Phytochemistry, Vol. 22, No. 4, pp. 813–816, 1983.
Printed in Great Britain.

0031–9422/83/040813–04$03.00/0

TRYPSIN INHIBITOR FROM BAMBARA PEA

MICHÈLE DI MARTINO-FERRER and ALAIN FERRER*†

Département de Biologie Végétale, Laboratoire de Physiologie Végétale, Faculté des Sciences, Université de Dakar, Sénégal; *Laboratoire d'Histologie, Embryologie et Cytogénétique, Faculté de Médecine et de Pharmacie, Université de Dakar, Sénégal

(*Revised received* 22 *June* 1982)

Key Word Index—*Voandzeia subterranea*; Leguminosae; bambara pea; protein; trypsin-like inhibitor; plant tissues.

Abstract—An antitryptic activity has been identified in the flour of dry non-germinated seed of an African leguminous plant, the bambara pea (*Voandzeia subterranea*). This inhibitor has been purified by trichloracetic acid and ammonium sulphate precipitations followed by gel filtration on Sephadex G25, ion exchange chromatography and gel filtration on Sephadex G75. Antitryptic activity increased 50-fold. Its purity has been verified by electrophoresis on polyacrylamide gel and gel chromatography. Its MW is 13 200 in the denatured and reduced forms and 26 300 in the native form. It is resistant to thermal denaturation and appears to be in monomeric form when entirely denatured.

INTRODUCTION

The presence of proteinase inhibitors in animal or plant tissues has been abundantly verified [1–4]. In spite of the considerable number of publications which has been dedicated to them, their physiological function is still unclear. Some authors suggest a function in the control of endogenic proteinases, or a protective role against exogenic proteinases on microbial or insect attack [4, 5].

These inhibitors have a low MW, are strongly resistant to thermal denaturation and retain inhibitory activity over a large pH range [6–11]. In addition, they show a characteristic absorption spectrum in the near UV because of their low tyrosine and phenylalanine content and the almost complete absence of tryptophan in their structure [12, 13]. Generally, these properties are used for the extraction and purification of these inhibitors.

This paper describes the purification and characterization of a bovine antitrypsin in the ungerminated seeds of bambara pea (*Voandzeia subterranea*) [14].

RESULTS AND DISCUSSION

Measurements of the lytic activity of bovine trypsin with time have permitted us to plot a straight line whose slope is equal to 18 mmol/min. ml of hydrolysed BAPNA. The addition of aqueous extract (100 μl or 2 mg protein) to the reaction medium reduced the reaction rate *ca* five-fold (3.3 mmol/min. ml).

Extraction and purification

We used the method described by Ventura and Filho [12] based on the fact that proteinase inhibitors are highly stable in acid medium. [13–15]. After treatment of the aqueous extract with TCA, the precipitate does not show any antitryptic activity. The supernatant (TCA fraction) is treated with ammonium sulphate and the majority of inhibitory substances are precipitated (sulphate fraction). Desalting of the sulphate fraction by dialysis was abandoned due to the loss of 90% of activity, probably due to permeability of the dialysis membrane to the inhibitor. Gel filtration allows the retention of maximum activity. After gel filtration of the sulphate fraction on Sephadex G25, the fraction which contains antitryptic activity was collected (G25 fraction) and lyophilized. This fraction was chromatographed on an ion exchange column (DEAE-cellulose). Part of the G25-fraction is excluded (A). Sodium chloride concentration gradient (0–1 M) permits the observation of a large peak eluted at 0.2 M (B). A smaller peak (C) is eluted at 1 M sodium chloride. Only peak B, corresponding to the largest fraction, shows antitryptic activity. This fraction constitutes the DEAE fraction. This was treated with ammonium sulphate at 380 g/l. for 12 hr at 4°. The precipitate was purified on a dextran gel (Sephadex G75). The eluate showing antitryptic activity was recovered and constitutes the G75-fraction which was lyophilized. Table 1 summarizes the different steps of purification. After Sephadex G75 chromatography, considerable increase in antitryptic activity was obtained (*ca* 50-fold).

Characterization of the inhibitor

The purity was analysed by polyacrylamide gel electrophoresis (PAGE). Fractions corresponding to the three peaks obtained after G25 fraction purification by ion exchange chromatography as well as the G75 fraction were analysed by electrophoresis on dodecylsulphate polyacrylamide gels. Many bands were found after purification on DEAE-cellulose (A–C). On the other hand, the G75 fraction migrates homogeneously and shows only one protein band after staining with amido black.

