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Bowman–Birk Proteinase Isoinhibitor Complements of Soybean Strains

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When immunological tests to identify proteins that show cross-reaction with antiserum from rabbits immunized with Bowman–Birk soybean trypsin inhibitor were used, chromatographic and electrophoretic analyses of the Bowman–Birk proteinase inhibitors in eight soybean strains reveal four to seven isoinhibitor species in each strain. There were ten different isoinhibitors altogether. These were designated BBSTI-A, A′, A″, B, B′, C, C′, D, E, and E′ from the slowest to the fastest migrating on 10% polyacrylamide disc gel electrophoresis in the Davis system.

The utilization of the rich protein resource in soybean is limited in part by antinutritional components. Unless partially destroyed by moist heat, these components cause growth inhibition, pancreatic hypertrophy, and inability to utilize the bean protein in nonruminant animals (Rackis, 1981; Rackis and Gumbmann, 1981). A significant portion of this antinutritional toxicant effect is due to the proteinase inhibitors, proteins which form complexes with mammalian pancreatic serine proteinases and inhibit their enzymatic activity. There are two classes of proteinase inhibitors, the Kunitz (KSTI) and the Bowman–Birk (BBSTI) inhibitors. Within each class, there are isoinhibitor forms (Wilson, 1981; Laskowski and Kato, 1981; Orf and Hymowitz, 1979).

Three isoinhibitors of the Kunitz class have been described. These have been designated Ti^a, Ti^b, and Ti^c (Orf and Hymowitz, 1979). Equilibrium constants for the complex formation of the Kunitz isoinhibitors with bovine trypsin differ by as much as three orders of magnitude (Freed and Ryan, 1980). Soybean strains differ in the type of Kunitz isoinhibitors present in the seed. An extensive study of this has been done by Orf and Hymowitz (1979).

The identification of Bowman–Birk isoinhibitors in soybeans has not been as extensive. Odani and Ikenaka (1977a) purified five Bowman–Birk inhibitors from a Japanese strain of soybeans Sode-furi. These were designated as inhibitors A, B, C-II, D-II, and E-I. Inhibitor A, the major component, was the same as the classical Bowman–Birk inhibitor purified by Bowman and Birk and whose amino acid sequence was elucidated by Odani et al. (1972) and by Odani and Ikenaka (1972). This inhibitor inhibited one molecule of bovine trypsin and one molecule of bovine chymotrypsin simultaneously. Species B was found to be identical with species A, except for the deamidation of one or more asparaginyl or glutaminyl residues. Species C-II sequenced by Odani and Ikenaka (1977b) was double-headed also, having one reactive site for either bovine trypsin or bovine chymotrypsin, the other active against bovine elastase. Inhibitor D-II inhibited only bovine trypsin and very weakly at that. Inhibitor E-I, having essentially the same inhibitor activity as D-II, was found to be a variant of D-II, lacking nine amino acid residues at the N-terminal region (Odani and Ikenaka, 1978).

Hwang et al. (1977) found five Bowman–Birk inhibitors in soybean cultivar Tracy. PI-V, the major component, was the same as the classical Bowman–Birk inhibitor. PI-I to PI-IV belonged to another set. Three of these are thought to be derived from the fourth by proteolysis. Unlike PI-V, none of these four inhibits bovine chymotrypsin and their inhibition of trypsin was weaker than that of PI-V. Recently, Foard et al. (1982) mentioned that the amino acid sequence of PI-II and PI-IV matched the sequence of E-I and D-II (Odani and Ikenaka, 1978), respectively.

Stahlhut and Hymowitz (1983) found five isoinhibitors in strain Amsoy 71 and named these I, II, III, IV, and V. Inhibitor III was found to be the classical Bowman–Birk inhibitor. They classified 470 strains of soybean according to the presence or absence of inhibitors III and V.

In an earlier report, we studied the isoinhibitors in cultivar Fiskeby V (Tan-Wilson et al., 1982). The classical Bowman–Birk inhibitor was designated BBSTI-E. Another inhibitor which was present in trace amounts in seed but increased in proportion to BBSTI-E in the cotyledon of the day 4 to day 6 seedlings was BBSTI-D. In addition, there were other isoinhibitors which eluted prior to BBSTI-E from a DEAE-cellulose column in a pH gradient from pH 6.5 to pH 5.

