ELSEVIER

Food Chemistry 78 (2002) 419–423

Food Chemistry

www.elsevier.com/locate/foodchem

Thermal inactivation of tepary bean (*Phaseolus acutifolius*), soybean and lima bean protease inhibitors: effect of acidic and basic pH☆

Magdi A. Osman*, Phyllis M. Reid, Charles W. Weber

Department of Nutritional Sciences, The University of Arizona, PO Box 210038, Tucson, AZ 85721-0038, USA

Received 17 September 2001; received in revised form 25 January 2002; accepted 25 January 2002

In memory of Bobby L. Reid, Professor and Head, Department of Nutritional Sciences, The University of Arizona

Abstract

Thermal stability of the protease inhibitor of tepary bean (*Phaseolus acutifolius*) was studied by comparing the antitryptic activities of a crude extract and the purified inhibitor (TBPI) to those of three purified protease inhibitors, soybean trypsin inhibitor (Kunitz; KSTI), soybean trypsin and chymotrypsin inhibitor (BBI) and lima bean trypsin inhibitor (LBTI). Three purified inhibitors, TBPI, BBI and LBTI, were found to be heat-stable when subjected to boiling (100 °C) at either neutral or acidic pH, or to autoclaving at neutral pH. The tepary bean crude extract and KSTI were found to be heat labile when boiled or autoclaved at neutral pH. At alkaline pH, all the purified protease inhibitors were heat labile, losing antitryptic activity rapidly upon heating to 100 °C. These data suggest that the trypsin inhibitor of TBPI is heat-stable, and similar to the Bowman–Birk family of inhibitors, and can be rapidly inactivated by heat under alkaline conditions. © 2002 Published by Elsevier Science Ltd.

Keywords: Tepary bean; *Phaseolus acutifolius*; Protease inhibitors

1. Introduction

Protease inhibitors are common in animal tissues, microorganisms and plants. Leguminous seeds are particularly rich sources of protease inhibitors; some of these have been isolated and their chemical, physiological and inhibitory properties have been studied (Liener & Kakade, 1980). Protease inhibitors from legumes are generally classified as either Kunitz or Bowman–Birk types (Laskowski & Kato, 1980). Kunitz inhibitors are relatively high molecular weight (around 20,000 daltons) with only one reactive site specific for trypsin. The lower molecular weight (around 9000 daltons) Bowman–Birk inhibitors are double-headed, inhibiting two serine proteases, trypsin and chymotrypsin, simultaneously. Bowman–Birk inhibitors are heat stable; while Kunitz inhibitors are heat-labile.

Osborne and Mendel (1917) were the first to report that heat treatment improved the nutritional value of soybean protein. Since then heat treatment has been successfully used to reduce the level of protease inhibitors in legumes, improving their nutritional quality. Heat-inactivation of protease inhibitors in legume seeds has been shown to be a function of several variables including particle size, temperature, moisture content and duration of heat treatment (Rackis, 1965).

Tepary bean (*Phaseolus acutifolius*) is a drought-tolerant legume native to the southwestern region of North America, where it has been used in Native American diets for centuries (Nabhan & Feleger, 1978). Studies on the nutritive value of the tepary bean showed that it contains 15–32% protein, 0.9–1.7% fat, and 65.3–69.1% carbohydrate (Thorn, Tinsley, Weber, & Berry, 1983). The presence of antinutritional factors in tepary bean was first reported by de Muelenaere (1964). Analysis of uncooked tepary bean samples for antinutritional factors revealed high levels of lectin and trypsin inhibitors (Thorn et al., 1983). Kabbara, Abbas, Scheerens, Tinsley, and Berry (1987) reported that trypsin inhibitor activity could be destroyed easily by soaking

Abbreviations: BBI, soybean trypsin and chymotrypsin (Bowman–Birk) inhibitor; KSTI, soybean trypsin (Kunitz) inhibitor; LBTI, lima bean trypsin inhibitor; TBPI, tepary bean protease inhibitor.

☆ Presented at the annual meeting of the Institute of Food Technologists, 10–14 July 1993, Chicago, IL, USA.

