Pak. j. sci. ind. res., vol. 34, no. 4, April 1991

CHARACTERISATION OF TRYPSIN INHIBITOR FROM MILLET (*PENNISETUM TYPHOIDEUM*)

T. K. OBIDAIRO* AND H. N. OBASUYI

Department of Biochemistry, University of Benin, Benin City, Nigeria

(Received July 7, 1990; revised June 15, 1991)

Trypsin inhibitors were shown to be present in bulrush millet (*Pennisetum typhoideum*).Chromatography of the crude extract first on a DEAE-cellulose column and then on a Sephadex G-75 column revealed the presence of four trypsin inhibitors. Inhibitor A_4 was the most active and was found to lose most of its activity on heating at 100° for 40 mins. Its molecular weight was estimated using molecular sieve chromatography and found to be 12,950.

Key words:Trypsin inhibitor, Chromatography.

Introduction

Proteins which inhibit the activity of proteolytic enzymes have been isolated from the tissues of a variety of animals and plants species. Their distribution and properties were presented in a recent general review and proceedings of research conferences [1-4]. Investigations have also shown that these inhibitors are also present in plant tissues such as barley, peanuts, sweet potatos, sugar beat, many fruits and vegetables [5].

Although studies on trypsin inhibitor (TI) in sorghum seeds and in barley have been carried out, cereals like millet have not been investigated [6]. It is, therefore of interest to investigate the TI in millet. Since millet constitutes an im-portant source of dietary protein in Nigeria, especially in the Northern parts of Nigeria where it is second only to sorghum, and in view of the apparent dearth of information on inhibitors from millet we have undertaken to isolate, purify and study them. Such studies will contribute at least in part to an understanding of their significance in animal nutrition. In this paper, the isolation and purification of the inhibitors is described.

Materials and Methods

The sample of millet seed was obtained from Benin City (Bendel State of Nigeria). The sample was ground and extracted with ice-cold 1% sodium chloride heated at 100° for 10 mins. and precipitated with ammonium sulphate at 100% saturation. This crude extract was dialysed against several changes of distilled water. The crude inhibitors were separated by anion exchange chromatography on DEAE-cellulose using 10 mM Tris buffer pH 7.5. The DEAE-cellulose column (1.6 x 40 cm) was equilibrated with 10mM. Tris-HCl buffer pH 7.5 and maintained at a flow rate of 30ml/hr. Elution was done using a linear sodium chloride gradient 10mM-100mM in Tris-HCl buffer pH 7.5. Fractions of 10ml each were collected. Further purification of the pooled fractions from the DEAE-column was achieved by separation on a column of Sephadex G-75 (1.6 x 95cm) using 0.1 M sodium acetate buffer pH 4.9, as described later under molecular weight estimation.

Trypsin inhibitor activity was determined by the method of Hummel as modified by Collins et al. [7]. The method measures the rate of substrate hydrolysed by trypsin. N-benzoyl- L-arginine ethyl ester (BAEE) was used as substrate for trypsin.

A solution of N-benzoyl-L-arginine ethyl ester (BAEE), 0.001M in distilled water and stock trypsin solution 40µg/ml in 0.001M HCl and 0.0115M $CaCl_2$ H_2O in 0.004M Tris buffer pH 7.5 were prepared. Standard solution of varying concentrations (0.5 - 3.5 µg/ml) of soyabean trypsin inhibitor in 0.125M H_2SO_4 were prepared.

For the assay of tryptic activity, 2.5ml buffer and 0.6ml BAEE solution were taken and 0.1ml standard trypsin added. The rate of tryptic hydrolysis was measured by recording the change per min. in absorbance at 255 nm with a Pye Unican SP-1800 spectrophotometer. To measure the inhibitory activity, a known amount of inhibitor in 0.1ml was pre-incubated with 0.1ml enzyme followed immediately by addition of 2.4ml Tris-HCl buffer pH 7.5 and 0.6 ml BAEE solution so that the total volume of the reaction mixture was 3.2ml. The residual tryptic activity was measured. Percentage inhibition I is expressed as follows:

$$I = \frac{T - T^*}{T} \times 100$$

where T = change in absorbance at 255nm for the un-inhibited sample and T* = change in the absorbance for the inhibited sample.

