ISOLATION OF A TRYPSIN INHIBITOR FROM ALFALFA MEAL

by

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INTRODUCTION

It has been reported that aqueous extracts of fresh alfalfa, ladino clover, and soybeans inhibit the *in vitro* digestion of casein by trypsin. A similar inhibition of trypsin by extracts of commercial dehydrated alfalfa also has been reported, indicating that the inhibitor withstands the heat of the dehydration process.

Many authors have shown that alfalfa meal has a growth depressing effect on chicks when it is present in broiler rations at levels of 10 per cent or more. The presence of a trypsin inhibitor might be responsible for a part of this effect. To determine the role of this inhibitor in chick growth depression, it will be necessary to isolate it in sufficient quantity that *in vivo* studies can be made. This report is concerned with a method of isolation of the trypsin inhibitor of alfalfa and with attempts to determine its chemical nature.

REVIEW OF LITERATURE

Numerous observations have been made since 1900 that naturally occurring substances are capable of inhibiting the action of trypsin. The modern approach to the study of trypsin inhibition began in 1931 with the crystallization of trypsin by Northrop and Kunitz (22, 23). The availability of the pure enzyme has made possible a better understanding of the specificity and mechanism of action of these inhibiting substances. A few of these inhibitors have been isolated and have been shown

to be proteins or polypeptides. They exert their effect by forming stable complexes with trypsin (12).

Trypsin inhibitors are distributed widely in nature. Willstatter and Rhodewald (27) detected an inhibitor in pancreas. Kunitz and Northrop (12) isolated a trypsin inhibitor from bovine pancreas in crystalline form. They complexed it with trypsin and isolated the complex in crystalline form also. Kazal et al. (10) isolated a similar substance in crystalline form from bovine pancreas which differed from the inhibitor of Kunitz and Northrop by being electrophoretically heterogeneous. Other inhibitors of animal origin have been found in human and bovine colostrum (17), blood plasma (16), urine (24, 8), and egg white (6). The latter was isolated by Balls and Swenson (1) and was characterized as an ovomucoid by Meyer et al. (21), and by Lineweaver and Murray (20).

Trypsin inhibitors of plant origin have been studied intensively in recent years. An inhibitor was discovered in 1944 in soybean seeds by Ham and Sandstedt (9) and by Bowman (4). Kunitz succeeded in isolating it in crystalline form (13, 14). He also isolated a stable crystalline soybean inhibitor--trypsin complex (15).

Borchers et al. (3) detected a trypsin inhibitor in lima bean seeds. Tauber et al. isolated a protein from this source which was a potent inhibitor, and which was heat resistant (26). Fraenkel-Conrat et al. (7) obtained an amorphous product from lima beans which had two and one-half times the inhibitory effect of Tauber's preparation.

Trypsin inhibitors of plant origin are not restricted to seeds. Kendall (11) reported their existence in fresh leaves of soybeans, ladino clover, and alfalfa. Beauchene and Mitchell (2) reported trypsin inhibition by aqueous extracts of dehydrated alfalfa meal. However, the chemical nature and mode of action of the inhibitors of plant leaves are unknown, since they have not been isolated.

A comprehensive review of the literature concerned with naturally occurring trypsin inhibitors was made by Laskowsky and Laskowsky in 1954 (18).

EXPERIMENTAL

The inhibitory activity of extracts of alfalfa, and of the fractions obtained from them, was determined by measuring the decrease in the amount of amino acids released during the *in vitro* digestion of casein by trypsin when the extracts or fractions were present. The decrease is proportional to the inhibitory activity of the added material. The method of measuring amino acid concentration was that of Spies and Chambers (25), which depends upon the reaction of amino acids with cupric ions under proper conditions to produce complexes having a blue color.