Although complete amino acid composition and sequence analysis for the isoinhibitors in these four strains of soybeans are not available, a cursory survey seems to point toward some isoinhibitors being common to all four strains and some isoinhibitors being in one but not another soybean strain. Since the studies were all conducted by separate research groups with different separation schemes and electrophoretic systems, we do not know just how extensive the number and variation of Bowman–Birk

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isoinhibitors is. In this paper we report the comparison of eight soybean strains with respect to their complements of Bowman–Birk type proteinase inhibitors. We identified the Bowman–Birk isoinhibitors by cross-reaction to anti-Bowman–Birk inhibitor antibody and inhibition of bovine trypsin. We looked for those which were common across strains by their chromatographic behavior on DEAE-ion exchange at pH 6.5–5 and by their electrophoretic migration on polyacrylamide gel electrophoresis at alkaline pH. Although each of the eight strains had six to seven isoinhibitors, there were many that were common to the strains so that we identified ten isoinhibitors altogether in the eight soybean strains.

EXPERIMENTAL SECTION

Materials. *Plant Materials.* Soybean, *Glycine max* (L.) Merrill, cv. Altona, Envy, Black Jet, Panther, and Wilkin, were obtained from Johnny's Selected Seed Company, Albion, ME. Amsoy 71 was from the May Seed and Nursery Company, Shenandoah, IA. Disoy was from Gurney Seed and Nursery Company, Yankton, SD, while Fiskeby V was from Stokes Seed Company, Buffalo, NY.

Reagents. Bovine trypsin, bovine α-chymotrypsin (3x crystallized), α-*N*-benzoylarginine ethyl ester, α-*N*-benzoyltryosine ethyl ester, congo red-elastin, *N,N'*-methylenebis(acrylamide), and *N,N,N',N'*-tetramethylethylenediamine were from Sigma Chemical Company. Sephadex G-75 was from Pharmacia Fine Chemicals. DE-52 DEAE-cellulose ion exchange resin was from Whatman.

Anti-Kunitz trypsin inhibitor antiserum was obtained from rabbits immunized with Kunitz trypsin inhibitor. Anti-Bowman–Birk trypsin inhibitor antiserum was from rabbits immunized with glutaraldehyde-polymerized Bowman–Birk inhibitor. Both antigens were electrophoretically pure and were judged to be pure also by amino acid composition. Antisera were specific for each class of inhibitor. Purification and immunization protocols have been described previously (Tan-Wilson and Wilson, 1982).

All pH measurements were made at room temperature (21 ± 1 °C). Doubly distilled water was used throughout.

Procedures. *Assays.* Trypsin inhibitor activity was determined by using α-*N*-benzoylarginine ethyl ester as substrate. One unit of inhibitor was defined as the amount of inhibitor which inhibits 1 mg of active trypsin. The values reported have been corrected for the amount of inactive trypsin in commercial preparations (Kassell, 1970).

Chymotrypsin inhibitor activity was assayed with bovine α-chymotrypsin by using α-*N*-benzoyltyrosine ethyl ester as the substrate (Hummel, 1959; Kress et al., 1968).

Elastase inhibitor activity was assayed by using congo-red elastin as the substrate (Naughton and Sanger, 1961).

The amounts of KSTI and BBSTI protein in column fractions was determined by radial immunodiffusion (Mancini et al., 1965) with modifications (Tan-Wilson et al., 1983). The Ti^a form off KSTI (Orf and Hymowitz, 1979) and the E form of BBSTI (Tan-Wilson et al., 1982) were used as standards. The concentrations of inhibitor were determined spectrophotometrically at 280 nm with $E^{1\%}_{1\ cm}$ = 10.01 and 4.6 for Ti^a and BBSTI-E, respectively (Kassell, 1970).

Soybean Bowman–Birk Trypsin Inhibitor Preparation. Seed coats were removed and seeds extracted with Tris-Cl pH 8 buffer containing phenylmethylsulfonyl fluoride and sodium iodoacetate. Bowman–Birk trypsin inhibitor was separated from other seed proteins by ammonium sulfate precipitation and by acid fractionation. Separation from Kunitz inhibitor was accomplished by Sephadex G-75 gel filtration, with radial immunodiffusion used for the measurement of KSTI and BBSTI concentrations in the fractions. All details have been described in an earlier report (Tan-Wilson et al., 1982).

