

Heat Inactivation of Trypsin Inhibitor in Kintoki Bean (*Phaseolus vulgaris*)

I. TSUKAMOTO, M. MIYOSHI, and Y. HAMAGUCHI,¹ Department of Food Science and Nutrition, Nara Women's University, Nara 630, Japan.

ABSTRACT

Cereal Chem. 60(3):194-197

Crude extract from kintoki bean (*Phaseolus vulgaris*) lost approximately 90% of the trypsin inhibitory activity after heating at 100°C for 60 min, but purified trypsin inhibitor was heat-stable. Heat treatment caused inactivation of the purified trypsin inhibitor when protein such as albumin

or high molecular weight proteins from bean extract was present. The extent of the inactivation increased with the concentrations of the trypsin inhibitor and total protein.

Heat treatments alter nutritive value and functional properties of proteins and products derived from them. The proteins in raw soybeans have low nutritive value, and various heat treatments cause an improvement of this value and loss of trypsin inhibitory activity (Birk and Gertler 1961, Kakade et al 1974, Liener 1958, Rackis 1966). Temperature, duration of heating, particle size, and moisture content have been examined as factors in the beneficial destruction of the trypsin inhibitor (Albrecht et al 1966; Liener and Kakade 1969; Rackis 1965, 1966, 1974). Dielectric heating (Borchers et al 1972, Simovic et al 1972), infrared cooking (Faber and Zimmerman 1973), microwave processing (Wing and Alexander 1971), acid or alkali treatment (Colvin and Ramsey 1969, Liener and Kakade 1980), and Ca(OH)₂ treatment (Cravioto et al 1951) of soybeans have also been studied as ways to inactivate the trypsin inhibitor.

The trypsin inhibitory activity of soybean protein in meat

products is more labile to heat inactivation due to some component in the meat (Nordal and Fossum 1974). Substances that accelerate thermal inactivation of trypsin inhibitors are present in aqueous extracts of soybeans, peanuts, and kidney beans (Ellenrieder et al 1980).

For accurate estimation of food value and prevention of risk, conditions and mechanisms of inactivation of deleterious components, such as trypsin inhibitor, need to be investigated. We investigated the behavior of the trypsin inhibitor in kintoki beans during heat treatment, focusing on the effect of coexisting proteins. Kintoki (the Japanese term for red) beans are a subspecies of kidney beans (*Phaseolus vulgaris* L.).

MATERIALS AND METHODS

Materials

Bovine pancreas trypsin, salt-free and twice crystallized, bovine serum albumin (BSA), RNA from tolula yeast, and DNA from herring sperm were purchased from Sigma Chemical Co. α -N-Benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) and soluble

¹Deceased.

starch were obtained from Nakarai Chemicals Co., Ltd., DEAE-cellulose from Whatman Ltd., and Sephadex G-75 and G-100 from Pharmacia Fine Chemicals. All other reagents were analytical grade.

Trypsin Inhibitory Activity

Trypsin was incubated with test solution for 5 min at 37°C before trypsin inhibitory activity was determined by a modified method of Erlanger et al (1961).

The reaction mixture (1.6 ml) buffered at pH 8.2 with 0.1M Tris-HCl contained 0.01M CaCl₂, trypsin (20 μg), or trypsin and test solution. After preincubation for 5 min, 15 μl of BAPNA in dimethyl sulfoxide (44 mg/ml) was added. The reaction was allowed to proceed for 15 min and was then stopped by addition of 1 ml of 10% acetic acid. Liberated *p*-nitroaniline was estimated from the absorbance at 410 nm by using a molar extinction coefficient of 8,800M⁻¹cm⁻¹ (Erlanger et al 1961). One unit of trypsin was the amount that liberated 1 μmole of *p*-nitroaniline per minute. One inhibitor unit (IU) was defined as the amount of inhibitor that decreased the activity of one trypsin unit. Specific activity (SA) was expressed as inhibitor units per milligram of protein. Protein content was determined by the method of Lowry et al (1951) with BSA as a standard.

