Food Chemistry **40** (1991) 9–23

Kinetics of Heat-inactivation of Trypsin Inhibitors from the African Yam Bean (*Sphenostylis stenocarpa*)

Eugene N. Onyeike, Bene W. Abbey & Emmanuel O. Anosike

Department of Biochemistry, Faculty of Science,
University of Port Harcourt, PMB 5323, Port Harcourt, Nigeria

(Received 21 April 1989; accepted 10 May 1990)

ABSTRACT

Six different solvents were investigated for their efficiency in the extraction of trypsin inhibitors from the African yam bean. Of these, sodium hydroxide extract gave a marginally higher specific trypsin inhibitory activity ($4{\cdot}32 \times 10^{-2}$) than those of sodium chloride ($4{\cdot}10 \times 10^{-2}$) and distilled water ($3{\cdot}93 \times 10^{-2}$). The K_m and V_{max} for trypsin in the presence of a certain amount of trypsin inhibitor activity decreased as process temperature increased.

At a fixed trypsin concentration of 100 μg ml^{-1}, the rate of inactivation of trypsin inhibitor activity increased as substrate concentration increased from 0·230 mM to 0·575 mM. The rate, however, decreased as substrate concentration was further increased from 0·690 mM to 1·150 mM due to substrate inhibition. At a constant period of heating (30 min), trypsin inhibitor activity (TIA) decreased from 2·21 to 0·332 mg pure trypsin inhibited per gram of sample as temperature of heat treatment increased from 80–180°C, and the determined values of the first order inactivation rate constants increased.

As the assay pH decreased from 10·5 to 5·50, the inactivation energy decreased from 21 155–10 496 J mol^{-1}. At a constant temperature of heat treatment, trypsin inhibitor activity decreased over time.

INTRODUCTION

The seeds of the African yam bean are consumed directly by both man and animals. They form an important source of dietary proteins in most African countries, including Nigeria, especially in the Southern States (Imo,

Food Chemistry 0308-8146/91/$03·50

Anambra, Rivers, Cross River and Akwa Ibom). In Nigeria, beans (legumes) form an important group of foodstuffs because they provide a cheap source of dietary proteins and are now being successfully used in child-feeding programmes (Ologhobo & Fetuga, 1983). Legumes are widely grown throughout the world as a source of protein, and their contribution to the world's protein requirement is 18% (Jalil & Tahir, 1973).

Trypsin inhibitors were first recognised as anti-nutritional factors in legume seeds by Osborne and Mendel (1917). They associate with proteinases in a definite ratio to produce complexes which show no proteolytic activity (Laskowski & Laskowski, 1954*a*), and thus inactivate the proteolytic enzymes capable of degrading proteins into amino acids (Feeney & Allison, 1969). Although trypsin inhibitors as a group are said to be highly resistant to heat denaturation compared to other proteins, they are inactivated by heat and, generally, this results in an enhanced nutritive value of the protein (Liener, 1962). Not all trypsin inhibitors are equally sensitive to heat. Generally, the low molecular weight inhibitors (molecular weight up to 10000) are more heat-stable than the inhibitors with molecular weight greater than 20000 (Laskowski & Laskowski, 1954*b*).

The very high degree of stability which some trypsin inhibitors exhibit is thought to be due to the high percentage of disulphide bonds in the molecule, and these are of considerable significance, especially when the nutritional values of these legumes are considered for humans and animals (Laskowski & Sealock, 1971). The growth-inhibiting effect of raw navy beans fed to rats has been attributed to the presence of trypsin inhibitors as well as a methionine deficiency (Kakade & Evans, 1965*a*, *b*), and activity of intestinal microflora (Jayne-Williams & Hewitt, 1972). The disproportionately high concentration in the inhibitor of total cystine which is resistant to enzymatic attack has also been offered as explanation (Kakade *et al.*, 1969). The trypsin inhibitors are thus the most studied toxicants in plants.

This study was therefore initiated to supply basic information on the best extractant for, and on the heat-inactivation of, the African yam bean trypsin inhibitors, and this has been followed through a kinetic approach.

MATERIALS AND METHODS

Materials

The African yam bean seeds were purchased from Mile 3 Market, Diobu, in Port Harcourt. The following chemicals were purchased from British Drug Houses (BDH) Poole, Dorset: Tris (hydroxymethyl amino methane), calcium chloride dihydrate, hydrochloric acid, sodium chloride, dimethyl

sulphoxide, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, trichloroacetic acid (TCA) and sodium potassium tartrate.

Sodium hydroxide and copper (II) sulphate pentahydrate were bought from M and B; acetic acid from Hopkin and Williams; sulphuric acid from PHYWE, West Germany.

The substrate α-*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), the crystalline salt-free bovine trypsin and the bovine serum albumin (BSA) were purchased from Sigma Chemical Company, USA.

METHODS

Preparation of samples

The dry seeds were finely ground and powdered in a laboratory mill to pass through a 150-mesh laboratory test sieve. The sieved samples were then stored in air-tight containers until they were required for analysis.

Heating of sample

A crucible with lid was heated to the desired temperature. One gram of the sample was weighed out and placed in it, and it was stirred while being heated to the desired temperature on a magnetic stirrer-hot plate.

