JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Novel Method for the Production of Pure Glycinin from Soybeans

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A novel method for the purification of glycinin from soy meal is presented. The method is based on the isoelectric precipitation of glycinin by using carbon dioxide as a volatile precipitant. Gaseous CO_2 was pressurized into the protein solution, thus lowering the pH and initiating glycinin precipitation. Pressurization and, consequently, acidification were done in a slow and controlled manner, with the end point of pH 6.4. The acidity of the protein solution was well controlled via the pressure of gaseous CO_2. In this way simultaneous precipitation of other soybean proteins was prevented and very pure glycinin was obtained. Approximately 40% of the glycinin present in the protein solution was recovered with purity as high as 98%. The purification process was successfully performed on both small and large scales, without affecting glycinin purity.

KEYWORDS: Soybean proteins; purification; glycinin; carbon dioxide; isoelectric precipitation

INTRODUCTION

Soybean Proteins. Soybeans contain a broad range of proteins, which are classified in different terms (*1*). On the basis of their solubility they are classified as globulins or albumins. Glycinin is the most abundant protein found in soybeans. It represents ∼25−35% of total seed protein and accounts for >40% of the total soybean globulin. It is widely used in the food industry as a filling agent due to its favorable gelatinous properties in aqueous solutions (*2*), as well as for its emulsifying and foaming activities (*3*). Although it has been known and used for years in the food industry, to this date a fast, efficient, and inexpensive process for obtaining pure glycinin from soy meal has not yet been developed.

Nowadays pure proteins appear in different forms, mostly depending on their application and current state of the art of their production. In general, spray-dried proteins, protein crystals, and liquid protein preparations are found. Dry products have the advantage of stability over a long period of time. When it comes to production of pure protein in dry form, in most cases crystallization is a key step (e.g., in the production of pure enzymes). With finely tuned process parameters, crystallization enables recovery of a protein in a solid form with high purity grade.

Glycinin is a hexameric protein comprised of five subunits (*4*). Each subunit comprises an acidic (molecular mass ∼40 kDa) and a basic (molecular mass ∼20 kDa) polypeptide, linked by a single disulfide bridge (*5*). These polypeptides are highly heterogeneous (*6*). Different glycinin subunits and corresponding glycinin genes have been identified and reported by various authors (*3*). Furthermore, different soybean cultivars appear to differ in content of the polypeptides constituting glycinin molecules (*4*). Glycinin molecules from different origins were proved to have different properties with respect to amino acid composition, molecular weight, and isoelectric point (*7*). It was also reported that glycinin from the same origin exhibited different compositions and properties. Due to the high heterogeneity of glycinin molecules, they are not prone (and have not been reported) to crystallize from natural sources. A satisfying alternative for this essential step does not seem to be easily developed.

Another abundant globulin protein found in soybeans is β-conglycinin, which represents ∼35% of soluble soy proteins. β-Conglycinin consists of three subunits, α, α', and β, with molecular masses of 76, 72, and 53 kDa, respectively. The subunits associate randomly into trimers of molecular masses of 150−220 kDa (*6*). Both glycinin and β-conglycinin are acid precipitable. Glycinin has an isoelectric point in the pH range from 5.2 to 4.9, and that of β-conglycinin is in the pH range of 5.0−4.7. However, it has been observed by many authors (*8*−*10*) and confirmed in this work as well (**Figure 1**) that both proteins precipitate readily and abundantly as soon as the pH of the solution decreases below 6.5.

Conventionally strong mineral acids, such as hydrochloric and sulfuric acid, are used for isoelectric protein precipitation of soybean proteins. Simultaneous precipitation of glycinin and β-conglycinin during mineral acid precipitation is inevitable due to the occurrence of local pH undershoot in the precipitation vessel despite considerable mixing introduced to the system.

