JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2002, 50, 4275−4281 4275

Differences in Denaturation of Genetic Variants of Soy Glycinin

CATRIONA M. M. LAKEMOND,[†,‡] HARMEN H. J. DE JONGH,[‡,§]
HARRY GRUPPEN,[†,§] AND ALPHONS G. J. VORAGEN*,[†,§]

Center for Protein Technology TNO-WU, Wageningen, The Netherlands;
Wageningen Center for Food Sciences, Wageningen, The Netherlands; and
Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences,
Wageningen University, P. O. Box 8129, 6700 EV Wageningen, The Netherlands

In heat denaturation studies conducted in the past the genetic variants of glycinin have been considered as a homogeneous group of proteins. In this work the validity of this assumption was tested. It was found by calorimetric studies that glycinin denatures heterogeneously at pH 7.6. When the temperature of isothermal treatment is increased from 70 to 82 °C the proportion of glycinin remaining native progressively decreases from 95% to 5% while the denaturation temperature of the glycinin remaining native increases from 88.5 to 95 °C. Similar trends were found for pH 3.8. Fractionation and subsequent analysis (MALDI-TOF and CE) of isothermally treated samples demonstrated that at pH 7.6 the heterogeneous denaturation is caused by differences in thermal stability of the genetic variants of glycinin. The stability increases in the order G2/G3/G1 $<$ A_4 $<$ G5 $<$ G4.

INTRODUCTION

Soy proteins have found applications in many different food products. Generally, such food products are heated to inactivate anti-nutritional factors, for food preservation reasons, and to obtain desired functional properties. The major seed storage protein in soybeans is glycinin. It represents generally about 30% of the total protein in soybean, but it may vary from 25 to 50% depending on the soybean variety (*1*). Fischer and Goldberg (*2*) described three glycinin genes (G1−G3) and Scallon et al. (*3*) reported the existence of two additional genes (G4 and G5). The five genes have diverged into two subfamilies that are designated as Group I and Group II glycinin genes (*4*). The different genetic variants of glycinin are also referred to as "subunits" throughout the literature and in this work. The different glycinin subunits were identified on the basis of their amino acid sequences (*5*−*8*). Each subunit consists of an acidic and a basic polypeptide (*7*) linked by a single disulfide bridge (*9*), except for the acidic polypeptide A_4 present in G4 (*7*). The two polypeptide chains result from posttranslational cleavage of proglycinin precursors (*10*). **Table 1** shows the molecular weights of different glycinin subunits as calculated from the reported DNA sequences of the different glycinin genes.

Indications exist that more than five different variants (subunits) of glycinin are present (*12, 16, 17*). Nielsen et al. (*12*) suggested the presence of more than five genes, but it was also suggested that glycinin undergoes, like other 11S storage proteins, a complex series of posttranslational events (*18*). Although glycosylation of glycinin is still questioned (19), some studies report that a small portion of glycinin is glycosylated (*20, 21*), but this is contradicted by others (*22*). It is, however, important to realize that the precise subunit composition depends on the soybean variety used (*16*).

It was found that glycinin associated into 7S or 11S aggregates depending on the type of glycinin subunits present (*23, 24*). Considering 7S glycinin denatures at lower temperatures than 11S glycinin (*25, 26*), it could be speculated that there is a relation between denaturation temperatures and subunit composition. However, in articles in which models for glycinin heat denaturation are proposed (for example in Peng et al. (*27*) and Yamauchi et al. (*1*)) glycinin has never been considered to be heterogeneous. This complication may have contributed to the fact that the glycinin heat denaturation mechanism has not been elucidated fully.

Table 1. Molecular Weights (exclusive signal and propeptides) of the Five Different Glycinin Subunits and Their Polypeptides (A, acidic; B, basic) as Calculated from Their Amino Acid Sequences as Derived from the DNA Sequence of the Five Different Glycinin Genes[a]

subunit	MW (kDa) of subunits	polypeptides	molecular weight (kDa) of polypeptides
G1	52.6	$A_{1a}B_2$	32.6; 20.0
G2	51.3	A_2B_{1a}	31.5; 19.8
G3	51.3	$A_{1b}B_{1b}$	31.5; 19.8
G4	61.2	$A_5A_4B_3$	10.5; 30.0; 20.7
G5	54.4	A_3B_4	36.4; 19.0

[a] Based on Scallon et al. (*11*); Nielsen et al. (*12*); Sims and Goldberg (*13*); Thanh et al. (*14*); and Cho and Nielsen (*15*).

