, and β-conglycinin hydrolysates The aggregation behavior of the hydrolysates and nonhydrolyzed solutions was studied as the (in-)solubility at a protein concentration of 0.8% (w/w; I=0.03 M). For the solubility of the nonhydrolyzed samples, the heated solutions, as also used for the hydrolysis experiments, were used.

The ionic strength of the heated protein solution was arbitrary regarded as 0 M. The ionic strength of 0.03 M was defined by the total amount of NaOH added during the hydrolysis and NaCl was added after hydrolysis. During hydrolysis, the ionic strength of the solution increases due to the liberation of charged groups. The increase in ionic strength during hydrolysis is assumed to be equal to the amount of NaOH added. This results in an increase of 0.003 and 0.009 M for hydrolysates with a DH of 2.2 and 6.5, respectively. To end up with a final protein concentration of 0.8% and an ionic strength of 0.03 M, Millipore water and 0.5 M NaCl were added. The solutions were acidified stepwise from pH 8.0 to pH 3.0 using 0.2 M HCl. A sample was taken every 0.5 pH units. The samples, set at various pH values, were mixed in a head-over-tail rotator for 1 h followed by centrifugation (20 min, 22,000×g, 20°C). The proportion of the total amount of peptides present that remained in the supernatant after centrifugation was defined as solubility. The solubility was determined by diluting the supernatant 20 times with 1% (w/v) SDS in Millipore water, followed by determination of the absorbance at 280 nm (A_{280}), using a spectrophotometer (UV 1601, Shimadzu, Japan). A 0.95% SDS solution served as a blank. In addition, all the (20 times) diluted samples were diluted 10 times with 1% SDS, followed by analysis of their absorbances at 220 nm (A_{220}). The measured absorbances were corrected for dilutions during acidification with HCl.

The solubility was calculated by expressing the A_{220} or A_{280} of the supernatant as proportion of the A_{220} or A_{280} of the total hydrolysate at pH 8.0. For SPI 64/36 and glycinin, the solubility curves were prepared in duplicate and were observed to have an average standard deviation <5% of the mean for each data point when obtained at pH 4.0 and higher, whereas at pH 3.0 and 3.5 standard deviations up to 20% were obtained.

The pellets, containing the aggregating peptides, were washed once with Millipore water and again centrifuged (20 min, 22,000×g, 20°C). The pellets were resuspended in 0.4% (v/v) trifluoroacetic acid (TFA) in Millipore water followed by freeze drying. TFA was added to cause a pH decrease sufficient to irreversibly inhibit the remaining minor activity of subtilisin Carlsberg. In this study, we define aggregating peptides as those peptides present in the pellets after centrifugation, although we are fully aware of the possibility that soluble aggregates, composed of peptides, remain present in the supernatant.

High-performance size-exclusion chromatography SPI 64/36, glycinin, β-conglycinin, and their hydrolysates and pellets were dissolved in 500 μL of 0.15 M Tris–HCl buffer (pH 8.0) containing 100 mM 1,4-dithiothreitol and 8 M guanidinium chloride. The final protein concentration was ~3 mg/mL. After mixing in a head-over-tail rotator for 1 h at ambient temperature, 215 μL of acetonitrile, containing 2% (v/v) TFA, was added and the mixture was mixed in a head-over-tail rotator for another 1 h. The solutions had a clear appearance, but were centrifuged as a precaution. After centrifugation (15 min, 22,000×g, 20°C), 20 μL of the supernatants was applied to a Shodex Protein KW-803 column (300×8 mm; Showa Denko K.K., Tokyo, Japan). The column was equilibrated and run with 6 M urea containing 30% (v/v) acetonitrile and 0.1% (v/v) TFA. The flow rate was 0.5 mL/min and the absorbance of the eluate was monitored at 220 nm. The column was calibrated using various proteins in a molecular mass range of 0.308–669 kDa (glutathione, bradykinin, angiotensin, insulin chain B, aprotinin, ribonuclease A, β-lactoglobulin, chymotrypsin, aldolase, ovalbumin, albumin, catalase, ferritin, and thyro-

globulin). The void volume of the column was ~5.8 mL, as determined from the volume at which thyroglobulin (669 kDa) eluted. The included volume was estimated using glutathione (308 Da) and was observed to be 12.7 mL.

To enable comparison between the size distribution of the nonhydrolyzed proteins with those of hydrolysates and the aggregating peptides, the total surface under the chromatograms was normalized. To facilitate comparison, the total surface under the chromatograms of the aggregated peptides was corrected for the proportion of peptides that was aggregated.

To determine the glycinin/β-conglycinin ratios in the SPIs, glycinin, and β-conglycinin samples, the surface under the peaks (corrected for the difference in absorbance between glycinin and β-conglycinin at 220 nm) in the SEC chromatograms at 220 nm was used. On a weight basis, the absorbance of glycinin appeared to be a factor 1.25 higher than the absorbance of β-conglycinin. Proteins eluting between 6.1–7.0 and 7.0–10 ml were considered to be β-conglycinin and glycinin, respectively, as described previously.[6] Due to the high solubility of trypsin inhibitors over a broad pH range,[23] their presence in the SPIs, glycinin, and β-conglycinin samples is unlikely and is, therefore, neglected.