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10.1021/jf0478206 CCC: $30.25 © 2005 American Chemical Society
Published on Web 05/26/2005

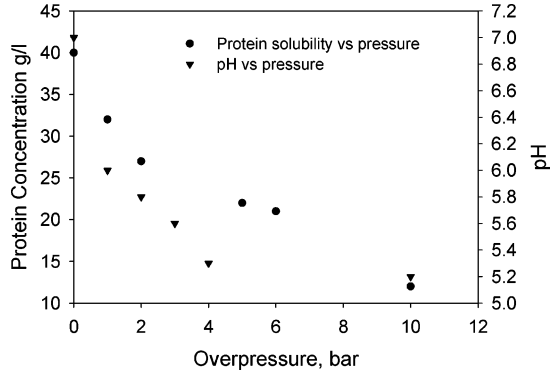

Figure 1. Overall solubility of soybean globulins as a function of pH and pressure.

Herein lies the main difficulty for recovery of pure glycinin from soy meal.

State of the Art of Glycinin Purification. Production of pure glycinin is attractive for several reasons. First, the ability to easily produce very pure glycinin on a laboratory scale would enable extensive research on the protein properties. Second, it would be possible to investigate the behavior of glycinin under various conditions, which would enable prediction of glycinin behavior under different process conditions. For example, not much is yet known on glycinin behavior at a molecular level under the conditions present in food. This behavior is largely dependent on various parameters such as ionic strength, pH, process temperature, and presence of other molecules. The influence of these parameters on glycinin solubility and, hence, gelation, emulsifying, and foaming activities could be studied in more detail, provided that enough glycinin of analytical purity was obtainable. Finally, with a sufficient amount of very pure glycinin easily available, its application potential could be explored more widely, not just in the food industry, where it could be used in pure form (and not as enriched precipitate), but also in other industries (pharmaceuticals, to name one), as well as for the development of specific product formulations.

Recovery of pure glycinin has been reported by a number of authors. The most common method is the isoelectric fractionation procedure described by Thanh and Shibasaki (*11*). Briefly, defatted soybean meal is extracted with 0.03 M Tris buffer, pH 8.0. containing 0.01 M 2-mercaptoethanol at room temperature for 1 h. The extract is adjusted to pH 6.4 with 2 M HCl. The glycinin fraction is collected by centrifuging and dialyzing the extract at pH 6.4 for 3 h. According to the authors, the purity of the obtained glycinin was 90%, with an additional 10% representing the β-conglycinin contamination.

Lakemond et al. (*2*) have reported an exhaustive procedure for glycinin purification, which represents a modified method of Thanh and Shibasaki (*11*). A soy protein isolate was obtained from defatted soy by extraction at pH 8.0 and acidic precipitation at pH 4.8. The precipitate was resolubilized at pH 7.8. Crude glycinin was obtained by acidic precipitation at pH 6.4, and the pellet was resolubilized in 10 mM potassium phosphate buffer at pH 7.8 containing 10 mM 2-mercaptoethanol. Further purification was achieved by salting out with ammonium sulfate and extensive dialysis against Millipore water. The final product was freeze-dried, and the purity of the glycinin, determined by SDS-PAGE, was estimated to be 95%.

Neither of the two references gives clear indication of the yield achieved.

Glatz et al. (*12*) investigated the influence of the acidification rate and mixing during isoelectric fractionation of glycinin and β-conglycinin. They reported obtaining two enriched protein fractions (~50% purity), at pH 6 and 4.8, using 0.1 M HCl as a precipitant. According to the authors, neither the acid addition rate nor the mixing rate had any influence on the overall protein yield and fraction composition.

Table 1. Henry's Coefficient for CO_2 at Different Pressures and Temperatures [from Hofland et al. (*14*)]

	5 °C, 1 bar	5 °C, 25 bar	25 °C, 1 bar	25 °C, 25 bar
H_{CO_2} (kg·bar/mol)	15.44	15.96	29.08	30.01

Wu et al. (*13*) scaled up the soybean fractionation process to the pilot plant scale, where different soy protein fractions (glycinin, intermediate mixture, and β-conglycinin) were obtained in amounts in the order of kilograms. Protein-rich fractions (>90% of protein) were obtained by means of acidification with 2 M HCl to different pH end points (6.4 for glycinin and 4.5 for β-conglycinin). The end product was extensively desalted by means of diafiltration. The authors reported glycinin purity of 90.4%, with yield of 10% per obtained fraction.

