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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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runs were made 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) at 20 °C.

Amino Acid Analysis. Hydrolysis was performed according to the method of Penke et al. (1974). Each protein sample (2–3 mg) was hydrolyzed with 1 mL of 3 N mercaptoethanesulfonic acid in an evacuated sealed tube at 110 °C for 24, 48, and 72 h, respectively. After hydrolysis the tubes were cooled and opened, 2 mL of 1 N NaOH was added to the solution, and then the content of the tubes was washed into a 5-mL volumetric flask. Aliquots of 1 mL were used for the amino acid analysis. Amino acid analysis was performed with a Hitachi KLA-5 automatic amino acid analyzer. For cystine and cysteine analysis, hydrolysis was performed for 20 h at 110 °C with 6 N HCl in the presence of 0.3 M dimethyl sulfoxide by using 2 mg of each protein in an evacuated sealed tube according to the method of Spencer and Wold (1969).

N-Terminal Analysis. Prior to dansylation, ~1 mg of each protein was oxidized with performic acid and lyophilized. About 200 μg of the oxidized sample was used for dansylation according to the method of Gray (1972) and Gros and Labouesse (1969). The dansylated protein was hydrolyzed in 100 μL of 6.1 N HCl for 16 h. The dansylated amino acids were identified by two-dimentional thin-layer chromatography on polyamide sheets (Cheng Chin Trading Co., Taipei, Taiwan): the solvents used were water–90% formic acid (200:3 by vol, first dimension) and benzene–acetic acid (9:1 by vol, second dimension).

Molecular Weight. Molecular weights (M_r) of KSTI and the proteins from α_2 and α_4 were determined by using substantially the following two methods.

(1) Sedimentation and Diffusion Constant. At first, the determination of M_r was performed by introducing the sedimentation constant (s), diffusion constant (D), and partial specific volume ($\bar{V}$) into the well-known equation

$$M_r = RT/(1 - \bar{V}\rho)s/D \quad (1)$$

where R was the gas constant (8.314×10^7 erg mol^{-1} deg^{-1}), T was the absolute temperature, and ρ was density of the medium (1.0159 g/mL). The sedimentation and diffusion constants of KSTI and the proteins from α_2 to α_4 are shown in Table II. The partial specific volume was assumed to be 0.75 mL/g for each protein.

(2) Sedimentation Equilibrium. For the determination of M_r by sedimentation equilibrium the following three methods were used. (1) The Yphantis method (Yphantis, 1960) was first used as a convenient sedimentation equilibrium method with a eight-channel cell. The Yphantis procedure was conducted at 12 290 rpm and 20 °C in μ = 0.5 buffer. (2) The second procedure was the meniscus depletion method (Yphantis, 1964). The selected operating speed was 36 561 rpm at 20 °C for 24 h using a 12-mm six-channel cell in μ = 0.5 buffer. (3) The low-speed sedimentation equilibrium method was used as the third method (Richards et al., 1968). The rotor speed was 12 930 rpm at 20 °C using a 12-mm six-channel cell in μ = 0.5 buffer.

Diffusion Constant. The diffusion constant was determined from sedimentation boundary curves according to the "height–area" method of Kawahara (1969) with the analytical centrifuge using a synthetic boundary cell at a rotor speed of 12 290 rpm.

Determination of Protein Concentration. The purified proteins were spectrophotometrically determined at 280 nm by using $E_{1cm}^{1\%}$ shown in Table II.

Isoelectric Point. Isolectric point was determined by isoelectric focusing with an LKB 8100 electrofocusing column of 110-mL capacity according to the method of a previous paper (Koshiyama, 1972).

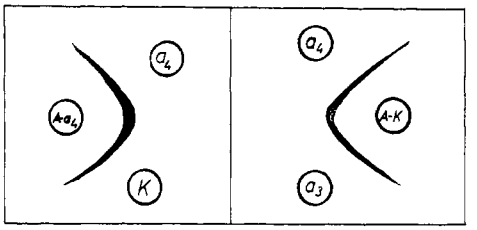

Figure 1. Double gel immunodiffusion of α_3 and α_4 proteins and KSTI against α_4 protein and KSTI antiserum. K, KSTI; A-α_4, α_4 protein antiserum; A-K, KSTI antiserum.

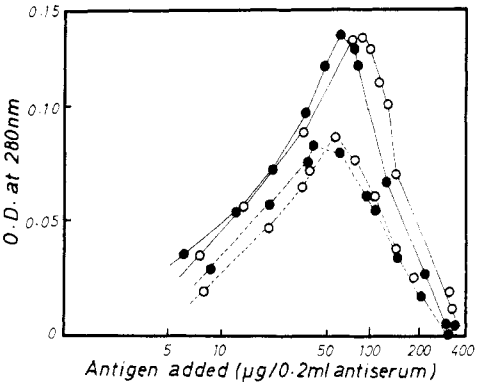

Figure 2. Quantitative precipitin curves of α_4 protein and KSTI with α_4 protein and KSTI antiserum. Increasing amounts of α_4 protein (●) and KSTI (○) were added to 0.2 mL of α_4 protein (—) and KSTI (---) antiserum. Absorbance at 280 nm indicates the amount of antigen–antibody complex.