Measurement of Inhibition

A modification of the procedure of Beauchene and Mitchell (2) was employed. A 3 per cent solution of casein in pH 8.4 phosphate buffer was prepared and was adjusted to pH 8.4 with dilute NaOH. Five ml. of the casein solution were placed in each

of four 25-ml. beakers. Two ml. of water were added to beakers 1 and 2, and 2 ml. of the inhibitor solution were added to beakers 3 and 4. The pH again was adjusted with dilute NaOH to 8.4, if necessary, by means of a Beckman pH meter equipped with microelectrodes. To beakers 1 and 3 were added one ml. of a solution containing 30 mg. of trypsin per 100 ml. of water. A portion of the trypsin solution was heated to boiling, and 1 ml. of this solution was added to beakers 2 and 4. The following mixtures thus were obtained:

Beaker 1. casein, trypsin.

Beaker 2. casein, inactivated trypsin.

Beaker 3. casein, trypsin, inhibitor.

Beaker 4. casein, inactivated trypsin, inhibitor.

Beaker 2 thus served as a control for beaker 1, and beaker 4 as a control for beaker 3.

The beakers were covered with small watch glasses and were placed in a water bath maintained at 37° C. After four hours, 5 ml. of the solutions were placed in test tubes and the undigested casein was precipitated by adding 5 ml. of a 10 per cent aqueous solution of trichloroacetic acid. The tubes were allowed to stand for five minutes, after which the liquid portion was obtained by filtration through Whatman No. 50 filter paper.

The pH of each filtrate was adjusted to 5.5 with 40 per cent NaOH, and 5 ml. of the filtrate were placed in 15 ml. graduated conical centrifuge tubes. Three ml. of a suspension of copper phosphate in pH 9.1 borate buffer were added. The contents of the tubes were mixed, allowed to stand five minutes, and were

centrifuged at 2500 r.p.m. for five minutes to sediment the excess copper phosphate. The intensity of the blue color of the supernatant solutions was measured at 620 mu with a Beckman DU spectrophotometer. A standard curve was prepared by carrying known amounts of alanine through the procedure and plotting absorbances against concentration expressed as milligrams of amino nitrogen per tube.

Isolation of Trypsin Inhibitor

Concentrates of the trypsin inhibitor were prepared from both commercially dehydrated alfalfa and alfalfa dried at low temperature in the laboratory. The methods of isolation from the two sources differed slightly.

The alfalfa meals used in the study were prepared from first cutting alfalfa in the early bloom stage of growth. Low temperature meal was prepared by drying the alfalfa at 50° C. overnight in a circulating air oven. High temperature meal was prepared by drying the alfalfa in a commercial dehydrator which operated with an air inlet temperature of approximately 900° C. and an air outlet temperature of about 175° C. The dried samples were ground to pass through a 20-mesh screen.

Preliminary extraction studies were performed by extracting 25-gram portions of high temperature meal in a Waring Blendor with 200 ml. of various solvents. After blending for 10 minutes, the samples were filtered through a cloth held in a Buchner funnel. When organic solvents were used, the solvent was removed by evaporation on a steam plate under a current of air from an

electric fan, and the residue was extracted with water. The final volume in each case was adjusted to 200 ml. and the solutions were centrifuged.

Dialyzability of the inhibitor was determined by placing a water extract in a cellophane dialysis bag and dialyzing against distilled water for several days. The water was changed frequently, and the dialysates so obtained were combined and concentrated to the same volume as the original extract.

The inhibitory activity of the various extracts was determined by adding 2 ml. to a solution containing trypsin and casein and incubating at 37° C., as described earlier. The results (Table 1) show that the inhibitor was soluble in water but was not extracted by acetone or 95 per cent ethanol. However, aqueous ethanol extracted the substance, there being a progressive increase in inhibition as the ethanol concentration was decreased. The dialyzed extract was as inhibitory as the undialyzed extract, while the dialysate was non-inhibitory. Hence, the substance responsible for trypsin inhibition is water soluble and non-dialyzable.

Having established that the inhibitor is soluble in H_2O, non-dialyzable, and precipitable from aqueous solutions by addition of ethanol, the procedures described below were employed for its isolation.