Substrate solution

Forty milligrams of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) were dissolved in 1·0 ml of dimethyl sulphoxide (DMSO), and diluted to 100 ml with tris buffer previously warmed to 37°C. This reagent was kept at 37°C in a water-bath while waiting for use and was freshly prepared each day.

Trypsin solution

Ten milligrams of accurately weighed crystalline salt-free bovine trypsin were dissolved in 0·001M HCl and made up to 200 ml with the acid. The solution was stored in the refrigerator for 2 to 3 weeks at 4°C without appreciable loss in activity.

Trypsin inhibitor extraction

The method of Smith *et al.* (1980) was adopted with slight modifications. Unheated and heated samples were used. One gram of the finely ground and

sieved sample was briefly shaken with 50 ml of 0·01M NaOH, and the sample readily dispersed. The pH of the resulting suspension was then adjusted to between 9·40 and 9·60 with 1·0M HCl and left overnight at 4°C.

Before use for trypsin inhibitor activity determination, it was shaken and diluted (five times for heated samples and about seventy-five times for unheated sample) with distilled water.

Determination of trypsin inhibitor activity (TIA) in unheated and heated samples

The method of Smith *et al.* (1980) was used. The following were pipetted into a series of 10 ml test tubes in duplicates:

(a) Reagent blank: 2·0 ml distilled water.
(b) Standard (100 μg trypsin): 2·0 ml standard trypsin solution plus 2·0 ml distilled water.
(c) Sample blank(s): 1·0 ml diluted sample extract plus 1·0 ml distilled water.
(d) Sample(s): 1·0 ml diluted sample extract plus 1·0 ml distilled water and 2·0 ml standard trypsin solution.

After mixing and pre-heating to 37°C for 10 min, 5·0 ml substrate solution (previously warmed to 37°C) was pipetted into each test tube and mixed. After exactly 10 min incubation at 37°C, each tube received 1·0 ml acetic acid (30% v/v) to terminate the reaction. Standard trypsin (2·0 ml) was added to the reagent blank (a) and sample blank (c) tubes. The contents of the tubes were then filtered using Whatman No. 1 filter paper. The absorbance of the clear tint of yellow-coloured solution obtained was measured spectrophotometrically at 410 nm. The blank used to zero the spectrophotometer prior to the measurement of absorbances at 410 nm was prepared by adding 1·0 ml of 30% v/v acetic acid to the test tube containing 2·0 ml trypsin solution and 2·0 ml distilled water before 5·0 ml substrate solution.

Survey of extractants

Five gram samples of the finely ground and sieved meal were extracted by stirring with 50 ml of each of the following solutions for 2 h at room temperature (28–30°C): 0·10M NaCl, pH 6·50; 0·10M phosphate buffer, pH 8·00; 0·05M H_2SO_4, pH 1·50; 0·05M NaOH, pH 11·5; 2·50% TCA, pH 1·80 and distilled water, pH 5·50. The resulting suspension was then centrifuged for 30 min at 4000 *g*. The volume of the supernatant was thereafter measured, and later assayed for total protein using the Biuret method (Gornall *et al.*, 1949), and for trypsin inhibitor activity using the

method of Smith *et al.* (1980). Trypsin inhibitor activity is expressed as trypsin units inhibited per milligram.

It has been reported that 1·0 mg trypsin inhibited per gram of sample is equal to 1·90 [TI] units per milligram (Kakade *et al.*, 1974). From this relationship, the values of the TIA in milligrams of pure trypsin inhibited per gram of sample were computed in terms of trypsin units inhibited per milligram.

Determination of K_m and V_{max} in the presence of TIA

One gram of the sieved sample was weighed out into seven sample bottles. Each was heated for 30 min at 100°C, extracted with 0·01M NaOH, and the trypsin inhibitor activity (TIA) determined using the method of Smith *et al.* (1980) with BAPNA as substrate. The enzyme concentration used for each assay was kept constant at $100\,\mu g\,ml^{-1}$.

For each of the substrate concentrations, the rate of reaction of the residual trypsin with the added substrate was determined by measuring the change in absorbance at 410 nm. From the rates measured at different substrate concentrations, the K_m and V_{max} for trypsin in the presence of certain amounts of TIA were calculated using Lineweaver-Burk (1934) double reciprocal plots. This was repeated for samples heated for 30 min at 125°C and at 150°C.

Variation of trypsin inhibitor activity with temperature at a constant period of heating

One gram of the ground and sieved sample was weighed out into six sample bottles and labelled A_1, A_2 ... A_6. Each of them was subjected to heat treatment for 30 min with the temperature varying from 80–180°C through 100°C, 120°, 140° and 160°C. After heating, the samples were then extracted with 0·01M NaOH, and the pH of each resulting suspension was adjusted to between 9·40 and 9·60. Trypsin inhibitor activity in each of them was then determined by the method of Smith *et al.* (1980).

Effect of pH and temperature on the values of inactivation energy (E_a) and the first order inactivation rate constants (K)

Six samples, each 1·0 g, were heated for 30 min at temperatures of 80°, 100°, 120°, 140°, 160° and 180°C, respectively. They were then extracted with 0·01M NaOH and the assay pH was adjusted. For the first run, each of the six samples was adjusted to pH 10·5 and, for the second run, each sample was adjusted to pH 9·50. For the third, fourth and fifth runs, the samples were,

respectively, adjusted to pH 8·50, 7·50 and 5·50. The trypsin inhibitor activities of the various samples were then determined using the method of Smith *et al.* (1980).