Table I. Antibody/Antigen Ratio of α_4 Protein and the Kunitz Trypsin Inhibitor (KSTI) against α_4 Protein and the Kunitz Trypsin Inhibitor Antiserum

	antibody/antigen
against α_4 protein antiserum	
α_4 protein	1.15
KSTI	0.62
against the KSTI antiserum	
α_4 protein	0.87
KSTI	0.63

Disc Electrophoresis. Disc electrophoresis was performed in the presence and absence of sodium dodecyl sulfate ($NaDodSO_4$) according to the method of previous papers (Koshiyama and Fukushima, 1976; Hayashi and Oba, 1972).

Determination of Protease Inhibitory Activities. Trypsin inhibition (TI) and α-chymotrypsin inhibition (CTI) activities were determined according to the preceding paper (Koshiyama et al., 1981). Protease inhibitory activities against all the other proteases except trypsin and α-chymotrypsin were also measured by substantially the same method as the casein digestion method which was used for TI and CTI.

RESULTS AND DISCUSSION

Immunological Homology among α_3 and α_4 Proteins and KSTI. On double gel immunodiffusion, α_3 and α_4 proteins and KSTI were entirely immunologically identcal as shown in Figure 1. The same results were also supported by the results of quantitative precipitin of α_4 and KSTI antiserum with KSTI and α_4 protein as illustrated in Figure 2. The precipitin curve shows a typical antigen–antibody reaction. At the point of maximum precipitation, it was found as shown in Table I that approximately 1 mol of α_4 protein and also KSTI combine with 1 mol of the rabbit antibody against α_4 protein and also KSTI, respectively. The same immunological reactivities

Table II. Physicochemical Properties of the Proteins from α_2 to α_4 and the Kunitz Trypsin Inhibitor (KSTI)

properties	α_2	α_3	α_4	KSTI
sedimentation constant ($s^0_{20,w}$), S	2.18	2.54	2.64	2.36
diffusion constant ($D_{20,w}$), $\times 10^{-7}$ cm^2 s^{-1}	6.23	10.43	8.19	10.41
isoelectric point (pI), pH	4.50	4.53	4.65	4.50
extinction coefficient ($E_{1cm}^{1\%}$) at 280 nm	9.08	8.08	7.97	7.20
molecular weight				
$s^0_{20,w}$, $D_{20,w}$	31 800	21 900	29 100	21 400
sedimentation equilibrium				
Yphantis method	32 500		27 800	
meniscus depletion method			27 300	
low-speed equilibrium method		19 800	26 500	

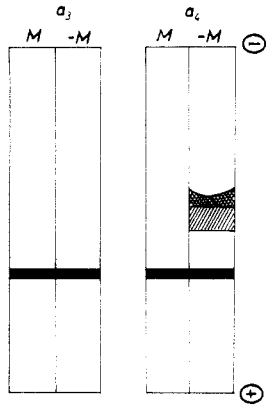

Figure 3. NaDodSO$_4$ slab gel electrophoretic patterns of α_3 and α_4 proteins in the presence and absence of ME. M, in the presence of ME; -M, in the absence of ME.

for both protein inhibitors makes conceivable the similarities in their amino acid sequences and their conformations as described by Reichlin (1975).

Some Physicochemical Properties. The physicochemical properties of the proteins from α_2 to α_4 and KSTI are summarized in Table II. In Table II, the sedimentation constants ($s^0_{20,w}$) of the four proteins were determined by extrapolation to zero concentation of protein, but the concentration dependence of the sedimentation constants could not be found in the all proteins.

Among the physicochemical properties between the three proteins of α_2, α_3, and α_4, the M_r's of α_4 protein obtained by the different four methods were larger in either case than those of α_3 protein (KSTI). The fact seems to support the possibility of the size isomers between α_3 and α_4 proteins as shown in the preceding paper (Koshiyama et al., 1981).

As shown in Figure 3, the electrophoretic patterns of α_3 protein on slab gel electrophoresis had nothing to do with the presence of 2-mercaptoethanol (ME), but the fairly large tailing part having the slower mobility was observed for α_4 protein in the absence of ME. There must be some large fragments which probably bind through the disulfide bond in α_4 protein, and the fragment part may contribute to the cause that the M_r of α_4 protein is larger than that of a α_3 protein.

The extinction constant at 280 nm for KSTI seemed to be smaller than that obtained by Yamamoto and Ikenaka (1967), but the discrepancy could not be explained from this experiment.