<u>Isolation from Low Temperature Meal</u>. Twenty-five grams of meal and 200 ml. of water were placed in the cup of a Waring blendor and the blendor was operated at half speed for 10 minutes. The resulting slurry was filtered through a cloth supported in a

Table 1. Effect of extracting solvent, alcohol precipitation, and dialysis on inhibitory activity as measured by inhibition of *in vitro* hydrolysis of casein by trypsin.

Preparation added	Amino nitrogen: per tube mg.	Inhibition per cent
None	1.09	0
Acetone extract	1.07	2
95 per cent ethanol extract	1.08	2
60 per cent ethanol extract	0.71	35
40 per cent ethanol extract	0.68	38
Water extract	0.62	43
Dialyzed water extract	0.63	42
Dialysate	1.06	2
Supernatant after ethanol precipitation	1.08	2

Buchner funnel, and the residue was washed several times with water. The filtrate was heated almost to boiling to coagulate protein and was centrifuged. The clear amber supernatant liquid was concentrated to a small volume under reduced pressure at 40° C. The concentrate was placed in a cellophane dialysis sack and was dialyzed against continuously replenished tap water for 12 hours. A portion of the amber color was eliminated in this manner. A slight turbidity which developed during dialysis was eliminated by centrifuging at 14,000 r.p.m. for 30 minutes in a supercentrifuge.

The clear amber solution was concentrated to about 30 ml., and 250 ml. of absolute ethanol were added. After standing in a refrigerator for a few hours, the precipitate which formed was collected by centrifugation. On drying, the precipitate was amber in color and had a glassy appearance. It was readily soluble in water, and could be purified further by repeating the centrifugation, dialysis, and ethanol precipitation. The amber color was never completely eliminated, however.

Isolation from High Temperature Meal. Water extracts of high temperature dehydrated alfalfa do not contain soluble heat coagulable protein (5). Hence, the heat coagulation step of the above procedure was unnecessary. Centrifugation at 3000 r.p.m. of the initial filtrate eliminated coarse suspended material. For more complete removal of turbidity, the supernatant was filtered under vacuum through Whatman No. 50 paper covered with a layer of Supercel. Concentration, dialysis, and ethanol precipitation yielded a product which was similar in appearance to that obtained from low temperature meal.

The material which was obtained by the procedures described above strongly inhibited the *in vitro* digestion of casein by trypsin. The activity of a typical preparation is shown in Table 2.

Properties of the Inhibitor

Effect of Heat. Although extracts of high temperature dehydrated alfalfa meal are inhibitory, it should not be assumed that the inhibitor is heat stable. It is conceivable that the

Table 2. Activity of the trypsin inhibitor of alfalfa.

Substances added to casein solution	Absorbance at 620 mu	Amino nitrogen per tube mg.	Net	Inhibition per cent
Trypsin	.215	1.10	1.09	0
Boiled trypsin	.003	.01	--	--
Trypsin, 2 ml. of extract*	.190	.96	.62	43
Boiled trypsin, 2 ml. of extract*	.070	.34	--	--
Trypsin, 4 mg. of isolated inhibitor	.114	.58	.56	49
Boiled trypsin, 4 mg. of isolated inhibitor	.005	.02	--	--

* 25 g. meal per 200 ml. water.

duration of heating during dehydration is too short to have an appreciable effect.

Heat stability of the isolated inhibitor was studied by dissolving it in water and heating the solution at 98° C. on a steam plate. Equal aliquots were removed after 0, 0.5, 1, 3, and 7 hours of heating, and were tested for inhibitory power. The results, presented in Table 3, show that inhibitory activity had decreased at 0.5 hour, and was almost completely absent after three hours. It was concluded, that, although the substance is heat labile, the time required for drying alfalfa commercially is too short for inactivation to occur.

<u>Electrophoretic Behavior</u>. One hundred mg. of the inhibitor were dissolved in pH 8.6 borate buffer, and the solution was

Table 3. Effect of heating at 98° C. on the activity of the inhibitor.