From the values of the TIA for each heat-treated sample under each pH condition and the value for the TIA at zero time (TIA for the unheated sample subjected to the same analytical procedure), the first order inactivation rate constants were calculated using the relationship reported by Perkins and Toledo (1982). That is, $\log C/C_0 = -K_t$ where C/C_0 is the fraction of the active trypsin inhibitor after time, t, and K is the first order inactivation rate constant.

From the values of the rate constants at different temperatures (in K), the inactivation energy (E_a) was then determined from Arrhenius (1889) plots. From the graphs which were negatively displaced, the inactivation energy at each pH was computed using slope $= -E_a/2{\cdot}303R = -E_a/19{\cdot}14$ where R is the gas constant.

Variation of trypsin inhibitor activity with time at a constant temperature

One gram of the ground and sieved sample was weighed out into each of 28 sample bottles and labelled. The first seven samples were heated for 20, 30, 40, 50, 60, 70 and 80 min, respectively, at the same temperature of 120°C. The heated samples were extracted with 0·01M NaOH as already described and trypsin inhibitor activity determined. The enzyme concentration used was constant at $100\,\mu g\,ml^{-1}$ while the substrate concentration was 0·460 mM throughout.

The heat treatment was repeated for the other samples (seven in each case) at 100°C, 140°C and 80°C with time varying from 20–80 min. The trypsin inhibitor activity (TIA) was then determined in each case.

RESULTS AND DISCUSSION

A total of six extractants were surveyed for the isolation of the African yam bean trypsin inhibitors. The results are shown in Table 1. NaOH extract contained the highest protein followed by phosphate buffer extract, while TCA extract showed the least protein content. Furthermore, NaOH extract gave a marginally higher specific trypsin inhibitory activity ($4{\cdot}32 \times 10^{-2}$) than sodium chloride extract ($4{\cdot}10 \times 10^{-2}$) and distilled water extract ($3{\cdot}93 \times 10^{-2}$). Thus, the survey established sodium hydroxide as the best extractant which was then chosen for the isolation of the African yam bean trypsin inhibitors in subsequent studies.

Sodium hydroxide has been used as the extractant in the determination of

TABLE 1
Survey of Extractants for the African Yam Bean Trypsin Inhibitors[a]

Extractant	*pH*	*Total protein*[b] *(mg)*	*Total [TI]*[c] *$\times 10^{-3}$ units*	*Specific activity*[d] *$\times 10^{-2}$*
0·05M NaOH	11·5	334·1	14 442	4·32
0·10M NaCl	6·50	241·3	9 882	4·10
Distilled water	5·50	232·0	9 122	3·93
0·10M phosphate buffer	8·00	278·4	9 882	3·55
2·50% TCA	1·80	204·2	4 560	2·23
0·05M H_2SO_4	1·50	259·8	3 040	1·17

[a] Extraction from 5·0 g bean meal.
[b] Total protein determined by the Biuret method (Gornall *et al.*, 1949).
[c] Trypsin inhibitor units determined by the methods of Smith *et al.* (1980) and Kakade *et al.* (1974) using α-*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) as substrate.
[d] Specific activity defined as the number of tryptic units inactivated.

trypsin inhibitor levels in foodstuffs (Smith *et al.*, 1980), and in the determination of TIA of soy products (Kakade *et al.*, 1969; 1974; Ellenrieder *et al.*, 1981; Hamerstrand *et al.*, 1981). In their study on the inactivation of trypsin inhibitors in aqueous soybean extracts by direct steam infusion, Johnson *et al.* (1980) assayed for TIA by the procedure of Swartz *et al.* (1977) in which distilled water was used as the extractant.

Boisen and Djurtoft (1981) employed 0·10M sodium acetate buffer, pH 4·90, to isolate trypsin inhibitors from rye endosperm. TIA was also assayed in peanut protein using 0·05M HCl as the extractant (Perkins & Toledo, 1982). Tsukamoto *et al.* (1983) purified and characterized three trypsin inhibitors from kintoki beans by chromatofocusing technique, and the inhibitors were extracted with distilled water, pH 5·30. Gomes *et al.* (1979) isolated trypsin inhibitors from navy beans by affinity chromatography in which 1·0M NaCl was used to extract the toxic protein. Organic solvents such as acetone and ethanol have also been used, but seem to be of limited applicability (Birk, 1961).

Most of the inhibitors are soluble and stable in acid medium and the properties of solubility and stability have been widely used in the isolation and purification of the low molecular weight inhibitors.

The result of variation of trypsin inhibitor activity (TIA) with substrate concentration at varying temperatures is presented in Table 2. As process temperature increased at a constant period of heating, the rate of trypsin inhibitor inactivation increased.

