Heat Inactivation of the Kunitz and Bowman–Birk Soybean Protease Inhibitors

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The most abundant soybean protease inhibitors (SBPI) are the Kunitz inhibitor (KSTI) and the Bowman–Birk inhibitor (BBI). With use of enzymatic and immunochemical techniques to distinguish between the two inhibitors, inactivation of KSTI and BBI during various types of heat treatment was investigated. Solutions of purified KSTI and BBI were heated (100 °C) along with a crude extract of raw soy flour. The soy extract lost inhibitory activity most rapidly, while purified KSTI lost inhibitory activity at an intermediate rate and purified BBI lost inhibitory activity very slowly. When the inhibitors, in pure form or in situ, were heated (75–95 °C) within a soy flour matrix, dramatically different results were obtained. SBPI in situ still lost inhibitory activity most rapidly. However, purified BBI was inactivated more quickly than purified KSTI. This result conflicts with the commonly held belief that BBI is a heat-stable protease inhibitor regardless of heating conditions.

It has long been known that soybeans contain protease inhibitors that are capable of acting as antinutrients. Though heat treatment is often used to inactivate these inhibitors, soy products often contain 5–20% of the inhibitory activity found in raw soy (Rackis and Gumbmann, 1981). There is concern that the residual amount of soybean protease inhibitors (SBPI) in soy products may be nutritionally significant (Liener, 1986).

There are two major types of SBPI, the Kunitz inhibitor (KSTI) and the Bowman–Birk inhibitor (BBI) [see review by Liener and Kakade (1980)]. KSTI is a protein with a molecular weight of 20 100. It has few disulfide bonds, inhibits trypsin strongly, and is a weak inhibitor of chymotrypsin. BBI is a protein with a molecular weight of 8000. It has many disulfide bonds and inhibits both trypsin and chymotrypsin strongly at independent binding sites.

KSTI is usually described as the "heat-labile" inhibitor, while BBI is often referred to as the "heat-stable" inhibitor. In most cases, descriptions of BBI as "heat stable" are derived from the early work of Birk (1961). In that work, purified BBI retained its antiproteolytic activity after being heated in an aqueous solution at 100 °C for 10 min. The original experimental evidence that led to the labeling of KSTI as heat labile was that of Rackis (1966). In that study, the inactivation of SBPI, which had been purified by ion-exchange chromatography, was examined. Since the purified SBPI was rapidly inactivated by heat, it was assumed to be KSTI rather than BBI. In later years, this led to the belief by researchers in general that KSTI is more heat labile than BBI. Obara and Watanabe (1971) presented direct evidence that purified BBI was more heat stable than purified KSTI when they were heated in aqueous solutions at temperatures ranging from 36 to 70 °C. However, the conditions used in their experiments do not represent conditions found during the actual heating of soy flour. Therefore, relying on results of experimentation with purified inhibitors in solution as a measure of heat stability in other situations may be improper.

The amount of active SBPI in a soy product is generally evaluated by measuring overall trypsin inhibitory activity. While this does offer useful information about the overall presence of protease inhibitory activity in a sample, it gives no indication about which type of SBPI is responsible for the inhibitory activity. Distinguishing between activity due to KSTI and BBI is important because the inhibitors may have different antinutritive effects once they are ingested. For example, it has been shown that lima bean inhibitor, an inhibitor very similar to BBI, is resistant to digestion by pepsin while KSTI is not (Krogdahl and Holm, 1981). Thus, specific identification of residual SBPI activity as KSTI or BBI is essential to properly evaluate its potential antinutritive effect.

It is possible to distinguish between SPBI activity due to KSTI and that attributable to BBI. Since KSTI inhibits only trypsin strongly while BBI inhibits both trypsin and chymotrypsin strongly, antichymotryptic activity provides a good measurement of BBI activity. Using antisera to KSTI, specific measurement of KSTI can be achieved by rocket immunoelectrophoresis (RIEP).

This study involved two phases of work. In the first phase, we investigated the ability of heat treatment to inactivate purified KSTI and purified BBI, as compared to the inactivation of SBPI activity in an extract of raw soy flour receiving the same heat treatment. The purpose of this phase of work was to attempt to confirm Birk's finding that purified BBI is heat stable and to compare its behavior with that of pure KSTI and unpurified SBPI. In the second phase of work, we investigated the inactivation of SBPI during heat treatment within a soy flour matrix. In this case, the inhibitors were either purified KSTI, purified BBI, or SBPI in situ. The purpose of this phase of work was to examine the effect of various parameters such as time, temperature, and water activity (a_w) on inactivation of the inhibitors. In addition, we wanted to determine whether behavior of purified KSTI and BBI (as detemined in the first phase) was predictive of their behavior in the soy matrix itself.