* Corresponding author. Department of Food Science, Nutrition, King Saud University, PO Box 2460, Riyadh, Saudi Arabia 11451. Fax: +9661-467-8394.

E-mail address: magdios@ksu.edu.sa (M.A. Osman).

0308-8146/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0308-8146(02)00144-9

and cooking, and concluded that the trypsin inhibitor of tepary bean is heat-labile. Similar results were reported by Gonzalez de Mejia, Grajeda Cota, Celada, and Valencia (1988) who found that soaking and boiling both black and white tepary beans for 140 min completely destroyed trypsin inhibitor activity. Idouraine, Sathe, and Weber (1992) observed that about 95% of the antitryptic activity of tepary bean flour was destroyed by autoclaving (120 °C) for 20 min, whereas tepary extract retained over 50% of its antitryptic activity. They suggested that tepary bean trypsin inhibitor is heat-stable, and may play a major role in the toxicity of raw tepary bean, because hemagglutinin was completely destroyed. This controversy as to whether tepary bean trypsin inhibitor is heat-stable or heat-labile, seems to have arisen because all of the investigations were carried out using raw tepary bean or crude extract, but not with pure inhibitor isolated from the bean.

The objectives of this study were: (1) to investigate the thermal stability of the antitryptic activity of purified tepary bean protease inhibitor (TBPI) as compared with that of an extract of raw tepary bean flour under two heat treatment conditions (boiling at 100 °C or autoclaving) at neutral pH; (2) to compare the heat-induced inactivation of purified TBPI with that of three other purified protease inhibitors: soybean protease inhibitor (Kunitz; KSTI), soybean trypsin and chymotrypsin inhibitor (Bowman–Birk; BBI), and lima bean trypsin inhibitor (LBTI) under acidic, neutral and basic pH conditions.

2. Materials and methods

2.1. Materials

Tepary beans (a brown seeded variety) were donated by a commercial producer (W.D. Hood, Coolidge, AZ). The beans were ground in a hammer mill to pass through a 40 mesh screen and the flour stored in a sealed container at −20 °C until use. Bovine trypsin, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide, commercially purified soybean trypsin inhibitor (KSTI), soybean trypsin and chymotrypsin inhibitor (BBI) and purified lima bean trypsin inhibitor (LBTI) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Methods

2.2.1. Preparation of tepary bean extract

A 1-g sample of tepary bean flour was extracted with 50 ml of 0.01 M NaOH for 2 h at room temperature using a magnetic stirrer, followed by centrifugation at 10,000 g for 30 min. The supernatant was collected and adjusted to pH 7.0 using 1.0 M HCl.

2.2.2. Purification of tepary bean protease inhibitor

Tepary bean protease inhibitor was extracted from the raw flour using a 0.05 M Tris–HCl buffer, pH 7.5 containing 0.5 M NaCl, 1 mM EDTA and 1 mM NaN_3. This crude extract was heat treated at 80 °C for 10 min in a boiling water bath, and the inhibitor precipitated from the supernatant by ammonium sulfate addition to 70% saturation. Final purification was achieved by sequential chromatography on DEAE-Sephadex, phenyl-Sepharose, CM-cellulose and G-75 Sephadex (Osman & Weber, 1994).

2.2.3. Protease inhibitor solutions

Tepary bean, soybean and lima bean protease inhibitors were each dissolved in three different pH buffers: 0.05 M acetate, pH 3; 0.05 M Tris–HCl, pH 7.0, and 0.05 M glycine/NaOH, pH 11. Each buffer also contained 0.1 M NaCl. TBPI, BBI and LBTI solutions were prepared at concentrations of 150 μg/ml. KSTI was prepared at a concentration of 200 μg/ml.

2.2.4. Heat treatments

Aliquots (1.0 ml) of the tepary bean extract and each protease inhibitor solution were placed in screw-cap vials (10×45 mm Pyrex with teflon-lined caps). The vials were all placed in a boiling water bath at the same time. A study was carried out at pH 7 for 360 min with one vial of each solution being removed from the boiling water bath and transferred to an ice water bath at 60 min intervals (DiPietro & Liener, 1989). This procedure was repeated for all the purified protease inhibitor solutions at pH 3 and pH 11 for 120 min with sampling times 20 min apart. An autoclaving study was conducted at pH 7 in which the tepary extract and each of the protease inhibitor solutions were heated (121 °C and 15 psi) for 20, 40 and 60 min durations.