TABLE 2
Variation of Trypsin Inhibitor Activity (TIA) with Substrate Concentration at Different Temperatures of Heat Treatment

Substrate concentration (mM)	*TIA (mg pure trypsin inhibited per gram sample)*		
	Temperature and time of heating		
	100°C for 30 min	*125°C for 30 min*	*150°C for 30 min*
0·230	2·95	2·48	1·18
0·345	3·59	3·16	1·31
0·460	3·82	3·36	1·55
0·575	4·39	3·76	1·60
0·690	2·73	1·83	1·37
0·920	2·07	1·39	1·03
1·150	1·03	0·51	0·32

At a particular temperature, TIA increased as substrate concentration increased from 0·230 mM to 0·575 mM, but the TIA decreased when substrate concentration was increased from 0·690 mM to 1·150 mM, probably due to substrate inhibition. Furthermore, at any particular substrate concentration, the rate of trypsin inhibitor inactivation and consequently trypsin inhibitor activity decreased as temperature of heat treatment increased. There was also a consistent decrease in the values of K_m and V_{max} determined for trypsin in the presence of certain amounts of trypsin inhibitor activity as shown in Table 3.

A linear relationship between temperature of heat treatment and the determined values of K_m and V_{max} is discernible (Fig. 1). The decrease in K_m and V_{max} may be attributable to increase in the rate of trypsin inhibitor inactivation. Increasing either temperature or moisture content has been

TABLE 3
K_m and V_{max} Values for Trypsin in the Presence of TIA at Different Temperatures of Heat Treatment

Heat treatment	K_m (mM^{-1})	V_{max} (*units* min^{-1})
30 min at 100°C	0·278	0·417
30 min at 125°C	0·263	0·345
30 min at 150°C	0·189	0·139

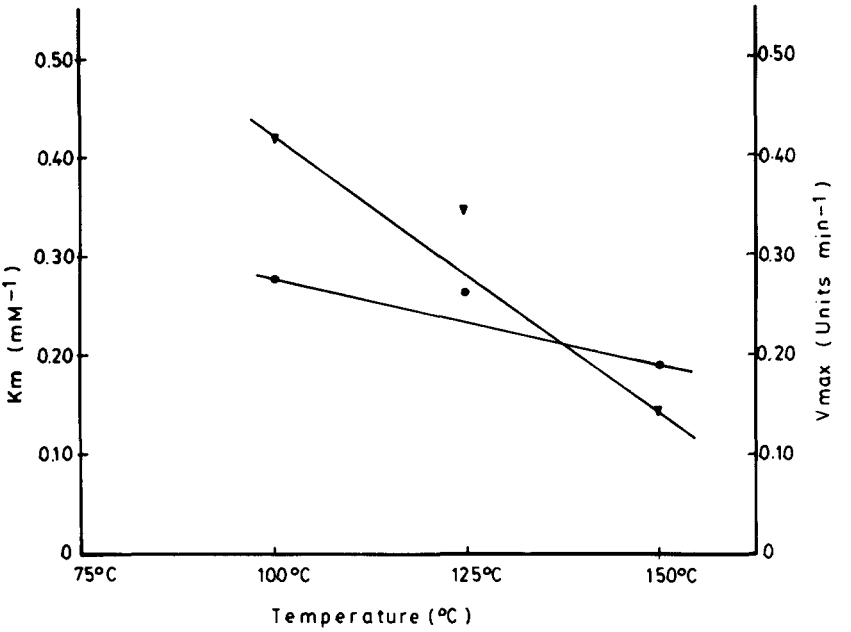

Fig. 1. Plots of K_m (●——●) and of V_{max} (▲——▲) against temperature of heat treatment (period of heating was 30 min in each case).

reported to increase the rate of trypsin inhibitor inactivation (Phillips *et al.*, 1983).

Figure 2 shows the results of the variation of trypsin inhibitor activity with temperature at a constant period of heating of 30 min.

It was observed that TIA decreased as temperature of heat treatment increased due to increase in the rate of trypsin inhibitor inactivation. These findings are consistent with those of Chang and Tsen (1981) who worked on characterization and heat-stability of trypsin inhibitors from rye, triticale and wheat samples. Triticale trypsin inhibitor was the most heat-resistant among the inhibitors which they tested. They also observed that heating up to 80°C for 60 min caused minimal conformational perturbation of the inhibitors, and the perturbed molecules tended to go back to their original conformation upon cooling. On the other hand, heating for 60 min at 100°C inactivated only 7% of triticale trypsin inhibitors while heating at 125°C for 60 min altered the conformation of the inhibitors and permanently inactivated all of them.

The results of varying the assay pH and temperature on the values of the first order inactivation rate constants (K) and the inactivation energy (E_a) are shown in Table 4. As the assay pH decreased, the energy of inactivation also showed a consistent decrease.

