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PURIFICATION OF A TRYPSIN INHIBITOR OF ALFALFA*

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Abstract—A study was made of a trypsin inhibitor in the vegetative portion of alfalfa. Alfalfa meal was extracted with acetone and ethanol to remove lipids and chlorophyll. The inhibitor then was isolated by extraction with acidic water and precipitation with ammonium sulfate. It was purified further by DEAE-cellulose chromatography. A double reciprocal study indicated the substance to be a non-competitive inhibitor. It was inactivated slowly by heating in aqueous solution. Its electrophoretic behavior and its positive reaction with Folin–Ciocalteau reagent and with phenol–sulfuric acid indicated it to be a polypeptide–carbohydrate complex or a glyco–protein.

INTRODUCTION

TRYPSIN-inhibiting substances are distributed widely in nature, occurring in both animal and plant tissues. Those of plant origin have been of particular interest to nutritionists because of the possibility that they may reduce *in vivo* protein digestion if present in feeds.[1,2] Their occurrence in seeds is well established, but little is known of their occurrence in plant vegetative tissues. Kendall[3] observed trypsin inhibition by aqueous extracts of fresh alfalfa forage. Mitchell *et al.*[2,4] prepared crude concentrates from alfalfa meal and suggested that the inhibitor was a non-coagulable polypeptide or small protein which was slowly heat labile. Mooijman[5] obtained an inhibitor from alfalfa meal which was extremely heat stable and which appeared to be a saponin-peptide complex. Thus, alfalfa forage may contain two distinct trypsin inhibitors.

Because of possible nutritional involvement, our earlier attempts to isolate and characterize a trypsin inhibitor from alfalfa forage were extended in this study.

RESULTS

DEAE-cellulose chromatography at pH 3·0 separated the crude inhibitor preparation into four protein fractions (Fig. 1). The components represented by peaks A, B, C and D were assayed for inhibitory activity and protein content (Table 1). Only fraction A showed appreciable inhibitory activity. Based on its protein content, the fraction represented a 3·6-fold purification of the inhibitor when compared to the crude preparation. However, disc electrophoresis of the fraction indicated it contained four protein components.

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[1] P. C. HAINES and R. L. LYMAN, *J. Nutr.* **74**, 445 (1961).
[2] J. S. RAMIREZ and H. L. MITCHELL, *J. Agr. Food Chem.* **8**, 393 (1960).
[3] K. A. KENDALL, *J. Dairy Sci.* **34**, 499 (1951).
[4] R. E. BEAUCHENE and H. L. MITCHELL, *J. Agr. Food Chem.* **5**, 762 (1957).
[5] J. G. J. M. MOOIJMAN, *Dissertation Abstr.* **25**, 4339 (1965).

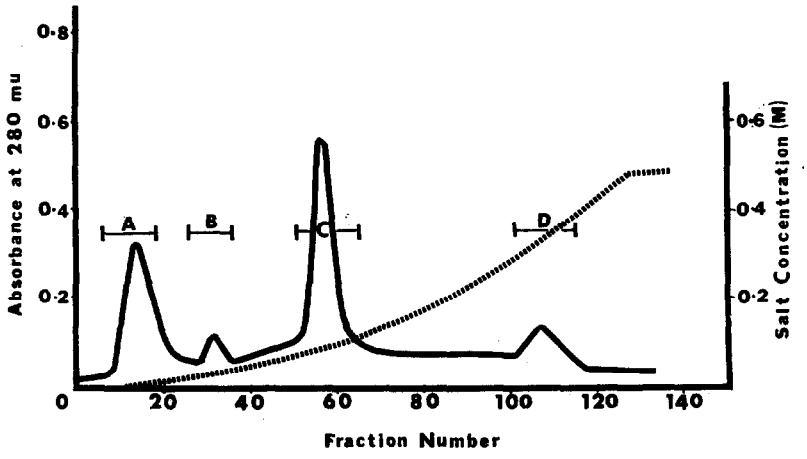

FIG. 1. CHROMATOGRAPHIC PATTERN OF CRUDE PREPARATION ON DEAE-CELLULOSE COLUMN. Elution achieved by 0·01 M citrate buffer, pH 3. The broken line indicates increasing salt concentration.

