The small fragments (P) were detected in glycinin-T (tryptic digestion intermediate of glycinin) by acetic acid-urea gel electrophoresis. Urea or 0.5 M acetic acid was needed for dissociation of them from glycinin-T. In the presence of urea, P fragments were separated from ISTs by use of solubility difference and Sephadex G-50 chromatography. Two bands of P fragments were observed on acetic acid-urea gels whereas a single band was on SDS-urea gels suggesting that the molecular weights of the two are almost the same. The fragments were also separable on a column of DEAE Sepharose CL-6B. They were isolated and the molecular weight of them was 9600.

Digestion properties of glycinin have been studied in relation to the conformation and the subunit structure.\(^{1-7}\) Native glycinin resists proteolytic digestion. On the other hand, denatured glycinin is degraded to polypeptides.\(^{3,6,7}\) However, even native glycinin is well degraded under low ionic strength condition, whereas it is split at a few peptide bonds under high ionic strength condition (0.5 M NaCl) yielding a digestion intermediate.\(^{4,5}\) Recent studies show that the acidic subunit of glycinin is degraded faster than the basic subunit by trypsin in its native conformation.\(^{2,4,5}\) Especially, under high ionic strength condition, the basic subunit is almost intact.\(^{4,5}\)

The previous investigation of the action of trypsin on native glycinin showed that the degradation process of glycinin could be described by the sum of two first-order reactions with very different velocity constants.\(^{5}\) Stable digestion intermediates were expected from this reaction type.\(^{8}\) Under high ionic strength condition, only a type of intermediate (glycinin-T) existed and was isolated.\(^{5}\)

Glycinin-T was isolated by gel filtration and its electrophoretical mobility (non-dissociative system) and elution volume of the gel filtration were similar (not identical) to those of glycinin.\(^{5}\) Therefore, the quaternary structure is almost intact in glycinin-T. Most of the fragments are associated together like native glycinin. Glycinin is composed of intermediary subunits (IS)\(^{9}\) and the degradation sequence of ISs was studied.\(^{4}\) Initially, ISs converted into IST-1 and IST-2 and then they were cleaved yielding IST-3 and IST-4. ISTs form the quaternary structure. The fragments other than ISTs were also expected to exist, but it was not clear whether they are associated with glycinin-T or dissociated from it.

The present report is concerned with the small fragments of glycinin-T. They were isolated from glycinin-T by dissociation with urea, fractional precipitation of IST, gel filtration and ion exchange chromatography. The number and molecular weights of the fragments were analyzed by acetic acid-urea and SDS-urea gel electrophoreses.

**MATERIALS**

Soybean (var. Raiden) harvested at Iwanuma, Miyagi, Japan in 1977 was used throughout this work. All the reagents were of the highest grade. Sepharose 6B, Sepharose CL-6B, DEAE Sepharose CL-6B, Sephadex G-50 and G-25 were obtained from Pharmacia Fine Chemicals. TPCK-trypsin was obtained from Worthington Biochemical Corp.
METHODS

Protein sample. Glycinin was prepared and purified as described previously\(^4\) by ammonium sulfate fractionation and NEM treatment.\(^{10}\)

Preparation of glycinin-T. One gram of purified glycinin was dissolved in 100 ml of 0.01 M Tris-HCl buffer (pH 8.0, 0.5 M NaCl, 0.02% NaN\(_3\)). Trypsin (0.2 mg/ml) was added and incubated at 30°C for 5 hr. Soybean trypsin inhibitor (0.3 mg/ml) was added to stop the reaction. The solution was concentrated by ultrafiltration and applied to Sepharose 6B column (4.3 \(\times\) 135 cm). Main peak was dialyzed and lyophilized.