The amount of active inhibitor present in the solution is obtained by extrapolation from a standard plot of commercial trypsin inhibitor (µg) against percentage inhibition (Fig. 1).

Effect of pH.The effect of pH on the inhibitor activity of A_4 was determined according to the method of Betz *et al.* [8]. Measurements were carried out at 25°.

*Lagos State University, Apapa, Lagos, Nigeria.

*Estimation of molecular weight by gel filtration.*The molecular weight was determined from the elution volume obtained after molecular sieve chromatography on a calibrated column of Sephadex G-75. The column 1.6 x 95cm was equilibrated with 0.1 M sodium acetate buffer pH 4.9 and maintained at a flow rate of 20ml/hr. The void volume was determined with blue dextran 2000 to be 60ml. The column was calibrated with the following proteins of known molecular weights; myoglobin (17,200), cytochrome C (12,000), casein (23,000), egg albumin (45,000), haemoglobin (65,000) and catalase (225,000). In all cases, 5ml of the sample was applied to the column and 3ml fractions collected. The elution position of each sample protein was determined by measurement of absorbance at 280 mm.

The elution data were expressed in terms of Kav from the formula.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Where Vo is the elution volume of blue dextran, Ve is the elution volume of the inhibitor or calibrating protein and Vt is the total volume of the gel bed. The molecular weight was then extrapolated from a plot of Kav against log M of the known proteins.

Results and Discussion

When an aliquot of crude inhibitor is fractionated on a column of DEAE-cellulose and eluted with a linear sodium chloride gradient in Tris-HCl buffer pH 7.5 an elution profile such as shown in Fig. 2 is obtained. Apart from the two protein peaks, A and B, shown, no other 280 nm-absorbing material can be detected. Fraction A was found to contain 70 µg/ml while fraction B contained 105 µg/ml of protein. They both show inhibitory activity on trypsin.

*Sephadex gel filtration of fraction A.*When aliquots of fraction A is chromatographed on a column of Sephadex G-75 (1.6 x 95cm) and eluted with 0.1 M acetate buffer pH 4.9, a chromatograph pattern such as that presented in Fig. 3 is obtained. Four protein peaks, fractions A_1, A_2, A_3 and A_4 are obtained. From a standard curve obtained by plotting commercial soybean (µg) against percentage inhibition of bovine trypsin, the concentration of TI in each fraction is obtained. (Fig. 1, Table 1).

From Table 1, fraction A_4 is found to be the most active among all the fractions. All the peaks differ widely with respect to their specific activities. Specific activity is defined as µg trypsin inhibited by 1 µg of inhibitor [9]. A_4 has the highest protein and TI contents and also gives the highest inhibitory activity. The protein contents in the other peaks are directly proportional to their trypsin inhibitor contents as well as their inhibitory activities. It is also seen that fractions A_1, A_2, A_3 and A_4 constitute 20.2mg of trypsin inhibitor per kg of the bulrush millet.

Figure 4 shows the effect of pH changes on the inhibitory activity of A_4. It is most active at pH 7.5.

A_4 was also found to retain its full activity when heated to 75°. AT 100° most of the activity is lost. Using Sephadex G-75 column and protein molecules as markers its molecular weight was found to be 12, 590.

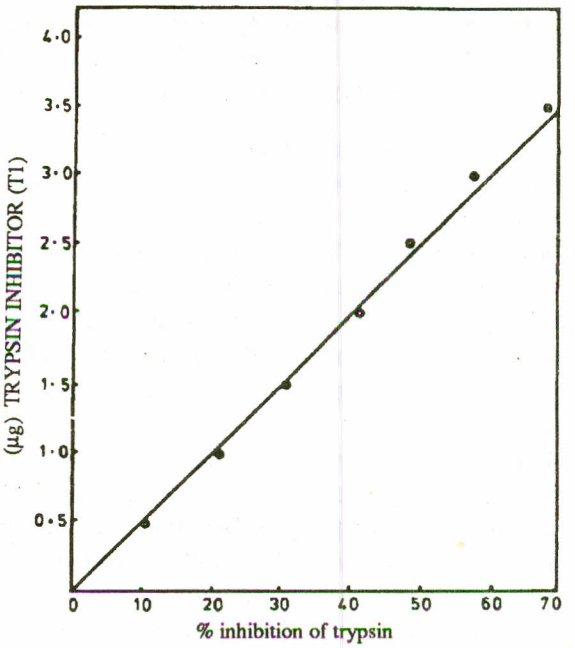

Fig. 1. A graph of commercial soyabean trypsin inhibitor (TI) (µg) against percentage inhibition.