2.2.5. Inhibitor assays

Trypsin activity was measured using α-*N*-benzoyl-DL-arginine-*p*-nitroanilide as a substrate (Kakade, Rackis, McGhee, & Puski, 1974). Chymotrypsin activity was measured using*N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) as the substrate (Erlanger, Edel, & Cooper, 1966). Inhibitor activity was expressed as a percent inhibition (I) of a control assay using the equation:

$$I\ (\%) = ((T - T^*)/T) \times 100$$

where T and T* are protease activities in the absence and presence of inhibitor, respectively.

3. Results and discussion

3.1. Thermal stability at pH 7.0

Purified TBPI was very heat stable at 100 °C and neutral pH (7) compared with the tepary bean extract

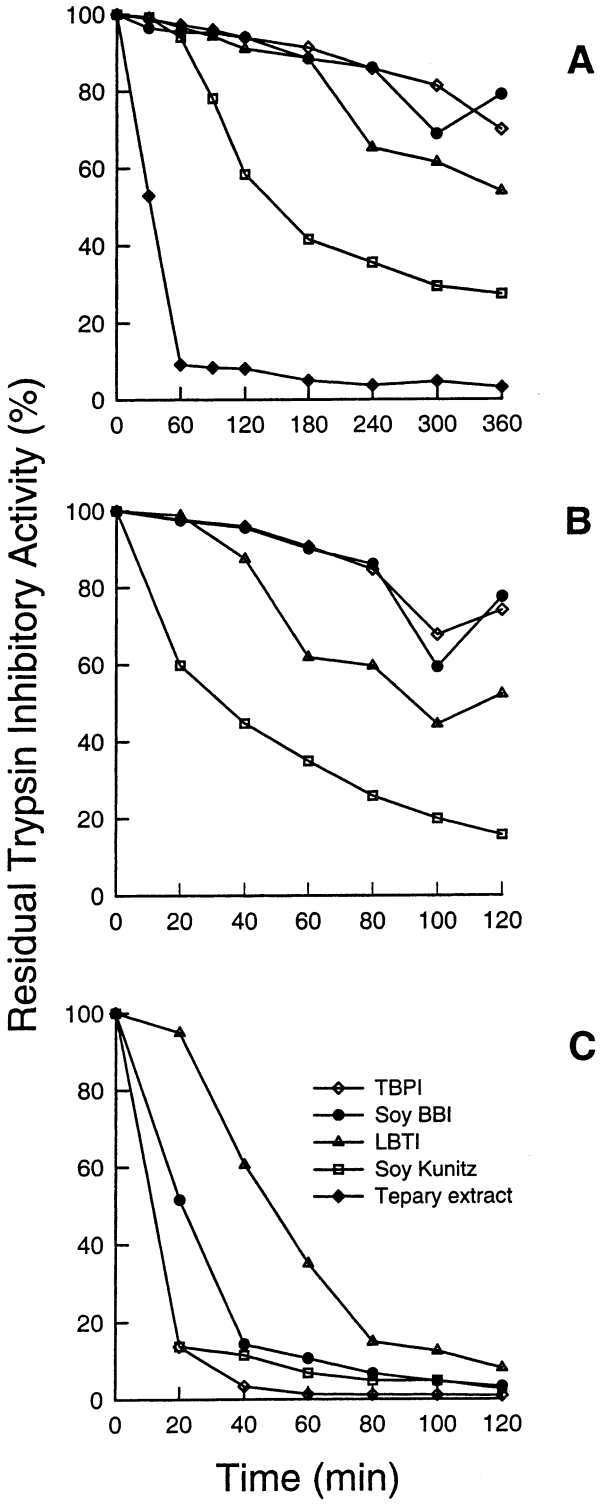

Fig. 1. Thermal inactivation of the trypsin inhibitor of tepary bean extract and of purified trypsin inhibitors at neutral (A), acidic (B) or alkaline (C) pH. Residual trypsin activity (% of trypsin activity in the absence of inhibitor) after heating neutral (pH 7) solutions of tepary bean extract and purified tepary bean (TBPI), soybean (KSTI and BBI), and lima bean (LBTI) protease inhibitors at 100 °C for durations up to 6 h is shown in panel A. Residual trypsin activity after heating acidic (pH 3) or alkaline (pH 11) solutions of TBPI, KSTI, BBI and LBTI at 100 °C for durations up to 2 h is shown in panels B and C, respectively.

