2S Globulins of Soybean Seeds. 1. Isolation and Characterization of Protein Components

Ikunori Koshiyama,* Mamoru Kikuchi, Kyuya Harada, and Danji Fukushima

The 2S globulins of soybean seeds have been recognized to be storage proteins without biological activities for a long time. There are two immunologically different antigens and six bands by disc electrophoresis. Among them, three major bands occupying ~80% of the globulins were isolated by DEAE-Sephadex chromatography. In opposition of the recognition so far, all peaks from the chromatography had some inhibitory activities against trypsin and/or α-chymotrypsin. Two of three major fractions designated as α_3 and α_4 protein were immunologically identical with each other but entirely different from the remaining one, designated as α_2 protein. From the distribution of the three major fractions in the 2S globulins, Japanese varieties belonged almost entirely to the α_3 or α_4 type but American varieties belonged almost entirely to the α_3 type. α_3 protein was identical with the Kunitz trypsin inhibitor, and α_4 was estimated to be a size isomer of α_3 protein.

Ultracentrifugal investigations have classically shown that soybean storage proteins (soybean globulins) consist of four components with sedimentation constants equal to about 2, 7, 11, and 15 S. Soybean globulins have been recognized to be storage proteins because of no biological activities and the location in cotyledonous subcellular particles called "protein bodies". Among them, the major components in the 7S and 11S fractions have been well investigated in many laboratories. The 2S fraction is not composed of only one protein component, but some protein components have been suggested to exist in the fraction (Vaintraub, 1965; Wolf and Sly, 1965). Moreover, two ultracentrifugally pure proteins, 2.3S and 2.8S globulins, by Vaintraub and Shutov (1969) and an antigenic protein, α-conglycinin, by Catsimpoolas and Ekenstam (1969) have been isolated from the 2S fraction of soybean globulin. α-Conglycinin has been reported to have no activities of protease inhibitiors. However, the 2S fraction is not always still well understood.

Recently, we have found that there is a strong allergenicity for food hypersensitivity of soybean proteins mediated by immunoglobulin E (IgE) antibody using a radioallergosolvent test (RAST) in the 2S fraction (unpublished results). Accordingly, it is very important to clarify the protein components in the 2S fraction of soybean globulins.

This paper reports the fractionation of the 2S fraction and the characterization of the fractionated components.

EXPERIMENTAL SECTION

Materials. Soybeans of Japanese varieties were obtained from Chushin Agricultural Experimental Station, Nagano-ken Agricultural Research Center, and Kariwano Branch, Tohoku National Agricultural Experimental Station, Japan, and those of American varieties were obtained from the Iowa State University, Ames, IA. Commercial soybean trypsin inhibitors which correspond with the Kunitz trypsin inhibitor (Kunitz, 1946, 1947) were purchased from Sigma Chemical Co., Merck, Boehringer Manheim GmbH, BDH Chemicals Ltd., and Miles Laboratory Ltd. and kindly supplied by Professor T. Ikenaka, Protein Laboratory, University of Osaka, Osaka, Japan. Trypsin (bovine pancreas, 2× crystallized, Type III) and α-chymotrypsin (bovine pancreas, 3× crystallized, Type II) were purcahsed from Sigma Chemical Co.

Methods. *Preparation of the 2S Fraction.* The 2S fraction was prepared from soybean (*Glycine max* var. Yagishirahana) by the following two methods. The first was according to the method by Catsimpoolas and Ekenstam (1969). The second was done as follows: the water-extractable soybean proteins were first obtained by extraction of the defatted flakes with water (flakes:water ratio, 1:10) at 25 °C and centrifugation to clarify the supernatant liquor. The extract was then adjusted to pH 5.8 with acetic acid and centrifuged. The supernatant was adjusted to pH 4.5 with acetic acid and centrifuged. The precipitate (the pH 5.8–4.5 globulins) was dissolved in μ = 0.5 buffer (32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, and 0.4 M NaCl, pH 7.60) and dialyzed against the buffer for 3 days at 4 °C. After removal of the insoluble fraction, the clear protein solution was applied to a 5 × 90 cm Sephadex G-100 column previously equilibrated with the buffer. Elution was performed with the buffer and 20-mL fractions were collected. The effluent absorption was measured by using a Hitachi 034-UV-VIS effluent monitor. The protein in selectively pooled fractions was dialyzed against water and lyophilized. It was confirmed by disc electrophoresis that all 2S globulins are contained in the pH 5.8–4.5 globulins. also, the possibility of subunits of other proteins except the 2S globulins in the 2S fraction (designated as S_2) are scarcely considered because the dissociation of the other proteins to their subunits hardly occurs under the mild condition of this fractionation procedure.