* To whom correspondence should be addressed. Fax +31 317 484893; tel +31 317 483209; e-mail Fons.Voragen@chem.fdsci.wag-ur.nl.
[†] Center for Protein Technology TNO-WU.
[‡] Wageningen Center for Food Sciences.
[§] Wageningen University.

10.1021/jf0110405 CCC: $22.00 © 2002 American Chemical Society
Published on Web 06/14/2002

The present work studied whether the different genetic variants of glycinin display different thermal stabilities. We compare heat denaturation at pH 7.6, a pH often used in soy protein in the literature, with that at pH 3.8, which is more representative for conditions present in food (pH 3−7) (*36*). Because glycinin is insoluble between pH 4 and 6 at the ionic strength (0.2) used (*26*), which gives experimental complications, intermediate pH values between were not studied.

MATERIALS AND METHODS

Glycinin Purification and Preparation of Glycinin Dispersions. Glycinin was purified from William's 82 soybeans (harvest 1994). Milling and defatting of the soybeans was performed according to Lakemond et al. (*28*). Because the impact of the de-fatting procedure on the protein's native state is often disputed, we chose to perform this fat extraction process at low (15 °C) temperature. Glycinin was extracted and purified according to a modified method of Thanh and Shibasaki (*29*).

A soy protein isolate was obtained after extraction of protein at pH 8.0 from the defatted soy meal and subsequent acidic precipitation at pH 4.8. The precipitate obtained was resolubilized at pH 7.8, and subsequently a crude glycinin fraction was obtained by acidic precipitation at pH 6.4. The pelleted glycinin was resolubilized in 10 mM potassium phosphate buffer at pH 7.8 containing 10 mM 2-mercaptoethanol. For further purification ammoniumsulfate was added to the resolubilized crude glycinin fraction up to 50% saturation. After this was centrifuged for 30 min at 12 000*g* and 4 °C, more ammoniumsulfate (70% saturation) was added to the supernatant. The precipitate obtained after centrifugation (30 min, 12 000*g*, 4 °C) was resolubilized in a 10 mM potassium phosphate buffer at pH 7.8 in the presence of 10 mM 2-mercatoethanol and 20% glycerol. Batches of this fraction, purified glycinin, were stored at −40 °C. After defrosting part of the purified glycinin, it was extensively dialyzed against Millipore filtered water, and then freeze-dried (storage at −20 °C). The purity of glycinin was determined by SDS−PAGE under reducing and nonreducing conditions on a Phast System (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Gradient gels (10−15%) were used, which were stained with Coomassie brilliant blue. The gels were calibrated with low-molecular-weight markers ranging from 14 to 94 kDa (Pharmacia). The purity of glycinin was estimated to be over 95% by densitometric analysis of the gels.

Prior to an experiment, 1% (w/v) glycinin was suspended in 35 mM potassium phosphate buffer at pH 7.6 containing 0.1 M NaCl. For experiments to be performed at pH 3.8, the pH was adjusted by adding HCl. The ionic strength was increased by ∼0.015 by the amount of HCl needed.

Differential Scanning Calorimetry (DSC). For experiments carried out at pH 7.6, DSC thermograms were recorded on a micro DSC III (Setaram, Caluire, France) using 0.9-mL vessels. For the experiments carried out at pH 3.8, a VP-DSC MicroCalorimeter (MicroCal Incorporated, Northhampton MA) with 0.5-mL cells was used because the micro DSC III was not sensitive enough to carry out the experiments at pH 3.8. Glycinin suspensions (1% w/v) at pH 7.6 and 3.8 were (partly) heat denatured consecutively by heating from 20 to 53−83 °C at a rate of 1 K/min, keeping the temperature constant at set temperatures between 53 and 83 °C for 16 h and cooling to 20 °C at a rate of 1 K/min. This sequence of heating conditions is further denoted as isothermal treatment. The total time for the isothermal treatment step was set at 16 h to ensure the denaturation process was in an equilibrium state. Next, the samples were reheated from 20 to 115 °C at a rate of 1 K/min and subsequently cooled to 20 °C to determine the proportion of denaturation and the denaturation temperature of the protein fraction unaffected by the isothermal treatment (referred to as glycinin "remaining native"). The denaturation temperature T_d is defined as the temperature at maximum heatflow of the endothermic transition. The proportion of denaturation was calculated from the enthalpy remaining after isothermal treatment divided by the total enthalpy of unheated glycinin. At pH 3.8 the inaccuracy of the data obtained was about 10% (σ_{n-1}), and at pH 7.6 it was about 3% (σ_{n-1}), as was derived from duplicate experiments.