(Fig. 1 A). The purified inhibitor retained nearly 70% of its antitryptic activity after 360 min of heat treatment, whereas the extract lost most of its antitryptic activity after 60 min. Among the purified inhibitors, TBPI and BBI and LBTI showed greater thermal stability than KSTI (Fig. 1A). After 360 min of exposure to 100 °C at neutral pH, the residual trypsin inhibitory activities of TBPI, BBI and LBTI were greater than 60%, whereas only 25% of the antitryptic activity of KSTI remained under the same conditions.

The effects of autoclaving on the inactivation of the protease inhibitor of tepary bean extract and purified TBPI, KSTI, BBI and LBTI were also investigated at neutral pH. It was evident that the antitryptic activity of the tepary bean extract was reduced much faster at autoclaving conditions (121 °C and 15 psi) than was the purified protease inhibitor (Fig. 2). Autoclaving the tepary bean extract for 20 min completely inactivated the trypsin inhibitor, while the purified TBPI retained 50% of its trypsin inhibitory activity after autoclaving for 60 min. TBPI, BBI and LBTI were again more thermally stable than KSTI (Fig. 2). After 60 min of autoclaving about 50% of the antitryptic activity of TBPI, BBI and LBTI was retained, while KSTI lost almost all (96%) of its trypsin inhibitory activity.

The loss of chymotryptic inhibitory activity during heat treatment of tepary extract, TBPI and BBI is presented in Fig. 3. Compared with either TBPI or tepary extract, BBI was very heat stable, retaining 78% of its antichymotryptic activity after 360 min of boiling at neutral pH; however, TBPI retained 40% of its chymotrypsin inhibitory activity under these conditions. The

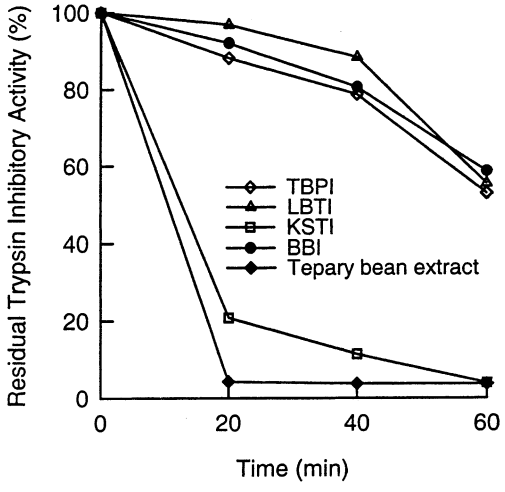

Fig. 2. Inactivation of the trypsin inhibitor of tepary bean extract and of purified trypsin inhibitors under autoclaving conditions at neutral pH. Residual trypsin activity (% of trypsin activity in the absence of inhibitor) after autoclaving neutral (pH 7) solutions of tepary bean extract and purified tepary bean (TBPI), soybean (KSTI and BBI), and lima bean (LBTI) protease inhibitors at 121 °C and 15 psi for durations up to 1 h.

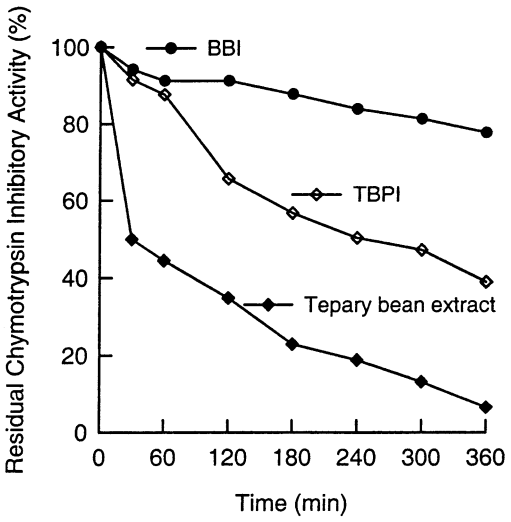

Fig. 3. Heat-induced loss of chymotrypsin inhibition at neutral pH. Residual chymotrypsin activity (% of chymotrypsin activity in the absence of inhibitor) after heating neutral (pH 7) solutions of tepary bean extract, pure tepary bean (TBPI), and soybean (BBI) protease inhibitors at 100 °C for durations up to 6 h.

tepary extract lost its antichymotryptic activity at a much faster rate than either BBI or TBPI.