Preparation of Crude Extract, Dialysate, and Purified Trypsin Inhibitor

Finely ground beans (300 g) were suspended in 10 volumes (w/v) of H₂O and centrifuged at 13,000 × *g* for 30 min. The supernatant was used as the crude extract (SA = 0.14 IU/mg of protein). Ammonium sulfate was added to the crude extract to 30% saturation, followed by centrifugation. The supernatant was brought to 70% saturation with ammonium sulfate and centrifuged. The precipitate was dissolved in H₂O and dialyzed against H₂O for two days. After the precipitate was removed, the supernatant was used as the dialysate of the crude extract (SA = 0.26 IU/mg of protein). The dialysate was chromatographed on Sephadex G-100 (7 × 95 cm), which had been equilibrated with 50 mM Tris-HCl buffer, pH 7.0. The inhibitor fractions were

combined and brought to 70% saturation with ammonium sulfate. After centrifugation, the precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.0, and dialyzed overnight against the same buffer. The precipitate was removed by centrifugation, and the clear supernatant was subjected to chromatography on a DEAE-cellulose column (1.8 × 30 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0. The trypsin inhibitor was eluted with 0.1M NaCl in 50 mM Tris-HCl buffer, pH 7.0. The inhibitor fractions were combined and brought to 70% saturation with ammonium sulfate. After centrifugation, the precipitate was dissolved in H₂O and dialyzed against H₂O. The dialysate was lyophilized and used as the purified trypsin inhibitor (SA = 2.0 IU/mg of protein).

High Molecular Weight Proteins

The dialysate of the crude extract was chromatographed on Sephadex G-75 (3 × 60 cm), which had been equilibrated with H₂O. The protein fractions that were eluted before the trypsin inhibitor were collected and lyophilized. The resulting sample was used as high molecular weight (HMW) proteins.

Heat Treatment

The solution containing the trypsin inhibitor was divided into two portions. One was used as an untreated sample, and the other was heated at 100°C for 60 min in boiling-water bath, unless otherwise stated. After centrifugation at 27,000 × *g* for 30 min at 4°C, the supernatant of the heated solution was used as the heated sample. The pH, trypsin inhibitory activity, and protein content of unheated and heated samples were determined. Residual inhibitory activity of the heated sample was expressed as percent of IU of the unheated sample.

RNA and DNA in the crude extract were extracted according to the method of Ogur and Rosen (1950) and estimated by the orcinol reaction and the diphenylamine reaction, respectively (Schneider 1945).

Carbohydrate in the crude extract was determined by the phenol sulfuric acid method with D-glucose as a standard (Dubois et al 1956).

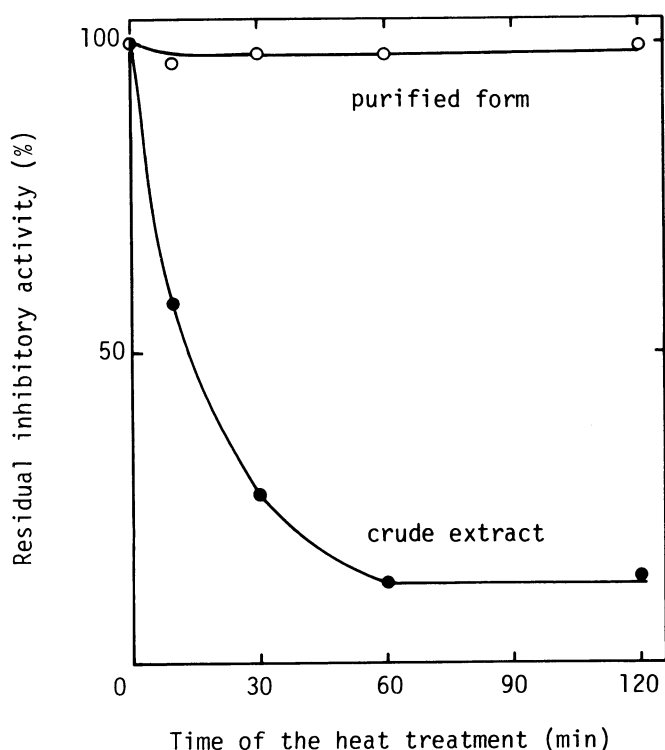


Fig. 1. Changes in trypsin inhibitory activity during heating of crude extract and purified trypsin inhibitor dissolved in H₂O (1.5 mg of protein per milliliter).