TABLE 1. PROTEIN CONTENT AND INHIBITORY ACTIVITY OF THE CRUDE INHIBITOR AND ITS FRACTIONS OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY AT pH 3·0 (AMINO NITROGEN DECREASE REFLECTS INHIBITION OF CASEIN HYDROLYSIS)

Fraction	Protein content (mg per ml)	ml per assay	Amino nitrogen decrease	
			mg per ml of fraction	mg per mg of protein
Crude preparation	0·440	5	0·108	0·245
Peak A	0·068	8	0·060	0·882
Peak B	0·008	8	0·003	0·375
Peak C	0·032	8	0·003	0·094
Peak D	0·060	8	0	0

In an effort to achieve further purification, fraction A was placed on a DEAE-cellulose column and was eluted with 0·05 M Tris–HCl buffer, pH 8·2 and NaCl gradient. Two fractions were obtained, both of which possessed appreciable inhibitory activity (Fig. 2 and Table 2). However, inhibitory activity per mg of protein was considerably greater for fraction A′, indicating some additional resolution had been achieved. Disc electrophoresis of this fraction showed the presence of two proteins. This fraction was accumulated from several columns and was dialyzed, lyophilized and used to study the nature of the inhibitor.

Inhibitory behavior was studied by adding constant amounts of the inhibitor to varying amounts of casein. A plot of the data by the double reciprocal method of Lineweaver *et al.*[6] showed it to be a non-competitive inhibitor.

The inhibitor was slowly heat labile, losing about half of its activity after 0·5 hr of heating at 98° (Table 3). A qualitative test by the method of Dubois *et al.*[7] showed the presence of carbohydrate. Thus, the inhibitor appears to be a glycoprotein, as are many of the trypsin inhibitors isolated from other sources.

[6] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).
[7] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH, *Anal. Chem.* **28**, 350 (1956).

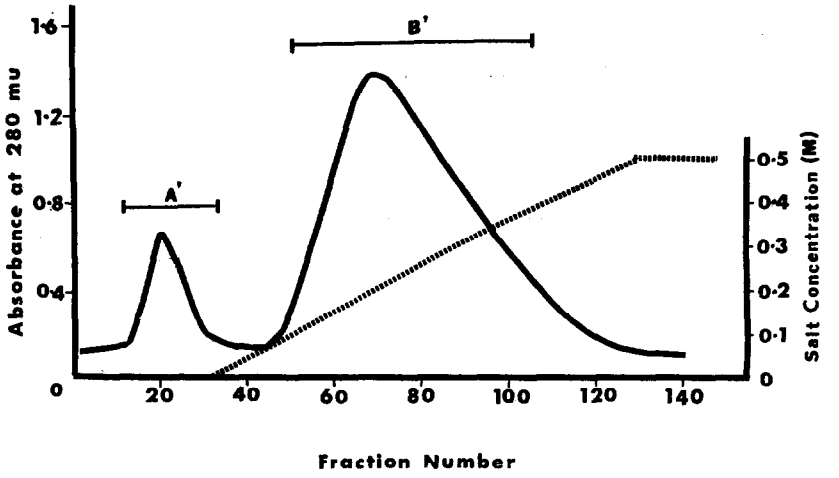

FIG. 2. CHROMATOGRAPHIC PATTERN OF FRACTION A ON DEAE-CELLULOSE COLUMN.
Elution was achieved with 0·05 M Tris–HCl buffer, pH 8·2. The broken line indicates increasing salt concentration.

TABLE 2. PROTEIN CONTENT AND INHIBITORY ACTIVITY OF PEAK A AND ITS FRACTIONS OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY AT pH 8·2

Fraction	Protein content (mg per ml)	ml per assay	Amino nitrogen decrease	
			mg per ml of fraction	mg per mg of protein
Peak A	0·112	4	0·108	0·982
Peak A′	0·018	8	0·058	3·220
Peak B′	0·054	8	0·063	1·165

TABLE 3. EFFECT OF HEATING ON ACTIVITY OF INHIBITOR

Heating time (hr)	Inhibitor used in assay (ml)	Trypsin solution	Absorbance at 620 nm	Inhibition (%)
0	0	Heated	0	
ditto	ditto	Unheated	0·248	
ditto	4	Heated	0	
ditto	ditto	Unheated	0·152	39
0·5	ditto	Unheated	0·202	19
1	ditto	Unheated	0·221	11
3	ditto	Unheated	0·242	2
5	ditto	Unheated	0.237	4
7	ditto	Unheated	0·243	2