Thermal denaturation of the inhibitor in an aqueous solution (0.3 mg/ml) was studied kinetically at 90°. This inhibitor is very resistant to thermal denaturation since after an exposure at 90° for 10 min, it retains half its initial activity. Within 20 min, 80% of the initial inhibitory activity disappears. After 40 min, 97% of the trypsin activity present in the experiment is recovered.

†To whom correspondence should be addressed.

Table 1. Antitrypsin activity of *Voandzeia subterranea* ungerminated seed extract

Extract	Amount of protein (g)	Antitrypsin activity	Purification factor
Aqueous extract	6.760	0.034	1
TCA fraction	0.700	0.068	2
Sulphate-fraction	0.288	0.211	6
G25 fraction	0.062	0.476	14
DEAE-cellulose fraction	0.028	0.920	27
G75 fraction	0.014	1.713	51

The MW of the inhibitor was determined by PAGE and by gel chromatography. For SDS-PAGE, standard proteins were denatured at 90° for 5 min in 2.5% dodecylsulphate and 5% β-mercaptoethanol. After denaturation at 90° in the same medium for 45 min, followed by 12 hr at room temperature, the bambara pea trypsin inhibitor migrates as one band at a MW of 13 200. For Sephadex G100 chromatography, a buffer of sodium acetate was studied; the MW of the native inhibitor and inhibitor denatured by heating (90°, 45 min) computed from the elution volume were, respectively, 26 300 and 13 500 (Figs. 1a, b).

Generally, trypsin inhibitors of low MW have few aromatic amino acids and a large number of amino acids containing sulphur atoms [16]. The absence of A maximum at 280 nm of an aqueous solution of the inhibitor, which is typical of proteins [9–17], is due to its weak tyrosine and tryptophan content [7]. The method of Bensze and Schmid described by Justisz [18] allowed us to determine the tyrosine (four residues) and tryptophan (no residue) composition per molecule of inhibitor, on the basis of a MW of 26 300.

Characterization of the trypsin–trypsin inhibitor complex

The inhibitor, reduced and denatured by heating, has a MW (evaluated by PAGE and gel chromatography) of 13 200–13 500. In order to define conditions of the EI complex formation, inhibitor denatured by heat and native inhibitor was analysed by gel chromatography on a dextran gel (Sephadex G100 fine). The ionic strength of the elution solution is 0.5. V_e/V_0 ratios were computed and MWs determined from a calibration curve (Fig. 1c).

The native inhibitor shows a V_e/V_0 ratio of 2.60. After heat treatment (90°, 20 min), A at 280 nm (Fig. 1 b) indicates that two kinds of molecule are present: one (I) active towards trypsin shows a V_e/V_0 of 2.60, the other (II), at a considerably higher concentration has no antitryptic activity ($V_e/V_0 = 3.65$). Comparison of these ratios with the calibration curve (Fig. 1 c) allowed us to compute the MW of native and denatured inhibitor of types I and II. Native inhibitor is excluded at a MW of 26 300. Denatured inhibitor of types I and II shows MWs of 26 300 and 13 500, respectively. Native and type I inhibitor with the same MW have identical specific activities. Type I probably corresponds to part of the inhibitor which has not been denatured, since after 20 min of treatment at 90°, a residual activity of 20% is still present. An analogous experiment was carried out with an elution solution at an ionic strength of zero and gave the same results.

The existence of monomeric and dimeric forms in the presence of denaturing agents [guanidine hydrochloride or urea (6 M)] [19, 20], or in terms of the pH of the incubation medium [21, 22], have been described. Denatured inhibitor shows a MW of 13 500. The MW of native inhibitor is 26 300. This is consistent with a separation of the native molecule into two pieces of the