Separation of Isoinhibitor Forms by Ion-Exchange Chromatography. The Bowman–Birk trypsin inhibitor pool was fractionated on a DEAE-cellulose column (DE-52) in 0.05 M ammonium acetate, pH 6.5, buffer. The inhibitors were eluted with a linear gradient of 0.05 M ammonium acetate pH 6.5 as the initial buffer and 0.5 M ammonium acetate pH 5 as the final buffer. Details of the procedure have been published (Tan-Wilson et al., 1982).

Electrophoresis. Two systems of polyacrylamide disc gel electrophoresis were used. One was the Davis (1964) system with 10% acrylamide slab gels. The other was the Davis system with 15% acrylamide slab gels and with the addition of 4 M urea in the gel and the reservoir buffer. The gels were stained with 1.5% (w/v) Amido-schwartz in 7% (v/v) acetic acid. Gels were destained by diffusion with 7% (v/v) acetic acid.

When multiple bands appeared, the unstained gels (without urea) were sliced and eluted and each band checked for Bowman–Birk trypsin inhibitor by radial immunodiffusion as described previously (Tan-Wilson and Wilson, 1982).

Amino Acid Analysis. Samples for amino acid analysis were hydrolyzed in 5.7 N HCl in vacuo at 110 °C for 20 h. Analysis was carried out on a Glenco MM-70 amino acid analyzer equipped with a ninhydrin detection system. All values reported reported are the average of at least two determinations.

RESULTS

Chromatographic Analysis of Bowman–Birk Isoinhibitor Forms. Elution profiles of the Bowman–Birk trypsin inhibitor pools from each of the seven soybean strains from ion exchange chromatography on DEAE-cellulose are compared in Figure 1. The elution profile for the Bowman–Birk inhibitors from Fiskeby V has been published earlier (Tan-Wilson et al., 1982). The fractions were monitored for both trypsin inhibitor activity and cross-reaction to anti-Bowman–Birk antibodies and the peaks for both were coincident in the elution profiles. Fractions in each peak were pooled and concentrated by lyophilization prior to comparison by polyacrylamide gel electrophoresis.

Electrophoretic Analysis of Bowman–Birk Isoinhibitor Forms BBSTI-A+A'+A". The electrophoretic migration patterns of the isoinhibitors eluting with less than 1 L of the gradient at a pH of 6 and with 0.05 M ammonium acetate buffer were compared. All soybean strains studied had at least two bands in this region.

The strain Wilkin showed a third band. The same multiple bands appeared after urea gel electrophoresis. All three protein bands were found to cross-react with anti-BBSTI antiserum when gel slices were examined. These three isoinhibitors are designated as BBSTI-A, A', and A". Electrophoretic migration patterns of sample pools containing these three isoinhibitors can be seen in Figures 2 and 3.

BBSTI-B+B'+C+C'. The Bowman–Birk inhibitors from the many peaks eluting with 1.5–2.5 L of the gradient at pH 5 and 0.15 to 0.2 M ammonium acetate run very closely on polyacrylamide gel electrophoresis. Four distinct forms could be discerned. Sliced gels were eluted and the protein bands were coincident with cross-reactivity to anti-Bowman–Birk antibodies. The four distinct forms also appear on urea gel electrophoresis although migration patterns change. These inhibitors are designated as BBSTI-B, B', C, and C'. Electrophoretic migration pat-