Electrophoreses. Acetic acid gel electrophoresis in 5 M urea and 8% acrylamide gels was performed according to the method of Kitamura et al.\(^9\) SDS-urea gel electrophoresis\(^{11}\) was done with 0.1 M Tris-acetic acid buffer (pH 6.8) and 8 M urea in 12.5% acrylamide gels. Molecular weight was determined using the following standards: myoglobin (17,200), cytochrome C (12,300), BrCN fragment of cytochrome C (C1, 7760; C2, 2780), insulin (A, 2400; B, 3500) and BrCN fragment of myoglobin (M3, 2550).

Column chromatography. Sepharose CL-6B column (2.2 \(\times\) 88 cm) was eluted with 0.01 M sodium phosphate buffer (pH 5.8) containing 6 M urea. Sepharose 6B column (4.3 \(\times\) 135 cm) was eluted with 0.01 M Tris-HCl buffer (pH 8.0, 0.5 M NaCl, 0.02% NaN\(_3\)). Sephadex G-50 column (2.6 \(\times\) 100 cm) and Sephadex G-25 column (1.1 \(\times\) 10 cm) were eluted with 0.5 M acetic acid. DEAE Sepharose CL-6B column (1.8 \(\times\) 19 cm) was eluted with 0.01 M Tris-HCl buffer (pH 8.0, 6 M urea) and ionic strength linear elution was achieved by adding 0.5 M NaCl solution to a mixing chamber containing 500 ml of the buffer. Fractions were monitored at 280 nm.

Desalting of the small fragments. The fragments in the buffer containing urea were dialyzed against 0.5 M acetic acid by use of a hollow fiber dialyzer (KL-2-30, C type, KURARAY Co. Ltd.). The obtained samples were concentrated by rotary evaporator and then gel filtrated on a column of Sephadex G-25.

RESULTS

Small fragments of glycinin-T

Glycinin-T was prepared according to previous reports\(^5\) including Sepharose 6B gel filtration. Fig. 1 shows acetic acid-urea polyacrylamide gel electrophoretic pattern of glycinin-T. Gel 1a, glycinin-T, shows two bands of ISTs and additional two bands (P frag-

![Fig. 1. Acetic Acid-Urea Polyacrylamide Gel Electrophoresis of Glycinin-T.
Glycinin-T (a) and glycinin-T reduced by 2-ME (b).](image)

![Fig. 2. Sepharose CL-6B Chromatography of Glycinine-T in Urea System.
A column of Sepharose CL-6B (2.2 \(\times\) 88 cm) was equilibrated with 0.01 M sodium phosphate buffer (pH 5.8) containing urea. Each tube contained 9.5 ml of effluent. The absorbance at 280 nm was measured.](image)
Small Fragment of Glycinin-T

A column of Sepharose CL-6B in the presence of 6 M urea. Three peaks were obtained and P fragments were contained in the peak 2. Peak 1 and peak 3 mainly consisted of ISTs and small peptides, respectively. However, other fragments (ISTs, and Ts generated from the acidic subunits whose disulfide bridges were already reduced during glycinin preparation) were contaminated in the peak 2. Therefore, certain procedures for removing the contaminated fragments are needed.

Isolation of P fragments

Application of fractional precipitation to the fragments fractionation was attempted. The peak 1 which mainly consists of ISTs and the peak 2 which contains P fragments were dissolved (0.02%) in 0.01 M acetic acid. The pH of the solution was adjusted with 0.01 M to 0.1 M NH₄OH to the range of pH 3 to pH 10. Turbidity of the samples was measured at 600 nm. The results are shown in Fig. 3. The peak 1 showed minimum solubility at pH 6, whereas the peak 2 was almost soluble in all the pH range. Therefore, ISTs can be reduced by adjusting the pH of the solution to pH 6 and centrifuging off. The fragments, however, could not be fractionated by simple adjustment of pH, because they associate to form the quaternary structure in glycinin-T (data not shown) by noncovalent bonds. These bonds must be split before fractionation.