At any particular pH, an increase in the temperature of heat treatment at a

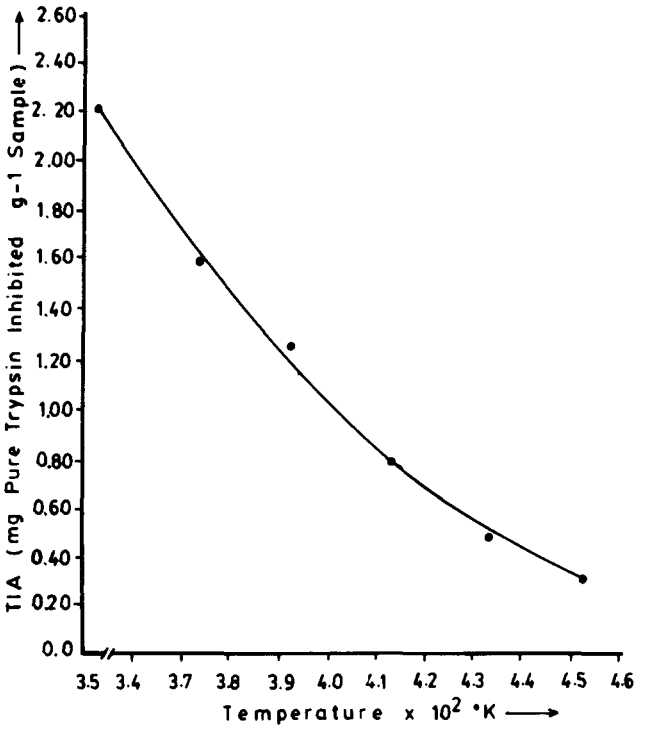

Fig. 2. Graph of the variation of trypsin inhibitor activity with temperature at a constant period of heating of 30 min.

TABLE 4

Effect of Varying the Assay pH and Temperature on the Values of Inactivation Energy (E_a) and the First Order Inactivation Rate Constants (K)

Assay period and temperature	*pH 10·5*		*pH 9·50*		*pH 8·50*		*pH 7·50*		*pH 5·50*	
	TIA	*K*	*TIA*	*K*	*TIA*	*K*	*TIA*	*K*	*TIA*	*K*
30 min at 80°C	2·53	6·65	2·22	8·58	2·05	9·66	1·42	15·0	1·11	18·6
30 min at 100°C	2·05	9·66	1·74	12·1	1·58	13·5	1·26	16·7	0·95	20·8
30 min at 120°C	1·74	12·1	1·26	16·7	1·11	18·6	0·79	23·5	0·63	26·7
30 min at 140°C	0·95	20·8	0·95	20·8	0·63	26·7	0·63	26·7	0·47	30·9
30 min at 160°C	0·71	25·0	0·47	30·9	0·47	30·9	0·40	33·5	0·32	36·8
30 min at 180°C	0·40	33·5	0·33	36·0	0·32	36·8	0·32	36·8	0·24	40·9
E_a (J mol^{-1})	21 155		18 566		18 374		12 384		10 496	

TIA = Trypsin inhibitor activity in mg pure trypsin inhibited per gram of sample.

K($\times 10^{-3}$ min^{-1}) = First order inactivation rate constant (min^{-1}) determined by the method of Perkins & Toledo (1982).

E_a = Inactivation energy obtained by making Arrhenius (1889) plot of log k against the reciprocal of absolute temperatures.

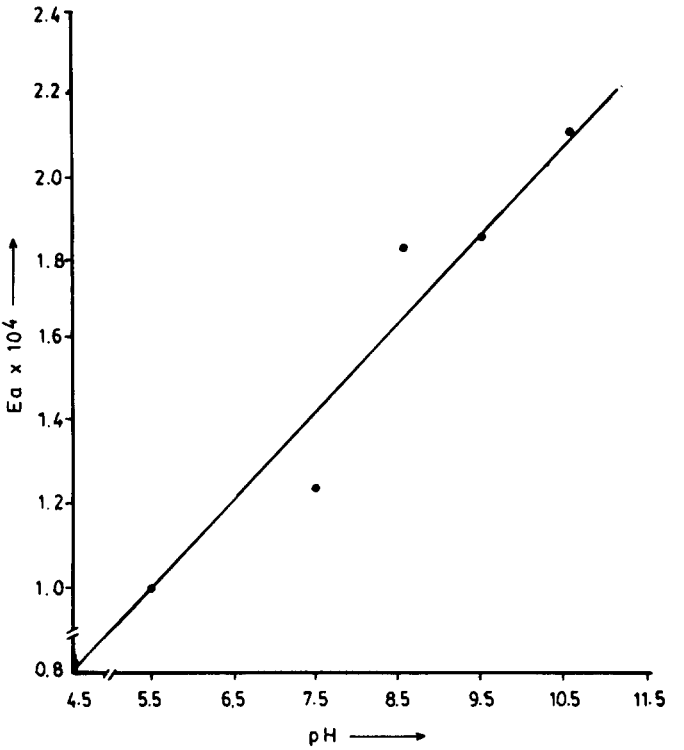

Fig. 3. Plot of inactivation energy against pH of the assay medium.

constant period of heating resulted in an increase in the values of the first order inactivation rate constants and a decrease in the trypsin inhibitor activity.

Figure 3 shows the plot of E_a against the assay pH which is a straight line passing through the origin. It indicates that E_a is directly proportional to the pH of the assay. Johnson *et al.* (1980) reported an energy of inactivation of 48, 409 J mol^{-1} and a rate constant of $1{\cdot}40 \times 10^{-4}$ s^{-1} for the inactivation of kunitz trypsin inhibitor in soymilk. Values of inactivation energy of 142, 835 J mol^{-1} and a first order inactivation rate constant of $2{\cdot}59 \times 10^{-13}$ min^{-1} were calculated for trypsin inhibitor inactivation in moist peanuts (Perkins & Toledo, 1982). In this work, values of E_a (J mol^{-1}) of 21 155, 18 566, 18 374, 12 384 and 10 496 were calculated. These values for the E_a vary and are lower compared to the values reported by other investigators mentioned above. The variation may be attributed to the differences of source of the inhibitors and also to the differences in the assay conditions.