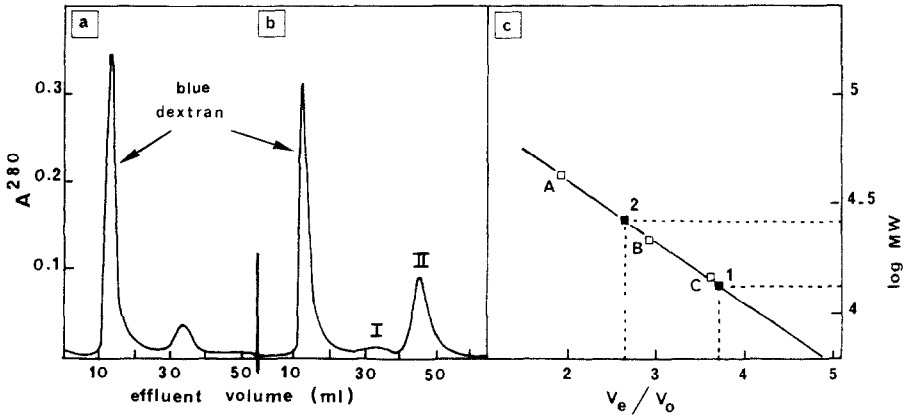

Fig. 1. Molecular study of the thermal denaturation of the inhibitor. Trypsin inhibitor (0.5 mg/test) was analysed by exclusion diffusion chromatography (Sephadex G100, 1 × 20 cm). a, Native inhibitor; b, heat denatured inhibitor; c, calibration curve. The logarithms of the MWs of native or denatured proteins were plotted against the ratio V_e/V_0. A, pepsin (35 000); B, trypsin (23 000); C, lysosyme (14 300); 1, denatured inhibitor; 2, native inhibitor.

same size, or, more probably into a monomer (MW 13 500) and a dimer (MW 26 300).

Inhibition stoichiometry was determined by the addition, at a constant concentration of substrate (1 nmol BAPNA), of increasing quantities of inhibitor and measurement of the ratio, $a = V_i/V_0$ [19], where V_0 is the initial rate of the enzymatic reaction without inhibitor and V_i, the initial rate with inhibitor. On the basis of a MW of 26 300, corresponding to native inhibitor, complete inhibition is obtained (Fig. 2) for a concentration ratio $(I^0)/(E^0)$ of 0.53 (equivalence point). The EI complex is formed from two molecules of enzyme and one of inhibitor. The stoichiometry of association is clearly different from 1 : 1; if the binding sites are independent, the intrinsic dissociation constant may be computed from the following equation:

$$\frac{I^0}{1-a} = \frac{1}{a}\cdot\frac{K_i}{n} + \frac{(E^0)}{n}\ [19].$$

where (E^0) and (I^0) are total concentrations of enzyme and inhibitor and n, the number of binding sites. If $n = 2$, from the slope it is possible to determine the K_i which is equal to 1.28×10^{-8} M.

The protein showing antitryptic activity identified in ungerminated seed of bambara pea corresponds to the typical proteinase inhibitors of plant origin [7–10, 23–25]. Some properties of this inhibitor are reported in Table 2.

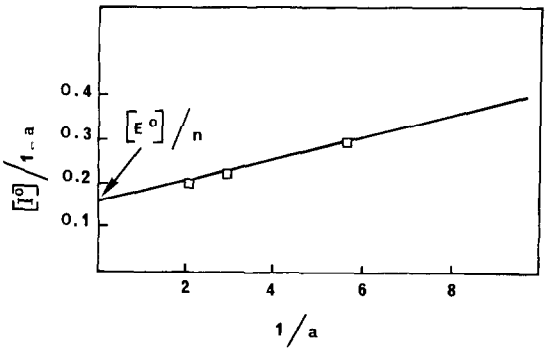

Fig. 2. Determination of the stoichiometry of trypsin–trypsin inhibitor association. Final concentrations in the test were: 1 mmol BAPNA in DMF, 7.25×10^{-3} nmol trypsin, 35 mM Tris–HCl, pH8, 35 mM $CaCl_2$.

EXPERIMENTAL

Trypsin (bovine pancreas type III), α-chymotrypsin, (bovine pancreas type II), pepsin (porcin stomach mucosa), α-*n*-benzoyl-L-tyrosine ethyl ester (BTEE) and hemoglobin bovine were provided by Sigma; filtration gels (Sephadex G25, G75 and G100) by Pharmacia; DEAE-cellulose was from Whatman.

Extraction. The extraction method represents a procedure described in ref. [12] modified in ref. [26].

Step 1: 100 g bambara pea was ground and the flour was stirred in 400 ml H_2O for 12 hr at room temp. The homogenate was passed through many sheets of gauze and the filtrate maintained for 18 hr at 4°. Insoluble residues were removed by centrifugation (aq. extract).

Step 2: A 50% aq. soln of TCA was added to the supernatant until the concn reached 2.5% (w/v). The mixture was stirred for 12 hr at 4° and the ppt removed by centrifugation and discarded. The supernatant was neutralized with 1 M NaOH. The new ppt at pH 6 ± 0.1 was filtered and the final soluble fraction, adjusted to pH 6.5, constitutes the TCA fraction.