DEAE-Sephadex Chromatography. The 2S fraction was fractionated by using a 1.0 × 20 cm column of DEAE-Sephadex A-50 equilibrated wtih μ = 0.1 buffer (32.5 mM K_2HPO_4 and 2.6 mM KH_2PO_4, pH 7.60). Approximately 200 mg of the fraction dissolved in 10 mL of the starting buffer was put on the top of the column. Elution was performed with the same buffer containing sodium chloride in gradient concentrations of 0–0.5 M. A linear gradient system was started by using the mixer chamber containing 250 mL of μ = 0.1 buffer and the reservoir chamber with an equal volume of the buffer containing 0.5 M NaCl adjusted to pH 7.60. Detection of the protein fractions (5 mL) was performed as described in the gel filtration experiment.

Immunological Methods. Preparation of antisera was performed by the method of a previous paper (Koshiyama

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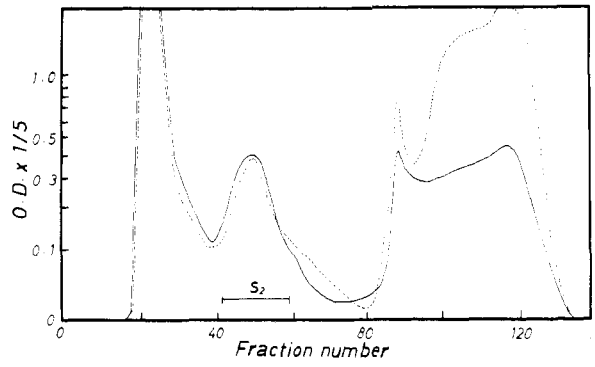

Figure 1. Gel filtation of the 2S fraction of soybean seeds on a Sephadex G-100 column. The underlined part (designated as S_2) was isolated as the 2S fraction. (—) 280 nm; (- - -) 260 nm.

and Fukushima, 1976). Immunoelectrophoresis and double gel immunodiffusion were performed according to the previous method (Koshiyama and Fukushima, 1976). For the preparation of 2S globulin antiserum, the 2S fraction obtained by the first method was used.

Electrophoresis. Disc electrophoresis was carried out as described by a previous paper (Koshiyama and Fukushima, 1976). Slab gel electrophoresis was performed with a constant current of 30 mA by the reason that the electrophoretic patterns were directly comparable under completely identical conditions. The concentration of acrylamide was 15%. Densitometric tracings were determined by using a Gilford Model 240 spectrophotometer equipped with a Model 2410 linear transport.

Estimation of Trypsin and α-Chymotrypsin Inhibition Activities. Trypsin inhibition (TI) and α-chymotrypsin inhibition (CTI) activities were determined by the following two methods. The first was determined for TI and CTI by the casein digestion method using 0.05 M Tris-HCl buffer containing 2 mM calcium acetate at pH 8.0. After the reaction was performed at 30 °C for 10 min, it was stopped by adding 4% trichloroacetic acid, and absorbancy of the filtrate at 280 nm was measured. TI and CTI were also determined by the spectrophotometric method of Rhodes et al. (1960) using toluenesulfonyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTE) as the substrate, respectively.

Analysis of the Protease Inhibitor Compositions in Various Kinds of Soybean Seeds. The defatted flakes were prepared from one sliced seed by extraction with ethyl ether for 3 days. Subsequently, the protein fractions of the flakes were extracted with water at pH 5.80 and centrifuged. The supernatant was done at pH 4.50 and centrifuged. The precipitate was dissolved in water at pH 7.60. After removal of the insoluble fraction, the clear protein solution was charged to slab gel electrophoresis.