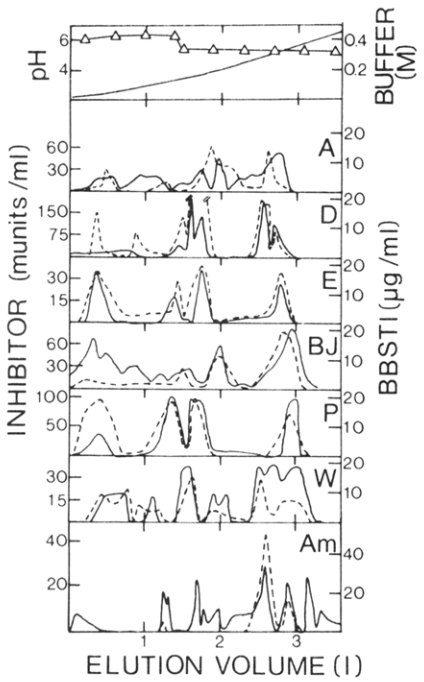

Figure 1. Comparison of elution profiles on DEAE-cellulose (2.5 × 100 cm) eluted with a 5.4 L gradient with 0.05 M ammonium acetate pH 6.5 as the initial buffer and 0.5 M ammonium acetate pH 5 as the final buffer at a flow rate of 60 mL/h. Bowman–Birk inhibitor pools from Sephadex G-75 columns were charged on these columns. Fractions were monitored for trypsin inhibitor activity (—) and Bowman–Birk concentration as determined by radial immunodiffusion (– –). The top panel shows the gradient in pH (–Δ–) and buffer molarity (—); the latter was determined from measurements of conductivity.

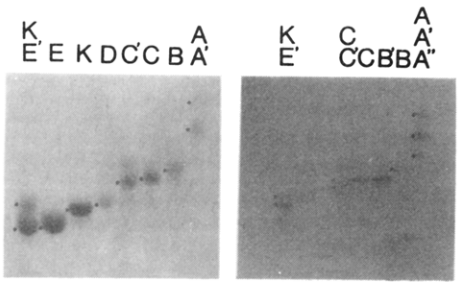

Figure 2. Polyacrylamide gel electrophoresis of sample pools which together display the ten Bowman–Birk and the Kunitz Ti[a] isoinhibitors. Dots to the left of each band mark the position of the isoinhibitor identified by the appropriate letters.

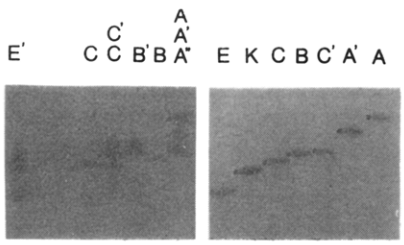

Figure 3. Polyacrylamide gel electrophoresis in 4 M urea of sample pools which together display the nine Bowman–Birk and the Kunitz Ti[a] isoinhibitors. Dots to the left of each band mark the position of the isoinhibitor identified by the appropriate letters.

terns of sample pools containing these four isoinhibitors can be seen in Figures 2 and 3. Some had electrophoretic bands corresponding to BBSTI-A″ since this isoinhibitor elutes just before BBSTI-B.

BBSTI-E+E′. With one exception, all strains had one Bowman–Birk isoinhibitor eluting at 2.5–3 L of the gradient at pH 5 and 0.3 to 0.5 M ammonium acetate buffer. These isoinhibitors from the different strains exhibited identical migration on polyacrylamide gel electrophoresis with and without urea. We did note that varying high amounts of the inhibitor from the same soybean strain tended to exhibit faster migration with an increase in the inhibitor quantity on gels without urea. This isoinhibitor is designated as BBSTI-E.

Cultivar Disoy had a Bowman–Birk isoinhibitor eluting just past BBSTI-E on the ion exchange column and migrating faster than BBSTI-E on gels with and without urea. This is designated as BBSTI-E′. Electrophoretic migration patterns of BBSTI-E and BBSTI-E′ are shown in Figures 2 and 3.

Bowman-Birk Isoinhibitor Distribution in Soybean Strains. Figure 2 shows two gels of sample pools which together display the whole range of Bowman–Birk inhibitors from the fastest migrating BBSTI-E′ through BBSTI-E, D, C′, C, B′, B, A″, A′, and finally to the slowest migrating BBSTI-A.

BBSTI-D is from day 6 cotyledons of cultivar Fiskeby V, an isoinhibitor derived from BBSTI-E by proteolysis during germination (Tan-Wilson et al., 1982).

Figure 3 shows the same isoinhibitors when run in 4 M urea gels. The sequence is slightly different: BBSTI-E′, E, D, C, B, C′, B′, A″, A′, and A from the fastest to the slowest migrating inhibitor.

Table I indicates the isoinhibitors found to be present in each strain studied and identified by three separate characteristics—elution from DEAE-cellulose at pH 6.5–5 and polyacrylamide gel electrophoresis at pH 8.9 with and without urea.