Results

Differences in solubility of the glycinin, β-conglycinin, and SPI 64/36 hydrolysates

To elucidate the individual contributions of glycinin- and β-conglycinin-derived peptides in the SPI hydrolysates, first the aggregation behavior of hydrolysates from glycinin and β-conglycinin was compared with the aggregation behavior of hydrolysates from SPI 64/36.

The most remarkable observation in this study was that the glycinin hydrolysates had much lower solubilities in the pH range from 7.0 to 8.0 than nonhydrolyzed glycinin, as indicated by the arrow in Figure 1a. Before ionic strength adjustment at pH 8.0, the glycinin hydrolysate at DH 2.2% already showed a white haze. It became even more turbid upon ionic strength adjustment to 0.03 M. The glycinin hydrolysate at DH 6.5% was already turbid when the desired DH was reached, and no extra aggregation could be visually observed upon adjustment of the ionic strength. After stepwise acidification of the glycinin hydrolysate, the solubility of the DH 2.2% sample slightly decreased until pH 4.5, whereas it increased upon further acidification. With decreasing pH, the glycinin hydrolysate at DH 6.5% showed almost no further decrease in solubility until pH 4.5. Below this pH, the solubility increased slightly during acidification to pH 3.0. In the pH range from pH 4.0 to 8.0, the solubility at DH 6.5% is higher that at DH 2.2%.

In contrast to the glycinin hydrolysates, the β-conglycinin hydrolysates had a clear appearance, before and after ionic strength adjustment at pH 8.0, which is reflected in the ~100% solubility at this pH (Figure 1b). Upon decreasing the pH of the hydrolyzed β-conglycinin, the pH at which the solubility started to decrease increased with increasing DH.

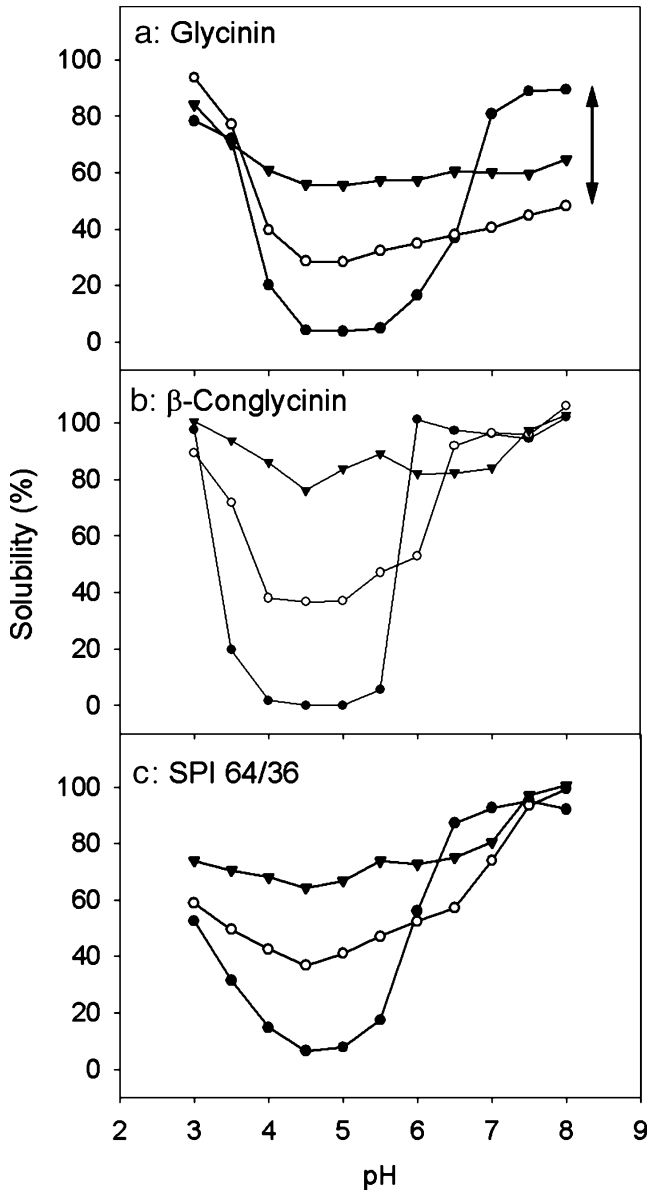

Fig. 1. Solubility curves for DH 0% (•), 2.2% (○), and 6.5% (▾) for glycinin (a), β-conglycinin (b), and SPI 64/36 (c) at 0.8% (w/w) protein concentration (I=0.03 M). The solubility was determined from the absorbance at 220 nm. The arrow indicates the decrease in solubility of the glycinin hydrolysates compared with the non-hydrolyzed glycinin.