MATERIALS AND METHODS

Sources of Inhibitors. *KSTI.* Commerically available purified KSTI (Sigma Chemical Co.) was used in both phase 1 and 2 heating studies. It was further purified on DEAE–cellulose (Freed and Ryan, 1978) before use in the phase 1 study, for antibody development or as a standard for RIEP.

BBI. BBI was purified by a slight modification of the procedure described by Birk et al. (1963) involving the extraction of raw defatted soy flour with 60% ethanol and

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precipitation by 2 vol of acetone. Final purification was achieved by sequential chromatography on DEAE- and CM-cellulose.

Soy Flour. Raw defatted soy flour was obtained from Cargill, Inc., Cedar Rapids, IA.

Inhibitor Assays. *Enzymatic Assays.* Trypsin inhibitory activity was measured in a manner similar to that of Kakade et al. (1969) using α-*N*-benzoyl-DL-arginine-*p*-nitroanilide as a substrate. Chymotrypsin inhibitory activity was measured in a manner similar to that of Bundy (1963) using *N*-benzoyl-L-tyrosine-*p*-nitroanilide as a substrate.

Generation of Antisera. Antisera to KSTI were generated by subcutaneous injection of the inhibitor into New Zealand white rabbits. Specificity of the sera was verified by immunoelectrophoresis (Weeke, 1973; Mayer and Walker, 1984).

RIEP for KSTI. RIEP was performed according to Freed and Ryan (1978) with some adaptations including the following. Rather than agarose gels, Whatman 3MM paper was employed as wicks for the electrodes. After the first hour of electrophoresis, plugs of fresh agarose were placed in the sample wells and then electrophoresis was continued for an additional 5 h.

Phase 1 Heating Study. *KSTI and BBI Solutions.* Buffered saline (0.05 M imidazole, 0.155 M NaCl, pH 7.0) was used to dissolve the purified inhibitors. KSTI was prepared at a concentration of 200 μg/mL. BBI was prepared at a concentration of 150 μg/mL.

Soy Extract. An extract of raw defatted soy flour was made by weighing 1 g of the flour into a 150-mL glass beaker. After the addition of 50 mL of 0.01 N NaOH, the flour was mixed for 2 min with a high-shear mixer. The sample was immediately centrifuged at 10000*g* for 30 min. The supernatant was collected and adjusted to pH 7.0 by dropwise addition of 1.0 M HCl.

Heat Treatment. Pyrex screw-cap vials (10 mm × 45 mm) with Teflon-lined caps were filled with 0.75 mL of the appropriate inhibitor solution. All of the vials were simultaneously placed in a boiling water bath. Samples were removed after each predesignated time period and then rapidly cooled in an ice water bath.

Phase 2 Heating Study. *Equilibration of Soy Flour to Various a_w.* Constant relative humdity chambers were set up according to the guidelines set forth by Labuza (1984). For this study, slushes of the salts $MgCl_2$, $MgNO_3$, and NaCl were used to generate chambers with constant a_w of 0.32, 0.50, and 0.75, respectively.

About 100 g of soy flour was placed in a baking dish (16.5 cm × 12 cm) inside each of the chambers. When the flour reached a constant weight, it was removed from the chamber and stored in sealed glass containers until needed. Equilibration normally took 14–21 days. Moisture content of the flours was determined by extracting the flours with ethanol and analyzing the moisture content of the extract with a Photovolt Corp. Aquatest IV Karl Fischer moisture analysis apparatus.

Autoclaved Soy Flour. To produce soy flour with less than 3% of the original inhibitory activity, a 1-cm layer of raw defatted soy flour was spread on a metal tray. The flour was left uncovered and was autoclaved at 15 psi (121 °C) for 10 min. It was allowed to cool to room temperature before addition of the purified inhibitors.

