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Alfalfa Trypsin Inhibitor

Trypsin inhibitor was isolated from defatted alfalfa by extracting with 0.2 M NaCl, adsorbing on a trypsin–Sepharose affinity column, and eluting with a glycine buffer. Subsequent CM-cellulose chromatography did not improve its purity appreciably. Its amino acid composition was compared with that of Bowman-Birk soybean trypsin inhibitor and with an average composition of other proteins. Inhibitor content increased as plants matured. The inhibitor is present in greatest concentration in leaves.

Trypsin inhibitors are distributed widely in plants. Although their nutritional effects on animals are poorly understood generally, a trypsin inhibitor of soybean seeds depresses rat growth (Rackis, 1965; Kakade et al., 1973), while isolated corn grain inhibitor does not (Mitchell et al., 1976). Alfalfa, a major forage crop, also contains a trypsin inhibitor (Chien and Mitchell, 1970), but its nutritional effects on animals are not known. To determine its nutritional effects, it will be necessary to isolate the inhibitor in pure form and in appreciable quantity. The feasibility of such an isolation by affinity chromatography is reported here.

EXPERIMENTAL SECTION

Analytical Methods. Protein concentration was measured by the method of Lowry et al. (1951). Trypsin inhibitory activity was determined by the benzoyl DL-arginine p-nitroanilide assay (method II) of Erlanger et al. (1961) to measure trypsin activity. Preliminary work with crude extracts revealed a linear relation between inhibitor concentration and percent inhibition up to about 75%. It was convenient, therefore, to define an inhibitor unit as the amount of inhibitor which inhibits 50% of trypsin activity as measured by the Erlanger Assay procedure.

Isolation of Inhibitor. Chopped, fresh alfalfa was dehydrated by two extractions with acetone. The tissue was ground and again extracted with acetone to remove lipids and chlorophyll. The meal (200 g) was stirred in 1 L of 0.2 M NaCl for 4 h. The liquid was collected by vacuum filtration, and the residue was extracted again for 4 h with 600 mL of the salt solution. The extracts were combined and centrifuged at 10000*g* in a refrigerated centrifuge. The supernatant was adjusted to 0.05 M Tris–HCl, pH 8.2, 0.02 M $CaCl_2$, and 1 M NaCl and again centrifuged to remove an inactive precipitate that formed. The inhibitor was adsorbed from the extract onto a trypsin–Sepharose affinity column (Swartz et al., 1977) on which 1 g of bovine trypsin had been attached. The column was washed thoroughly with the starting buffer to remove inactive material, and 0.1 M glycine–HCl buffer, pH 1.5, was then applied. Inhibitor activity eluted in a single sharp peak of about 100 mL. Progress of the washing and elution was monitored by measuring absorbance at 220 nm of the column effluents. Exposure of the inhibitor to pH 1.5 was limited to about 12 h at 4 °C.

Table I. Purification of Alfalfa Trypsin Inhibitor

stage	inhibitor units	protein, mg	sp act.	recovery, %
crude extract	11600	17000	0.67	100
affinity column	9600	85	113	83
Sephadex G-10 column	8400	68	122	72
CM-cellulose column	6800	55	122	59

The inhibitor activity was found to be dialyzable. Therefore, to exchange buffers in preparation for ion-exchange chromatography, active material from the affinity column was applied to a 3.5 × 45 cm Sephadex G-10 column that had been equilibrated with 0.01 M Tris–HCl, pH 7.5. The column was developed with the same buffer. The inhibitor activity eluted as a single early peak, presumably in the exclusion volume. It was followed by two smaller peaks of absorbance at 220 nm. The inhibitory fraction was applied to a 2.5 × 25 cm CM-cellulose column, which was developed with a linear gradient from 0 to 0.5 M NaCl in 0.01 M Tris–HCl buffer, pH 7.5. Only one peak of absorbance at 220 nm was obtained, and it was inhibitory (Figure 1).

The progress of purification through the various stages is shown in Table I. Most of the inhibitor was removed from the crude extract by the affinity column and was recovered from that column (83%). The gel chromatography step resulted in only a slight increase in specific activity, which indicates that the last two peaks in the elution profiles probably were not protein. No further increase in specific activity was obtained by CM-cellulose chromatography.