Interestingly, Figure 1c shows that around pH 8.0, the solubility of the nonhydrolyzed SPI 64/36 and its hydrolysates was almost 100%. The overall solubility profile of SPI 64/36 and their hydrolysates as a function of pH was, therefore, similar to that obtained for β-conglycinin and its hydrolysates, with the main difference that the pH at which the solubility started to decrease was now the same for both DHs studied.

All the hydrolysates had a minimum solubility in the pH 4–5 range. The solubility in this range increased with increasing DH, which agrees with the general observation that hydrolysis increases protein solubility.[24,25] Upon further decrease of the pH to 3, the solubility increased with decreasing pH. Glycinin and β-conglycinin and their hydrolysates reached a solubility of ~80% or higher at pH 3.0, whereas the solubility of SPI 64/36 and its hydrolysates at pH 3.0 remained below 80%.

Contribution of aromatic amino acids to the composition of aggregating peptides

The solubility as a function of pH for SPI 64/36, glycinin, and β-conglycinin and their hydrolysates as calculated from the A_{220} and A_{280} absorbances are shown in Figure 2.

For both glycinin hydrolysates, the solubility measured as A_{280} was lower than the solubility measured as A_{220} over the whole pH range for the two hydrolysates, whereas for β-conglycinin hydrolysates, as well as for all three non-hydrolyzed preparations, the solubility measured as A_{280} was similar to the solubility measured as A_{220}. This indicates that the aggregating peptides from glycinin seem to be enriched in aromatic amino acids compared with the aggregating peptides from β-conglycinin.

For the hydrolysates of SPI 64/36, the solubility measured as A_{220} is the same as the solubility measured as A_{280} at neutral