RESULTS AND DISCUSSION

Composition of the 2S Fraction in Soybean Globulins. Figure 1 shows a typical elution pattern of the pH 5.8–4.5 globulins on a Sephadex G-100 column. The second peak (the underlined part, designated as S_2) was isolated as the 2S fraction. The 2S fraction was fractionated into six bands by disc eletrophoresis as shown in Figure 2. Among them, bands from 1–3 were the major protein components. The integration of the area under the trace of their peaks was almost equal to ~80%. These results were completely identical between the 2S samples prepared by the different two methods described under Experimental Section. However, immunologically there were only two antigenic components as shown in Figure 3.

Fractionation of the 2S Fraction. Figure 4 shows a typical chromatogram of the 2S fraction on a DEAE-

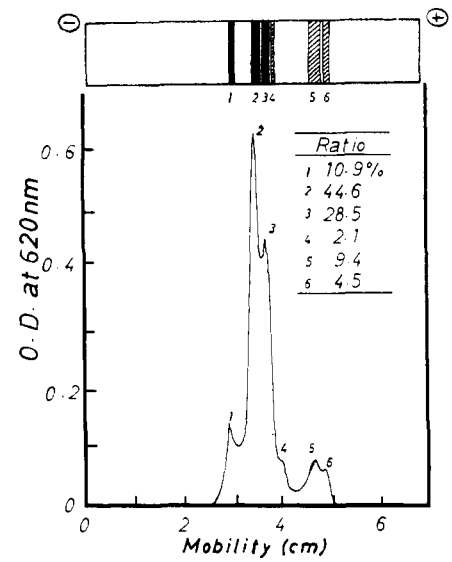

Figure 2. Disc electrophoretogram of the 2S fraction. The small table shows the concentration ratio of each band calculated from the area of each peak under the densitometric tracing.

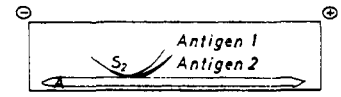

Figure 3. Immunoelectrophoretogram of the 2S fraction against the 2S globulins antiserum. A, the 2S globulin antiserum; S_2, the 2S fraction.

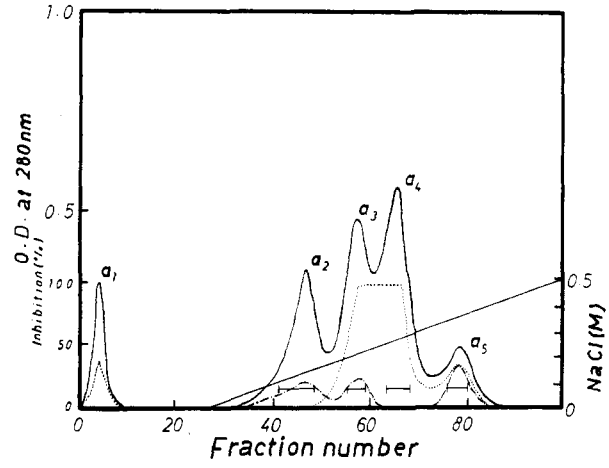

Figure 4. Chromatogram of the 2S fraction of a DEAE-Sephadex column. (- - -) Trypsin inhibitory actibity; (-·-) α-chymotrypsin inhibitory activity. The underlined parts in each peak were isolated as the samples of each fraction.

Sephadex A-50 column. The elution peaks were designated as α_1 to α_5 accordingly to the elution order. Among them, the α_2 peak had only CTI activity and the main peak of α_4 only TI activity in the determination of TI and CTI activities by using the casein digestion method. The peaks of α_3 and α_5 had the both activities of CTI and TI. The peak of α_1, eluted before application of the gradient, was of only TI activity, but it was not determined if α_1 was derived from the excessive charge of the 2S fraction or not in this experiment.

The fact that all peaks fractionated by DEAE-Sephadex chromatography have some activities of TI and/or CTI contradicts the recognition that the 2S globulins were believed to be storage proteins without biological activities.