The use of CO_2 as a precipitant for isoelectric precipitation of soybean proteins has been previously reported by Hofland et al. (*14*). The authors also reported the use of CO_2 for a large-scale isoelectric fractionation of the two major soybean globulins, glycinin and β-conglycinin (*10*). The method involved three-step CO_2 precipitation resulting in three fractions: enriched glycinin (with a maximum purity of 95%), a mixture of the glycinin and β-conglycinin, and enriched β-conglycinin (with a maximum purity of 80%). The reported glycinin yield was 28%. The possibility of obtaining glycinin of very high purity and with higher yields by using CO_2 has not been reported yet.

In this paper we present a method based on CO_2-aided protein precipitation. Glycinin is obtained in a series of consecutive precipitation steps. In comparison with the above-described methods, CO_2-aided protein precipitation relies on only one precipitant, gaseous CO_2. Use of volatile acid brings several advantages, such as mild conditions during precipitation and no protein denaturation. More importantly, when using CO_2, the end product does not contain any salts or acids, so extensive downstream treatments such as dialysis are avoided as are considerable salt waste streams after the product removal.

CO_2-Aided Precipitation. CO_2 is a volatile electrolyte, with a low water solubility (4.9 mg/kg at atmospheric pressure) and low dissociation constant in water ($K_a = 4.7 \times 10^{-7}$ at 25 °C). Solubility of CO_2 is often presented in terms of Henry's coefficient, as shown in **Table 1**. These properties enable mild and reversible acidification, using the pressure as a well-controllable process parameter. During CO_2 acidification the pH of the solution is determined by the gas−liquid phase equilibrium; thus, no local undershoot of pH occurs. Additionally, acidification with CO_2 happens relatively slowly as compared to the acidification with mineral acids, which enables the process conditions to be finely tuned toward production of the desired product with highest purity. CO_2 can be successfully used as an acidifying agent in the pH range of 4−8.5. This makes it an appropriate precipitation agent for glycinin, which has a p*I* in the pH range of 5.2−4.9. A detailed study of the CO_2 pressure−pH relationship was made by Hofland et al. (*14*).

As has been observed and described before by other authors (*15*), when using CO_2 as an acidifying agent, time and length scales of CO_2 mass transfer from gaseous to liquid phase, liquid mixing, and precipitation do not overlap, due to the fact that the three mechanisms take place at significantly different rates.

Table 2. Characteristic Times of the Underlying Mechanisms during CO_2 Precipitation

mechanism	characteristic time	eq
macromixing	$t_{macro} = V/(r_c N_q d_s N)$	1
micromixing	$t_{micro} = 0.1(\nu^{3/2}/\epsilon^{1/2}D)$	2
precipitation	$t_p = \pi/(4\alpha\phi_{vol}G)$	3
mass transfer	$t_{tr} = 1/(k_l a)$	4

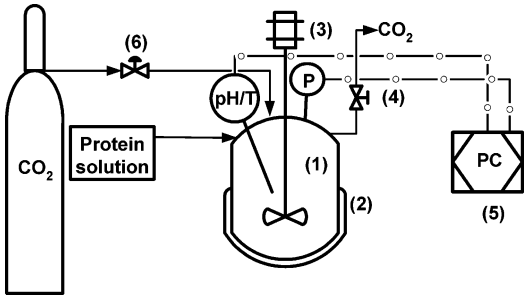

Figure 2. Schematic diagram of the 50 mL precipitation setup: (1) 50 mL reactor; (2) protective net; (3) stirrer; (4) depressurization valve; (5) personal computer; (6) pressure controller.

Therefore, it is anticipated that the purification process could be scaled up without drastically affecting the product quality.