Isolation of Glycinin Remaining Native after Isothermal Treatment. At pH 7.6 glycinin samples of typically 1 mL were (partly) heat denatured by heating according to the temperature profile described above for the isothermal treatment. To isolate the glycinin fractions remaining native, first a supernatant was obtained by centrifugation (30 min at 15 800*g*) at 20 °C. The native protein fraction was separated from the soluble fraction by gel permeation chromatography (described below). Protein that did not dissociate or aggregate (corresponding to 11S and 15S glycinin) after heat treatment was considered to be native and will be referred to as "purified native glycinin". These fractions were analyzed for their subunit composition by anion exchange chromatography. It should be noted that it cannot be excluded that nondenatured proteins could get encapsulated in aggregates of denatured proteins and are as a result not included in the fraction defined as "remaining native".

Gel Permeation Chromatography. Gel permeation chromatography was performed at 20 °C on a Superdex 200 column 10/30 (Pharmacia, Uppsala, Sweden) attached to an FPLC system (Pharmacia). Typically 200-μL samples were applied on the column. The elution buffer consisted of 35 mM potassium phosphate and 0.1 M NaCl at pH 7.6. The flow rate was 1 mL/min and the eluent was monitored at 280 nm. The column was calibrated with a high-molecular-weight gel filtration calibration kit (Pharmacia; 152−669 kDa).

Separation and Identification of the Genetic Variants of Glycinin. At pH 7.6 anion exchange chromatography was used to separate unheated glycinin into several fractions. Anion exchange chromatography was performed at 20 °C on a mono Q HR 5/5 column (Pharmacia) attached to an FPLC system (Pharmacia). First, the protein was brought onto the column with 10 mM potassium phosphate buffer containing 6 M urea at pH 6.6 and eluted afterward in the same buffer. The protein bound to the column was eluted using a linear gradient from 0 to 0.5 M NaCl in the same buffer over 17.5 column volumes. The flow rate was 1 mL/min and the eluent was monitored at 280 nm. The different fractions pooled were analyzed for subunit composition by MALDI-TOF MS spectroscopy and by capillary electrophoresis (CE). These techniques are all described below in more detail. Prior to MALDI-TOF MS analysis the acidic polypeptides were separated from the basic polypeptides with anion exchange chromatography as described previously (*28*). These fractions were also analyzed with CE.

MALDI-TOF MS Spectroscopy. MALDI-TOF MS spectroscopy was performed using a Voyager DE RP instrument (PerSeptive Biosystems, Framingham, MA). To prepare the matrix, 10 mg/mL of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma D-7927, St. Louis, MO) in 0.6% (v/v) aqueous trifluoroacetic acid was mixed with acetonitrile in a ratio of 7:3 (by volume). The samples were diluted 10 times with matrix and allowed to crystallize for 30 min on a gold-plated welled plate. External calibration was performed according to the description of the manufacturer using insulin (5734 Da), cytochrome C (12361 Da), myoglobulin (6952 Da), and BSA (66431 Da) to obtain an inaccuracy of <50Da.

Capillary Electrophoresis. Capillary electrophoresis was performed on a Beckman P/Ace system 5500 equipped with a diode array detector (Beckman Instruments, Fullerton, CA). Separation was obtained using a 50-μm hydrophilic-coated capillary of 57 cm (Celect P-150, Supelco, Bellefonte, PA). The fractions were analyzed at a concentration of 5 mg/mL in a 10 mM potassium phosphate buffer containing 6 M urea and 4.5 mM DTT at pH 6.6. The running buffer contained 8 M urea, 0.38 M citric acid, and 0.05% methyl-hydroxy-ethyl-cellulose at pH 2.7. The separation voltage was 25 kV, the temperature was 45 °C, detection was at 214 nm (data collection rate 1 Hz), and injections were carried out by pressure (injection time 10 s). Replicate experiments were performed.