Electrophoretic purity of the isolated peaks were shown in Figure 5. The peaks from α_2 to α_4 showed a single band, respectively, but the peak of α_5, a braod band, corre-

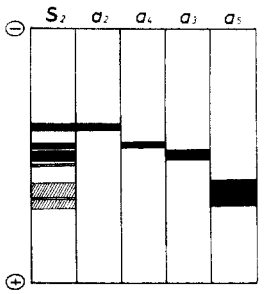

Figure 5. Disc electrophoretic patterns from the peak α_2 to the peak α_5. S_2, the 2S fraction.

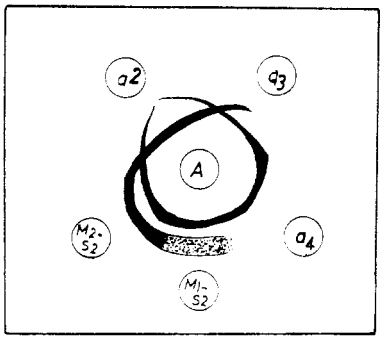

Figure 6. Double gel immunodiffusion from α_2 to α_4 proteins and the 2S fractions against the 2S globulins antiserum. A, the 2S globulins antiserum; M_1-S_2 and M_2-S_2, the 2S fractions prepared by the first and second method, respectively.

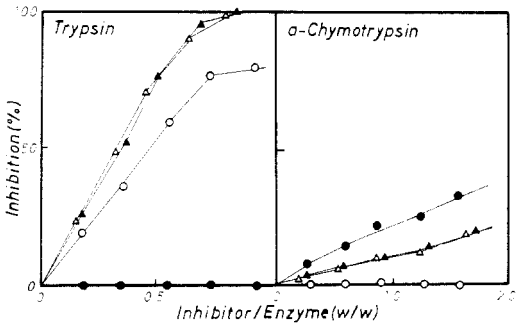

Figure 7. Inhibiting behaviors of α_2, α_3, α_4, and KSTI for trypsin and α-chymotrypsin by the casein digestion method. (△) KSTI; (●) α_2; (▲) α_3; (○) α_4.

sponded with the bands 4 and 5. The elution order between the peaks α_3 and α_4 reversed with disc electrophoresis.

Each protein from the peaks of α_2 to α_4 was immunologically pure as shown in Figure 6. Among them, α_3 and α_4 proteins were completely fused against the 2S globulins antiserum. Accordingly, they are immunologically the same proteins. However, α_2 protein was immunologically completely different from α_3 and α_4 proteins.

Inhibiting behaviors of α_2, α_3, and α_4 proteins against trypsin and α-chymotrypsin are compared with the Kunitz trypsin inhibitor (KSTI) of Sigma Chemicals Co. in Figures 7 and 8. Their inhibiting behaviors parallel each other in the determination of TI and CTI activities by using casein and also synthetic compounds as the substrate except that α_2 protein gradually inhibits the activity of trypsin with TAME in the ratio of inhibitor to enzyme of more than 0.6. From the results, α_3 protein agreed well with KSTI. However, α_2 and α_4 proteins were apparently different from KSTI in their specific activities as summarized in Table I.

Relationship between Commercial KSTI's and the Inhibitors in the 2S Fraction. As shown in Figure 9,

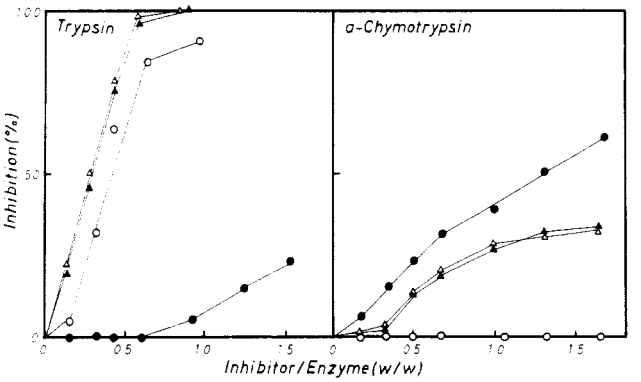

Figure 8. Inhibiting behaviors of α_2, α_3, α_4, and KSTI for trypsin and α-chymotrypsin by the spectrophotometric method. (△) KSTI; (●) α_2; (▲) α_3; (○) α_4.