Time scales of gas−liquid mass transfer, mixing, and precipitation were calculated on the basis of the equations shown in **Table 2**. The symbols used in the equations have the following meanings: $k_l a$ is the volumetric mass transfer coefficient; G is the shear rate; α is a factor indicating the probability of a successful collision; Φ_{vol} is the volume fraction of particles; V is a liquid volume; r_c is the circulation ratio; N_q is the pumping number of the impeller; d_s is the impeller diameter; N is the stirring rate; D is the molecular diffusivity; υ is the kinematic viscosity; and ϵ is energy dissipation. For more detailed explanation, the reader is referred to Hofland et al. (*15*).

MATERIALS AND METHODS

Precipitation Setup. High-pressure experiments were done in two different setups. Initially, a 50 mL reactor (**Figure 2**) made of pressure-resistant glass (Buchiglas, Uster, Switzerland) was used. The inner diameter of the vessel was 51 mm. The impeller was a six-bladed disk turbine 34 mm in diameter, mounted at 5 mm from the bottom. After successful small-scale glycinin purification, the procedure was conducted in a 1 L stainless steel reactor equipped with two sight-glasses (Buchiglas). The inner diameter of the vessel was 84 mm. The impeller was a four-bladed pitched blade (45°) 46 mm in diameter, mounted at 28 mm from the bottom. Both reactors were equipped with magnetically coupled stirrers. In both setups pressure-resistant pH electrodes were used. In the 1 L setup it was a high-pressure glass combination electrode (Mettler-Toledo, Giessen, Germany), and in the 50 mL setup it was a specially customized small solid-state pH electrode (Sentron, Zug, Switerland). Carbon dioxide was introduced via a pressure controller. Pressure, temperature, and pH were measured on-line and recorded on a personal computer, with Labview software (Halotec, Gouda, The Netherlands).

Extraction of Soy Meal. The soy protein solution was prepared according to a modified procedure of Chan et al. (*8*). Defatted soy meal (Sigma Aldrich, S-9633) was dissolved in 30 mM Tris buffer (pH 8.0), containing 10 mM 2-mercaptoethanol to give a 10% (w/w) concentration. The pH was adjusted to 8.0 with 1 M NaOH. After 60 min of stirring, the dispersion was centrifuged at 8500 rpm for 45 min at 20 °C. The obtained supernatant, the so-called total water extract (TWE), contained water-soluble soy proteins including glycinin.

Solubility of Soybean Globulins. The overall solubility of soybean globulins was determined by precipitation experiments with gaseous CO_2. Carbon dioxide was pressurized into the TWE, containing 40 g/L of globulin proteins, to the different end-pH values. The experiments were done at room temperature, with intense stirring. After equilibration of the solution, the suspension was centrifuged for 30 min at 4000 rpm, and the protein content of the supernatant was determined using the bicinchoninic acid (BCA) protein assay of Pierce (Rockford, IL).

Purification Experiments. Purification was done by sequentially applying CO_2 precipitation in three runs. The reactor was filled with TWE, and CO_2 was carefully introduced into the solution. Pressurization of the TWE was done in such a manner that the final pH was 6.4 in order to ensure that only glycinin precipitated from the protein solution. The duration of the CO_2 pressurization as well as the pressure applied differed in the two setups. In the 50 mL setup acidification happened at a faster rate as compared to the 1 L setup due to their somewhat different configurations and capacities (50 mL setup, $D_{vessel}/H_{vessel} = 0.78$; 1 L setup, $D_{vessel}/H_{vessel} = 0.45$). The CO_2 overpressure applied in the small precipitation vessel was 3−4 bar and the acidification time was 5−6 min, with a stirring rate of 250 rpm. In the 1 L precipitation vessel 9 bar of CO_2 overpressure was applied, and acidification took 10−15 min, with a stirring rate of 300 rpm. After the precipitation, the suspension was depressurized, removed from the reactor, and centrifuged at 8500 rpm, at 4 °C for 45 min (small scale) or at 4600 rpm at room temperature for 40 min (large scale). The supernatant was discarded, and the precipitate was resolubilized in 30 mM Tris buffer (pH 8.0) with 10 mM 2-mercaptoethanol, added to prevent agglomeration of glycinin molecules. The dissolved and dispersed precipitate was quantitatively transferred to the reactor and subjected to the next precipitation step. The obtained solution was placed in the precipitation vessel and subjected to CO_2 precipitation, after which centrifugation and resolubilization of the precipitate took place in the same manner as in the first run. The obtained precipitate from the second run was once again resolubilized and subjected to CO_2 precipitation with an end point of pH 6.4. After centrifugation, the precipitate was washed with Milli-Q water, freeze-dried for 72 h, and kept in the freezer.