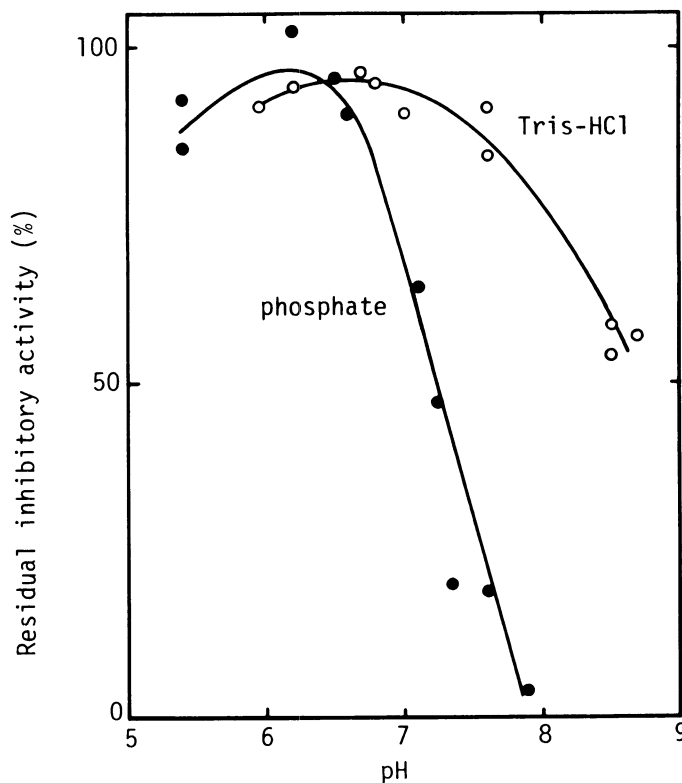


Fig. 2. Effect of pH on the heat stability of purified trypsin inhibitor dissolved in 50 mM Tris-HCl buffer, pH 5.9-8.7, or 50 mM phosphate buffer, pH 5.4-7.9 in a concentration of 1 mg/ml.

RESULTS AND DISCUSSION

Figure 1 shows the time course of inactivation of the trypsin inhibitor in the crude extract and of the purified form. Loss of inhibitory activity was not observed in the purified form even after heating for 2 hr, but in the crude extract, inhibitory activity decreased with the heating time. These observations suggest that water-extract of kintoki bean contains factors that inactivate the trypsin inhibitor during heating.

The effect of pH on the heat stability of the purified trypsin inhibitor is shown in Fig. 2. The trypsin inhibitor maintained about 90% of its inhibitory activity after heating in 50 mM Tris-HCl buffer, pH 6-7, and was gradually inactivated at higher pH. In 50 mM phosphate buffer, the trypsin inhibitor was heat-stable between pH 5.5 and 6.5. When the trypsin inhibitor at higher pH was heated, however, a remarkable loss of inhibitory activity was observed (60% loss at pH 7.0; 95% loss at pH 8.0). The pH of the

TABLE I
Effect of Ionic Strength on Heat Inactivation of Trypsin Inhibitor^a

NaCl Concentration (M)	Trypsin Inhibitory Activity ^b (heated/unheated, %)
0.0	98
0.005	90
0.05	95
0.25	91
0.5	93

^aPurified trypsin inhibitor (final concentration, 0.6 mg/ml) was dissolved in NaCl solution (0-0.5M) for the heat treatment.

^bValues are averages of triplicate determinations.

crude extract was within the heat-stable region for both buffers (pH 6.3-6.5). Heat inactivation of trypsin inhibitor in the crude extract was therefore not accounted for by the pH. The pH profiles of the two buffers differed markedly. Because the purified trypsin inhibitor dissolved in H₂O was stable, the difference may have been caused by ionic strength.

As shown in Table I, approximately 90% of the inhibitory activity remained after heating in 0.005, 0.05, 0.25, or 0.5M NaCl solution (pH 5.5-6.5). Therefore, the ionic strength of solution did not appear to affect heat inactivation of the trypsin inhibitor in this wide range.

Figure 3 shows the effect of protein concentration on the heat inactivation of the trypsin inhibitor. The heat stability of the trypsin inhibitor was independent of its concentration when only the trypsin inhibitor was heated. Dilution of the crude extract decreased the extent of heat inactivation. The dialysate of the crude extract also showed loss of inhibitory activity and dilution dependence. These results suggest that, combined with heating, HMW substances such as protein, polysaccharide, and nucleic acid are inactivating factors.