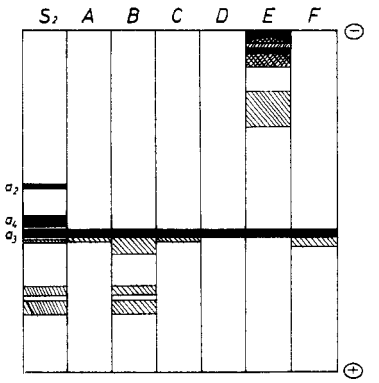

Figure 9. Slab gel electrophoretogram between commercial KSTI's and the inhibitiors in the 2S fraction. A, Miles Laboratory Ltd.; B, Boehringer Manheim GmbH; C, Sigma Chemicals Co.; D, Ikenaka's KSTI; E, BDH Chemicals; F, Merck; S_2, the 2S fraction.

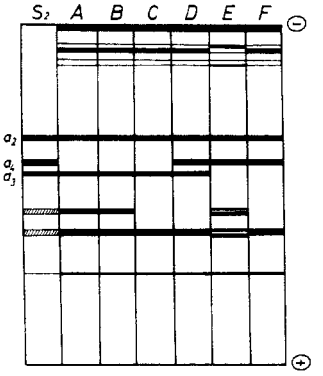

Figure 10. Distribution of the protease inhibitors in Japanese varieties of soybean seeds. A, Yagi No. 1; B, Kitaminagaha; C, Tokachinagaha; D, Yagishirahana; E, Yogetsu No. 1; F, Bonminori.

Table I. Specificity Differences of Protease Inhibition

	μg of enzyme inhibited/μg of inhibitor	
	trypsin	α-chymotrypsin
α_2 protein		0.18
α_3 protein	1.24	0.13
α_4 protein	1.11	
the Kunitz trypsin inhibitor	1.46	0.12

five kinds of commercial KSTI's gave an almost single band on a slab gel electrophoretic chromatogram except the preparation from BDH Chemicals Ltd. Their mobilities were almost the same as that of α_3 protein. Par-

Table II. Distribution of the Protease Inhibitors from Soybean Seeds of American Varieties

type	varieties
α_3	Amsoy, Braggs, Clark, Chippewa, Dare, D-60, Harosoy, Hawkye, Hill, Lee, Hark, Traverse, Wayne
α_4	Kent

ticularly, the purest preparation from Dr. Ikenaka also gave the same mobility as that of α_3 protein. Accordingly, α_3 protein is identical with KSTI.

Distribution of the Inhibitors in the 2S Fraction in Japanese and American Varieties of Soybean Seeds. In Figure 10 is shown the distribution of the inhibitors in the 2S fraction in Japanese varieties of soybean seeds. From the results, Japanese varieties could be separated into three groups: (1) the α_3 group (Yagi No. 1, Kitaminagaha, and Tokachinagaha), (2) the α_4 group (Yogetsu No. 1 and Bonminori), and (3) the α_3 plus α_4 group (Yagishirahana). However, American varieties used in this experiment belonged only to the α_3 group except that of Kent which had an α_4-type inhibitor as shown in Table II.

Relationship between α_3 and α_4 Inhibitors. A naturally occurring genetic variant of KSTI of which its electrophoretic mobility is slightly different was reported by Hymowitz and Hadley (1972) and Hymowitz (1973). Recently, four types of KSTI have been identified by Orf and Hymowitz (1979). Three of the types, designated Ti^a, Ti^b, and Ti^c, are electrophoretically distinguishable from one another by their different R_f values of 0.79, 0.75, and 0.83, respectively, using 10% polyacrylamide gel. The three types are inherited as codominant alles in a multiple allelic system at a single locus.