Addition of Inhibitors to Soy Flour. Purified KSTI and BBI were dissolved in the appropriate amount of distilled deionized water (DDW). The amounts of soy flour, inhibitor(s), and DDW used to produce the four different samples are shown in Table I. Purified KSTI and BBI were added so that their final concentration was similar to what was found in the raw soy flour used here, on the basis of analysis by chymotrypsin inhibition and KSTI RIEP. The solubilized inhibitors were mixed with soy flour for 2 min with a high-shear mixer. Once the samples had been mixed and then lyophilized, they were reground with a mortar and pestle. Reground samples were equilibrated to a_w 0.75 in a constant-a_w chamber, as previously described.

Table I. Composition of Soy Flour with Purified SBPI Added

sample	soy flour	amt flour, g	inhibitor, mg		DDW, mL
			KSTI	BBI	
raw	raw	15	0	0	60
KSTI	autoclaved	15	225	0	60
BBI	autoclaved	15	0	105	60
KSTI + BBI	autoclaved	15	225	105	60

Heat Treatment. Heat treatment of the soy flours took place in a water bath set at 75, 85, or 95 °C. Approximately 2 g of the appropriate flour was placed in a Pyrex screw-cap vial (122 mm × 13 mm) with Teflon-lined caps. The recorded heating time began immediately after the vials were placed in the water bath. Vials were removed after their assigned heating time and cooled rapidly in an ice water bath.

Extraction of Heated Soy Flour for Inhibitor Assays. Extracts of heated soy flours were prepared as described for the preparation of soy extract except that after centrifugation the pH was not adjusted.

Statistical Analysis. Data from phase 2 were subjected to repeated measures ANOVA (Weisberg and Koehler, 1982). Regression analysis was performed as described by Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Phase 1 Heating Study. One of the objectives of this research was to examine the inactivation of KSTI and BBI during heat treatment. In phase 1, the thermal inactivation of pure KSTI and BBI in solution and of SBPI in a 2% extract of raw defatted soy flour was compared.

Figure 1A shows the loss of antitryptic activity during heat treatment of the three different solutions. Under these conditions, purified BBI was extraordinarily heat stable as compared to purified KSTI or SBPI activity in soy extract. While BBI retained over 75% of its antitryptic activity after 360 min of heat treatment, KSTI lost most of its activity after 180 min and the soy extract after just 30 min of heat treatment.

Figure 1B shows the loss of antichymotryptic activity during heating. Since KSTI does not significantly inhibit chymotrypsin, no data are presented for this inhibitor. As might be expected from the results of the antitryptic activity, BBI retained nearly 75% of its antichymotryptic activity even after 360 min of heat treatment. In this case, the soy extract lost much of its inhibitory activity in the first 180 min of heating, much faster than the purified BBI.

Birk (1961) had also reported that purified BBI was stable when heated in aqueous solution at 100 °C for 10 min. The results presented here thus confirm the heat stability of purified BBI in solution.

Other researchers have reported that protease inhibitors are less susceptible to thermal inactivation when they are in purified form. Ellenreider et al. (1980) heated soy extracts and purified inhibitors at 96 °C. They reported that the purified material was more resistant to the heat treatment than the SBPI activity in the extracts. Tsukamoto et al. (1983) investigated inactivation of a protease

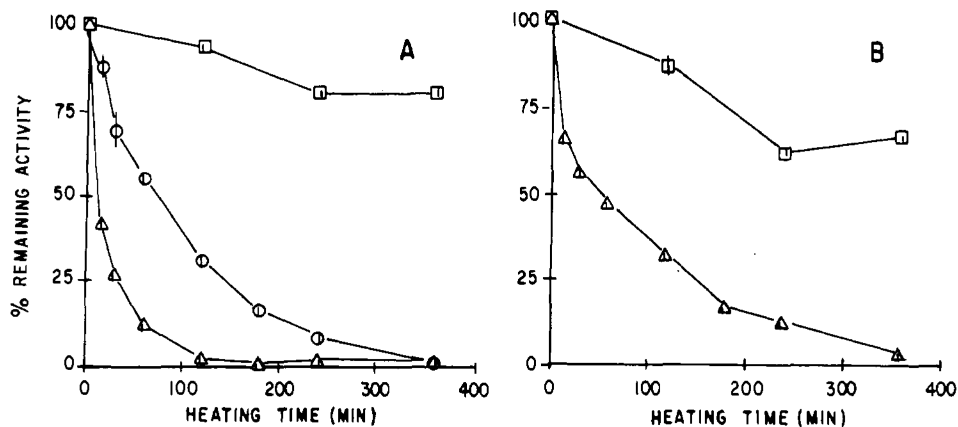

Figure 1. Loss of inhibitory activity of SBPI during heat treatment in aqueous solutions and in soy extract. Inhibitory activity (percent of original) versus heating time at 100 °C: (A) trypsin inhibitory activity; (B) chymotrypsin inhibitory activity. Error bars indicate the standard deviation of three replicates. Purified KSTI (○) concentration was 200 μg/mL. Purified BBI (□) concentration was 150 μg/mL. Soy extract (△) was a 2% extract of raw defatted soy flour.