The crude extract contained 13.1 mg of protein, 14.4 mg of carbohydrate, and 1.2 mg of RNA per milliliter. DNA was not detectable in the crude extract. When purified trypsin inhibitor was heated in the solutions (1.6 mg/ml) of starch, RNA from yeast or the crude extract, or DNA from herring sperm that had been adjusted to pH 6 with NaOH, no loss of inhibitory activity was observed. But heating the trypsin inhibitor in BSA solution (1.6 mg/ml) caused approximately 40% loss of the inhibitory activity. These results strongly suggest that proteins are inactivation factors during heating.

The effect of protein concentration on trypsin inhibitor was examined using BSA. As shown in Fig. 3, the inactivation increased

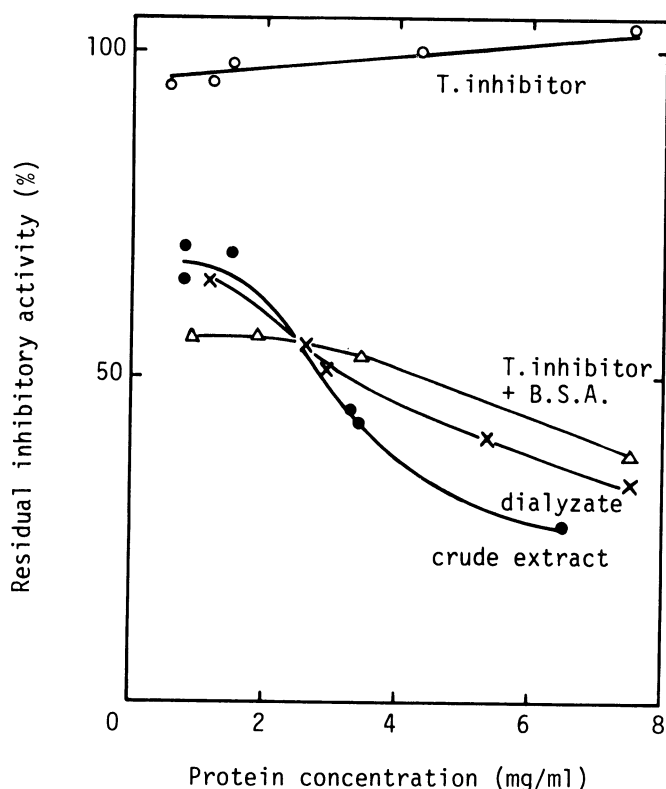


Fig. 3. Effect of protein concentration on heat inactivation of trypsin inhibitor. Purified trypsin inhibitor was dissolved in H₂O in a concentration of 0.6-7.5 mg/ml. The crude extract was diluted with H₂O to 0.8-6.5 mg of protein per milliliter. The dialysate of the crude extract was diluted with H₂O to 1.2-7.5 mg of protein per milliliter. The purified trypsin inhibitor and bovine serum albumin (BSA) were dissolved, 1:14, in H₂O to obtain a specific activity similar to that of the crude extract. The mixture was diluted with H₂O in protein concentrations of 0.9-7.5 mg/ml.