Figure 4 shows the fractionation procedure of the P fraction. Urea was used in order to dissociate glycinin-T into the fragments. Two hundred mg of glycinin-T was dissolved in 10 ml of 8 M urea and was allowed to stand for an hour. Fifty ml of cold methanol was added and stored at 5°C overnight. The solution was centrifuged (10,000 rpm, 20 min, 5°C) and the precipitate was extracted overnight with water at pH 6. The supernatant (crude P fraction) was lyophilized.

Figure 5 shows gel filtration profile of crude P fraction. The crude P fraction was dissolved in 0.5 M acetic acid and applied on a column (2.6 x 100 cm) of Sephadex G-50. The second peak consisted of P fragments and ISTs elute at void volume. The second peak was lyophilized. It seems that P fragments and ISTs elute at void volume.

Fig. 3. Effect of pH on Solubility of the Sepharose CL-6B Peaks.

The peak 1 (●) and the peak 2 (○) were dissolved (0.02%) in 0.01 M acetic acid and the pH was adjusted with NH₄OH. Turbidity of the samples was measured at 600 nm.

Glycinin-T
- Dissolved in 8 M urea
- 5 fold of MeOH
- Stored at 5°C overnight
Precipitate
- Extracted with water (pH 6)
Supernatant
- Lyophilized
Crude P fraction

Fig. 4. Fractionation Profile of the P Fraction.

Fig. 5. Sephadex G-50 Chromatography of the Crude P Fraction.
A column of Sephadex G-50 (2.6 x 100 cm) was equilibrated with 0.5 M acetic acid. Each tube contained 10 ml of effluent. The absorbance at 280 nm was measured.
ISTs can be separated simply by the Sephadex G–50 chromatography, but glycinin-T had high viscosity when it was dissolved in 0.5 M acetic acid and gel filtration profile was disturbed.

Figure 6b shows electrophoretic patterns on acetic acid-urea gel of the P fraction. Two major bands were observed in the gel. Two to three minor bands were also seen and their electrophoretical mobilities coincide with those of T fragments. On the other hand, SDS-urea gel electrophoresis of the P fraction (Fig. 6c) showed a single main band suggesting that P fragments have similar molecular weight each other. The molecular weight was estimated using the standards as described in the method. Obtained molecular weight, 9600, is the smallest value among ISTs (29,000–48,000)\(^4\) Ts (13,500–35,000)\(^5\) and P fragments.

![Fig. 6. Polyacrylamide Gel Electrophoresis of the P Fraction.](image)

Electrophoretic patterns of glycinin-T (a) and P fraction (b) in acetic acid-urea system, and the P fraction in SDS-urea system (c) are shown.

Figure 7 shows the ion exchange chromatography of the P fraction. The lyophilized sample was dissolved in 0.01 M Tris-HCl buffer (pH 8.0) containing 6 M urea. Possible disulfide bonds were reduced with 1 mM DTT and blocked with 5 mM NEM. The sample was applied on a column (1.8 × 19 cm) of DEAE Sepharose CL–6B. When the buffer containing no urea was used, the elution profile had some peaks and all peaks contained P fragments and other contaminants (T fragment) suggesting that P fragment and T fragment associate to form molecular aggregates under this condition (data not shown). Therefore,

![Fig. 7. DEAE Sepharose CL–6B Chromatography of the P Fraction.](image)

A column of DEAE Sepharose CL–6B (1.8 × 19 cm) was equilibrated with 0.01 M Tris-HCl buffer containing 6 M urea. Each tube contained 10 ml of effluent. The absorbance at 280 nm was measured. Ionic strength linear elution was achieved by adding 0.5 M NaCl solution to a mixing chamber containing 500 ml of the buffer.

![Fig. 8. Acetic Acid-Urea Gel Electrophoretic Patterns of the DEAE Sepharose CL–6B Peaks.](image)

Glycinin-T (a), P fraction (b), F–1 (c), F–2 (d) and F–3 (e).
urea was used to dissociate the fragments. The non-adsorbed fraction is DTT used for reduction. Three eluted peaks (F-1~F-3) were obtained and they were desalted with hollow fiber dialyzer and Sephadex G-25 column (1.1 × 10 cm). These fractions were analyzed by acetic acid-urea gel electrophoresis.