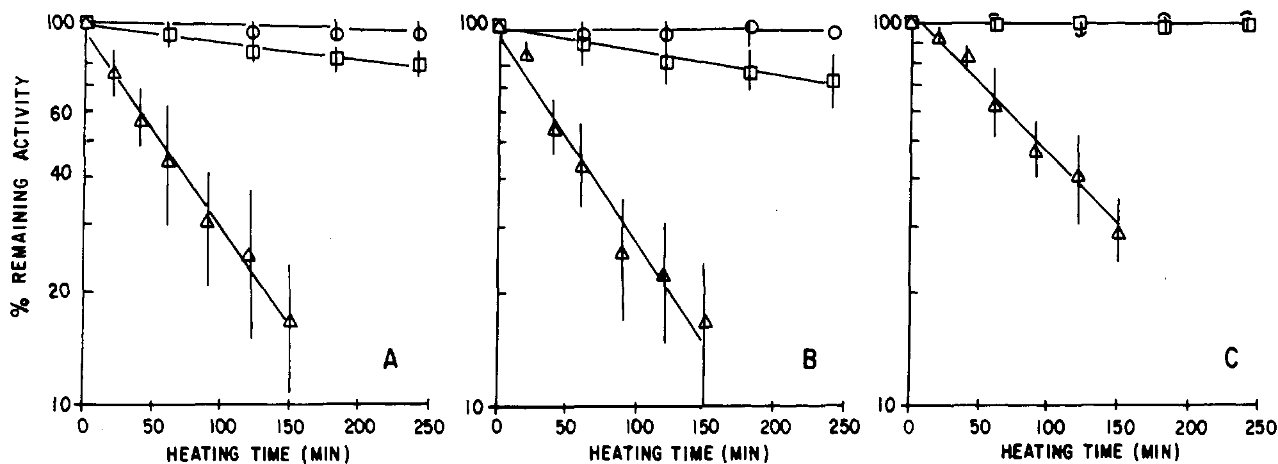

Figure 2. Loss of inhibitory activity of SBPI during heat treatment of soy flour at different a_w. Inhibitory activity (percent of original) versus heating time at 95 °C: (A) trypsin inhibitory activity; (B) BBI activity (as measured by chymotrypsin inhibition); (C) KSTI activity (as measured by RIEP). Error bars indicate the standard deviation of four replicates. Raw defatted soy flour was equilibrated to a_w 0.32 (○), 0.50 (□), and 0.75 (△).

inhibitor from *Phaseoulus vulgaris*. Heat treatment of the pure inhibitor at 100 °C for 1 h did not cause it to lose its inhibitory activity while a crude extract of *Phaseolus* rapidly lost its inhibitory activity when heated under the same conditions. Thus, the results presented here for heat inactivation of SBPI in solution or in a soy extract are generally in agreement with the results of similar previous reports.

Because the soy extract loses its ability to inhibit trypsin and chymotrypsin faster than KSTI or BBI in solution, we can assume that some interaction of the inhibitors with other materials in the extract promotes the inactivation of the inhibitors. For example, as was hypothesized by Nordal and Fossum (1974) and as shown by Friedman et al. (1982, 1984), the presence of free thiol groups available for disulfide bond interchange can aid in inactivation of the inhibitors. In addition, soy extract has a higher overall protein concentration as compared to pure inhibitor solutions. This would increase the possibility of noncovalent protein–protein interactions, which may lead to loss of inhibitory activity through denaturation and/or precipitation. In any case, it is important to note that studies of the destruction of inhibitory activity performed in purified solution may not be indicative of the behavior of the inhibitors in a more complex system.

Phase 2 Heating Study. In this phase of work, defatted soy flour was heated at different moisture and temperature conditions. In a portion of this work, purified inhibitors were added to the soy flour to see whether their sensitivity to heat treatment in a pure solution (as determined in phase 1) was predictive of their behavior when heated in a soy flour matrix.