The rapid heat inactivation of the tepary bean extract trypsin inhibitor compared with TBPI is similar to reported observations in soybean (DiPietro & Liener, 1989; Ellenreider, Geronazzo, & de Bojarski, 1980), in kintoki bean (Tsukamoto, Miyoshi, & Hamaguchi, 1983) and in wing bean (de Lumen & Belo, 1981). Ellenreider et al. (1980) showed that a soybean trypsin inhibitor, purified by gel permeation, had much greater thermal stability than the trypsin inhibitor of a soybean extract, and suggested the presence of a protein substance which accelerated thermal inactivation of the inhibitor. In studies of kintoki bean (*Phaseolus vulgaris*) trypsin inhibitor inactivation, Tsukamoto et al. (1983) found that 90% of the antitryptic activity of a kintoki bean extract was lost after heating at 100 °C for 60 min. The purified kintoki bean inhibitor was heat stable, but easily inactivated in the presence of a high molecular weight protein such as bovine serum albumin. DiPietro and Liener (1989) observed that while heating at 100 °C inactivated a soybean extract and KSTI after 30 and 180 min, respectively, BBI retained over 75% of its antitryptic activity after 360 min. It has been demonstrated, many times since the first report of Birk, Gertler, and Khalef (1963), that Bowman–Birk inhibitors are more heat stable than Kunitz inhibitors, and the results of the present study provide another demonstration of this well-known fact.

Our results with the autoclaved tepary bean extract were not exactly the same as those reported earlier by Idouraine et al. (1992) which indicated that tepary bean extract was relatively heat stable in the dry state. Many researchers have reported that protease inhibitors are less susceptible to thermal inactivation when they are in the dry form. DiPietro and Liener (1989) and Baintner (1981) independently reported that purified KSTI in the soybean flour matrix was more heat stable than KSTI in solution. Kapoor and Gupta (1978) showed that autoclaving of soybean seeds for 30 min or steaming for 60 min completely inactivated the inhibitor activity, whereas soaking for 8 h, prior to steaming, considerably reduced the time for inactivation to 15 min. Similarly, the trypsin inhibitor of whole soybean, with an initial moisture content of 20%, was completely destroyed by steaming for 15 min, and when the moisture content was raised to 60% by overnight soaking, then boiling for only 5 min was sufficient for the inactivation of the inhibitor (Albrecht, Mustakas, & McGhee, 1966). Friedman, Gumbmann, and Grosjean (1984) postulated that the disulfide bond interchange between the trypsin inhibitor and several other proteins was responsible for increased thermal inactivation. In addition to agents that induce change in disulfide bonds, moisture and interaction with other constituents, such as carbohydrates, appear to contribute to the denaturation of the inhibitor (Oste, Brandon, Bates, & Friedman, 1990). These studies indicate that the presence of moisture or other agents may catalyze heat inactivation of the purified inhibitors, extract or the whole seed. In any case, it is important to note that studies on the destruction of protease inhibitory activity performed in purified solution may not be indicative of the behaviour of the inhibitor in a complex matrix.

3.2. Acidic and basic pH effects on thermal stability

Both TBPI and BBI were very stable to heat (100 °C) at pH 3.0 compared to LBTI and KSTI (Fig. 1B). Over 75% of the trypsin inhibitory activity of TBPI and BBI was retained after heating for 120 min, while LBTI and KSTI retained 49 and 15% of their antitryptic activities, respectively. All the protease inhibitors, except LBTI, were easily inactivated by boiling (100 °C) at pH 11, losing about 90% of their antitryptic activity within 40 min (Fig. 1C). During the first 40 min of boiling at pH 11, LBTI retained 60% of its trypsin inhibitory activity and lost 90% of the activity only after 120 min (Fig. 1C). The purified protease inhibitors were more heat-labile in alkaline pH than in acidic pH.