RESULTS

Progressive Denaturation of Glycinin. DSC measurements were performed to establish glycinin fractions denatured to different extents at pH 7.6 and pH 3.8. In **Figure 1** the influence of the isothermal treatment on the proportion of glycinin remaining native after isothermal treatment at pH 7.6 and 3.8 is presented using the enthalpies of the endothermic transitions

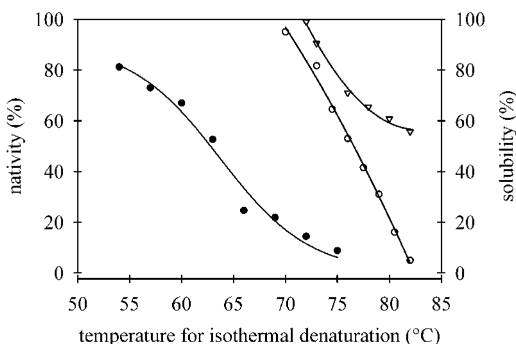

Figure 1. Influence of the isothermal temperature (heating during 16 h) on the proportion of glycinin remaining native at pH 3.8 (●) and pH 7.6 (○) (DSC measurements) and the solubility at pH 7.6 (△).

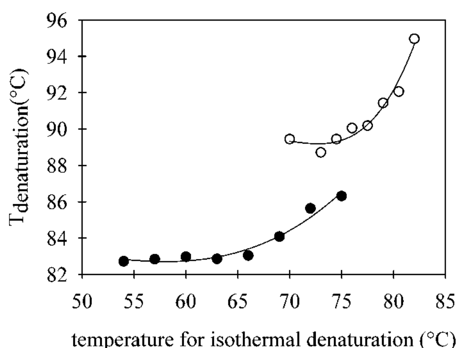

Figure 2. Denaturation temperatures of the protein remaining native after isothermal treatment at different temperatures at pH 3.8 (●) and pH 7.6 (○) as obtained with DSC.

of unheated glycinin as a reference value. At pH 7.6 it is found that when the heating temperature increases from 70 to 82 °C the proportion of glycinin remaining native progressively decreases from about 95 to 5%. At pH 3.8 it is found that when the isothermal heating temperature is increased from 54 to 75 °C the proportion of native glycinin decreases progressively from 81 to 9%. From 54 to about 66 °C only 7S glycinin denatures, and at higher temperatures the 11S form also starts to denature, since it is known from earlier work (*26*) that at pH 3.8 two endothermic transitions (at 68 and 82 °C) are observed that are related to the presence of both a 7S and an 11S form. In addition, at pH 7.6 only one endothermic transition is observed (at 87 °C) because at this pH glycinin is present in the 11S form only (*26*).

To investigate whether the denaturation process is measured in an equilibrium state, we compared heating for 16 h to heating for 24 h at 77.5 °C at pH 7.6. The prolonged incubation shows that the native state of the glycinin decreases with only additional 4%, which is within the experimental error of the enthalpy determination. Thus, the samples can be assumed to be in an equilibrium state. The fact that it is possible to obtain glycinin fractions that are denatured to different extents points at a heterogeneous heat denaturation process at both pH 7.6 and 3.8.

Furthermore, it was observed that at pH 7.6 the denaturation temperature of the protein remaining native increases from 88.5 to 95 °C as the temperature of isothermal treatment increases from 70 to 82 °C (**Figure 2**), suggesting differences in the thermostability. For pH 3.8 a similar trend is observed. Only the denaturation temperature of the 11S glycinin transition at low pH is shown in **Figure 2**, because the denaturation temperatures of the 7S form could not be determined accurately (the relatively smaller 7S endotherm is not separated well from the 11S endotherm).

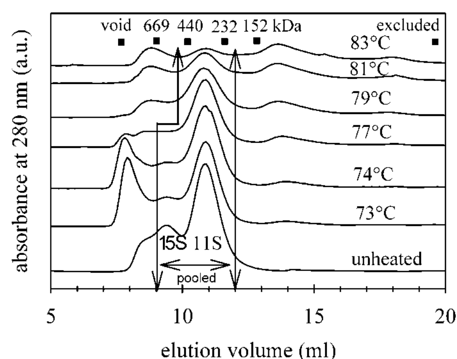

Figure 3. Elution patterns of the soluble fractions obtained after isothermal treatment at pH 7.6 at different temperatures on a Superdex 200 10/30 column. Fractions were pooled to obtain the purified native protein as indicated.