The appearance of a new band with electrophoretic mobility slightly lower than that of KSTI, which appeared about as equally intense as KSTI, was also found during germination of soybean seeds by Orf et al. (1977). The immunological identity of the newly formed protease inhibitor and KSTI was confirmed (Freed and Ryan, 1978).

The electrophoretic mobility of the new inhibitor seemed to be very similar to an inhibitor of the Ti series. Although the new form of KSTI may be a protease-modified molecule, the modified inhibitor must have an electrophoretic mobility higher than that of the unmodified molecule if the assumption is true as shown in a trypsin-modified KSTI by Bidlingmeyer et al. (1972). As mentioned above, α_3 and α_4 proteins are also immunologically isoinhibitors. In this paper, we did not determine the relationship between the isoinhibitors of the Ti series and α_3 (KSTI) or α_4 inhibitor, but the nature of a new form of KSTI (α_4) is very interesting.

There are some possibilities which explain the occurrence of the α_4 isomer from KSTI (α_3). The first is the enzymatic modification except for proteases. For example, the deamidation of the γ-amide of glutamine or the β-amide of asparagine in the α_3 and α_4 inhibitor, may be considered. However, at least, there was no difference by the enzymatic reaction for both proteins with peptidoglutaminase II (EC 3.5.1.44) (Kikuchi et al., 1971; Kikuchi and Sakaguchi, 1976) as shown in Figure 11.

The second possibility is that the new form is a dimer which has been found by Mamiya et al. (1973) using gel filtration with a sephadex G-75 column. However, both proteins could not be separated by gel filtration with a Sephadex G-100 column as shown in Figure 1.

The possibility of artifact formation caused by the persulfate catalyst on disc or slab gel electrophoresis may be also considered as reported by Brewer (1967) and Catsimpoolas et al. (1969). But, the effect of persulfate catalyst was not completely observed on the electrophoretograms.

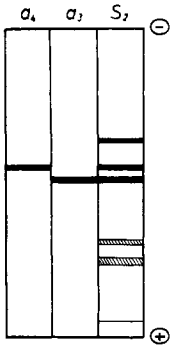

Figure 11. Slab gel electrophretogram of α_3 and α_4 proteins before and after enzymation by peptidoglutaminase II for 72 h. S_2, the 2S fraction.

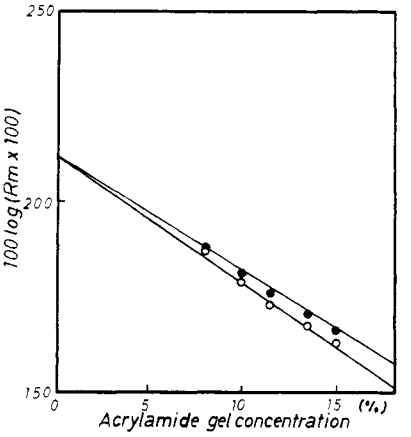

Figure 12. Effect of different gel concentrations on the mobility of α_3 and α_4 proteins. (○) α_3; (●) α_4.

Hedrich and Smith (1968) have established a method which distinguishes between size and charge isomer families of proteins utilizing disc electrophoresis. When the log of protein mobility relative (R_m) to the dye front was plotted vs. acrylamide gel concentration, size isomeric proteins gave a family of nonparallel lines extrapolating to a common point in the vicinity of 0% gel concentration. On the other hand, charge isomeric proteins gave a parallel family of lines. A plot of log R_m vs. gel concentration for the both proteins of α_3 and α_4 yielded two nonparallel lines as shown in Figure 12. The result indicates that the two proteins are size isomers.

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2S Globulins of Soybean Seeds. 2. Physicochemical and Biological Properties of Protease Inhibitors in 2S Globulins

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Some physicochemical and biological properties of the three major protein components from α_2 to α_4 proteins having protease inhibitory activities in the 2S globulins of soybean seeds have been determined and compared with those of the Kunitz trypsin inhibitor (KSTI). The significantly different physicochemical property was their molecular weights, 32 500, 21 900, and 27 800 for α_2, α_3, and α_4 proteins, respectively. Their amino acid compositions were also determined. Their N-terminal amino acids were all aspartic acid. Between them, both the α_3 and α_4 proteins were immunologically identical with KSTI. One mole of them combined with 1 mol of rabbit antibody. The protease inhibition spectra of α_3 protein agreed well with those of KSTI, but those of α_2 and α_4 proteins were different from them. The pH stability of α_2 to α_4 proteins was completely stable between pH 3 and pH 10. Thermal stability was the highest in α_4 protein, but that of α_2 protein was lost entirely at 79 °C.