Phillips *et al.* (1983) reported that TIA in cowpea flour containing 7·50% moisture decreased when heated to 100°, 125° and 150°C while the first order inactivation rate constants increased from $1{\cdot}06 \times 10^{-2}$ min^{-1} to 4·45 min^{-1}

through $9{\cdot}42 \times 10^{-2}\,min^{-1}$. Collins and Beaty (1980), studying the inactivation of trypsin inhibitors in mature green soybeans in boiling water, calculated a rate constant of $3{\cdot}40 \times 10^{-1}\,min^{-1}$ while Perkins and Toledo (1982) reported a rate constant of $2{\cdot}50 \times 10^{-1}\,min^{-1}$ at 100°C and $2{\cdot}60\,min^{-1}$ at 120°C for whole moist peanuts (approximately 70% moisture).

At any given temperature, TIA was found to decrease with time of heating as shown in Fig. 4. The results are consistent with previous reports by Perkins and Toledo (1982). Many enzymes follow first order kinetics with

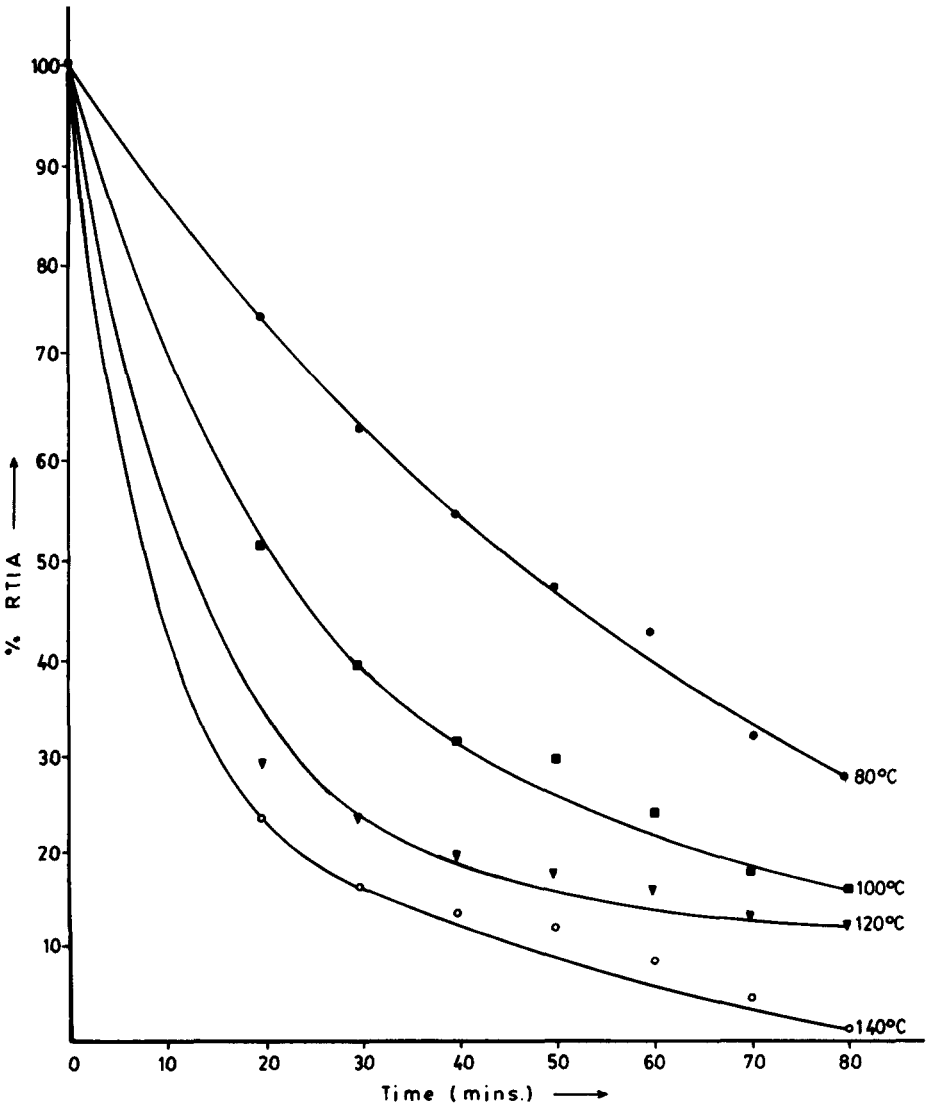

Fig. 4. Plots of percentage residual trypsin inhibitor activity against time of heating at constant temperatures.

respect to denaturation due to heating. This is true for the partially purified cocoyam (*Xanthosoma sagittifolium*) polyphenoloxidase (Anosike & Ojimelukwe, 1982), and for aerial yam (*Dioscorea bulbifera*) (Anosike & Ayaebene, 1981).