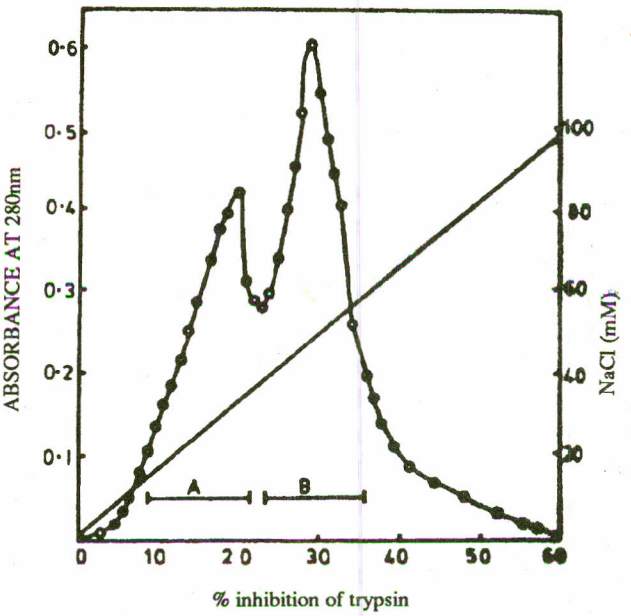

Fig. 2. Ion exchange chromatography of crude inhibitor on DEAE-cellulose column (1.6 x 40cm). Elution was with a linear NaCl gradient (10mM-100mM) in 10 mM Tris-HCl buffer pH 7.5.

TABLE 1. THE CONCENTRATION OF TRYPSIN INHIBITOR TI IN EACH FRACTION IS DETERMINED FROM THE STANDARD CURVE (FIG. 1) OBTAINED BY PLOTTING COMMERCIAL SOYABEAN µg AGAINST PERCENTAGE INHIBITOR. THE PROTEIN CONTENT WAS DETERMINED BY THE METHOD OF LOWRY [16] USING BOVINE SERUM ALBUMIN AS STANDARD. SPECIFIC ACTIVITY IS DEFINED AS µg OF TRYPSIN INHIBITED BY L µg OF INHIBITOR, (i.e. TRYPTIC UNITS).

Specific activity	% inhibition	TI µg/ml	Protein content µg/ml
A_1 2.2	35.55	1.75	6.00
A_2 1.74	13.30	0.65	3.60
A_3 1.86	43.00	2.15	8.40
A_4 6.15	45.60	2.30	9.20

The amount of inhibitor in each peak is estimated by extrapolation from Fig.1.

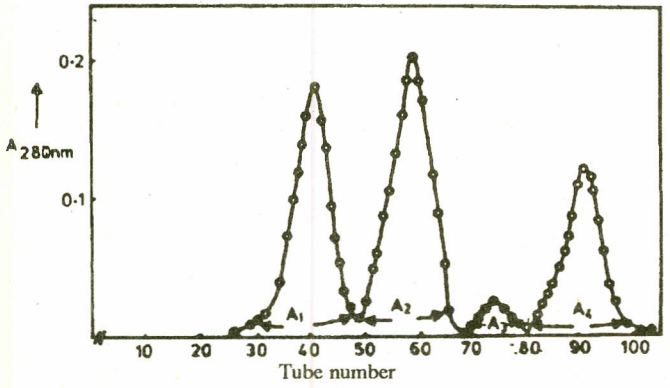

Fig. 3. Gel filtration on Peak A (from DEAE-cellulose column) on Sephadex G-75 column (1.6 x 95cm). Elution was with 0.1 M acetate buffer pH 4.9.