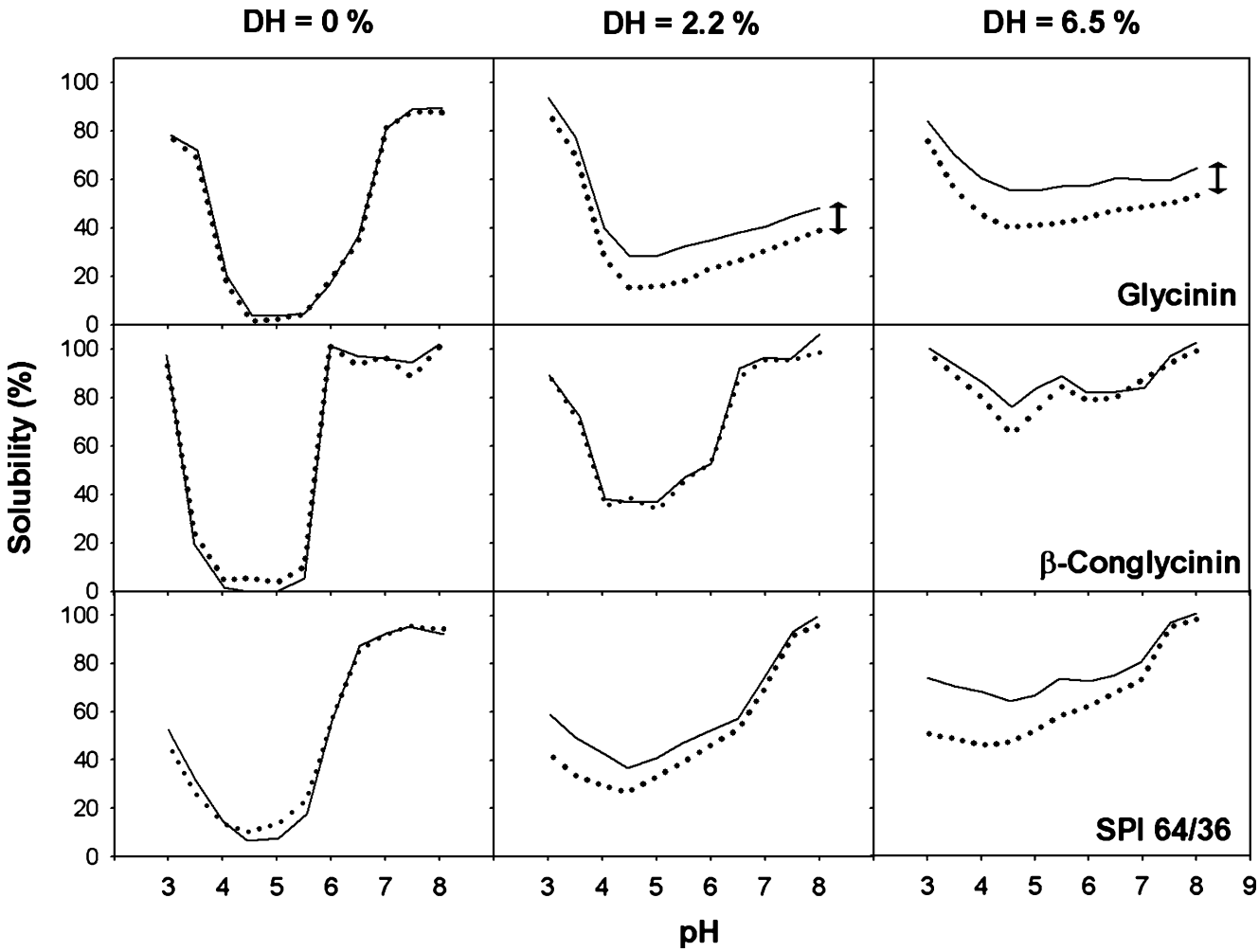

Fig. 2. Solubility curves as a function of pH for glycinin, β-conglycinin, and SPI 64/36 at 0.8% (w/w) protein (I=0.03 M), with DH values of 0%, 2.2%, and 6.5%. The solubility was determined from the absorbance at 220 nm (A_{220}; —) and 280 nm (A_{280}; ···). The arrows indicate the difference in solubility for glycinin when measured at different wavelengths.

pH, but the solubility measured as A_{280} decreases further with decreasing pH than the solubility measured as A_{220}.

Molecular size distribution of aggregating peptides

The HP-SEC chromatograms of the nonhydrolyzed proteins, of their hydrolysates at DH 2.2% and 6.5%, and of the peptides aggregating at pH 5.0 are shown in Figure 3. For all hydrolysates, it can be observed that the aggregating peptides represent all the larger peptides present in the hydrolysates. The aggregating peptides at DH 6.5% are smaller compared with the size of aggregating peptides at DH 2.2%. Peptides with a molecular mass <3 kDa do not precipitate at pH 5.0 (black arrows).

In Figure 3, only the chromatograms of the aggregating peptides at pH 5.0 are shown, because all pellets originating from one hydrolysate showed similar patterns at the various precipitation pH values (pH 3, 4, 5, 6, 7, and 8), and differed only in amount. The only exception to this were the peptide or peptides present in the peak eluting around 8.1 mL (~24 kDa; gray arrows), originating from β-conglycinin, which precipitated to a lower extent at the pH values tested above pH 5. Although we do not know whether this concerns one peptide, or various peptides with a similar mass, we denote peptides with an estimated mass of 24 kDa, as the 24-kDa peptide.

When comparing the chromatograms of the DH 2.2% hydrolysates with those of the DH 6.5% hydrolysates (Figure 3), it is remarkable to see that a higher proportion of the larger peptides seem to aggregate at a lower DH than at a higher DH. The 24 kDa peptide present in the β-conglycinin hydrolyzate is the best example of this phenom-