Thus, plots of the residual trypsin inhibitor activity (RTIA) as a percentage of the original against the time of heat treatment were found to be curvilinear (Fig. 4). However, at 80°C this was linear, indicating a first order process. From Fig. 5, a rate constant of $1{\cdot}48 \times 10^{-2}\,\text{min}^{-1}$ was

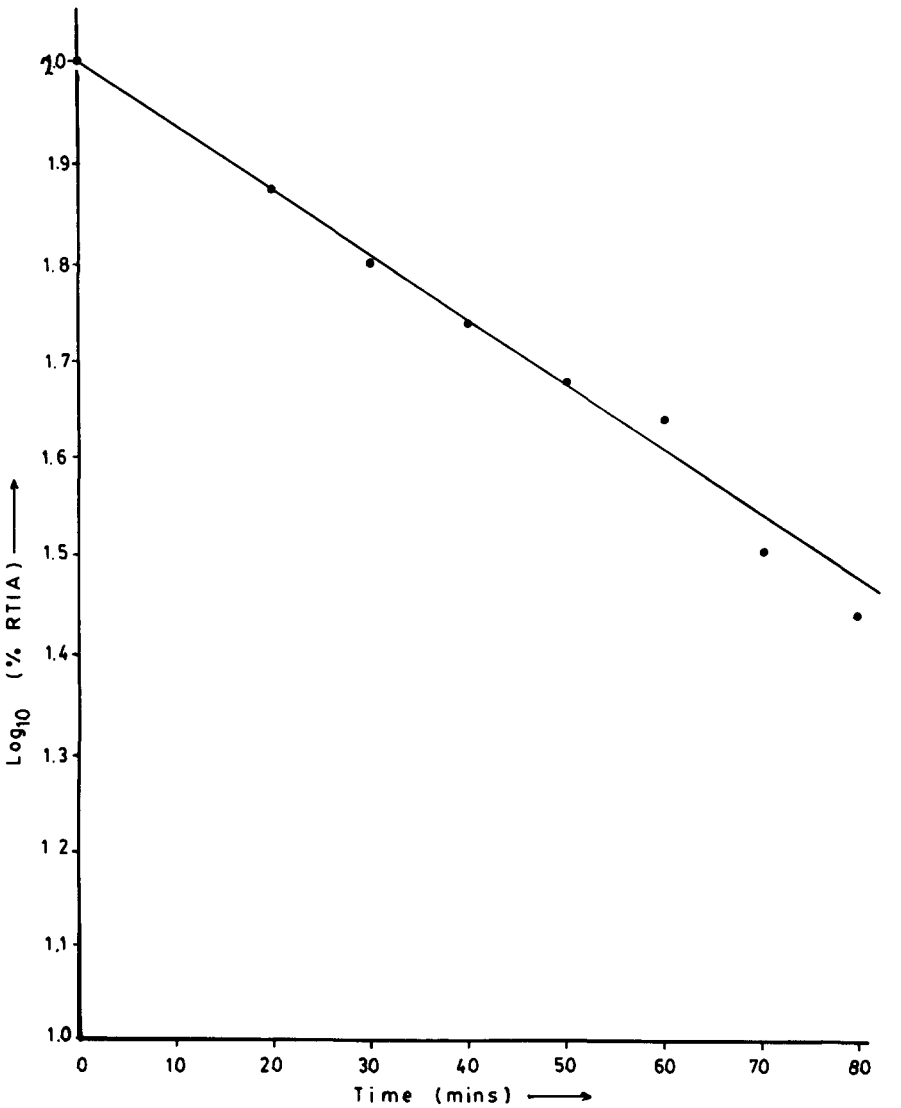

Fig. 5. Plot of log(% residual trypsin inhibitor activity) against time of heating at 80°C.

calculated. Similar plots for 100°C and 120°C were biphasic while that for 140°C was triphasic.

The results of this work would indicate that the African yam bean seed is best cooked at 140°C for 80 min for optimum inactivation of trypsin inhibitors in order to improve the protein quality of the final food product.

REFERENCES

Anosike, E. O. & Ayaebene, A. O. (1981). Purification and some properties of polyphenol oxidase from the yam tubers (*Dioscorea bulbifera*). *Phytochemistry*, **20**(12) 2625–8.

Anosike, E. O. & Ojimelukwe, P. C. (1982). Partial purification and characterization of polyphenol oxidase from cocoyam (*Xanthosoma sagittifolium*). *J. Experimental Bot.*, **33**(134), 487–94.

Arrhenius, K. (1889). Determination of the energy of activation of a reaction. In *A Biologist's Physical Chemistry*, by J. Gareth Morris, pp. 268–70.

Birk, Y. (1961). Purification and some properties of highly active inhibitor of trypsin and chymotrypsin from soybeans. *Biochem. Biophys. Acta.*, **54**, 378.

Boisen, S. & Djurtoft, R. (1981). Trypsin inhibitor from rye endosperm: Purification and properties. *Cereal Chem.*, **58**(3), 194–8.

Chang, C. R. & Tsen, C. C. (1981). Characterization and heat stability of trypsin inhibitors from rye, triticale and wheat samples. *Cereal Chem.*, **58**(3), 211–13.