The effects of alkaline and acidic pH on the thermal inactivation of legume trypsin inhibitors at elevated temperature have been reported by many investigators. Obara and Watanbe (1971) observed that purified soybean trypsin inhibitors were heat stable when heated at low or acidic pH and less heat stable at alkaline pH. Wallace, Bannatyne, and Khaleque (1971) found, at 98 °C, an increased rate of inactivation of the soymilk trypsin inhibitor as the pH of the soymilk was increased from neutral to alkaline. Similarly, the destruction of

trypsin inhibitor was accelerated by cooking soybeans in 1% NaOH, and unaffected by cooking in 1% HCl (Baker & Mustakas, 1973). Kwok, Qin, and Tsang (1993) reported that soymilk trypsin inhibitors were very heat-resistant at pH 2 and 93 °C, but the rate of inactivation increased as the pH was increased.

A possible explanation for the heat-lability of trypsin inhibitor in alkaline solution, is that rapid destruction of disulfide bonds, which are important for the heat stability of the inhibitor facilitates further denaturation of the inhibitor (Friedman et al., 1984). Dehydroalanine, formed during the alkaline cleavage of cystine, is available to cross-react with lysine residues in the inhibitor reactive site (Osman & Weber, 1994), modifying them to lysinoalanine, and thus permanently inactivating the inhibitor.

In the final analysis, although the protease inhibitor of the tepary bean extract solution is not heat-stable at pH 7, the trypsin inhibitor of the purified TBPI appears to be a heat stable protease inhibitor similar to that of BBI and LBTI. These data confirm our prior biochemical studies, which revealed that the tepary protease inhibitor belongs to the Bowman-Birk family of inhibitors (Osman & Weber, 1994). Furthermore, heat treatment of the purified TBPI in alkaline solution caused more rapid inactivation of inhibitory activity than in either neutral or acidic solution. This suggests that an alkali pre-soak of tepary bean seeds prior to heat treatment may provide a simple means of heat-processing without deleterious effects on other proteins.

References

Albrecht, W. J., Mustakas, G. C., & McGhee, J. E. (1966). Rate studies on atmospheric steaming and immersion cooking of soybeans. *Cereal Chemistry*, *46*, 400–407.

Baintner, K. (1981). Trypsin-inhibitor and chymotrypsin-inhibitor studies with soybean extracts. *Journal of Agricultural and Food Chemistry*, *29*, 201–203.

Baker, E. C., & Mustakas, G. C. (1973). Heat inactivation of trypsin inhibitor, lipoxygenase and urease in soybeans: effect of acid and base additives. *Journal of the American Oil Chemists Society*, *50*, 137–141.

Birk, Y., Gertler, A., & Khalef, S. (1963). A pure trypsin inhibitor from soya beans. *Biochemical Journal*, *87*, 281–284.

de Lumen, B. O., & Belo, P. S. Jr. (1981). Inhibitors of trypsin and chymotrypsin in winged bean (*Psophocarpus tetragonolobus*) tubers. *Journal of Agricultural and Food Chemistry*, *29*, 884–886.

de Muelenaere, H. J. H. (1964). Toxicity and hemagglutinating activity of legumes. *Nature*, *206*, 827–829.

DiPietro, C. M., & Liener, I. E. (1989). Heat inactivation of the Kunitz and Bowman-Birk soybean protease inhibitors. *Journal of Agricultural and Food Chemistry*, *37*, 39–44.

Ellenreider, G., Geronazzo, H., & de Bojarski, A. B. (1980). Thermal inactivation of trypsin inhibitors in aqueous extracts of soybeans, peanuts, and kidney beans: presence of substances that accelerate inactivation. *Cereal Chemistry*, *57*, 25–26.

Erlanger, B. F., Edel, F., & Cooper, A. G. (1966). The action of chymotrypsin on two new chromogenic substrates. *Archives Biochemistry and Biophysics*, *115*, 206–210.