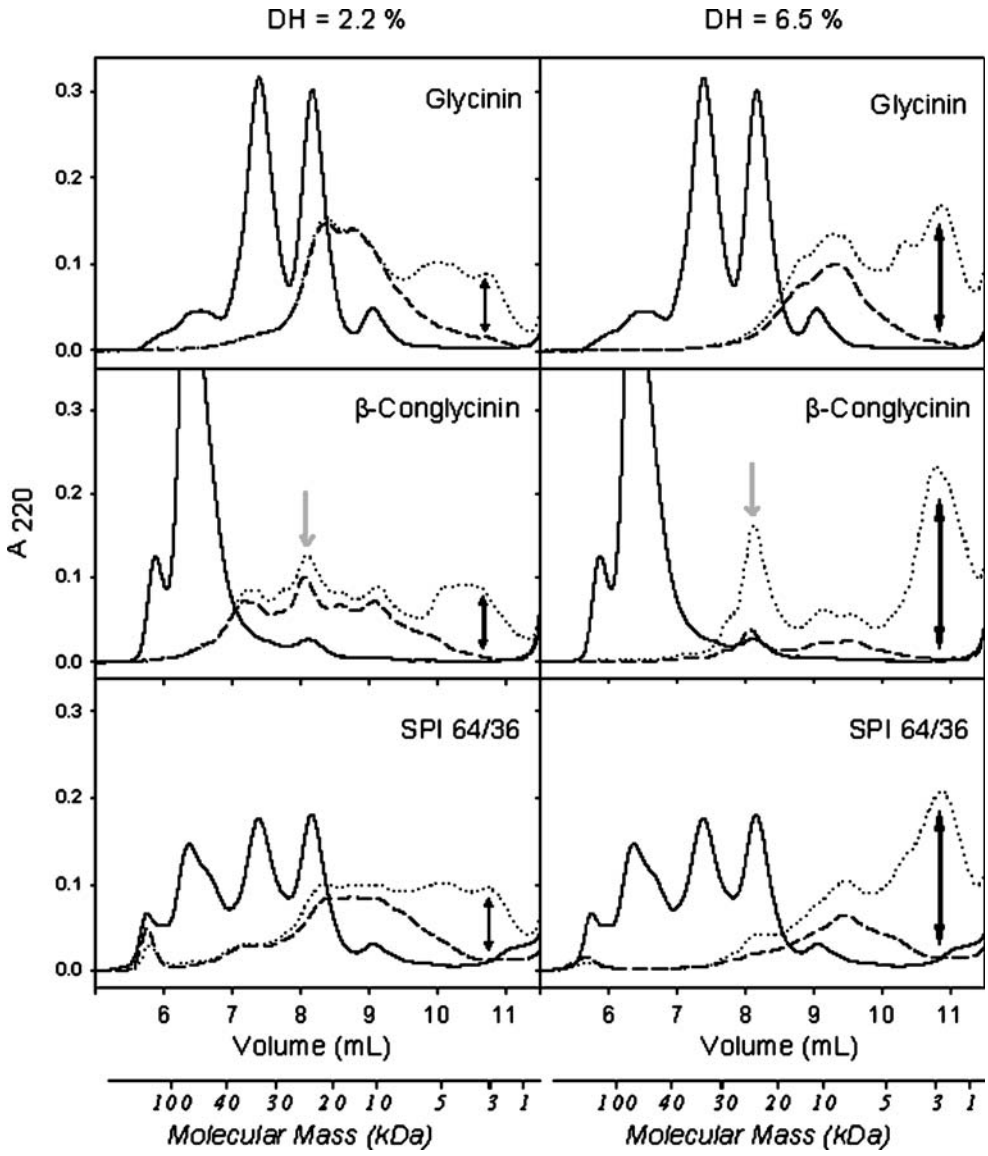

Fig. 3. High-performance size-exclusion chromatograms under denaturing conditions of glycinin, β-conglycinin, and nonhydrolyzed SPI 64/36 (—), hydrolysates (···) thereof at DH 2.2% and 6.5%, and aggregating peptides (- - -) of the hydrolysates at pH 5.0. The samples were reduced before analysis. The secondary *X*-axis shows the apparent molecular mass eluting at that retention time. The black and gray arrows indicate the high solubility of small peptides and the presence of the hydrolysis-resistant ~24 kDa peptide, respectively.

enon because it remained almost completely soluble at DH 6.5%, whereas at DH 2.2%, it precipitated almost completely.

Figure 4 shows the SDS-PAGE gel of nonhydrolyzed SPI 64/36, glycinin, and β-conglycinin and their hydrolysates. The absence of β-conglycinin bands in glycinin (lane 4) and the absence of glycinin bands in β-conglycinin (lane 7) indicate that glycinin and β-conglycinin had a high purity. It should be noted that in the β-conglycinin, the β-subunit (~48 kDa)[15] was present in a relatively low amount compared with SPI 64/36.

The DH 2.2% samples show that at this DH nonhydrolyzed protein was no longer present. In the hydrolysates, separate peptide bands were observed, corresponding to specific peptides that represent intermediate peptides in the degradation of the protein toward smaller peptides.

The 24 kDa peptide that seems to be resistant to enzymatic hydrolysis, as shown in Figure 3, can also be observed in the SDS-PAGE gel and is present in both hydrolysates of SPI 64/36 and β-conglycinin, although its intensity in SPI 64/36 is lower due to the lower amount of β-conglycinin present in the SPI 64/36, when compared with the β-conglycinin preparation itself.

Aggregation behavior of peptides in hydrolysates of soy protein isolates having different glycinin/β-conglycinin ratios

To determine how the individual aggregation behavior of glycinin and β-conglycinin peptides can be translated to "normal" SPI, SPIs with different glycinin/β-conglycinin ratios were prepared, as shown in Table 1. This table shows that an almost linear distribution of glycinin and β-conglycinin ratios was obtained over the six SPIs, which can also be observed from the SDS-PAGE gel as presented in Figure 5. This setup enabled us to control the protein composition and use glycinin/β-conglycinin ratios that vary more than the variation present in cultivated soybeans.[26–28]

The solubility curves as a function of pH obtained for hydrolysates of the various SPIs at DH 2.2% and 6.5% are shown in Figure 6. It can be clearly seen that the higher the glycinin content of the SPI, the higher the pH at which the solubility started to decrease (SPI 64/36, 49/51, 35/65, and 22/78) or the lower the solubility at pH 8.0 (SPI 92/8 and 79/21) as indicated with the arrow (Figure 6a). This phenomenon can also be observed at DH 6.5% (Figure 6b), but it is less clear than at DH 2.2%. All preparations displayed the same pH dependency around pH 4.5, followed by an increase in solubility upon further decrease of the pH.

Discussion

Differences in aggregation behavior between glycinin and β-conglycinin hydrolysates

The most remarkable result in this study was the difference in aggregation behavior of glycinin- and β-conglycinin-derived peptides. Around neutral pH, glycinin-derived peptides have a much stronger tendency to aggregate than β-conglycinin-derived peptides (Figure 1). This tendency of glycinin-derived peptides to form aggregates seems, therefore, to be the driving force in the previously observed shift in aggregation pH (i.e., the pH at which visible aggregation occurs) for hydrolysates of SPI when compared with nonhydrolyzed SPI.[6] This was confirmed by the solubility behavior of hydrolysates of the