Time of heating hours	Inhibition per cent
0	35
0.5	13
1	11
3	2
7	0

subjected to a current of 20 milliamperes for 30 minutes in an Aminco portable electrophoresis apparatus. Two moving peaks were detected (Fig. 1). This exploratory experiment indicates a lack of homogeneity of the inhibitor as isolated by the procedure employed.

Nature of the Inhibition. The nature of the inhibition was studied by measuring the effect of a given concentration of inhibitor with varying concentrations of substrate. A 4 per cent casein solution was prepared by dissolving 12 g. of casein in 300 ml. of pH 8.4 phosphate buffer and adjusting the pH to 8.4 with a few drops of 40 per cent NaOH solution. Aliquots of this solution were diluted with buffer to yield solutions of 0.5, 1, 2, 3, and 4 per cent casein. A trypsin solution was prepared by dissolving 30 mg. of the enzyme in 100 ml. of water. The inhibitor was dissolved in pH 8.4 phosphate buffer so that 5 ml. of the solution contained 25 mg. of the inhibitor. Three reaction mixtures were prepared in 100 ml. flasks for each substrate

Fig. 1. Electrophoretic pattern of the isolated trypsin inhibitor.

concentration, as follows:

Flask A. 20 ml. casein, 5 ml. buffer, 2 ml. trypsin.

Flask B. 20 ml. casein, 5 ml. inhibitor, 2 ml. trypsin.

Flask C. 20 ml. casein, 5 ml. inhibitor, 2 ml. boiled trypsin.

The enzyme solution was added at zero time, each flask was shaken vigorously, and was placed in a water bath maintained at 37° C. A 5 ml. aliquot was withdrawn and was added to 5 ml. of 10 per cent trichloroacetic acid to stop the reaction. Additional 5 ml. aliquots were treated similarly after two hours. Each sample so obtained was allowed to stand 15 minutes and was filtered through dry filter paper. The pH was adjusted to 7.0 with 40 per cent sodium hydroxide by means of the Beckman pH meter equipped with microelectrodes. Five ml. of the resulting solution were used for amino acid determination. Flask C was used as a blank to correct for small amounts of free amino acids.

The analytical data are presented in Table 4. From these values 1/S and 1/v were calculated, and a plot was prepared (Fig. 2) according to the double reciprocal method of Lineweaver and Burk (19). Failure of the two lines to intersect indicates that the inhibition was non-competitive.

Chemical Nature of the Inhibitor. Since the active principle was precipitated from water by dilution with a relatively large volume of ethanol, it was thought that it might be inorganic in nature. This was shown not to be true when a portion of the isolated substance was ashed and the residue was found to be non-inhibitory.

Table 4. Effect of inhibitor and substrate concentration on hydrolysis of casein by trypsin.

	(A) Inhibitor absent						(B) Inhibitor present					
(S) Casein concentration, per cent	5	4	3	2	1	0.5	5	4	3	2	1	0.5
(V) Absorbance at 620 mu after two hours at 37° C.	.238	.237	.201	.196	.178	.123	.133	.115	.102	.112	.095	.068
1/S	.20	.25	.33	.5	1	2	.20	.25	.33	.5	1	2
1/V	4.2	4.2	5.0	5.1	5.6	8.1	7.5	8.7	9.8	9.0	10.5	14.7