To specifically quantitate both KSTI and BBI in systems containing both inhibitors, KSTI RIEP was used in addition to trypsin inhibition and chymotrypsin inhibition. In previous work (DiPietro and Liener, 1985), it was demonstrated that, during thermal inactivation of KSTI, loss of antitryptic activity was accompanied by a loss of antibody recognition. Thus, KSTI RIEP is an appropriate technique to use to measure active KSTI.

At Different a_w and a Fixed Temperature. Soy flour that had been equilibrated at a_w 0.32, 0.50, and 0.75 (5.3%, 7.7%, and 14.7% moisture, respectively) was heated at 95 °C. The loss of inhibitory activity during heat treatment is shown in Figure 2, and a kinetic analysis of these data is presented in Table II.

The loss of inhibitory activity was obviously more rapid in soy flour at a_w 0.75 as compared to soy flour at either of the lower a_w. It is no surprise that, with an increase in the availability of solvent water, the opportunity for inhibitors to denature or to otherwise become inactivated also increases.

Further examination of these data leads to some conclusions about the differences in the heat stability of KSTI and BBI. For a_w 0.50 and 0.75, a contrast of the means of BBI activity and KSTI activity showed that they were

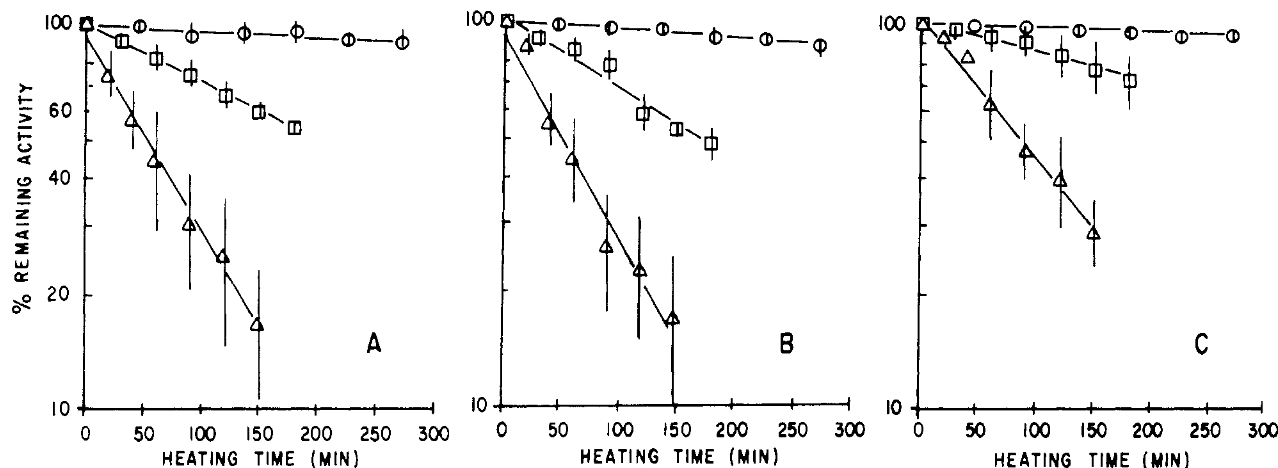

Figure 3. Loss of inhibitory activity of SBPI during heat treatment of soy flour at different temperatures. Inhibitory activity (percent of original) versus heating time: (A) trypsin inhibitory activity; (B) BBI activity (as measured by chymotrypsin inhibition); (C) KSTI activity (as measured by RIEP). Error bars indicate the standard deviation of four replicates. Raw defatted soy flour was heated at 75 °C (○), 85 °C (□), and 95 °C (△) after equilibration to a_w 0.75.

Table II. First-Order Reaction Constants[a] for Inactivation of SBPI at Different a_w

a_w	activity	k, min^{-1}	ln $[I_i]$	r^2 [b]
0.32	trypsin inhibitory	−0.0003	4.6	0.80
	BBI[c]	−0.0001	4.6	0.05
	KSTI[d]	−0.0001	4.6	0.17
0.50	trypsin inhibitory	−0.0011	4.6	0.94
	BBI[c]	−0.0014	4.6	0.97
	KSTI[d]	−0.0001	4.6	0.53
0.75	trypsin inhibitory	−0.0116	4.5	0.99
	BBI[c]	−0.0124	4.6	0.97
	KSTI[d]	−0.0086	4.7	0.99