EXPERIMENTAL

Analytical Methods

Trypsin inhibition was measured by the method of Ramirez *et al.*,[2] which consists of determining the amount of casein hydrolyzed by trypsin in the presence and in the absence of the inhibitor. Trypsin activity was measured by adding a copper phosphate suspension to the deproteinized enzymic hydrolysates and

measuring the absorbance of the resulting blue solution at 620 nm.[8] The protein content of each inhibitor preparation was determined by Miller's modification[9] of the method of Folin and Lowry.[10,11] Bovine serum albumin was used for preparation of a standard curve.

Extraction

2 kg of commercial dehydrated alfalfa (*Medicago sativa*) meal were defatted by one extraction with 8 l. of acetone and three extractions with 8 l. of 80% aqueous ethanol. 10 l. of 0·25 N H_2SO_4 were added and the mixture was allowed to stand overnight. The extract was collected by filtration through cheese-cloth and was clarified by centrifugation at 2000 × *g* for 20 min. The inhibitor was precipitated from the clear extract by adding $(NH_4)_2SO_4$ to 70% saturation. After standing overnight, the precipitate was recovered by centrifugation at 4000 × *g* for 10 min. The precipitate was dissolved in a minimum amount of distilled water and the solution dialyzed against running tap-water. Finally, the extract, which will be referred to as "Crude preparation", was frozen and kept in that state while awaiting further fractionation by column chromatography.

Chromatography

A 1 × 9 in. column of DEAE-cellulose was prepared and used as described by Peterson and Sober.[12] The crude inhibitor preparation was equilibrated against the starting buffer and centrifuged. A portion of the solution was placed on the column and the column was eluted with NaCl and 0·01 M citrate buffer, pH 3·0, using an open volume NaCl gradient.[13,14] The eluate was collected in 5-ml fractions and the absorbance of each fraction was measured with a Beckman DU spectrophotometer at 280 nm with a 0·6 mm slit. The fractions then were combined in such a manner that the observed peaks were contained in separate solutions. These were dialyzed and then concentrated by hanging the dialysis tubes in front of an electric fan. The protein content and inhibitory activity of each fraction and of the crude concentrate were determined. The fold of purification was calculated as follows:

$$\text{Fold} = \frac{\text{Amino N decrease/mg of protein of a fraction}}{\text{Amino N decrease/mg of protein of crude preparation}}$$

where "amino N decrease" is the difference between the amount of amino N released from casein by trypsin and the amount released by trypsin in the presence of the inhibitor.

Thermostability

A solution containing 1·5 mg/ml of lyophilized inhibitor was heated at 98° in a water bath and under a reflux condenser. Samples were withdrawn after 0, 0·5, 1, 3, 5 and 7 hr of heating. The solutions were cooled to room temperature and their inhibitory activities were determined.

Electrophoresis

Disc electrophoresis was performed with a Canalco Model 6 electrophoretic apparatus. The inhibitor was dissolved in sample gel at the rate of 1 mg per ml and 0·4 ml of the solution was placed on the top of the spacer gel. Electrophoresis was performed at pH 8·3 with a current of 5 mA per column. The gel column was removed from the apparatus and was fixed by immersing it in 20% sulfosalicylic acid solution. It was stained by soaking in 0·25% Coomassie Brilliant Blue dye solution for 1 hr. Excess dye was then removed by soaking the gel column in tap water.

[8] R. E. BEAUCHENE, A. D. BERNEKING, W. G. SCHRENK, H. L. MITCHELL and R. E. SILKER, *J. Biol. Chem.* **214**, 731 (1955).
[9] G. L. MILLER, *Anal. Chem.* **31**, 964 (1959).
[10] O. FOLIN and V. CIOCALTEAU, *J. Biol. Chem.* **73**, 627 (1927).
[11] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[12] E. A. PETERSON and H. A. SOBER, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 5, p. 3, Academic Press, New York (1962).
[13] L. R. SNYDER, *Chromatog. Rev.* **7**, 1 (1965).
[14] D. F. H. WALLACH and G. L. NORDBY, *Biochim. Biophys. Acta* **70**, 188 (1963).