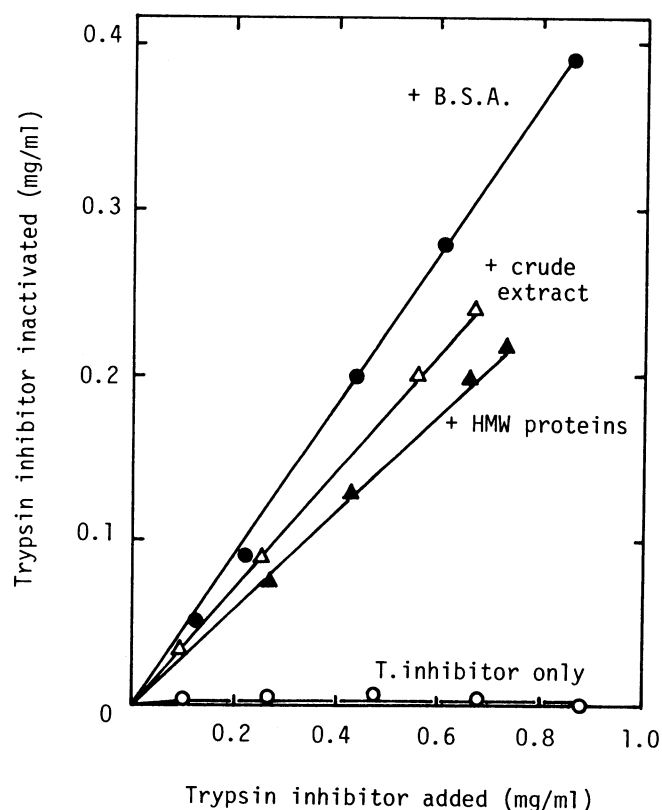


Fig. 4. Effect of trypsin inhibitor concentration on heat inactivation of the trypsin inhibitor in the presence of protein. Bovine serum albumin (BSA), the crude extract, or high molecular weight (HMW) proteins was dissolved, 1.6 mg/ml of H₂O. The purified trypsin inhibitor (final concentration, 0.11-0.9 mg/ml) was added to the protein solutions or H₂O. The quantities of inactivated trypsin inhibitor were calculated from the inhibitory activities using the 2.0 as the specific activity of the purified trypsin inhibitor.

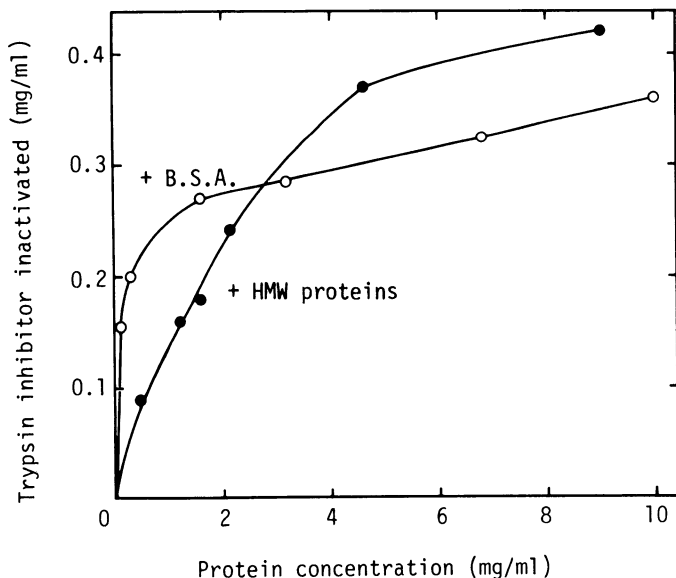


Fig. 5. Effect of protein concentration on inactivation of trypsin inhibitor during heating. Trypsin inhibitor (final concentration, 0.6 mg/ml) was mixed with bovine serum albumin (BSA, 0.1–10 mg/ml) or high molecular weight (HMW) proteins (0.43–9.0 mg/ml). The quantities of inactivated trypsin inhibitor were calculated from the inhibitory activities using the specific activity 2.0 as of the purified trypsin inhibitor.

with the protein concentration in a manner similar to that of the crude extract. Proteins such as cytochrome c or gelatin produced similar results. These observations indicate that a wide range of proteins inactivate trypsin inhibitor during heating.

The relation between the concentrations of the trypsin inhibitor and coexisting proteins was studied in detail. When trypsin inhibitor was heated without coexisting proteins, inactivation was not observed (Figs. 3 and 4). But when various concentrations of the trypsin inhibitor were heated in a fixed concentration (1.6 mg/ml) of BSA, the crude extract, or HMW proteins, the amount of inactivated trypsin inhibitor increased in proportion to the amount of added trypsin inhibitor (Fig. 4). In BSA solution, 45% of added trypsin inhibitor was constantly inactivated. Inactivations were 36 and 27% in the crude extract and HMW proteins, respectively. At this concentration (1.6 mg/ml), BSA was more effective than proteins from bean in inactivating the trypsin inhibitor. On the other hand, when the fixed concentration of the trypsin inhibitor (0.6 mg/ml) was heated in various concentrations of BSA or HMW proteins, the amount of inactivated trypsin inhibitor increased with the protein concentration (Fig. 5). The extent of inactivation increased rapidly at a low protein concentration and then gradually increased at higher levels. These results indicate that inactivation of the trypsin inhibitor during heating was caused by an irreversible interaction between the trypsin inhibitor and coexisting proteins, and the extent depended on the concentrations of both.