Aggregation State of Glycinin after Isothermal Treatment. To investigate the aggregation state of the glycinin fractions after isothermal treatment, the amount of protein remaining soluble was determined at pH 7.6 (**Figure 1**). The results indicate that upon increasing the temperature of isothermal treatment to 82 °C the solubility decreases progressively to about 50% (solubility of unheated glycinin was set at 100%), while the protein was denatured over 95%. Such values are in agreement with previous reports (*37, 38*). The soluble protein fraction consists of protein aggregates smaller than approximately 0.2 μm, as calculated using the Stokes equation from the *g*-force applied.

Gel permeation chromatography was used to separate aggregated or dissociated protein from native protein (11S and 15S glycinin). **Figure 3** shows the elution patterns of the different soluble fractions obtained after isothermal treatment at different temperatures. The elution patterns in **Figure 3** show that the unheated glycinin is predominantly present in the 11S form, which is in agreement with observations throughout the literature. Also a 15S fraction was present and a fraction consisting of even larger aggregates (together ∼20−30%). Upon increasing the temperature, the intensity of the 11S and 15S peaks decreases progressively. At 73 and 74 °C a large void peak is observed, containing aggregates with apparent molecular weights larger than 700 kDa. At higher temperatures these large aggregates have disappeared, and in the elution patterns also a protein peak with a molecular weight below 152 kDa occurs. This dissociation is probably linked to the disruption of the disulfide bridge between the acidic and basic polypeptides, after which the basic polypeptides precipitate and the acidic polypeptides stay soluble (*26*). The fractions containing native protein in an 11S or 15S structural organization are pooled for further analysis as indicated in **Figure 3**.

Analysis of the Purified Native Glycinin. The purified native protein fractions obtained by GPC after isothermal treatment at different temperatures were analyzed with anion exchange chromatography to study whether the different subunits of glycinin denature at different temperatures (at pH 7.6). In **Figure 4** the elution patterns of the different purified native glycinin fractions are presented. In the elution pattern of the unheated sample five major fractions are observed, one in the nonbound material (fraction a) and four in the bound material (fractions b−e). Fraction a is still present at a temperature at which fractions b−e can no longer be observed. When the temperature is increased the intensity of fraction c decreases somewhat faster than the intensity of fraction b. With increasing temperature, the intensity of fraction e decreases relatively faster than that

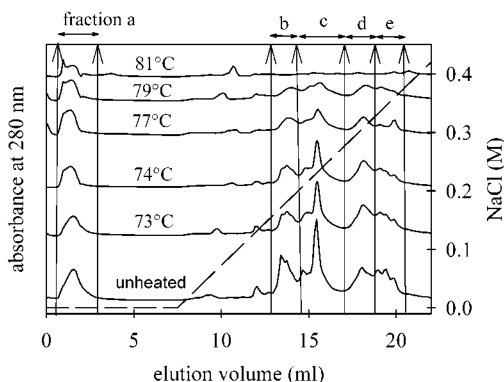

Figure 4. Elution pattern of purified native glycinin fractions obtained after isothermal treatment at different temperatures on a mono Q column. The arrows indicate the different protein fractions. The NaCl gradient is indicated with a dashed line.

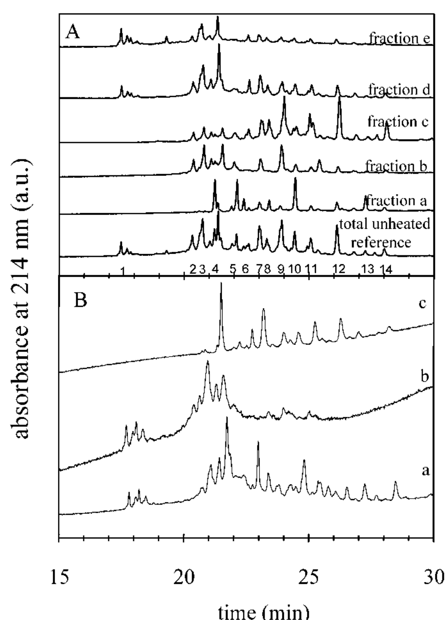

Figure 5. (A) Electropherograms of the different peaks that were obtained after separation of unheated glycinin on a mono Q column as shown in **Figure 4**, fraction a. (B) Electropherograms of all polypeptides (a), the acidic polypeptides (b), and the basic polypeptides (c) present in fraction (d) (see panel A) derived from unheated glycinin.

of fraction d. The intensity of fractions b and c decreases at lower temperatures compared to that of fractions d and e.