Table 2. Properties of trypsin inhibitor of bambara pea

Property	Value
MW	
by electrophoresis	13 200
by chromatography: (a) denatured form	13 000–13 500
(b) nature form	25 300–27 200
Isoelectric point (by isoelectric focussing)	6.8
Absorbance ($A^{1\%}_{1\,cm}$) at 280 nm	2.5
K_i (0.05 M Tris–HCl, pH 8, 0.05 M $CaCl_2$)	1.28×10^{-8} M
Anti-α-chymotryptic activity*	$V_e/V_i = 4$
Antipeptic activity (hemoglobin digestion)†	$V_e/V_i = 4$

*Carried out by the method of Bieth and Aubry [22].
†Carried out by the method of Ryle [23].
V_e = initial rate of enzymatic reaction without inhibitor; V_i = initial rate of enzymatic reaction with inhibitor. Experimental conditions are specified in the text.

Step 3: $(NH_4)_2SO_4$ was added to TCA-fraction to a final concn of 380 g/l. The ppt obtained after 12 hr at 4° was recovered by centrifugation and dissolved in the minimum possible quantity of H_2O (sulphate fraction).

Step 4: The sulphate fraction was desalted on a Sephadex G25 column (1 × 50 cm). The antitryptic activity of the eluate was assayed and fractions of 3 ml having a high activity were lyophilized and constituted the G25 fraction.

Step 5: The G25 fraction was analysed by DEAE-cellulose chromatography. The DEAE-cellulose column (1 × 50 cm) was equilibrated with 0.05 M Tris–HCl buffer, pH 7.6, containing 1 M NaCl, then washed with the same buffer soln without NaCl. Elution of the G25 fraction was carried out with a linear gradient of increasing NaCl concn (0–1 M) in the buffer. Fractions of 20 ml, showing high antitryptic activity, were recovered and named the DEAE fraction.

Step 6: The DEAE fraction was ppted with $(NH_4)_2SO_4$ by stirring for 18 hr at 4° (see step 3). After centrifugation at 8000 *g* for 30 min, the pellets were dissolved in the minimum quantity of 0.05 M Tris–HCl buffer, pH 7.6. The soln obtained was analysed by gel filtration (Sephadex G75). The Sephadex G75 column (1 × 50 cm) was equilibrated with 0.05 M Tris–HCl buffer, pH 7.6. The column was eluted with the same buffer. Fractions showing A at 260 nm and a maximum antitryptic activity were lyophilized (G75 fraction).

The protein concn was either determined by the method of ref. [27], using bovine serum albumin (fraction V) as a standard, or estimated, according to circumstances, by A at 280 nm in aq. medium.

Activity measurements. The BAPNA in DMF was synthetic substrate. Its hydrolysis was followed at 410 nm according to ref. [28]. The concn (μmol of nitroaniline released/ml·min) was computed using the coefficient of molar extinction λ^{BAPNA}_{max} (log E): 410 (8.80) [29]. The enzymatic assay contained 6×10^{-4} nmol trypsin, 1 nmol BAPNA in DMF, 0.05 M Tris-HCl buffer, pH 8, 0.05 M $CaCl_2$. The final vol. was 1 ml and the temp 23°.

The lytic activity of trypsin was determined from the linear portion of the hydrolysis reaction curve of BAPNA. One unit is equal to hydrolysis of 1 μmol BAPNA/min; this value was modified by a corrective factor (0.68 for bovine trypsin) de-

termined by active titration, in order to eliminate the inactive fraction of commercial trypsin. This correction allowed us to determine a K_m of 1 nM at 25°, pH 8, a value close to those found in the lit. [30]. The specific antitryptic activity is described as the No. of mg of inhibited bovine trypsin per mg of protein.

The measurement of anti- α-chymotryptic activity was carried out according to ref. [31]. BTEE in MeOH was used as a synthetic substrate and its hydrolysis was followed by A at 256 nm. The lytic activity of pepsin was determined by the method of ref. [32] using hemoglobin as substrate. The enzymatic assays contained: (a) for α-chymotrypsin: α-chymotrypsin 0.05 nmol, BTEE 0.025 μmol, T.I. 1 μg; (b) for pepsin: pepsin 0.13 nmol, hemoglobin 2% (w/v), T.I. 1 μg. Final vol. 1 ml, temp. 23°.

Electrophoresis. On 10% polyacrylamide gel and carried out by the method of ref. [25]. Gels were stained with 0.3% amido black in 7% HOAc and destained electrophoretically.

The MW of the inhibitor. Determined by exclusion diffusion chromatography (Sephadex G100) as described in the legend of Fig. 1. The perfusion soln was either 0.1 M NaOAc, NaCl (ionic strength 0.5), pH 5.8, or deionized H_2O.