The presence of two different antigenic proteins in the 2S globulins has been demonstrated in the preceding paper (Koshiyama et al., 1981). However, it has been simultaneously found by disc electrophoresis and chromatography with DEAE-Sephadex that the globulins are composed of several components which have the ability to inhibit trypsin and/or α-chymotrypsin. About 80% of the globulins was represented by the three components. One of the major three components designated as α_2 protein was an inhibitor which inhibited α-chymotrypsin stronger than trypsin. But, the other two, designated as α_3 and α_4 proteins, one (α_3 protein) of which was identical with the Kunitz trypsin inhibitor (KSTI; Kunitz, 1946, 1947), were immunologically identical with each other. α_4 protein which inhibited only trypsin was estimated to be a size isomer of α_3 protein (KSTI).

This paper describes their physicochemical and biological properties, and the properties are compared with those of KSTI.

EXPERIMENTAL SECTION

Materials. The three inhibitors from α_2 to α_4 proteins in the 2S globulins were prepared according to the preceding paper (Koshiyama et al., 1981). For determination of protease inhibition spectra, the various proteases were purchased as follows: α-chymotrypsin (bovine pancreas, 3× crystallized, Type II), trypsin (bovine pancreas, 2× crystallized, Type III), pepsin (hog stomach mucosa, 2× crystallized), papain (papaya latex, 2× crystallized), Pronase (*Streptomyces griseus*, Type VI), plasmin (fibrinolysin, porcine blood), and subtilisin (*Bacillus subtilis*, Type VIII) from Sigma Chemical Co.; thermolysin (3× crystallized) and acid protease of *Aspergillus niger* from Seikagaku Kogyo Co. Acid protease of *Aspergillus sojae* and *Aspergillus saitoi* and alkaline protease of *A. sojae* were kindly supplied by Dr. K. Hayashi of Central Research Laboratory, Kikkoman Shoyu Co. KSTI was obtained from Sigma Chemical Co.

Methods. *Immunochemical Methods.* Preparation of antisera and double gel immunodiffusion were performed by the method of a previous paper (Koshiyama and Fukushima, 1976). In the quantitative precipitin test, increasing amounts of KSTI and α_4 protein in 0.5-mL volumes of μ = 0.1 buffer (32.5 mM K_2HPO_4 and 2.6 mM KH_2PO_4, pH 7.60) were added to test tubes containing 0.2 mL of the antiserum. The reaction mixtures were incubated for 30 min at 37 °C and then stored overnight at 4 °C. The resultant precipitates were centrifuged, washed twice with pH 7 bufferen saline (0.87 g of $Na_2HPO_4 \cdot H_2O$, 0.38 g of KH_2PO_4, and 9 g of NaCl made to 1 L with water), and dissolved in 5 mL of 0.1 N NaOH. The absorbance of the solutions was measured at 280 nm with a Hitachi P-1 spectrophotometer. The molecular ratio of antigen to antibody present in the immunoprecipitate at equivalence was calculated from the extinction coefficients at 280 nm and molecular weights of α_4 protein, KSTI, and rabbit γ-globulin. $E_{1cm}^{1\%}$ values of 8.0 for α_4 protein, 7.2 for KSTI, and 14.0 for the rabbit γ-globulin (Porter, 1957) were used for approximate calculations. The molecular weights of α_4 protein, KSTI, and γ-globulin were taken as 27 800, 21 400, and 160 000 (Phelps and Putnam, 1960), respectively.

Sedimentation Analysis. Sedimentation analysis was performed with a Hitachi UCA-1 centrifuge. Routine assay

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