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Proteolysis of Kunitz Soybean Trypsin Inhibitor. Influence on Its Activity

Iosif A. Vaintraub* and Haram B. Yattara

Laboratory of Protein Chemistry, State University of Moldova,
60 Mateevici Str., Kishinev 277014, Republic Moldova

Action of papain, subtilysin, and pepsin on Kunitz soybean trypsin inhibitor initially splits the inhibitor into fragments retained in the molecule by noncovalent and disulfide bonds. A hypothetical scheme of the inhibitor scission under the action of papain is presented. Further hydrolysis degrades the inhibitor into short peptides. During this time the composition and the ratio of the fragments in the residual protein remain unchanged when hydrolysis was carried out by papain. Only minor changes were observed under the action of the two other proteinases investigated. The inhibitor, when split into fragments, retained in full its inhibitory activity. Decrease of the latter was proportional to the decrease of the residual protein. The results are discussed in the frames of Linderstrøm-Lang's theory of proteolysis.

Keywords: *Kunitz soybean trypsin inhibitor; type of proteolysis; kinetics of proteolysis; inhibitor inactivation; papain; subtilysin; pepsin*

INTRODUCTION

Kunitz soybean trypsin inhibitor (KSTI) is one of the major factors lowering the nutritional quality of soybean proteins (Liener, 1979). It must be inactivated or destroyed during protein processing. Proteolysis is largely used for improving the functional properties of vegetable proteins (Adler-Nissen et al., 1983). During proteolytic modification of soybean proteins, concomitant proteolysis of KSTI leading to inhibitor inactivation may be expected.

The rate of the proteolytic inactivation of any biological active protein depends on the characteristics of the proteolysis and its resuling products. Two extreme types of enzymatic proteolysis have been suggested, the "zipper" and the one-by-one types (Linderstrøm-Lang, 1952; Rupley, 1967). According to these authors a sharp decrease of biological activity of a native protein after splitting of a limited number of peptide bonds occurs during the zipper type proteolysis. With the one-by-one process, the decrease of activity is proportional to the degree of hydrolysis.

Recently a mixed type proteolysis of several proteins was shown to take place. The zipper process may finish at the beginning of proteolysis (Shutov et al., 1991), or it may continue simultaneously with the one-by-one process up to the complete degradation of protein by the latter process (Do Ngok Lanh et al., 1985; Vaintraub et al., 1993).

Though the structure and properties of KSTI have been investigated in detail, little attention has been given to its proteolysis. Limited proteolysis by trypsin and subtilysin, both of which lead to the scission of a single peptide bond, and its effect on the structure and activity of KSTI were reported (Laskowski et al., 1974). No studies of a more profound proteolysis of the native KSTI were performed. It was only mentioned that KSTI is hydrolyzed by pepsin (Birk, 1968).

In the present work the action of three proteinases (papain, pepsin, and subtilysin) on KSTI was studied to establish the type of proteolysis. The detection of the intermediate high molecular mass fragments by SDS gel electrophoresis is sufficient for the detection of zipper type proteolysis (Rupley, 1967). Additional studies of the kinetics of protein degradation and the determination of the molecular mass of the residual protein, e.g., by gel filtration, are needed to establish the parallel occurrence of the zipper and one-by-one proteolysis (Shutov et al., 1991). The determination of the type of proteolysis of KSTI will permit elucidation of the possibility of the inactivation of KSTI during the limited proteolysis of soybean proteins.

MATERIALS AND METHODS

Reagents. Commercial preparations of KSTI (Reanal, Hungary, or practical grade, Serva, Germany) were purified by hydrophobic chromatography (Senyuk et al., 1994). Pepsin (Olajne, Latvia), papain (twofold crystallized, Serva), subtilysin (Serva), trypsin (Spofa, Czech Republic), and benzoyl-DL-arginine *p*-nitroanilide (Merck, Germany) were used in this work.

Proteolysis. Solutions (0.5%) of KSTI were hydrolyzed by pepsin in 0.05 M acetate buffer at pH 4.5 and by papain and subtilysin in 0.05 M Tris-HCl buffer at pH 8.5 and 8.0, respectively. The enzyme–substrate ratio was 1:200 for pepsin and 1:100 for the two other proteinases. The incubation temperature was 37 °C. Hydrolysis was carried out until 80–90% of protein was degraded which required hydrolysis times from 25 to 50 h. NaN_3 was added to the incubation mixture at 0.02% concentration. Samples of hydrolysates were removed at definite time intervals to determine the degree of hydrolysis, performed by measuring the residual (precipitated by TCA) protein, and to carry out SDS electrophoresis and gel filtration. The proteolysis was repeated four to five times for each enzyme.