Analytical Procedures. The protein concentration of the TWE and the overall solubility of soybean globulins were determined using the BCA protein assay of Pierce.

The protein purity was quantitatively determined using a Protein 200 Plus Assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). The Bioanalyzer is based on a microchip assay for the sizing and quantification of proteins by means of capillary gel electrophoresis using a fluorescence dye for detection.

The particle morphology of the precipitate was characterized by scanning electron microscopy (SEM) (JEOL JSM5400, Tokyo, Japan) and by image analysis (IA), using an Olympus reverse microscope, type IMT-2 (Olympus Paes Nederland) and a Leica digital camera, DFC 320 (Leica Microsystems BV) along with its software Qwin Pro, version 3.1. The precipitate was analyzed for particle morphology both in suspension before centrifugation and in a dry form.

RESULTS AND DISCUSSION

Characteristic Time Analysis. Characteristic times were calculated for both setups and for the conditions applied during the purification process. For the characteristic time analysis eqs 1−4 were used. From the values presented in **Table 3**, it is clear that certain mechanisms happen at different time scales in both setups. The results also show that the characteristic time for mass transfer is considerably longer than that for mixing, reaction of CO_2 with H_2O, and precipitation. This means that the mass transfer from gaseous to liquid phase is the rate-limiting step.

Yield of the Purification Process. The purification yield was determined on the basis of the BCA protein assay and the Protein 200 Plus Assay on the Bioanalyzer of the TWE and pure glycinin. Samples from the 50 mL and 1 L reactors were compared, and it was determined that although purity did not change, a somewhat higher yield was obtained with scaling up of the purification process.

Table 3. Characteristic Times for Glycinin Precipitation

vessel liquid vol (L)	0.05	0.6
stirring rate (1/min)	250	300
power input (W/kg)	0.14	0.05
shear rate (1/s)	187	166
characteristic times (s)		
mass transfer	204	309
liquid mixing		
macro	0.25	1.03
micro	0.36	0.60
reaction CO_2 and H_2O[a]	0.05	0.05
precipitation[b]		
$\alpha = 1$	0.56	0.57
$\alpha = 0.1$	5.6	5.7
$\alpha = 0.01$	56	57

[a] Value taken from Hofland et al. (*15*). [b] α is the collision efficiency, which increases with decreasing pH (*15*).

Table 4. Comparison of Glycinin Purity from Small and Up-scaled Setup and from Reference Sample (WU)

sample	basic subunit (wt %)	Δwt%[a]	acidic subunit (wt %)	Δwt%	total glycinin (wt %)	Δwt%	*n*[b]
50 mL setup	32.25	2.66	62.73	2.79	97.98	1.48	8
1 L setup	35.0	0.74	62.2	1.37	97.2	1.64	6
reference (WU)	36.37	2.28	61.1	4.1	97.47	2.25	3

[a] Δwt % is ordinary standard deviation. [b] *n* is the number of analyses performed.

From 6.5 g of defatted soy meal was produced 0.35 g of pure glycinin using the small setup, equal to a yield of 34%, taking into account the fact that glycinin accounts for 40% of soluble soy proteins.