Separation and Identification of the Different Glycinin Subunits. To analyze the protein composition in the various anion exchange chromatography fractions obtained of unheated glycinin (**Figure 4**) both CE and MALDI-TOF MS were used. The CE electropherograms of the different fractions of unheated glycinin show that the different polypeptides in the fractions have retention times varying from about 17 to 28 min (**Figure 5A**). The number of peaks in the electropherogram (over 25 in the total unheated reference sample) exceeds the number of polypeptides identified from the literature (**Table 1**). Significant differences between CE electropherograms are observed when a comparison is made between the electropherograms of fractions a to e, indicating that the different fractions of the unheated glycinin contain multiple (genetic) variants of glycinin. It is possible to identify which peaks originate from basic or acidic polypeptides by separating the acidic from the basic polypeptides prior to capillary electrophoresis as described previously (*26*), as is shown for fraction d (**Figure 5B**). It can be concluded that the peaks present around 18 min originate from acidic polypeptides. Between 20 and 22 min about five peaks from acidic polypeptides, and one from basic polypeptides, are visible. Furthermore, the electropherogram of the basic polypeptides shows over seven peaks with retention times from 22 to 27 min.

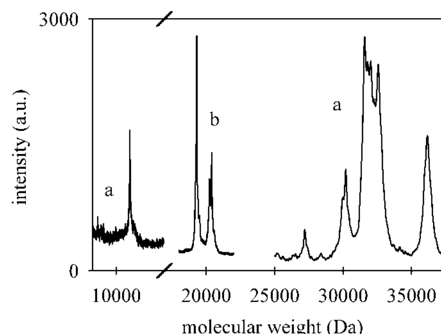

Figure 6. MALDI-TOF MS spectra of the acidic (a) and basic polypeptides (b) of total (nonfractionated) unheated glycinin.

MALDI-TOF MS spectrometry was used to analyze the subunit composition of the fractions obtained after anion exchange chromatography of unheated glycinin (**Figure 4**). The MALDI-TOF MS spectra (**Figure 6**) of the acidic polypeptides present in the total (nonfractionated) unheated glycinin sample show five major peaks at ~10.6, 30.2, 31.6, 32.6, and 36.2 kDa. The spectrum of the basic polypeptides shows three major peaks at 19.3, 20.2, and 20.4 kDa. In the spectra of different fractions obtained after anion exchange chromatography of unheated glycinin, however, a peak at 20.3 kDa was also clearly visible (spectra not shown).

The peaks could be assigned to specific acidic and basic polypeptides (**Table 2**) on the basis of their theoretical molecular weights (**Table 1**). Please note that the masses listed in **Table 2** refer to the values reported in **Table 1** rather than those determined by MS directly for the reason of clarity. Potential discrepancies in masses can be attributed to inaccuracies in the MS method and the fact that primary sequences were used to calculate the masses in **Table 1.** It was not possible to distinguish between the polypeptides in G2 and G3 because they have comparable molecular weights (**Table 1**). The peaks observed at 20.2 and 20.3 kDa could not be assigned unambiguously, because the observed molecular weights do not exactly match the theoretical molecular weights (**Table 1**).

From the MALDI-TOF MS spectra of the different fractions indicated in **Figure 4** it was derived which subunits or polypeptides were present as major or minor components (**Table 2**). It was assumed that the different acidic and basic polypeptides ionize similarly. Fraction a (**Figure 4**) contained mainly subunit G4 (without polypeptide A_4). In fraction b subunit G1 was present, whereas fraction c contained predominantly subunits G2 and/or G3. The main components of fraction d were subunit G5 and the acidic polypeptide A_4. Fraction e contained predominantly the acidic polypeptide A_4. This acidic polypeptide, which is part of glycinin G4, is not covalently bound to a basic polypeptide (*7*) and elutes, therefore, in a different fraction than the polypeptides A_5 and B_3, the other constituents of G4. Each fraction also contained minor components as indicated in **Table 2**.