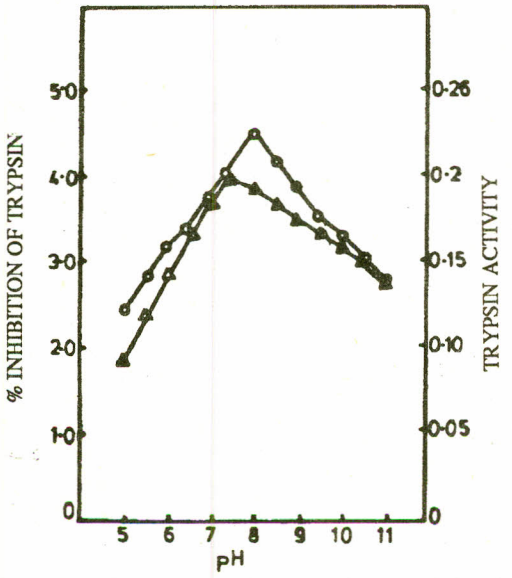

Fig. 4. The effect of pH on the trypsin activity (tryptic units) and on the activity of fraction A_4 from Sephadex G-75 column, in the absence ——o—— ——o——o—— and in the presence –Δ–Δ–Δ– of inhibitor respectively. The maximum activity was given at pH 7.5

The presence in bulrush millet of several components with inhibitory activity is not unique since many other protease inhibitors isolated are found to be heterogenous [10]. The multiplicity of inhibitors could not be due to chromatographic artifacts, since the elution positions of the individual peaks are reproducible upon rechromatography. This suggests the possibility that the distinct inhibitors could be constituents of the millet.

The isoinhibitors show differential activities towards trypsin (Table 1). In agreement with results obtained by othere workers [11,12], those fractions with high specific inhibitory activities represent native inhibitors while the rest are modified forms. The inhibitors in millet have certain properties in common with lima bean and chick peas inhibitors. They retain their activity after exposure to extreme temperature [13].

The nutritional significance of protease inhibitors in leguminous seeds is still controversial [14]. Nevertheless, there are numerous reports which indicate that the destruction of these inhibitors by controlled heat treatment is accompanied by improved nutritive value. The present investigation on stability has revealed that these inhibitors can be destroyed by boiling at 100° for 30 mins. They display maximum activity at pH 75., while their activity falls to about 20% at pH 5 or pH 11.

The estimated molecular weight for A_4(12,590) is comparable with a value of 14,200 for Mijyo barley [15].

References

1. R. Vogel, I. Trauchschold and E. Werle, *Natural Proteinase Inhibitors* (Academic Press, New York and London, 1968).
2. I.E. Liener and M.L. Kakade, *Toxic Constituents of Plant Foodstuffs*, I.E. Liener, ed. (Academic Press, New York, 1969), pp. 7-68.
3. T.K. Obidairo and O.O. Ogiemudia, Nig. J. Biochem., **3**, 44 (1986).
4. M. Laskowski and I. Kato, Ann. Rev. Biochem., **49**, 593 (1980).
5. R. Borchers and C.W. Anderson, Biochem., **13**, 291 (1947).
6. Jose Filho, J. Fd. Sci., **39**, 422 (1974).
7. T.L. Collins and G.G. Sanders, J. Fd. Sci., **41** (1), 168 (1976).
8. H. Betz, H. Hinze and H. Holzer, J. Biol. Chem., **249**, 4515, (1974).
9. M. Kunitz, J. Gen. Physiol., **30**, 291 (1947).
10. J. Porath and L. Sundberg, *In Protides of the Biological Fluids*, Proceeding of the 18th Colloguim, Brugee, 1970, Peters H. ed (Pergamon Press, Oxford and New York, 1971), pp. 401-407.
11. J. Krahn and F.C. Stevens, Biochemistry, **9**, 2646

(1970).
12. G. Feinstein and A. Gertler, Eur. J. Biochem., **31**, 25 (1972).
13. G. Jones, S. Moore and W.H. Stein, Biochemistry, **2**, 66 (1963).
14. Y. Birk, Acad. Sci., **146**, 388 (1968).
15. T. Ogiso, T. Noda, Y. Sako, Y. Kato and M. Aoyama, J. Biochem., **78**, 9 (1975).
16. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem., **193**, 265 (1951).