When using the 1 L setup, 7 g of pure glycinin was obtained from 100 g of defatted soy meal. The BCA protein assay of the TWE and the final product showed that 42% of glycinin present in the TWE was obtained in pure form. When the TWE and glycinin were analyzed on the Bioanalyzer, the calculated yield was 37%, confirming the result obtained with the BCA protein assay.

It has to be stressed that obtaining a higher yield was indeed easily achievable by simply further lowering the end-pH of the precipitation, however, at the cost of a decreased purity, which was a primary target in our work. With lower end-pH simultaneous precipitation of β-conglycinin would occur, thus contaminating the end product. This was successfully avoided with a higher end-pH and consequently lower yield.

Glycinin Purity. During the Protein 200 Plus Assay on the Bioanalyzer proteins are denaturated with sodium dodecyl sulfate (SDS) and stained with a fluorophore; hence, on the electropherogram the acidic and basic subunits of glycinin are detected. As a reference for the glycinin purity determination a sample of pure glycinin kindly donated by Dr. Ir. Harry Gruppen from Wageningen University [obtained according to the protocol described in Lakemond et al. (*2*)] was used. This reference sample is indicated throughout the further text as WU. The results from the Bioanalyzer assays are summarized in **Table 4** and shown in **Figures 3** and **4**. It can be seen that both samples from the 50 mL and 1 L precipitation setups have high purities of ca. 98 and 97 wt %, respectively. Samples were compared against the reference sample (WU), which showed a purity of ca. 97.5 wt %.

Glycinin Morphology. The precipitate morphology was determined with SEM and Image Analysis. Although the suspension revealed nicely shaped spherical particles, as shown in **Figure 5**, the freeze-dried precipitate consisted of more irregular shapes (**Figure 6**), most probably due to the agglomeration of the spherical aggregates during the drying process. A fine spherical structure of the dry product may be achieved with spray-drying.

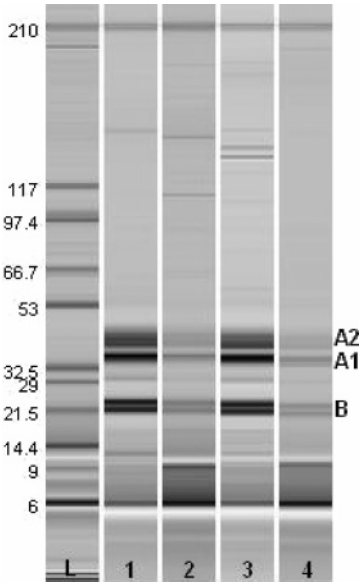

Figure 3. Electropherogram from Protein 200 Plus Assay converted into SDS-PAGE-like gel: (lanes 1 and 3) pure glycinin obtained in this work; (lanes 2 and 4) reference sample (WU). L, ladder; B, basic subunit; A_1, A_2, acidic subunits.

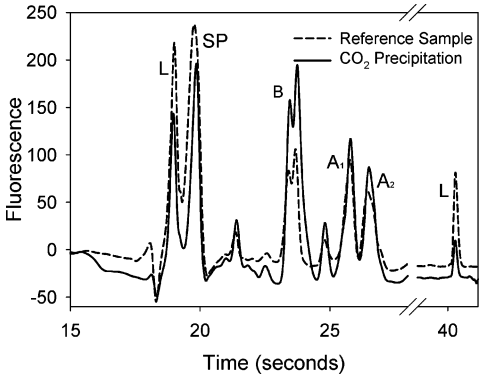

Figure 4. Compared electropherograms of glycinin from this work and reference sample (WU). L, ladder; SP, system peak; B, basic subunit; A_1, A_2, acidic subunits. Solid line corresponds to lane 1 in **Figure 3**. Dashed line corresponds to lane 2 in **Figure 3**.