We attempted to designate the different peaks in the CE electropherogram of the unheated sample (14 major peaks were identified) to specific basic or acidic polypeptides using the data from **Table 2** (based on MALDI-TOF MS spectra). The results

Table 2. Subunit Composition of the Different Purified Native Glycinin Fractions as Determined Using MALDI-TOF MS[a,b]

	$M_{w\ acidic\ polypeptides}$ (kDa)					$M_{w\ basic\ polypeptides}$ (kDa)				subunit/polypeptide	
	A_5	A_4	A_2/A_{1B}	A_{1A}	A_3	B_4	B_{1A}/B_{1B}	B_{1B}/B_2	B_3		
ref.	10.5	30.5	31.5	32.6	36.4	19.0	20.0	20.0	20.7	major	minor
fr. a	++							+	++	G4[c]	B/B_2
fr. b				++				++	+	G1	B_3
fr. c			++	+			++	+		G2/G3	G1
fr. d	+	++	+	+	++	++	+	+	+	G5, A_4	G1,G2/G3,G4
fr. e	+	++	+	+	+	+	+	+	+	A_4	G1,G2/G3,G4,G5

[a] The masses indicated refer to those reported in **Table 1** rather than the actual determined MS data. ++ = major component; + = minor component; A = acidic polypeptide; B = basic polypeptide. [b] The peaks (**Figure 6**) present in the unheated glycinin were assigned to specific basic and acidic polypeptides. Based on the compositions of the different fractions obtained, the subunits present in each purified native glycinin fraction were assigned. [c] Subunit G4 did not contain the acidic polypeptide A_4.

Table 3. Identification of the Different Peaks Present in the Capillary Electrophoresis Electropherogram of the Total Unheated Reference Sample (**Figure 5A**)

peak	polypeptide	peak	polypeptide
1	A_4	7	B_{1A}/B_{1B}
2	A_{1A}/A_5[a]	8	A_2/A_{1B}
3	A_{1A}	9a	B_2
4a	B_4	9b	A_2/A_{1B}
4b	B_3	10	A_5
4c	A_3	11	A_2/A_{1B}
4d	A_{1A}	12	B_{1A}/B_{1B}
5	B_3	13	A_5
6	B_4[a]	14	B_{1A}/B_{1B}

[a] Peak constituent uncertain.

are given in **Table 3**. Because the different fractions all contained minor constituents we assumed that a major component of a specific fraction gave rise to major contributions in the electropherograms.

DISCUSSION

Separation and Analysis of the Different Glycinin Subunits. To investigate the heterogeneous denaturation of glycinin, a procedure was developed to separate the different genetic variants of glycinin. It was proven that it is possible to separate the genetic variants of glycinin subjected to isothermal treatment with anion exchange chromatography by analyzing the different fractions obtained with capillary electrophoresis (**Figure 5**) and MALDI-TOF MS analysis (**Table 2**). The results are in line with the work of Staswick et al. (*7*), who identified the different genetic variants on the basis of their amino acid compositions.

The number of polypeptides identified by MALDI-TOF (5 acidic and 4 basic polypeptides; **Table 2**) is in line with the number of polypeptides identified in the literature (*2, 3*; **Table 1**). The number of peaks in the CE electropherogram (over 25 in the unheated glycinin sample) exceeds the number of polypeptides identified from MALDI-TOF MS spectrometry. The number of five genetic variants consisting of 11 different polypeptides in total (*2, 3*; **Table 1**) seems to be insufficient to explain the CE electropherograms, supposing that one specific polypeptide leads to one peak in the electropherogram. The large number of peaks in the CE electropherogram could be explained by the presence of more than five (genetic) variants as is also suggested by others (*12, 16*). These glycinin variants possibly do not differ much in molecular weight, which could explain the wide peaks in the MALDI-TOF data. CE is a very sensitive method and could be of future interest to analyze glycinin fractions for subunit composition on the condition that the peak composition in the electropherograms is studied more thoroughly. Conclusively, the above-described analyses are sufficient to investigate the possible relation between the genetic variants and the glycinin structural stability upon isothermal treatment.