[a] Constants fit first-order rate expressions of the form ln $[I_t]$ = ln $[I_i] - kt$, where $[I_t]$ = percent inhibitory activity remaining at time t, $[I_i]$ = percent original inhibitory activity (theoretically 100%), and t = heating time (minutes). [b] r^2 is the square of the correlation coefficient. [c] As measured by chymotrypsin inhibitory activity. [d] As measured by RIEP.

significanly different (p = 0.05). As can be seen in Table II, the rate for BBI inactivation was −0.0014 min^{-1} when the a_w was 0.50. In comparison, the rate for KSTI inactivation (under the same conditions) was about 10 times slower, −0.0001 min^{-1}. A similar effect occurs at a_w 0.75 where the rates for BBI and KSTI inactivation were −0.0124 and −0.0086 min^{-1}, respectively. These results are in conflict with the assertions of several researchers (Johnson et al., 1980a,b; Tan-Wilson and Wilson, 1986) that BBI can generally be regarded as more heat stable than KSTI.

At Different Temperatures and a Fixed a_w. Soy flour was humidified to a_w 0.75 before heat treatment at 75, 85, and 95 °C. The loss of inhibitory activity is shown in Figure 3, and a kinetic analysis of these data is presented in Table III. (Note: Data for 95 °C are the same data included in the previous section for a_w 0.75).

As expected, an increase in the heating temperature caused the inhibitors to be inactivated more quickly. As with the data from soy flour at different a_w, further examination of this data leads to the conclusion that KSTI is more heat stable than BBI under these conditions. A contrast of the means of BBI activity and KSTI activity showed that they were significantly different (p = 0.05) at all temperatures. As already pointed out for the data at different a_w, these results are in conflict with the assertions of researchers (Johnson et al., 1980a,b; Tan-Wilson and Wilson, 1986) that BBI was more heat stable than KSTI.

Table III. First-Order Reaction Constants[a] for Inactivation of SBPI at Different Temperatures

temp, °C	activity	k, min^{-1}	ln $[I_i]$	r^2 [b]
75	trypsin inhibitory	−0.0004	4.6	0.85
	BBI[c]	−0.0005	4.6	0.96
	KSTI[d]	−0.0001	4.6	0.17
85	trypsin inhibitory	−0.0035	4.6	0.99
	BBI[c]	−0.0044	4.6	0.97
	KSTI[d]	−0.0020	4.6	0.96
95	trypsin inhibitory	−0.0116	4.5	0.99
	BBI[c]	−0.0124	4.6	0.97
	KSTI[d]	−0.0086	4.7	0.99

[a] Constants fit first-order rate expressions of the form ln $[I_t]$ = ln $[I_i] - kt$, where $[I_t]$ = percent inhibitory activity remaining at time t, $[I_i]$ = percent original inhibitory activity (theoretically 100%), and t = heating time (minutes). [b] r^2 is the square of the correlation coefficient. [c] As measured by chymotrypsin inhibitory activity. [d] As measured by trypsin inhibitory activity.

Table IV. First-Order Reaction Constants[a] for Inactivation of SBPI Added to Soy Flour

flour	activity	k, min^{-1}	ln $[I_i]$	r^2 [b]
raw	trypsin inhibitory	−0.0184	4.4	0.98
	BBI[c]	−0.0194	4.3	0.95
	KSTI[d]	−0.0120	4.7	0.98
autoclaved + KSTI	trypsin inhibitory	−0.0056	4.6	0.99
	BBI[c]	e	e	e
	KSTI[d]	−0.0044	4.6	0.99
autoclaved + BBI	trypsin inhibitory	−0.0105	4.6	0.99
	BBI[c]	−0.0096	4.5	0.99
	KSTI[d]	e	e	e
autoclaved + KSTI, BBI	trypsin inhibitory	−0.0082	4.7	0.99
	BBI[c]	−0.0091	4.6	0.99
	KSTI[d]	−0.0055	4.6	0.98

[a] Constants fit first-order rate expressions of the form ln $[I_t]$ = ln $[I_i] - kt$, where $[I_t]$ = percent inhibitory activity remaining at time t, $[I_i]$ = percent original inhibitory activity (theoretically 100%), and t = heating time (minutes). [b] r^2 is the square of the correlation coefficient. [c] As measured by chymotrypsin inhibitory activity. [d] As measured by RIEP. [e] Not applicable.