Collins, J. L. & Beaty, B. F. (1980). Heat inactivation of trypsin inhibitor in fresh green soybeans and physiological response of rats fed the beans. *J. Food Sci.*, **45**, 542.

Ellenrieder, G., Blanco, S. & Bondoni, A. (1981). Thermal inactivation of trypsin inhibitors in aqueous extracts of soybeans: Studies on substances that accelerate inactivation. *Cereal Chem.*, **58**(4), 291–3.

Feeney, R. E. & Allison, R. G. (1969). *Evolutionary Biochemistry of Proteins. Homologous and Analogous Proteins from Avian Egg Whites, Blood Sera, Milk and Other Substances.* Wiley—Interscience, New York.

Gomes, J. C., Koch, U. & Brunner, J. R. (1979). Isolation of a trypsin inhibitor from navy beans by affinity chromatography. *Cereal Chem.*, **56**(6), 525–9.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). Determination of serum proteins by means of the Biuret method. *J. Biol. Chem.*, **177**, 751–66.

Hamerstrand, G. E., Black, L. T. & Glover, J. D. (1981). Trypsin inhibitors in soy products: Modification of the standard analytical procedure. *Cereal Chem.*, **58**(1), 42–45.

Jalil, M. E. & Tahir, W. M. (1973). World supplies of plant proteins. In *Proteins in Human Nutrition.*, ed. J. W. G. Porter & B. A. Rolls, Academic Press, London and New York, pp. 35–46.

Jayne-Williams, D. J. & Hewitt, D. (1972). The relationship between the intestinal microflora and the effects of diet containing raw navy beans on the growth of Japanese Quail (*Coturnix japonica*). *J. Appl. Bact.*, **35**, 331–444.

Johnson, L. A., Deyoe, C. W., Hoover, W. J. & Schwenke, J. R. (1980). Inactivation of trypsin inhibitors in aqueous soybean extracts by direct steam-infusion. *Cereal Chem.*, **57**(6), 376–9.

Kakade, M. L. & Evans, R. J. (1965*a*). Growth-inhibition of rats fed navy bean fractions. *J. Agr. Food Chem.*, **77**, 450–2.

Kakade, M. L. & Evans, R. J. (1965*b*). Nutritive value of navy beans. *Brit. J. Nutr.*, **19**, 269–76.

Kakade, M. L., Simons, N. & Liener, I. E. (1969). An evaluation of natural versus synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem.*, **46**, 518–26.

Kakade, M. L., Rackis, J. J., Mcghee, J. E. & Puski, G. A. (1974). Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. *Cereal Chem.*, **51**, 376–82.

Laskowski, M. & Laskowski, M. Jr (1954*a*). Naturally occurring trypsin inhibitors. In *The Enzymes*, ed. P. D. Boyer, Vol. 111, Academic Press, New York, pp. 376–473.

Laskowski, M. & Laskowski, M. Jr (1954*b*). Naturally occurring trypsin inhibitors. *Advances in Protein Chemistry*, **9**, 203–42.

Laskowski, M. Jr & Sealock, R. W. (1971). Protein-proteinase inhibitors—Molecular aspects. In *The Enzymes*, Vol. 3, 3rd edn. ed. P. D. Boyer. Academic Press, New York, pp. 375–473.

Liener, I. E. (1962). Toxic factors in edible legumes and their elimination. *Am. J. Clin. Nutr.*, **11**, 287–98.

Lineweaver, H. & Burk, D. (1934). Transformation of the Michaelis-Menten equation into a linear form for analysing data graphically and detecting deviations from the ideal behaviour. *J. Am. Chem. Soc.*, **56**, 658.

Ologhobo, A. D. & Fetuga, B. L. (1983). Investigations on the trypsin inhibitor, hemagglutinin, phytic and tannic acid contents of cowpea (*Vigna unguiculata*). *Food Chem.*, **12**, 249–54.

Osborne, T. B. & Mendel, L. B. (1917). The use of soybean as food. *J. Biol. Chem.*, **32**, 369–87.

Perkins, D. & Toledo, R. T. (1982). Effects of heat treatment for trypsin inhibitor inactivation on physical and functional properties of peanut proteins. *J. Food Sci.*, **47**, 917–23.

Phillips, R. D., Chhinnan, M. S. & Mendoza, L. G. (1983). Effect of temperature and moisture content on the kinetics of trypsin inhibitor activity, protein *in vitro* digestibility and nitrogen solubility of cowpea flour. *J. Food Sci.*, **48**, 1863–7.

Smith, C., Megen, W. V., Twaalfhoven, L. & Hitchcock, C. (1980). The determination of trypsin inhibitor levels in foodstuffs. *J. Sci. Food Agric.*, **31**, 321–50.

Swartz, M. J., Mitchell, M. L., Cox, D. J. & Reeck, G. R. (1977). Isolation and characterization of trypsin inhibitor from opaque—2 corn seeds. *J. Biol. Chem.*, **252**, 8105.

Tsukamoto, I., Miyoshi, M. & Hamaguchi, Y. (1983). Purification and characterization of three trypsin inhibitors from beans (*Phaseolus vulgaris*) 'kintoki', *Cereal Chem.*, **60**(4), 281–6.