* Author to whom correspondence should be addressed [fax (3732)240-655; e-mail vaintraub@university.moldova.su].

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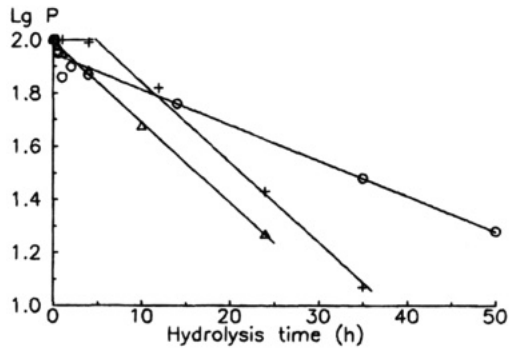

Figure 1. Kinetic curves of the KSTI proteolysis. The log of the mass concentration of the residual expressed as percent of the initial KSTI concentration is plotted on the *y*-axis. Hydrolysis was by papain (○), subtilysin (+), and pepsin (△). Hydrolysis conditions are detailed under Materials and Methods.

Protein determination was performed by a dye-binding method (Gofman, 1968). Samples (5 μL) of hydrolysate were applied on chromatographic paper, washed with 5% TCA, and dyed with 0.05% solution of bromophenol blue in 5% TCA. The excess of the dye was removed by washing with 5% TCA, and the paper was dried at room temperature. The bound dye was eluted by 1.5 mL of 0.01 M NaOH, and the absorbance at 591 nm was measured. Unhydrolyzed KSTI was used for the calibration. The absorbance of a sample with initial KSTI concentration of 5 mg/mL was equal to 0.25, and the calibration curve was linear up to a protein concentration of 8 mg/mL. Three to five determinations were performed for each hydrolysis time. The standard deviation did not exceed 0.01 absorbance unit. The activity of KSTI was determined by measuring its inhibitory action on the trypsin hydrolysis of benzoyl-DL-arginine *p*-nitroanilide (Kakade et al., 1969).

Gel filtration of the incubation mixture through a column (1.2 × 60 cm) of Sephadex G-50 fine was carried out for the evaluation of M_r of the residual protein. The elution was performed with 0.2 M CH_3COOH at an elution rate of 12 mL/h. The protein in the eluate was detected by its absorbance at 290 nm in the flow photometer (PUM-2, Minsk, Byelorus). Elution volume was determined by weighing the eluate.

SDS Electrophoresis. The protein in samples of the hydrolysate was precipitated by adding 3 volumes of acetone. After centrifugation, the precipitated protein was washed three times with acetone and then treated according to Laemmli (1970). SDS electrophoresis was performed in a vertical flat-bed (115 × 115 × 1 mm) 15% polyacrylamide gel. The protein bands were stained with Coomassie brilliant blue G250 (Holbrook and Leaver, 1976). KSTI, lysozyme, A-chain of soybean 11S protein, beef lung trypsin inhibitor, A- and B-chains of insulin, and bacitracin oligomers obtained using glutaric aldehyde as cross-linking agent were all used as standards for M_r determination.

The electrophoregrams were quantified by densitometry using the gel scanning attachment to the spectrophotometer (M-40, Carl Zeiss, Jena). The relative mass content of fractions was determined by weighing the peak areas. Each electrophoregram was analyzed two to three times by the densitometer. Each densitogram was copied twice, and the peaks of the two copies were cut and weighed. The standard deviation did not exceed 12%. The mole fraction of the residual unchanged KSTI and of its fragments was determined from the results of weighing and the respective values of their M_r.

RESULTS

Protein Degradation. All the proteinases used will hydrolyze KSTI to peptides soluble in TCA. In all cases linear dependence of the log of the residual protein concentration on the time of hydrolysis was observed. Hence, the protein degradation followed the pseudo-first-order reaction (Figure 1). A short lag period of several minutes, not visible in the figure, occurred at the beginning of papain hydrolysis. Under the action of subtilysin, lag time was prolonged for about 4 h.