Table I. Bowman–Birk Isoinhibitor Compositions of Soybean Strains

soybean strain	BBSTI species									
	A	A′	A″	B	B′	C	C′	D	E	E′
Altona	X	X		X	X	X	X		X	
Amsoy 71	X	X	X	X		X	X	X	X	
Black Jet	X	X				X			X	
Disoy	X	X	X	X		X			X	X
Envy	X	X		X		X	X		X	
Fiskeby V	X	X	X	X		X		X	X	
Panther	X	X	X	X		X			X	
Wilkin	X	X	X			X	X		X	

Table II. Amino Acid Compositions of BBSTI-E and BBSTI-C

amino acid	BBSTI-E		BBSTI-C			
	best	integer	A[b]	best	integer	PI-III[c]
Asx	10.9	11	11	11.1	11	11
Thr[a]	2.1	2	2	2.6	3	3
Ser[a]	8.6	9	9	8.3	8	8
Glx	6.7	7	7	7.2	7	6
Pro	5.7	6	6	4.0	4	5
Gly	0	0	0	1.1	1	1
Ala	4.2	4	4	1.2	1	1
$^1/_2$Cys	13.3	13–14	14	12.8	13	12
Val	1.0	1	1	0	0	0
Met	0.9	1	1	1.6	2	2
Ile	1.8	2	2	1.3	1	1
Leu	2.0	2	2	3.0	3	3
Tyr	1.9	2	2	1.9	2	2
Phe	1.8	2	2	1.0	1	1
Lys	5.0	5	5	3.9	4	4
His	0.9	1	1	0.9	1	1
Arg	2.0	2	2	4.0	4	4
total		70	71		66	65

[a] All data from 20-h hydrolyses. Thr and Ser values corrected for 5% destruction of Thr and 10% destruction of Ser. [b] Odani and Ikenaka (1972). [c] Hwang et al. (1977).

Amino Acid Analysis. The amino acid compositions of BBSTI-E and BBSTI-C were determined and compared to published data for selected inhibitors from cultivars Sode-furi and Tracy in Table II. The identity of BBSTI-E to inhibitor A (Odani and Ikenaka, 1977) and to PI-V (Hwang et al., 1977) is clearly seen. BBSTI-C is not exactly identical in composition to any of the other characterized isoinhibitors but is most closely related to the family represented by D-II, E-I (Odani and Ikenaka, 1977) and PI-II, PI-III, and PI-IV (Hwang et al., 1977). Of these, BBSTI-C mostly closely resembles PI-III.

Inhibitor Activity. Only BBSTI-E and BBSTI-D inhibited both bovine trypsin and chymotrypsin. The others inhibited only bovine trypsin. None of the ten inhibited bovine elastase.

DISCUSSION

We have defined the term Bowman–Birk inhibitor to be, for the purposes of this paper, a protein which inhibits bovine trypsin and which cross-reacts with antiserum from rabbits immunized with BBSTI-E. The forms which we have named BBSTI-A, A′, A″, B, B′, C, and C′ did not cross-react with antiserum produced initially by the rabbits. Booster injections with BBSTI-E widened the specificity of later antisera to include these other isoinhibitors although the cross-reaction is quite visibly weaker. Hwang et al. (1977) also found that their inhibitors PI-I to PI-IV did not cross-react with antiserum elicited by PI-V.