Ellenrieder et al (1980) reported the concentration-dependence of the thermal stability (heating at 96°C for 15 min) of trypsin inhibitory activity in aqueous extracts of soybeans, peanuts, and kidney beans. They suggest that destabilizing factors were HMW substances from the extracts, most likely proteins. Our experiments with starch, RNA, DNA, and BSA demonstrated that the HMW substances they mentioned were proteins. Furthermore, our results

that BSA, cytochrome c, or gelatin can inactivate the trypsin inhibitor in a concentration-dependent fashion suggest that many kinds of proteins, rather than a specific one, may participate in inactivating the trypsin inhibitor in the crude extract during heating.

LITERATURE CITED

- ALBRECHT, W. J., MUSTAKAS, G. C., and MCGHEE, J. E. 1966. Rate studies on atmospheric steaming and immersion cooking of soybeans. *Cereal Chem.* 43:400.
- BIRK, Y., and GERTLER, A. 1961. Effect of mild chemical and enzymatic treatment of soybean meal and soybean trypsin inhibitors on their nutritive and biochemical properties. *J. Nutr.* 75:379.
- BORCHERS, R., MANAGE, L. D., NELSON, S. O., and STETSEN, L. 1972. Rapid improvement in nutritional quality of soybeans by dielectric heating. *J. Food Sci.* 37:331.
- COLVIN, B. M., and RAMSEY, H. A. 1969. Growth of young calves and rats fed soy flour treated with acid or alkali. *J. Dairy Sci.* 52:270.
- CRAVIOTO, R. O., GUZMAN, J. G., and MASSIEU, H. G. 1951. Increasing the nutritive value of the tortilla. III. The effect of heat and calcium hydroxide on the antitryptic activity of soybean. *Ciencia* 11:81.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A., and SMITH, F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350.
- ELLENRIEDER, G., GERONAZZO, H., and DE BOJARSKI, A. B. 1980. Thermal inactivation of trypsin inhibitors in aqueous extract of soybeans, peanuts, and kidney beans: Presence of substances that accelerate inactivation. *Cereal Chem.* 57:25.
- ERLANGER, B. F., KOKOWSKY, N., and COHEN, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95:271.
- FABER, J. L., and ZIMMERMAN, D. R. 1973. Evaluation of infrared-roasted and extruder-processed soybeans in baby pig diets. *J. Anim. Sci.* 36:902.
- KAKADE, M. L., RACKIS, J. J., MCGHEE, J. E., and PUSKI, G. 1974. Determination of trypsin inhibitor activity of soy products. *Cereal Chem.* 51:376.
- LIENER, I. E. 1958. Effect of heat on plant proteins. Page 79 in: *Processed Plant Protein Foodstuffs*. A. M. Altschul, ed. Academic Press, New York.
- LIENER, I. E., and KAKADE, M. I. 1969. Protease inhibitors. Page 48 in: *Toxic Constituents of Plant Foodstuffs*. I. E. Liener, ed. Academic Press, New York.
- LIENER, I. E., and KAKADE, M. I. 1980. Protease inhibitors. Page 51 in: *Toxic Constituents of Plant Foodstuffs*, 2nd ed. I. E. Liener, ed. Academic Press, New York.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.
- NORDAL, J., and FOSSUM, K. 1974. The heat stability of inhibitors in meat products. *Z. Lebens. Unter. Forsch.* 154:144.
- OGUR, M., and ROSEN, G. 1950. The nucleic acids of plant tissues. I. The extraction estimation of desoxyribose nucleic acid and pentose nucleic acid. *Arch. Biochem.* 25:262.
- RACKIS, J. J. 1965. Physiological properties of soybean trypsin inhibitors and their relationship to pancreatic hypertrophy and growth inhibition of rats. *Fed. Proc.* 24:1488.
- RACKIS, J. J. 1966. Soybean trypsin inhibitors: Their inactivation during meal processing. *Food Technol.* 20:1482.
- RACKIS, J. J. 1974. Biological and physiological factors in soybeans. *J. Am. Oil Chem. Soc.* 51:161A.
- SCHNEIDER, W. C. 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxyribose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.* 161:293.
- SIMOVIC, R., SUMMERS, J. D., and BILANSKI, W. K. 1972. Heat treatment of full-fat soybeans. *Can. J. Anim. Sci.* 52:183.
- WING, R. W., and ALEXANDER, J. C. 1971. The heating of soybean meals by microwave radiation. *Nutr. Rep. Int.* 4:387.

[Received April 7, 1982. Accepted September 3, 1982]