Soy Flour with Purified Inhibitors Added. The loss of inhibitory activity during heat treatment of soy flour with or without added purified inhibitors is shown in Figure 4, and a kinetic analysis of these data is presented in Table IV. Four different samples of soy flour were equilibrated to a_w 0.75 and heated at 95 °C. The first sample was raw soy flour; the second, autoclaved soy flour with purified KSTI added; the third, autoclaved soy flour

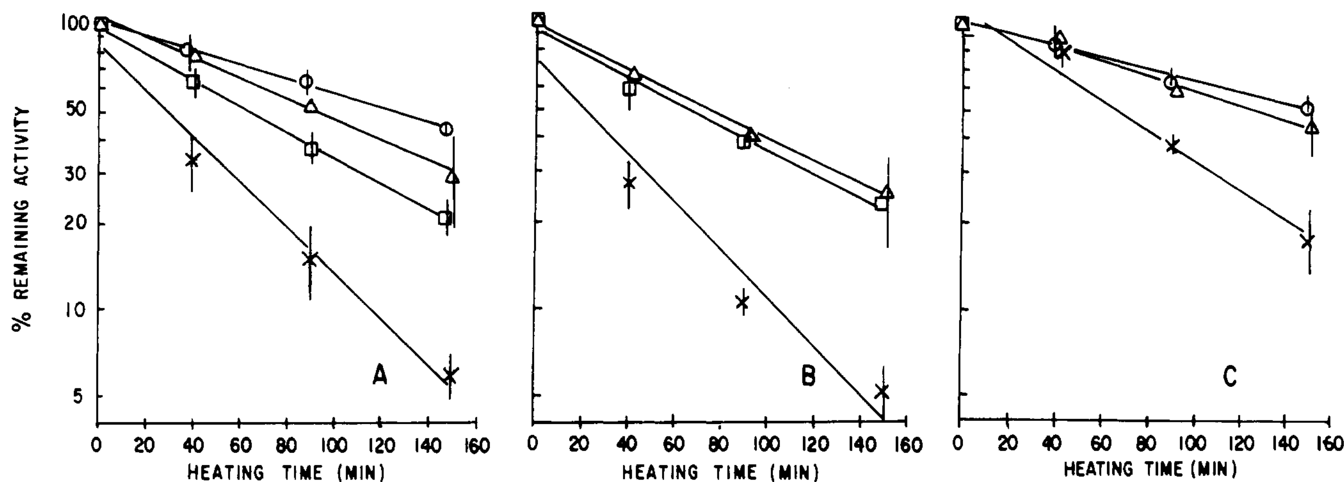

Figure 4. Loss of inhibitory activity of SBPI during heat treatment of soy flour with purified inhibitors added. Inhibitory activity (percent of original) versus heating time at 95 °C: (A) trypsin inhibitory activity; (B) BBI activity (as measured by chymotrypsin inhibition); (C) KSTI (as measured by RIEP). Error bars indicate the standard deviation of four replicates. The flours were raw defatted soy flour (×), autoclaved defatted soy flour with KSTI added (○), autoclaved defatted soy flour with BBI added (□), and autoclaved defatted soy flour with both KSTI and BBI added (△). All flours were equilibrated to a_w 0.75.

with purified BBI added; and the fourth, autoclaved soy flour with both KSTI and BBI added. For the second sample, only the results of trypsin inhibition and KSTI activity are presented since, as expected, only residual amounts of BBI were present. For the third sample, only the results for trypsin inhibition and BBI activity are presented since, as expected, only residual amounts of KSTI were present.

For each of the different samples, with or without purified inhibitors added, heating time had a significant effect on inhibitory activity ($p = 0.05$). This result is as expected since these samples were heated at water activity 0.75 and at 95 °C, conditions sufficient to cause inactivation of SBPI as described in the preceding experiments.

These results also indicate that BBI is inactivated more rapidly than KSTI during this type of heat treatment. A comparison of the reaction rates illustrates this point. Table IV shows that, for raw soy flour, KSTI was inactivated more rapidly than BBI (−0.0120 and −0.0194 min^{-1}, respectively). For autoclaved samples with purified inhibitors added, the rates for KSTI inactivation were −0.0044 and −0.0055 min^{-1} (when only KSTI or KSTI and BBI were added, respectively). The rates for BBI inactivation in corresponding samples were −0.0096 and −0.0091 min^{-1}, almost twice the rates for KSTI.