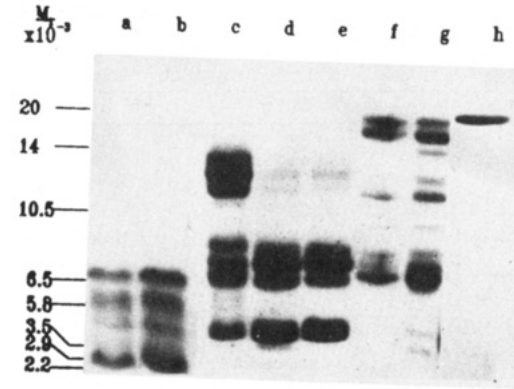

Figure 2. Composition of the residual protein during proteolysis of KSTI. Separation is by SDS electrophoresis under reducing conditions. M_r represents the molecular masses of marker proteins. Hydrolysis was by papain (a, b), subtilysin (c–e), and pepsin (f, g); unhydrolyzed KSTI (h). Hydrolysis times: 2 min (a, c, f), 3 h (d), 15 h (g), and 25 h (b, e).

Table 1. Molecular Masses of the Fragments Formed during KSTI Proteolysis[a]

	M_r		
fragment	papain	subtilysin	pepsin
F18			18.4
F17		16.7	16.9
F14		14.5	14.2
F13		13.1	12.9
F11			11.3
F10			10.2
F9		8.8	8.4
F8		7.7	7.6
F7	6.7	6.7	6.9
F6	5.9		
F5	4.8		5.0
F4		3.9	3.5
F3	2.7		

[a] The fragments are named after the rounded values of their M_r. For details of M_r determination, see Materials and Methods.

Studies of the Residual Protein. Concomitant with the hydrolysis of KSTI to small peptides, this protein was split into several fragments as indicated in SDS electrophoresis gels of the residual protein (Figure 2). The splitting occurred at the very beginning of enzyme action. The first samples for electrophoresis were taken off after 2 min of action of enzymes. The summary of the fragments formed and the values of their M_r are shown in Table 1. The differences in the values of M_r of identically designated fragments are considered to be within the limits of the error of M_r determination by SDS electrophoresis.

The simplest pattern of the fragments formed is observed during the papain hydrolysis. Only four fragments were detected from the very beginning of proteolysis (Figure 2a). Their relationship to the residual protein evaluated was quantitatively close to equimolar and remained constant within the limits of the error of determination during the proteolysis (Figure 2b and Table 2). The sum of M_r of all four fragments which equals 20.1 kDa fits the molecular mass of KSTI determined from its primary structure (Koide and Ikenaka, 1973).

Hydrolysis with subtilysin also brought about a rapid disappearance of the zone of the initial KSTI with consequent formation of seven fragments. Three fragments of higher molecular mass—F17, F14, and

Table 2. Molar Parts of the KSTI Fragments in the Residual Protein of Papain Hydrolysate[a]

hydrolysis time (min)	fragment			
	F7	F6	F5	F3
2	0.21	0.33	0.25	0.20
10	0.19	0.32	0.26	0.25
30	0.24	0.35	0.21	0.16
60	0.17	0.30	0.32	0.25
mean	0.20 ± 0.03	0.33 ± 0.02	0.26 ± 0.04	0.22 ± 0.04

[a] Mean of two independent hydrolysis experiments.

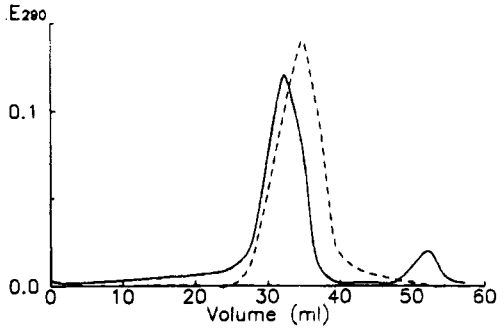

Figure 3. Gel filtration of unhydrolyzed (– – –) versus papain-hydrolyzed (—) KSTI through Sephadex G-50. Hydrolysis time: 20 min. For conditions, see Materials and Methods.

F13—observed after 2 min of hydrolysis (Figure 2c) are intermediate ones. They disappear before the end of the lag period (Figure 2d). Four other fragments are present in the residual protein during the subsequent proteolysis (Figure 2e). All fragments formed by subtilysin except fragment F7 differ from those obtained under the action of papain.