Figure 8 shows the acetic acid-urea gel electrophoretic patterns of the fractions obtained from DEAE Sepharose CL-6B chromatography. Figure 8b shows the pattern of the P fraction after Sephadex G-50 gel filtration. The P fraction gave two bands which were assigned to P fragments. F-1 and F-2 correspond to fast moving and slow moving P fragments, respectively. On the other hand, F-3 seems to correspond to T fragments. From these results, it was shown that two P fragments can be separated by the ion exchange chromatography.

DISCUSSION

During the course of the degradation of native glycinin, the large molecular weight fragments (ISTs and Ts) are generated and have been studied. On the other hand, it was not clear whether the fragments other than these dissociate from glycinin-T or not. SDS- or acetic acid-urea gel electrophoresis sometimes gave some small molecular weight bands besides the bands of ISTs or T fragments. This indicates that the small fragments are large enough to be detected on the gel electrophoresis. Isolation of the fragments was attempted.

The small fragments (P) are associated with ISTs even after isolation of glycinin-T by gel filtration. The connecting bonds are non-covalent because urea or 0.5 M acetic acid can separate them. The disulfide bridge between the acidic subunit and the basic subunit links the basic subunit and T fragment. There are no disulfide linkage between P fragment and the other fragments.

The solubility curve (Fig. 3) shows that IST fraction is not appreciably soluble at pH 5 to pH 10. On the other hand, glycinin-T is soluble in pH 8.0 buffer. These suggest that P fragments have important role on solubilization of glycinin-T. Contributions of P fragments to the solubility of glycinin-T may be based on two possibilities: 1) P fragments are needed for building up the three dimensional structure which is essential for the soluble form; 2) The hydrophilic property of P fragments is needed for solubilization of the protein.

P fragments were separated by ion exchange chromatography with the buffer containing urea to eliminate the contaminated T fragments. This contamination of T fragments could be reduced by careful NEM treatment of glycinin. NEM blocks the free sulfhydryl groups and the free sulfhydryl groups of glycinin cause disulfide exchange reaction. Through this reaction the intact disulfide bridges are exchanged or split. Further, by NEM treatment, we can exclude 2-ME for reduction of intermolecular disulfide bridges from the purification procedure of glycinin. This reagent may split part of intramolecular disulfide bridges. The prevention of the disulfide exchange and splitting reaction may decrease the split of disulfide bond between the basic subunits and T fragments. Therefore, the contamination of T fragments were reduced.

Without urea, P fragments and T fragments could not be separated by ion exchange chromatography at pH 8.0. Some peaks, which contain P fragment and T fragment, are observed (data not shown). These suggest that P fragment and T fragment tend to associate to form several soluble aggregates.

The acetic acid-urea polyacrylamide gel electrophoresis of P fraction shows two distinct bands and a few minor bands. The distinct bands correspond to P fragments. Although each original subunit of the fragments is not clear, P fraction should consist of three kinds of P fragment, since three kinds of IS were converted into three kinds of IST by trypic digestion. Two of these fragments presumably have similar electrophoretic mobilities. Therefore, only two bands were observed
Figure 9 shows the schematic representation of glycinin degradation. Glycinin is composed of three different intermediary subunits (IS-1 ~ IS-3) which consist of an acidic subunit and a basic subunit. Only the acidic subunits are degraded and the fragmentation processes of ISs are similar. Initially ISs are degraded into IST-1 or IST-2 followed by degradation to IST-3 or IST-4. It is not clear which step is related to P fragments generation. Detailed analyses are needed to reveal the definite fragmentation sequence. The control of the digestion point is being studied.

REFERENCES