Heterogeneous Denaturation of Glycinin Subunits. At pH 7.6, at which glycinin is present in the 11S form (*28*), the different genetic variants of glycinin display a different thermostability (**Figure 4**; **Table 2**). Although the intensity of genetic variants G2 and G3 (fraction c) decreases somewhat more progressively than the intensity of genetic variant G1 (fraction b) (**Figure 4**), this difference is not designated as significant. The free acidic polypeptide A_4 denatures at lower temperatures than G5, because the intensity of fraction e decreases relatively quicker than that of fraction d. The fact that the intensity of G1 and G2/G3 (fractions b and c) decreases less progressively compared to that of G5 and A_4 (fractions d and e) means that G1 and G2/G3 denature at lower temperatures than G5 and A_4. The intensity of fraction a, in which G4 (without the polypeptide A_4) is present, is still present at a temperature at which fractions b−e can no longer be observed. This means that the denaturation temperature of the glycinin subunits increases in the order G2/G3/G1 < A_4 < G5 < G4 at pH 7.6. It is interesting to observe that A_4 is less stable than A_5B_3, because this implies that G4 does dissociate while it has no impact on the thermostability of A_5B_3. The fact that it is possible to obtain glycinin fractions that are denatured to different extents (**Figure 1**), and that increasing denaturation temperatures of the native glycinin fractions were observed after increasing the temperature for isothermal treatment (**Figure 2**), also point at a heterogeneous heat denaturation of glycinin at pH 7.6.

At pH 3.8 (which is a pH more representative of conditions occurring in foods than pH 7.6), it is found that glycinin also denatures heterogeneously because (a) it is possible to obtain glycinin fractions that are denatured to different extents (**Figure 1**) and (b) the heat denaturation temperatures of the purified native glycinin fraction increases as the temperature of isothermal denaturation increases (**Figure 2**). It is likely that, in analogy to that at pH 7.6, the heterogeneity in heat denaturation at pH 3.8 is linked to different thermal stabilities of the genetic variants of glycinin. However, the situation at pH 3.8 is more complicated than that at pH 7.6, because this fraction does not only contain glycinin in the 11S form but also in the 7S form, which denatures at lower temperatures than the 11S form (*26*). Gel electrophoresis showed that at pH 3.8 the 7S form contained the acidic polypeptide A_3 and the 11S form did not (results not shown), which is in line with the results of others (*23, 24*). This means that a relation exists between aggregate size and subunit type at pH 3.8. It was not studied whether the order of denaturation is equal to that at pH 7.6.

Because heating took place for 16 h it is expected that in this study glycinin could become deamidated during isothermal treatment. For example, significant deamidation levels are reached after heating for only 1 h at 95 °C (22%) (*30*) and for 9 h at 70 °C (25%) (*31*). Although we cannot exclude that deamidation of glycinin occurs at the temperatures used, we believe it does not interfere with the interpretation of results, as capillary electrophoresis provides no indication of significant amounts of deamidation in the heat-treated materials (not shown). **Figure 2** shows that heating of glycinin for 16 h at 52−68 °C did not influence the denaturation temperature of 11S glycinin. In the literature also there was no evidence found for a relation between increasing denaturation temperatures and increasing deamidation levels (*32, 33*).

The genetic variant composition of glycinin is of industrial relevance because, for example, Mori et al. (*34*) and Tezuka et al. (*35*) found links between gelation properties and subunit composition. The fact that it is known that 7S glycinin denatures at lower temperatures (over 10 °C difference) than 11S glycinin, combined with the fact that glycinin associates into 7S or 11S aggregates depending on the types of genetic variants present (*23, 24*), possibly explains the relation that is found between subunit composition and gelation.

CONCLUSION

It is concluded that the thermal stability of the different genetic variants of glycinin increases at pH 7.6 in the order G2/G3/G1 < A_4 < G5 < G4 (without A_4). At pH 3.8 the genetic variants display a different thermostability. Therefore, glycinin cannot be considered as a homogeneous group of proteins as has been the case in many heat denaturation studies.

ACKNOWLEDGMENT

We thank Jolan de Groot of the Laboratory of Food Chemistry, Wageningen University, for performing the capillary electrophoresis experiments.

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Received for review August 6, 2001. Revised manuscript received April 3, 2002. Accepted April 17, 2002.

JF0110405