The complete amino acid sequence of KSTI (the primary structure) with 181 amino acid residues has been determined by Koide and Ikenaka (1973). Although the number of amino acid residues and the amino acid compositions of KSTI and α_3 protein were slightly different from those of Koide and Ikenaka, those of α_2 and α_4 proteins were fairly different from those of KSTI as shown in Table III. N-terminal amino acid was all aspartic acid

Table III. N-Terminal and Amino Acid Composition of the Proteins from α_2 to α_4 and the Kunitz Trypsin Inhibitor (KSTI)

	mol of amino acids/mol of protein for				
amino acid	α_2	α_3	α_4	KSTI	KSTI[a]
Asp	31	26	34	27	26
Thr	13	7	10	7	7
Ser	10	10	13	10	11
Glu	30	17	24	19	18
Pro	15	10	13	10	10
Gly	24	16	21	17	16
Ala	14	8	10	8	8
$^1/_2$-Cys	7	4	7	4	4
Val	22	14	19	14	14
Met	5	2	2	2	2
Ile	18	14	19	14	14
Leu	26	14	18	15	15
Tyr	10	5	4	5	4
Phe	16	10	13	10	9
Lys	15	10	13	10	10
His	2	2	2	2	2
Arg	11	9	11	9	9
Trp	6	2	3	2	2
total	277	180	236	185	181
molecular weight	32 500	21 900	27 800	21 400	20 100
N-terminal amino acid	Asp	Asp	Asp	Asp	Asp[b]

[a] Koide and Ikenaka (1973). [b] Yamamoto and Ikenaka (1967).

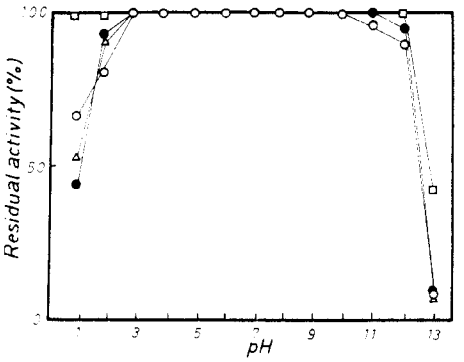

Figure 4. Effect of pH on the stability of the proteins from α_2 to α_4 and KSTI. (□) α_2 protein; (△) α_3 protein; (○) α_4 protein; (●) KSTI.

between them. The carbohydrate moiety could not be found in them.

Some Biological Properties. The pH stability of the proteins from α_2 to α_4 and KSTI are shown in Figure 4. The four inhibitors were completely stable, extending over the wide pH range from 3 to 10 at 35 °C for 3.5 h using 0.1 M CH_3COONa–HCl buffer for the pH range from 1 to 5 and 0.1 M glycine–NaOH buffer from 9 to 13. In particular, α_2 protein was entirely stable even at pH 1 and 12

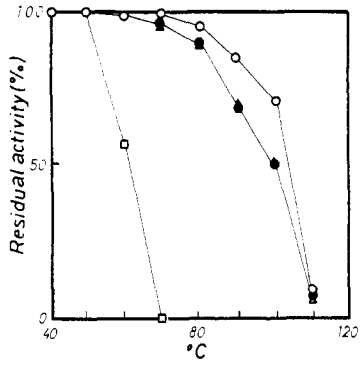

Figure 5. Thermal inactivation curves of the proteins from α_2 to α_4 and KSTI. (□) α_2 protein; (△) α_3 protein; (○) α_4 protein; (●) KSTI.

Table IV. Protease Inhibition Spectrum of Proteins from α_2 to α_4 and the Kunitz Trypsin Inhibitor (KSTI)

	α_2	α_3	α_4	KSTI
animal protease				
trypsin	−	+	+	+
α-chymotrypsin	+	+	−	+
plasmin	−	+	−	+
pepsin	−	−	−	−
plant protease				
papain	−	−	−	−
microbial protease				
Pronase	−	−	−	−
subtilisin BPN′	−	−	−	−
thermolysin	−	−	−	−
A. sojae alkaline protease[a]	−	−	−	−
A. sojae acid protease[b]	−	−	−	−
A. niger acid protease	−	−	−	−
A. saitoi acid protease[c]	−	−	−	−

[a] Hayashi et al. (1967). [b] Hayashi and Mizunuma (1975). [c] Ichishima and Yoshida (1965).

and ~50% of the inhibitory activity against α-chymotrypsin remained at pH 13. In general, the four proteins were highly stable in the acidic region, but the three proteins except α_2 protein almost lost their biological activities at pH 13.

Figure 5 shows the thermal stabilities of the four inhibitors in 0.05 M Tris-HCl buffer (pH 8.00). Among them, α_2 protein was completely inactivated at 79 °C. However, the remaining three inhibitors almost lost their inhibitory activities at 110 °C for 20 min. In particular, α_4 protein was highly thermally stable.

In Table IV, protease inhibition spectra of the four inhibitors are summarized. α_3 protein also agreed well biological with KSTI. It is of interest to note that the two inhibitors of α_2 and α_4 inhibit only α-chymotrypsin or trypsin, respectively, when casein was used as the substrate. However, α_2 inhibitor was not strictly an inhibitor against only α-chymotrypsin. Because the inhibitor reacted with trypsin in the high ratio of inhibitor to enzyme when toluenesulfonyl-L-arginine methyl ester was used as the substrate as described in the preceding paper (Koshiyama et al., 1981), Yamamoto and Ikenaka (1967) reported their Kunitz preparation inhibited only trypsin. The inhibitor may be identical with α_4 protein described here.

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