Friedman, M., Gumbmann, M. R., & Grosjean, O. K. (1984). Nutritional improvement of soy flour. *Journal of Nutrition*, *114*, 2241–2246.

Gonzalez de Mejia, E., Grajeda Cota, P., Celada, E., & Valencia, M. E. (1988). [Caracterizacion del potencial nutricional del frijol tepari (*Phaseolus acutifolius*) cultivado en Mexico.]. *Arch. Latin Am. Nutr.*, *38*, 907–924.

Idouraine, A., Sathe, S. K., & Weber, C. W. (1992). Biological evaluation of flour and protein extract of tepary bean (*Phaseolus acutifolius*). *Journal of Agricultural and Food Chemistry*, *40*, 1856–1859.

Kabbara, S. R., Abbas, I. R., Scheerens, J. C., Tinsley, A. M., & Berry, J. W. (1987). Soaking and cooking parameters of tepary beans: effect of cooking time and cooking temperature on hardness and activity of nutritional antagonists. *Plant Foods for Human Nutrition*, *36*, 295–307.

Kakade, M. L., Rackis, J. J., McGhee, J. E., & Puski, G. (1974). Determination of trypsin inhibitor activity of soy products: a collaborative analysis of an improved procedure. *Cereal Chemistry*, *51*, 376–382.

Kapoor, A. C., & Gupta, Y. P. (1978). Trypsin inhibitor activity in soybean seed as influenced by stage of its development and different treatments, and the distribution in its anatomical parts. *Indian Journal of Nutrition Dietetics*, *15*, 429–433.

Kwok, K. C., Qin, W. H., & Tsang, J. C. (1993). Heat inactivation of trypsin inhibitor soymilk at ultra-high temperatures. *Journal of Food Science*, *58*, 859–862.

Laskowski, M., Jr., & Kato, I. (1980). Protein inhibitors of proteinases. *Annu. Rev. Biochem.*, *49*, 593–626.

Liener, I. E., & Kakade, M. L. (1980). Protease inhibitors. In I. E. Liener (Ed.), *Toxic constituents of plant foodstuffs* (2nd ed.) (pp. 7–57). New York: Academic Press.

Nabhan, G. P., & Feleger, R. S. (1978). Teparies in southwestern North America. A biographical and ethnographical study of Phaseolus acutifolius. *Economic Botany*, *32*, 2–19.

Obara, T., & Watanabe, Y. (1971). Heterogeneity of soybean trypsin inhibitors. II. Heat inactivation. *Cereal Chemistry*, *48*, 523–527.

Osborne, T. B., & Mendel, L. B. (1917). The use of soy bean as food. *Journal of Biological Chemistry*, *32*, 367–377.

Osman, M. A., & Weber, C. W. (1994). Purification and characterization of serine proteinase inhibitor from tepary bean (*Phaseolus acutifolius*). *FASEB Journal*, *8*(A936) (Abstr.).

Oste, R. E., Brandon, D. L., Bates, A. H., & Friedman, M. (1990). Effect of Maillard reaction of the Kunitz soybean trypsin inhibitor on its interaction with monoclonal antibodies. *Journal of Agricultural and Food Chemistry*, *38*, 258–261.

Rackis, J. J. (1965). Physiological properties of soybean trypsin inhibitors and their relationship to pancreatic hypertrophy and growth inhibition of rats. *Federation Proceedings*, *24*, 1488–1493.

Thorn, K. A., Tinsley, A. M., Weber, C. W., & Berry, J. W. (1983). Antinutritional factors in legumes of the Sonoran desert. *Ecolology of Food and Nutrition*, *13*, 251–254.

Tsukamoto, L., Miyoshi, M., & Hamaguchi, Y. (1983). Heat inactivation of trypsin inhibitor in Kintoki bean (*Phaseolus vulgaris*). *Cereal Chemistry*, *60*, 194–197.

Wallace, G. M., Bannatyne, W. R., & Khaleque, A. (1971). Studies on the processing and properties of soymilk. II. Effect of processing conditions on the trypsin inhibitor activity and the digestibility in vitro of proteins in various soymilk preparations. *Journal of the Science of Food and Agriculture*, *22*, 526–531.