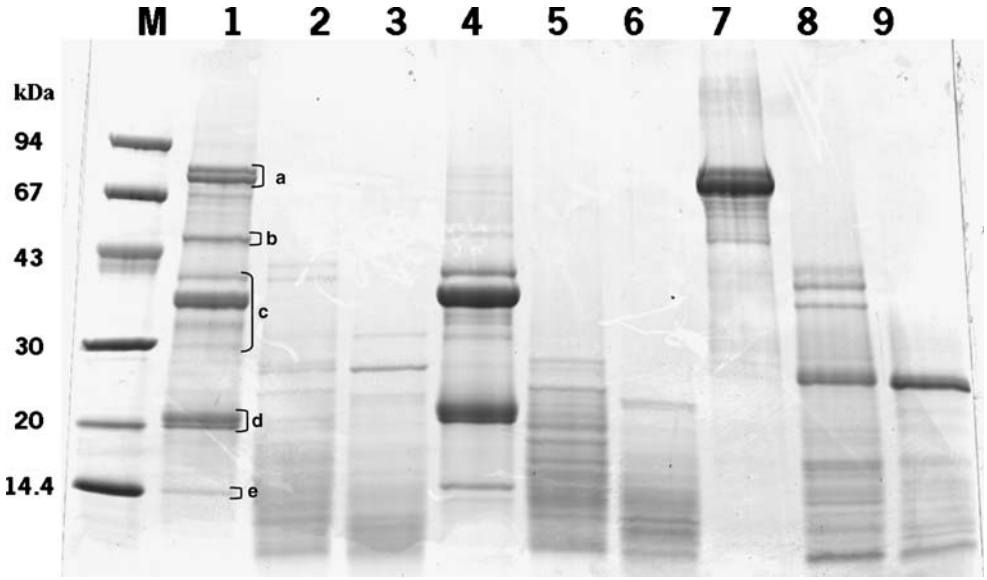

Fig. 4. SDS-PAGE gel under reducing conditions showing the marker (M); SPI 64/36 at DH 0%, 2.2%, and 6.5% (1–3); glycinin at DH 0%, 2.2%, and 6.5% (4–6); and β-conglycinin at DH 0%, 2.2%, and 6.5% (7–9), respectively. In the SPI lane 1, the α/α′ subunits of β-conglycinin (a), β subunit of β-conglycinin (b), the acidic polypeptides of glycinin (except A5; c), the basic polypeptide of glycinin (d), and the acidic polypeptide A5 of glycinin (e) are indicated.

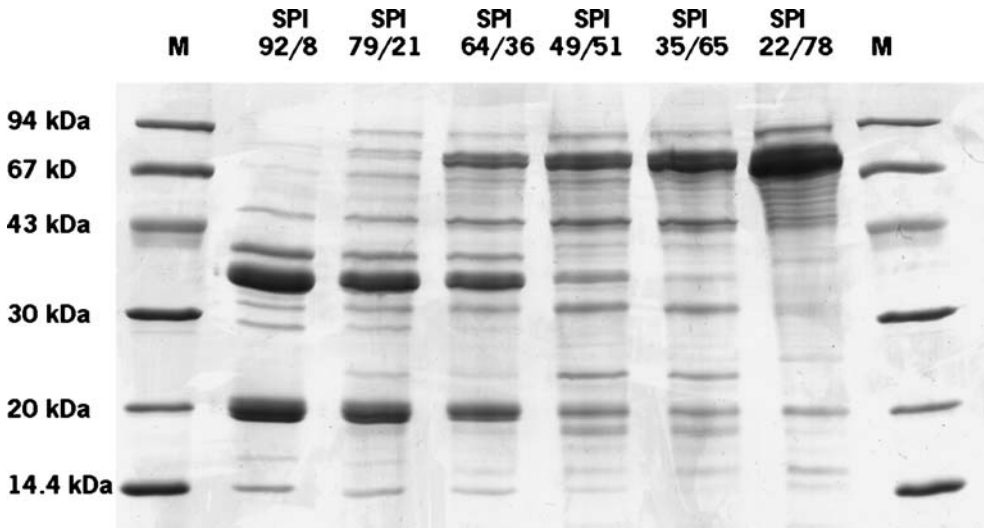

Fig. 5. SDS-PAGE gel under reducing conditions showing the marker (M), SPI 92/8, SPI 79/21, SPI 64/36, SPI 49/51, SPI 35/65, and SPI 22/78.

various SPIs (Figure 6), showing that the higher the glycinin content, the lower the solubility around neutral pH. When translating the behavior of hydrolysates of glycinin and β-conglycinin to SPI, it was observed that, when hydrolyzed separately, the hydrolysis of β-conglycinin is up to 15% slower than the hydrolysis of glycinin (results not shown), a difference that is not very large. When the SDS-PAGE patterns of the hydrolysates of SPI are compared with those of the hydrolysates of the glycinin and β-conglycinin (Figure 4), it can be observed that several peptides present in the SPI hydrolysate also seem to be present in the hydrolysates of glycinin and β-conglycinin. This may indicate that the two purified proteins were indeed hydrolyzed into the same fragments when hydrolyzed individually or in a mixture, such as in the SPI.