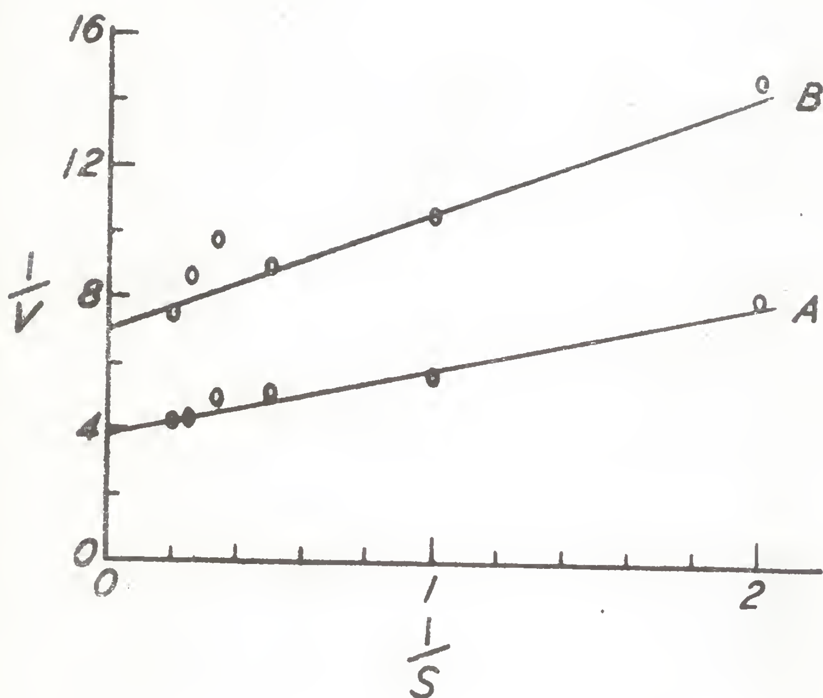

Fig. 2. Double reciprocal plot of the data of Table 4 to show the type of inhibition.

The possibility that it is a heat coagulable protein was eliminated by the fact that high temperature meal does not contain such material, while the soluble protein of low temperature meal was removed by heat coagulation prior to ethanol precipitation. However, it could be a polypeptide with a sufficiently low molecular weight that it would not be coagulated by heat. This possibility was investigated by refluxing 5 mg. of the inhibitor with 6N HCl overnight. The hydrolysate was concentrated almost to dryness under reduced pressure at 40° C., and water was added. Concentration under reduced pressure and dissolution in water was repeated six times to insure removal of HCl. The final residue was dissolved in one ml. of water and 25 ul. were spotted on a strip of Whatman No. 1 filter paper. The chromatogram was developed for 16 hours with 80 per cent phenol and 20 per cent water.

When the paper was sprayed with ninhydrin, several sharply defined spots were observed. A similar chromatogram of the unhydrolyzed inhibitor gave no spots with ninhydrin. Hence the spots obtained with the hydrolysate were due to amino acids which were released during hydrolysis rather than to free amino acids in the inhibitor.

The above experiment does not preclude the possibility that the peptide detected by hydrolysis is merely an impurity, rather than the active principle. A purification procedure suggested by the work of Kunitz and Northrop (12) was used to investigate this possibility.

Fifty mg. of the isolated inhibitor were dissolved in 10 ml. of water, and 30 mg. of trypsin were dissolved in 10 ml. of water. The two solutions were combined, mixed, and allowed to stand for an hour with occasional shaking. Sixty ml. of saturated ammonium sulfate solution were added and the mixture was allowed to stand for one hour in a refrigerator. The material which precipitated was removed by centrifuging and was dissolved in 10 ml. of water. This solution was dialyzed overnight. Ten ml. of a 5 per cent solution of trichloroacetic acid were added and the mixture was allowed to stand for 30 minutes in the refrigerator. The precipitated trypsin was removed by centrifuging, and the supernatant solution was dialyzed overnight. The solution was concentrated to 10 ml. by suspending the dialysis bag in front of an electric fan. One ml. of this solution caused 50 per cent inhibition when tested as described earlier. One ml. of the original inhibitor solution also caused approximately 50 per cent inhibition. Although such a comparison probably is not quantitative, it does indicate that the inhibitor formed a complex with trypsin which was precipitated with ammonium sulfate, and that the complex was decomposed by trichloroacetic acid without precipitation of the inhibitor. The inhibitor thus behaves in a manner quite similar to that of the crystalline polypeptide inhibitor of Kunitz and Northrop (12).