The action of pepsin was studied at pH 4.5 to avoid the possible influence of extreme acidity. A 2 min hydrolysis caused the decomposition of most of the KSTI with the formation of nine fragments (Figure 2f). Later, two new fragments with lower molecular mass appeared (Figure 2g). M_r of seven of the fragments formed by pepsin was similar to that of the fragments appearing under the action of subtilysin (Table 1). However, the high molecular mass fragments were observed during the whole hydrolysis, and the zone of the initial KSTI was still observed in the residual protein even after breakdown of more than 70% of protein.

Samples of hydrolysates with degrees of hydrolysis up to 70% were taken out for gel filtration. In spite of numerous fragments detected by SDS electrophoresis, only one high molecular mass peak was observed during gel filtration of KSTI hydrolysates through Sephadex G-50. The pattern of gel filtration of a papain hydrolysate is shown in Figure 3 as an example. Elution volume remains the same as that of the initial KSTI in the case of pepsin hydrolysate (V_e/V_o = 1.278 ± 0.009 and 1.287 ± 0.010, respectively). The elution volumes of residual proteins of papain and subtilysin hydrolysates were even a little smaller (V_e/V_o = 1.234 ± 0.006) but also unchanged during the proteolysis.

No appreciable changes of inhibitory activity of KSTI were observed under the action of all three proteinases studied after 2 min of hydrolysis. By that time KSTI is either completely split into fragments or almost completely under the action of pepsin. Moreover, in the case of subtilysin hydrolysis, no change of activity occurred during the entire prolonged lag period of the protein degradation. The inhibitory activity begins to decline when protein degradation starts. Its decrease is proportional to the decrease of the residual protein (Figure 4).

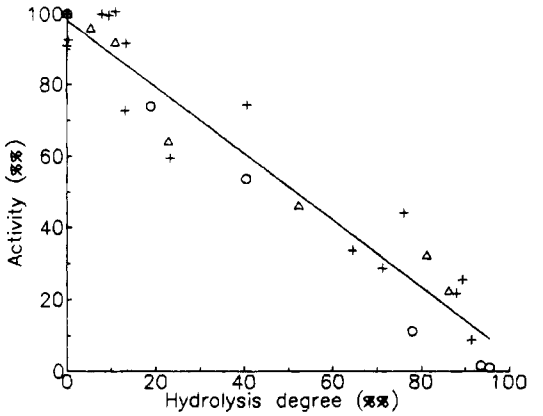

Figure 4. Changes of KSTI activity during its proteolysis. KSTI activity is expressed as percent of the initial activity, and proteolysis degree is the percent of the decrease of the residual TCA-precipitated protein. Hydrolysis was by papain (○), subtilysin (+), and pepsin (△).

DISCUSSION

As in the case of the storage 11S proteins (Shutov et al., 1991), the proteolysis of KSTI may proceed in two ways. Its polypeptide chain is split into several fragments by the zipper process. The splitting occurs mainly at the very beginning of the action of enzymes (≤2 min). A visual estimate of the SDS electrophoregrams obtained during further hydrolysis by papain showed no significant changes in the composition and ratio of the fragments when KSTI is almost completely degraded. Only minor changes occurred under the action of the other proteinases investigated. In the case of papain hydrolysis, these observations were confirmed by quantitative determinations. Formation of relatively stable intermediate fragments is characteristic of the zipper type proteolysis (Lunderstrøm-Lang, 1952; Rupley, 1967).

It was suggested that zipper proteolysis is accompanied by a decrease in the molecular mass of the protein proportional to the lowering of its mass concentration (Shutov et al., 1991). However, according to our gel filtration data, no significant changes of molecular mass of the residual protein occurred. Evidently, the fragments formed remained interlinked by noncovalent and disulfide bonds as was found with hydrolysates of ovalbumin (Vaintraub et al., 1993) and 11S seed storage proteins (Do Ngok Lanh, 1985; Shutov et al., 1991). In contrast to the latter study, no splitting off of short peptides which would lower the molecular mass of the protein occurred by zipper proteolysis of KSTI. Zipper proteolysis of KSTI proceeded, apparently, without any significant changes of its molecular mass. Thus, the above-mentioned suggestion is not of general character.

According to the definition of the zipper proteolysis (Rupley, 1967), each subsequent peptide bond is split considerably slower than the preceding one. Consecutive formation of the fragments results from this definition. However, under our conditions almost all fragments appeared simultaneously. Their consecutive formation may occur at a lower enzyme–substrate ratio. Indeed, conditions were found where only the first split occurred in KSTI under the action of subtilysin (Laskowski et al., 1974).