Acknowledgements—The present investigations were carried out in the Laboratoire de Biochimie, Faculté des Sciences de la Santé Cotonou (R.P. Bénin), with financial aid from the World Health Organization and from Ministère français de la Coopération et du Développement. We wish to thank Pr. A. Peyre and Pr. Y. Pares for interest in this work and for their support and Pr. P. Penon for stimulating discussions. We are greatly indebted to Mrs. H. Khoury for help in the preparation of the English translation of this paper and Mrs. B. Gueye for typing the manuscript.

REFERENCES

1. Laskowski, M. Sr. and Laskowski, M. Jr. (1954) *Adv. Protein Chem.* **9**, 203.
2. Vogel, R., Traustschold, I. and Werle, E. (eds.) (1968) *Natural Proteinase Inhibitor*. Academic Press, New York.
3. Kassel, B. (1970) in *Meth. Enzymol.* Vol. 19, 839.
4. Ryan C. A. (1974) in *Bayer-Symposium V, Proteinase Inhibitors*. (Fritz H., Tschesche, H., Greene, L. J. and Truscheite, E., eds.) p. 667. Springer, Berlin.
5. McFarland, D. and Ryan, C. A. (1974) *Plant Physiol.* **54**, 706.
6. Birk, L., Gertler, A. and Khalaf, S. (1963) *Biochem. J.* **8**, 281.
7. Jones, G., Moore, S. and Stein, W. H. (1963) *Biochemistry* **2**, 66.
8. Pusztai, R. and Feeney, R. E. (1966) *Biochem. J.* **101**, 379.
9. Haynes, R. and Feeney, R. E. (1967) *J. Biol. Chem.* **242**, 5378.
10. Wagner, L. P. and Reihm, J. P. (1967) *Arch. Biochem. Biophys.* **121**, 672.
11. Laskowski, M. Jr. and Sealock, R. N. (1971) in *The Enzymes* (Bayer, P. D., ed.) Vol. 3, p. 375. Academic Press, New York.
12. Ventura, M. M. and Filho, J. X. (1966) *An Bras. Cienc.* **38**, 553.
13. Breslow, E., Aaning, H. L., Abrosh, L. and Schimir, M. (1971) *J. Biol. Chem.* **246**, 5179.
14. Hepper, F. N. (1963) *West Afr. Kew Bull.* **16**, 398.
15. Kassel, B. (1970) *Meth. Enzymol.* **19**, 840
16. Wilson, K. A. and Laskowski, M., Sr. (1973) *J. Biol. Chem.* **248**, 756.
17. Gennis, L. S. and Cantor, C. R. (1976) *J. Biol. Chem.* **251**, 741.
18. Justisz, M. (1963) *Techniques de laboratoire* (Masson and Cie, eds.) 3rd edn, Vol. 1, p. 1094.
19. Sealock, R. W. and Laskowski, M. Jr. (1969) *Biochemistry* **8**, 3703.
20. Leach, B. S. and Fisch, W. W. (1977) *J. Biol. Chem.* **252**, 5239.
21. Melville, J. C. and Ryan, C. A. (1972) *J. Biol. Chem.* **247**, 3445.
22. Gennis, L. S. and Cantor, C. R. (1976) *J. Biol. Chem.* **251**, 747.
23. Chu, H. M. and Chi, C. W. (1965) *Sci. Sin.* **14**, 1441.
24. Wilson, K. A. and Laskowski, M. Sr. (1975) *J. Biol. Chem.* **250**, 4261.
25. Swartz, M. J., Mitchell, H. L., Cox, D. J. and Reeck, G. R. (1977) *J. Biol. Chem.* **252**, 8105.
26. Gennis, L. S. and Cantor, C. R. (1976) *J. Biol. Chem.* **251**, 734.
27. Lowry, O. H., Rosebrough, N. J., Farr A. L. and Randall R. J. (1951) *J. Biol. Chem.* **193**, 265.
28. Erlenger, B. F., Kikowski, N. and Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271.
29. Fric, P., Slaby, J., Kasafirek, E. and Malis, F. (1975) *Clin. Chim. Acta* **63**, 309.
30. Bieth, J., Métais, P. and Warter, J. (1966) *Ann. Biol. Clin.* **24**, 785.
31. Bieth, J. and Aubry, M. (1975) *Les colloques de l'Inserm* **40**, 157.
32. Ryle, A. P. (1970) *Meth. Enzymol.* Vol. 19, pp. 316. Academic Press, New York.
33. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.