The active fractions from the affinity and CM-cellulose columns were subjected to 15% polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol (Maizel, 1969). The protein bands were made visible by staining with amido black. Although the bands were rather diffuse, each gel contained only one band, which is consistent with little improvement in purity

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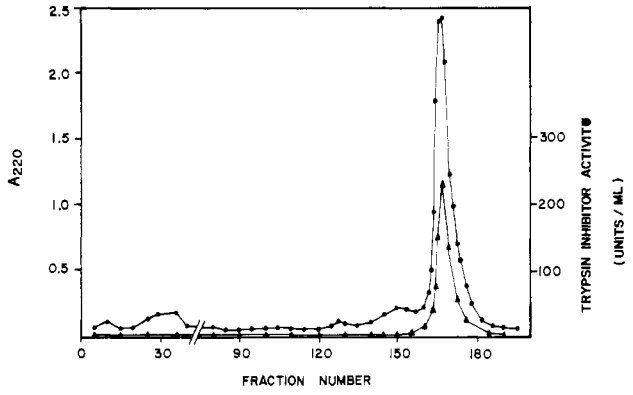

Figure 1. Chromatography of alfalfa trypsin inhibitor on CM-cellulose: (-●-) absorbance; (-▲-), inhibitor activity. Fraction volume, 2.5 mL. Salt gradient was applied at fraction 110. No significant absorbance was observed after fraction 200.

Table II. Comparison of Amino Acid Compositions of Alfalfa Trypsin Inhibitor, Bowman-Birk Soybean Trypsin Inhibitor, and Average of 207 Proteins

amino acid	alfalfa inhibitor, mol %	bowman-birk, mol %	protein av, mol %
lysine	4.2	7.0	6.5
histidine	2.0	1.4	2.2
arginine	1.9	2.8	4.4
aspartic acid	9.5	15.5	10.7
threonine	11.9	2.8	5.7
serine	5.0	12.7	6.3
glutamic acid	5.8	9.9	10.6
proline	12.1	8.5	4.8
glycine	2.4	0	8.1
alanine	4.8	5.6	8.5
half-cystine	25.1	19.9	2.3
valine	0.9	1.4	6.8
methionine	0	1.4	1.9
isoleucine	7.2	2.8	5.0
leucine	1.7	2.8	8.1
tyrosine	1.7	2.8	3.3
phenylalanine	3.9	2.8	3.7
tryptophan	N.D.	0	1.3

resulting from further chromatography of the active fraction from the affinity column.

Amino Acid Composition. Inhibitor obtained by CM-cellulose chromatography was hydrolyzed in 6 N HCl at 100 °C for 20 h in evacuated tubes. Triplicate amino acid analyses were conducted with a Beckman Model 120 C amino acid analyzer. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after hydrogen peroxide and performic acid oxidation (Hirs, 1967). Table II compares the amino acid composition of our alfalfa inhibitor with that of another small trypsin inhibitor, soybean Bowman-Birk inhibitor, whose amino acid composition was calculated from its amino acid sequence (Odani and Ikenaka, 1972). A comparison also is made with the average composition of 207 other proteins (Reeck and Fisher, 1973). The two inhibitors have some similarities that are rather distinctive in comparison to the protein averages—particularly, high contents of half-cystine and proline and low contents of glycine and valine. The ultraviolet absorption spectrum of the alfalfa inhibitor is consistent with the absence of tryptophan, which does not occur in the Bowman-Birk inhibitor.

Table III. Trypsin Inhibitor Activity of Alfalfa at Five Stages of Growth

plant height, in.	inhibitor units/g of dry wt		
	leaves	stems	whole plant
5	27.5	13	17
10	22.5	8	13
15	25	7	16.5
20	31	17	22
25	47	13	34.5

Changes during Plant Growth. Plants were collected periodically from a field during first cutting growth, beginning with early growth and continuing until early bloom. Some of the plants were separated into leaves and stems. Ten-gram samples were disintegrated in a Sorvall Omni-mixer operated at full speed for 1 min with 100 mL of 0.05 M Tris–HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$. Extracts were centrifuged and assayed for inhibitory activity (Table III). Inhibitory activity in all samples decreased through the second sampling date. Inhibitory activity increased rapidly thereafter with increasing age. Ryan and Shumway (1971) reported similar increases in inhibitor I of potato leaves. Stems of alfalfa had much less inhibitor than leaves.

We conclude that alfalfa trypsin inhibitor with acceptable purity for nutritional studies can be obtained directly from an affinity column. Additional purification does not improve purity appreciably, but reduces yields. Using only leafy portions of older plants will hold the amount of tissue to be processed to a manageable level.

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