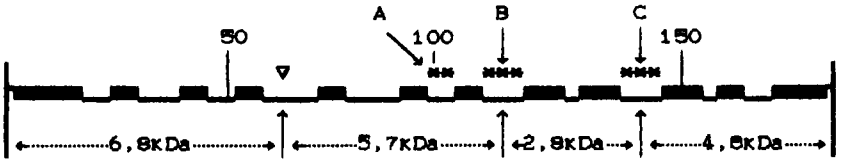

Figure 5. Hypothetical scheme of KSTI scission under the action of papain: (■) β-strands, (—) loops, (***) segments with low electron density, (▽) position of the active center, and (↑) expected sites of scission.

The number and the size of fragments found in the residual protein during the action of papain on KSTI are indicative of three sites of splitting. The known tertiary structure of Kunitz type trypsin inhibitors deduced from X-ray diffraction data (Onesti, 1992) permits to present a hypothetical scheme for the splitting of KSTI during the zipper hydrolysis by papain. The KSTI molecule consists of 12 short (4–8 amino residues) antiparallel β-strands connected by loops of variable length (4–15 amino acid residues). The localization of β-strands and the loops on the unfolded polypeptide chain of KSTI is shown in Figure 5. Three sites of low electronic density (A, B, and C) which possess a high mobility coincide with three of the loops. Owing to high mobility, these sites are more accessible to proteinases. Along with the loop containing the active center of the inhibitor which protrudes on the surface of the molecule, they are the most probable loci of splitting. If the splitting takes place at the low-electron density B and C sites and at the active center site, then the molecular masses of the fragments formed will coincide with the values obtained experimentally (cf. Table 1 and Figure 5). The assumed stability of the short loop A with seven residues is possibly due to the lack of amino acids specifically hydrolyzed by papain.

The action of two other proteinases resulted in a much more complex fragmentation pattern which makes their interpretation difficult. Formation of fragments with $M_r = 6.7–6.9$ under the action of all three proteinases indicates that in all cases one of the scission loci probably occurred near the active center of the inhibitor (see Figure 5). N-terminal sequencing of the fragments formed during zipper proteolysis is planned to support the proposed scheme of the papain action and to explain the action of subtilysin and pepsin.

The degradation of KSTI to short peptides soluble in TCA, the constancy of the fragment composition, and the molecular mass of the residual protein that occurred during further action of proteinases are all indicative of one-by-one type proteolysis (Shutov et al., 1991). Another criterion for the one-by-one proteolysis also fulfilled during the KSTI hydrolysis is that it followed first-order kinetics (Shutov et al., 1991; Vaintraub et al., 1994).

Zipper proteolysis that occurred during the lag period of the one-by-one proteolysis may elicit conformational changes needed for the exposure of the peptide bond whose scission is needed to unfold the KSTI molecule. After the unfolding, one-by-one proteolysis can proceed. A lag period was observed only during the action of papain and subtilysin. Some conformational changes must have occurred under the action of the same proteinases which resulted in a slight but significant decrease of elution volume of the residual protein during gel filtration on Sephadex. Thus, our results indicate that proteolytic action on KSTI is of a mixed type. Zipper type proteolysis is finished at the beginning of proteolysis and then superseded by the one-by-one type process.

According to Linderstrøm-Lang (1952) and Rupley (1967), one should expect a sharp drop in the inhibitory activity of KSTI at the beginning of the proteinase action if proteolysis proceeds by the zipper type. Evidently, this is not the case with KSTI. Its activity remained unchanged during the lag period when the inhibitor is already split into fragments. So, KSTI activity is unaffected by zipper proteolysis. The proportionality of the inhibitor activity to the concentration of the residual protein shows that it is destroyed only by the degradation of the protein to TCA-soluble peptides due to the one-by-one proteolysis. This result is unexpected. However, KSTI also retained its whole activity after splitting of two peptide bonds by consecutive action of subtilysin and trypsin (Laskowski et al., 1974).

A high degree of hydrolysis is required to obtain the isoelectric soluble soy protein hydrolysate used for protein-fortified beverages. Substantial inactivation of KSTI if present in the protein may be reached in this case. Limited proteolysis used to improve the functional properties of the soybean protein (Adler-Nissen et al., 1983) will have little influence on KSTI activity.

ABBREVIATIONS USED

KSTI, Kunitz soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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