There are certain precautions which we have found to be essential in an analysis of Bowman–Birk inhibitors. (1) Organic solvent extraction should be avoided. We have found that the use of acetone extraction to defat soybean meal or the use of acetone in organic solvent precipitation to separate Kunitz from Bowman–Birk inhibitor produces artifactual BBSTI species. Four BBSTI species differing in chromatographic properties on DEAE- and CM-cellulose but migrating as BBSTI-E on electrophoresis and having the same amino acid compositions were found in our initial pilot studies with organic solvents. We suspect that acetone treatment promotes the deamidation of BBSTI-E. (2) Soybean seeds contain proteolytic enzymes which utilize BBSTI-E and most possibly other isoinhibitors as substrates. The addition of phenylmethylsulfonyl fluoride and sodium iodoacetate to inhibit serine and sulfhydryl proteinases during extraction procedures aid in preventing the generation of new isoinhibitor forms not originally present in the seed or the disappearance of some original forms by complete proteolysis. The generation of isoinhibitors by proteolysis during seed maturation and storage, of course, can not be prevented. (3) Unless the separation of Kunitz from Bowman–Birk inhibitors is complete, chromatographic and electrophoretic analysis of Bowman–Birk isoinhibitor forms should include a method to distinguish the two classes of inhibitors. In our procedure, Kunitz inhibitor elutes just beyond BBSTI-E on the DEAE-cellulose column and migrates between BBSTI-E and BBSTI-D on the gels. We identify it by its cross-reaction to anti-KSTI but not to anti-BBSTI antisera. (4) Some Bowman–Birk inhibitors such as BBSTI-E and BBSTI-D tend to self-associate. This causes changing patterns of electrophoretic migration with increasing concentration of inhibitor. The tendency to self-associate appears to increase with length of storage of the preparations in solution. The addition of urea eliminates such anomalies. (5) BBSTI-E and BBSTI-D fix in gels only when freshly prepared Amido-schwartz stain is used.

Employing all these precautions, we have found a total of ten isoinhibitor forms of BBSTI in eight soybean strains (Table I). BBSTI-A, A′, C, and E were present in all strains. BBSTI-A″, B, and C′ were in about half the strains. BBSTI-B′ and E′ were found only in cultivars Altona and Disoy, respectively. BBSTI-D is not usually found in significant amounts in the dry seed but arises by proteolysis of BBSTI-E during seed germination (M. Madden, A. L. Tan-Wilson, and K. A. Wilson, manuscript submitted for publication).

Correlating our results with the published work of Odani and Ikenaka (1977), Hwang et al. (1977), and Stahlhut and Hymowitz (1983), we find at least one BBSTI common to all soybean strains that had been studied extensively. The classical Bowman–Birk inhibitor, designated inhibitor A, PI-V, inhibitor III, or BBSTI-E, appears as a major component. However, Stahlhut and Hymowitz (1983) did not find their inhibitor III in all the 470 strains that they surveyed. The strains were also classified according to the presence or absence of inhibitor V, an inhibitor which they had first established to be present in Amsoy 71. Analysis of the BBSTI complement in Amsoy 71 by our methods did not reveal the presence of an inhibitor with much faster migration than BBSTI-E in the 22% polyacrylamide gels used for the survey. Therefore, we can not identify inhibitor V with any of our ten isoinhibitor species. A major Bowman–Birk inhibitor that appears to be related to inhibitors E-I and D-II in Sode-furi and to PI-II, PI-III, and PI-IV in Tracy by amino acid composition is BBSTI-C found in the eight strains that we studied. Functionally, these inhibitors only inhibit bovine trypsin, not bovine chymotrypsin. Immunochemically, these inhibitors show weak or no cross-reaction to rabbit antibodies elicited by glutaraldehyde-polymerized BBSTI-E.

The other two isoinhibitors which we found in all strains were BBSTI-A and A′. None of the other published works has described inhibitors with the electrophoretic properties of BBSTI-A and A′. However, pool D-I in Odani and Ikenaka's work elutes off the DEAE cellulose column at pH 6.5 and at very low concentrations of ammonium acetate buffer as well. Pool D-I was not further characterized.

Other isoinhibitors, BBSTI-A″, B, B′, C′, D, and E′ were not found in all strains. The difference in the complement of Bowman–Birk isoinhibitor forms in soybean strains might occur due to differences in the array of inhibitor genes present, in expression of these genes during seed maturation, or in the possible occurrence of proteolysis during seed maturation and storage.

Bowman–Birk inhibitors, by virtue of their high cystine content, complement the low sulfur-containing amino acid storage proteins in the soybean. Studies of bean inhibitors have shown very significant differences in the inhibition of trypsins from different mammalian species (Wilson, 1981; Madar, 1979). The inclusion in livestock feed of soybean strains containing Bowman–Birk isoinhibitors with the weakest inhibition for a particular livestock species would optimize the nutritional advantage offered by this class of inhibitors.

Registry No. BBSTI, 37330-34-0.