There is one important anomoly in these results. In every case, the rate of inactivation of purified inhibitors added to autoclaved soy flour was slower than the rate for inactivation of the inhibitors in situ. When the purified inhibitors are added back to the soy flour, they cannot be returned to the localized area within the soybean matrix where they are normally located. Horisberger and Tacchini-Vonlanthen (1983a,b) reported that the inhibitors are found in the protein bodies and other specific sites within the soybean cotyledon. Adding the inhibitors back to soy flour may put them in a distinctly different microenvironment with respect to other metabolic and cellular components in situ, altering their sensitivity to heat inactivation. Also, chemical conditions within the soy flour may have been significantly altered by the autoclaving process. For example, Baintner (1981) has observed that, under some conditions, loss of inhibitory activity paralleled nonenzymatic browning. The autoclaving process may destroy most of the reducing sugars and/or free amino groups in the flour. If the inactivation of SBPI is related to nonenzymatic browning, this would explain why inactivation of SBPI was slower when added to autoclaved soy flour.

Further studies concerning the significance of SBPI should take into consideration that *both* KSTI and BBI are inactivated during moist heat treatments, and the conclusion that residual SBPI activity is due to "heat-stable" BBI alone is not justified.

ABBREVIATIONS USED

a_w, water activity; BBI, Bowman–Birk inhibitor; DDW, distilled deionized water; KSTI, Kunitz inhibitor; RIEP, rocket immunoelectrophoresis; SBPI, soybean protease inhibitor(s).

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N′-Acyl and *N*′-Nitroso Pyridine Alkaloids in Alkaloid Lines of Burley Tobacco during Growth and Air-Curing

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N′-Acyl and *N*′-nitroso pyridine alkaloids were quantified by GC in burley tobacco genotypes varying in accumulation of pyridine alkaloids. Leaves were sampled during field growth and air-curing. N′-Substituted alkaloid identities were confirmed by GC and GC–MS; *N*′-acetylanatabine was newly identified. Alkaloid derivatives in lamina were in the following order of decreasing content averaged over the sampling dates. Acylated compounds: formylnornicotine (FNN), *n*-octanoylnornicotine (ONN), *n*-hexanoylnornicotine (HNN), formylanatabine (FAT), acetylnornicotine (ANN), *n*-butanoylnornicotine (BNN), and acetylanatabine (AAT). Nitrosamines: nitrosoanatabine (NAT), nitrosonornicotine (NNN), and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Generally higher levels of N′-substituted pyridine alkaloids were found in lamina of the KY 78379 line (high nornicotine and total alkaloids) and B21 cultivar (high nicotine and total alkaloids) compared to LAB21 (low total alkaloids). *N*′-Acyl alkaloids occurred in green as well as cured leaves; *N*′-nitroso alkaloids were mainly present during curing. Thus, acylated alkaloids, unlike nitroso alkaloids, are apparent products of plant metabolism during late growth stages.

During investigations of the effects of controlled environmental air-curing and other postharvest processing procedures on tobacco-specific nitrosamines and minor alkaloids in burley tobacco leaf extracts (Andersen and Kemp, 1985; Andersen et al., 1987), several unidentified gas chromatographic peaks were observed that did not correspond to commonly encountered tobacco pyridine alkaloids such as nicotine, other commonly described pyridine alkaloids, or known tobacco-specific *N*′-nitroso pyridine alkaloids of burley leaf. Subsequently, it was determined that several of these components belonged to a series of nornicotine- and anatabine-related N′-acylated pyridine alkaloids differing in carbon chain length of the acyl substituent (Burton et al., 1988).

Although there is relatively little known about these *N*′-acyl compounds, several of this type were described earlier. Warfield et al. (1972) identified and quantified *N*′-formylnornicotine and *N*′-acetylnornicotine in aged, air-cured burley tobacco, and Bolt (1972) identified *N*′-*n*-hexanoylnornicotine and *N*′-*n*-octanoylnornicotine in flue-cured tobacco. Miyano et al. (1979, 1981) identified and determined quantities of *N*′-formylanatabine and *N*′-formylanabasine in aged air-cured burley tobacco and *N*′-(6-hydroxy-*n*-octanoyl)nornicotine in Japanese domestic tobacco. Matsushita et al. (1979) identified *N*′-*n*-butanoylnornicotine in flue-cured tobacco leaf, and later Matsushima et al. (1983) quantitatively analyzed *N*′-acyl pyridine alkaloids in tobacco lamina at the time of harvest

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