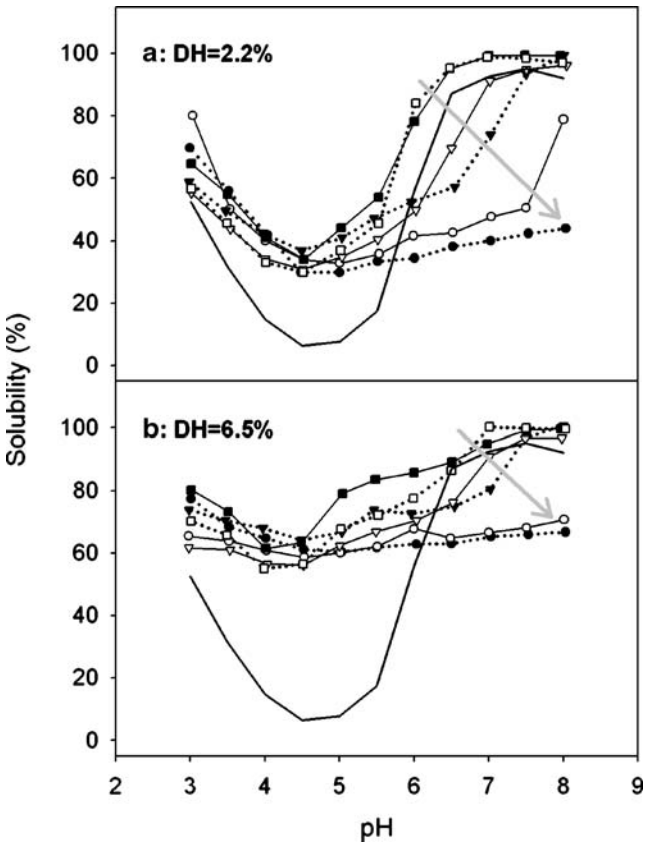

Fig. 6. Protein solubility as a function of pH for nonhydrolyzed SPI 64/36 (–), DH 2.2% (a) and 6.5% (b) for SPI 92/8 (•···•), SPI 79/21 (○—○), SPI 64/36 (▾···▾), SPI 49/51 (▿—▿) SPI 35/65 (▫···▫) and SPI 22/78 (▪—▪) at 0.8% (w/w) protein (I=0.03 M). The shift in aggregation pH is indicated with the arrows.

This difference in contribution to the total aggregation behavior between peptides derived from different proteins was also reported for the enzyme-induced gelation of a mixture of α-lactalbumin and β-lactoglobulin.[29] α-Lactalbumin-derived peptides were able to form much stronger gels than β-lactoglobulin-derived peptides. Moreover, the presence of β-lactoglobulin-derived peptides even inhibited gelation of α-lactalbumin.

Characteristics of peptides responsible for aggregation

Because subtilisin Carlsberg is a nonspecific protease, preferentially cleaving at the C-terminal side of neutral and acidic amino acids, a broad range of peptides could be expected. Nevertheless, by using SDS-PAGE, clearly distinctive bands above 10 kDa can be seen in the hydrolysates. This indicates that the degradation of the proteins develops according to a specific pathway, resulting in a limited set of intermediate peptides that are degraded upon further hydrolysis. The only exception to this seems to be a 24-kDa peptide derived from β-conglycinin, which seems to be a stable end-product instead of an intermediate peptide and seems to accumulate with increasing DH (Figures 3 and 4), because the intensity increases with increasing DH, whereas the intensity of bands at higher molecular weight decreases. This stepwise degradation, via intermediate peptides, was also observed during hydrolysis of α-lactalbumin with a *B. licheniformis*

protease, in which α-lactalbumin was degraded into various intermediates of which one specific peptide proved to be resistant to further hydrolysis.[30]

The broad specificity of subtilisin Carlsberg would theoretically result in a huge amount of peptides in the hydrolysates. Although, as described above, there seems to be some degree of specificity, it remains difficult to speculate on the aggregation mechanism of glycinin peptides based on their amino acid composition. Nevertheless, amino acids containing aromatic rings (Phe, Trp, and Tyr) seem to accumulate in the precipitate. This accumulation was mainly present in the aggregating peptides from glycinin, and almost absent in the precipitate of β-conglycinin (Figure 2). Because tryptophan and phenylalanine are relatively hydrophobic,[31] this may suggest that hydrophobic interactions play a dominant role in the aggregation of glycinin-derived peptides. This hypothesis can be confirmed by comparing the amino acid sequences of glycinin and β-conglycinin. If hydrophobic interactions play a dominant role in the aggregation of glycinin-derived peptides, then a relatively high proportion of aromatic amino acids should be present in the hydrophobic patches of glycinin. Therefore, hydrophobic patches were defined in all sequences of glycinin and β-conglycinin subunits, by calculating the hydrophobicity according to Eisenberg.[31] The calculation was performed using the ProtScale software from the Swiss Institute of Bioinformatics (http://www.expasy.org), using a "window size" of 15 (peptide range over which the average hydrophobic score is calculated). Those regions in the amino acid sequence for which the hydrophobic score was above 0, for at least 10 amino acids in a row, were regarded as hydrophobic patches. In these patches, as well as in the total protein, the proportion of aromatic amino acids (mol%) was calculated and is presented in Table 2.