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Degradation of 2,4,6-Trialkyltetrahydro-1,3,5-thiadiazines during Storage

Tetsuo Kawai,* Mamoru Irie, and Morihiko Sakaguchi

Degradation of 2,4,6-trialkyltetrahydro-1,3,5-thiadiazines (alkyl = methyl, III; alkyl = ethyl, IV) during storage was investigated. Both thiadiazines synthesized from aldehydes, ammonia, and hydrogen sulfide decomposed largely to 2,4,6-trialkyldihydro-1,3,5-dithiazines (alkyl = methyl, I; alkyl = ethyl, II) and *N,N'*-dialkylidene-1,1-diaminoalkanes (alkyl = ethyl, VII; alkyl = propyl, VIII) during storage. Some other compounds which simultaneously occurred were identified as *N*-ethylidene-1-aminoethene (X), *N*-ethylidene-1,1'-diaminoethane (XI), and *N*-propylidene-1-amino-1-propene (XII) by GC–MS. Diaminoalkanes VII and VIII were thermally decomposed further to XI and XII, respectively. A pathway was proposed for the degradation of III and IV to these compounds as well as to I and II. Odor profiles of I–IV were described in relation to pH alteration.

Ethanal, propanal, ammonia, and hydrogen sulfide are known as degradation products of lipids and amino acids during cooking and also as natural precursors of heated flavor components (Forss, 1967; Fujimaki et al., 1969).

A flavor substance, 2,4,6-trimethyldihydro-1,3,5-dithiazine (I) is generally formed from a mixture of ethanal, ammonia, and H_2S; the reaction to form I occurs almost spontaneously (Brinkman, et al., 1972; Kubota et al., 1982a,b). Wöhler and von Liebig (1847) first isolated I from the mixture of H_2S and 2,4,6-trimethylhexahydro-1,3,5-triazine (V). Later, I was known to occur as a major component in heated beef broth (Brinkman et al., 1972). The substance has so far been detected in beef (Wilson et al., 1973, 1976; MacLeod and Coppock, 1977), lamb fat (Buttery et al., 1977), mutton (Nixon et al., 1979), antarctic krill (Kubota et al., 1980), small shrimp (Kubota et al., 1982a), and soya bean (Sugawara et al., 1983). In red bean (Buttery et al., 1975) and small shrimp (Choi et al., 1983), I is also a major component of the volatile oil. In a reaction mixture of cysteine with xylose (Ledl and Severin, 1973, 1974), I was detected, and similarly in a thermally decomposed product of cysteine or cystine alone (Ledl, 1976).

2,4,6-Trimethyltetrahydro-1,3,5-thiadiazine (III), together with I, has been produced from a mixture of ammonia and bis(1-mercaptoethyl) sulfide prepared from ethanal and H_2S, as reported by Boelens et al. (1974). They also described that III decomposed rapidly when it was applied to GC. Ledl (1975) isolated 2,4,6-triethyldihydro-1,3,5-dithiazine (II) from a mixture of propanal, ammonia, and H_2S. No report on the isolation of 2,4,6-triethyltetrahydro-1,3,5-thiadiazine (IV) has been available yet.

The present paper reports synthetic pathways of I and II via III and IV. We also propose decomposition pathways of III and IV to I, II, and *N,N'*-dialkylidene-1,1-diaminoalkanes (alkyl = ethyl, VII; alkyl = propyl, VIII). Brief descriptions on some organoleptic characteristics of I and II have been known as heated meat or beef odor (Ledl, 1975; Kubota et al., 1980) and onion odor (Ledl, 1975); we report here some characteristics of III and IV together with I and II in more detail.

EXPERIMENTAL SECTION

Preparation of Substances. Dithiazine I was synthesized from ethanal, ammonia, and H_2S. H_2S gas was bubbled moderately through the mixture of 2.7 mol of ethanal (132 g, assay ca. 90%) and 2.7 mol of concentrated (25–28%) aqueous ammonium hydroxide (360 mL) with stirring, having kept the temperature of the mixture in a range of −5 to −10 °C until the reaction temperature ceased rising. The reaction consumed approximately two times as much as the theoretical weight of H_2S. After being left overnight at room temperature, the mixture was heated to 64 °C. The organic layer was separated, washed four times with hot water, dried over sodium sulfate, filtered, and stored at −20 °C. Recrystallization was performed

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