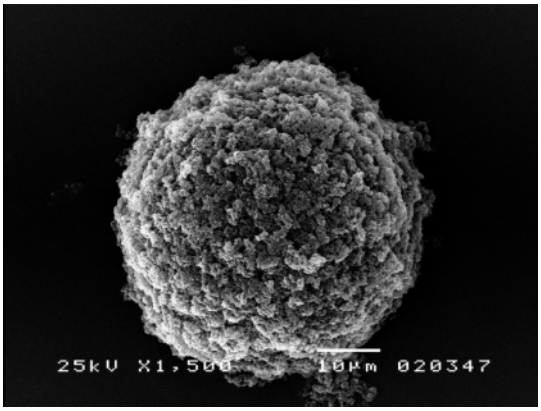

Figure 5. Morphology of a soybean protein aggregate using CO_2 as a precipitant.

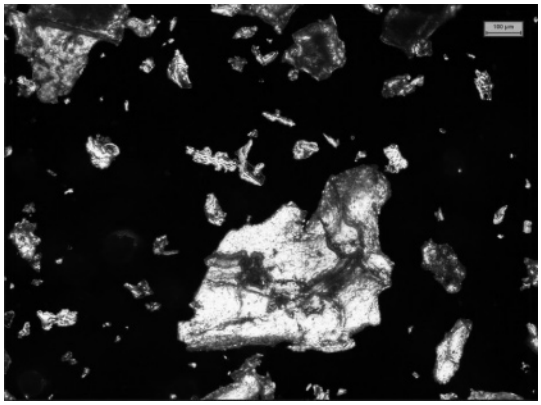

Figure 6. Picture of the freeze-dried glycinin using the Image Analyzer. The scale at the upper right corner represents 100 μm.

Glycinin Resolubility. The resolubility of the obtained glycinin was estimated on the basis of the amount of glycinin dissolved in a 10 mM Tris buffer, containing 10 mM 2-mercaptoethanol at pH 9.3, while stirring with a magnetic stirrer during 3 h. The solution was afterward centrifuged at 4000 rpm and room temperature for 30 min, and the protein content of the supernatant was determined with a BCA test. The results showed that resolubility of the pure glycinin was 80%, which indicates that the molecule structure was probably preserved during the purification procedure, although it has not been confirmed experimentally. However, it has been observed and reported by other authors, that even if a protein retains high resolubility after the purification process, its tertiary and even secondary structures might well be changed in the course of the purification process (*16*). It remains to be investigated in future research whether this is the case with glycinin recovered as described in this text.

To summarize, a novel, environmentally friendly, and fast process for the recovery of very pure glycinin has been developed. It was based on CO_2-aided precipitation of glycinin from the soybean protein solution (total water extract). The use of a strong mineral acid as well as a large amount of salts for the precipitation was elegantly avoided by using a volatile acid. By acidifying the solution carefully with carbon dioxide to the desired end-pH, the end product of very high purity has been obtained. The additional advantage of the process is the absence of extensive and costly downstream processes such as dialysis to remove vast amounts of salt or other precipitants otherwise present in the end product. The process yielded 40% of the glycinin present in the TWE, with a purity of 98%, as measured with the Bioanalyzer. Another feature of the end product is its high resolubility (80%). High resolubility implies that protein denaturation probably occurred at a very low rate, although this needs experimental verification. This is the consequence of the mild process conditions, such as room temperature, slow acidification rate, and mild volatile acidifying agent used.

As expected on the basis of the different time scales of underlying mechanisms of the purification process (liquid mixing, mass transfer, and precipitation), the process scale-up from 50 mL to 1 L was done successfully, without affecting yield or product purity. It is therefore anticipated that even larger scale-up would be possible, enabling fast and relatively cheap production of large amounts of pure glycinin.

ACKNOWLEDGMENT

We gratefully acknowledge Professor Dr. Ir. Harry Gruppen (Wageningen University) for the generous donation of the pure glycinin, Dr. Ir. Catriona Lakemond (Wageningen University) for comments, and Paul Durville (Laboratory for Process Equipment, TU Delft) for assistance with scanning electron microscopy.

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Received for review December 23, 2004. Revised manuscript received April 14, 2005. Accepted April 28, 2005.

JF0478206