The hydrophobic patches of the acidic polypeptides of glycinin and the basic polypeptide of glycinin 4 appeared to be more than 1.4 times higher than the average proportion of aromatic amino acids present in the complete protein (as indicated in gray). This indicates that the differences between the solubilities measured as the A_{220} and A_{280} could be due to the accumulation of the hydrophobic patches of the acidic polypeptides of glycinin and the basic polypeptide of glycinin 4 in the precipitates. For β-conglycinin and the basic polypeptides of glycinin 1, 2, 3, and 5, the aromatic amino acids are less accumulated in the hydrophobic patches, i.e., more evenly distributed over the sequence, resulting in a similar development of the solubility measured as A_{220} and A_{280}.

Besides aggregation of the hydrophobic patches, also peptides originating from the basic polypeptides of glycinin 1, 2, 3, and 5 were likely to be present in the precipitated glycinin-derived peptide fractions, because the basic polypeptides have a higher average hydrophobicity and p*I* than the acidic polypeptides as indicated in bold in Table 2.

Although for β-conglycinin, the proportion of aromatic amino acids, as well as the average hydrophobicity and the p*I*, are similar to those of the acidic polypeptide of glycinin, there are no indications that β-conglycinin-derived peptides show the same aggregation behavior at neutral pH.

Table 2 Proportion of aromatic amino acids in the proteins (mol%), average hydrophobicity and the p*I* of each polypeptide of glycinin and subunit of β-conglycinin, as well as the proportion of aromatic amino acids present in the defined hydrophobic patches (mol%)

	Subunit	Polypeptide	Proportion of aromatic amino acids in protein[a]	Proportion of aromatic amino acids in patches[a]	Average hydrophobicity[b]	p*I*
Glycinin	Gly 1	A1a	7.0	11.9	−0.20	5.2
		B2	8.3	8.6	**0.03**	**6.3**
	Gly 2	A2	6.8	10.6	−0.20	4.9
		B1a	8.3	7.9	**0.04**	**6.1**
	Gly 3	A1b	8.4	11.9	−0.19	5.1
		B1b	8.3	7.1	**0**	**8.0**
	Gly 4	A5	4.1	7.4	−0.03	5.5
		A4	5.1	11.6	−0.42	4.7
		B3	9.7	14.1	**0**	**9.3**
	Gly 5	A3	6.3	12.7	−0.24	5.0
		B4	8.1	11.1	**−0.02**	**9.5**
β-Conglycinin	α	–	7.6	7.1	−0.24	4.9
	α′	–	7.6	9.4	−0.24	5.6
	β	–	9.7	10.2	−0.11	5.2

[a] mol%.
[b] Average hydrophobicity of all the amino acids according to Eisenberg.[31]

Inhibition of aggregation

Glycinin hydrolysates have a solubility at pH 8.0 of ~40% and 60% for DH 2.2% and 6.5%, respectively, whereas the SPI and β-conglycinin hydrolysates are ~100% soluble (Figure 1). This is remarkable because glycinin accounts for ~64% of the total protein in SPI 64/36. Therefore, if it is assumed that glycinin, when present in SPI, is degraded into the same peptides as when hydrolyzed individually, a lower solubility of the SPI hydrolysate would be expected at pH 8.0. A possible explanation for this difference could be that in SPI, peptides derived from β-conglycinin are present that inhibit aggregation. This can also be observed in Figure 3, in which it can be seen that with increasing DH, the total amount of precipitating peptides in the SPI hydrolysate decreases relatively more than the total amount of larger peptides in the hydrolysate. This is illustrated by the peak representing the 24-kDa peptide in β-conglycinin. At DH 2.2%, the 24-kDa peptide is almost completely present in the pellet, whereas at DH 6.5% most of this peptide is present in the supernatant.

Therefore, it can be hypothesized that peptides are present that can inhibit or change aggregate formation in soy protein hydrolysates. This is in agreement with observations by Sodini et al.,[32] who observed that dairy yoghurts, supplemented with hydrolysates, showed a decreased complex viscosity with increasing supplementation. That peptides can change the properties of nonhydrolyzed proteins was also observed by Barbeau et al.,[33] who found that specific peptide fractions stabilized the structure of β-lactoglobulin and hypothesized that these peptides might have prevented interactions between denatured β-lactoglobulin and casein. The phenomenon that peptides can inhibit aggregation was also noticed in studies of blood platelet aggregation. It was found that milk- and soy-derived "bioactive" peptides could inhibit blood platelet aggregation.[12,13]

Our results indicate that in the case of soy proteins, the peptides derived from β-conglycinin are predominantly responsible for the inhibition of peptide aggregation, whereas the peptides derived from glycinin have the opposite effect. The presence of peptides that have the ability to prevent aggregation may be interesting for the food industry, for use in products where aggregation of proteins is unwanted.

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