DISCUSSION

It is necessary to include adequate blanks when assaying various preparations for inhibitory activity by the method employed in this work. Water extracts of alfalfa contain free amino acids, and a correction for them must be made when enzymic hydrolysis of casein in the presence of an extract is used to measure the degree of inhibition. Furthermore, crude plant extracts are amber in color. Often the intensity of this color is sufficient to cause appreciable light absorption at the wavelength (620 mu) used to measure the blue color of the amino acid-copper complexes.

Inspection of Table 2 will emphasize the variability of blanks. Both casein and trypsin are essentially devoid of free amino acids, and a very low absorbance was obtained in the boiled trypsin experiment. When the plant extract was added, a marked increase in absorbance of the blank resulted. The addition of 4 mg. of the isolated inhibitor had essentially no effect on absorbance. Hence, dialysis and ethanol precipitation eliminated the free amino acids and much of the substances responsible for the amber color.

The data reported above do not prove conclusively that the inhibitor is a polypeptide or a protein. However, its solubility characteristics, its loss on prolonged heating, its behavior when subjected to the complexing and recovery procedure of Kunitz, and the release of amino acids during acid hydrolysis are evidence that it is a polypeptide or a protein of low molecular

weight. Elucidation of the chemical nature of the substance will not be achieved until it is obtained with a greater degree of purity. Perhaps use of the Kunitz technique on a larger quantity of the crude isolate, and repeating the complexation two or three times will yield sufficient pure material to permit characterization.

Further study of the trypsin inhibitor of alfalfa also should include chick feeding studies to determine if the material is inhibitory under *in vivo* conditions. If the inhibitor were hydrolyzed or otherwise altered while it was in the crop and gizzard of the chick, it might not be inhibitory to trypsin after reaching the intestinal tract. Hence, its role in the growth depression resulting from high levels of alfalfa meal in chick rations will have to be determined by adding varying amounts of the purified material to the rations of chicks and observing the effect on rate of growth.

SUMMARY

A study was made of the trypsin inhibitor of alfalfa. The substance was isolated from alfalfa meal by extracting with water, dialyzing, and precipitating with ethanol. The isolate strongly inhibited the *in vitro* hydrolysis of casein by trypsin.

The inhibitor could be purified further by complexing it with trypsin, coagulating the complex with ammonium sulfate, and removing the trypsin portion of the complex by precipitation with trichloroacetic acid.

A kinetic study indicated the substance to be a non-competitive inhibitor. It was inactivated slowly when heated at 98° C. in water solution. Acid hydrolysis resulted in the liberation of amino acids. Its solubility characteristics, amino acid content, and electrophoretic behavior indicate it to be a polypeptide or a low molecular weight protein.

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Aqueous extracts of fresh alfalfa inhibit the *in vitro* digestion of casein by trypsin. A similar inhibition of trypsin by extracts of commercial dehydrated alfalfa meal also occurs, indicating that the inhibitor withstands the heat of the dehydration process. This investigation was undertaken to devise a procedure for isolation of the inhibitory substance and to investigate its chemical nature and mode of action.

The inhibitory activity of extracts of alfalfa was determined by measuring the decrease in the amount of amino acids released during the *in vitro* hydrolysis of casein by trypsin when the extracts were added. Amino acids were determined by adding a copper phosphate suspension to the deproteinized hydrolysates, thereby forming amino acid-copper complexes having a blue color. The intensity of the blue color was measured with a Beckman DU spectrophotometer at a wavelength of 620 mu.

Preliminary extraction studies showed that the inhibitor was not extracted by acetone or 95 per cent ethanol, but was extracted by water. Aqueous ethanol also extracted the substance, the degree of extraction increasing as the amount of water was increased. The water extracts did not lose their inhibitory activity when subjected to exhaustive dialysis.

These properties were utilized in devising a method of isolation which consisted of extracting alfalfa meal with water, dialyzing exhaustively, and precipitating the active principle by adding ethanol. The inhibitor could be purified further by complexing it with trypsin, coagulating the complex with ammonium

sulfate, and removing the trypsin portion of the complex by precipitation with trichloroacetic acid.

The inhibitory activity of the isolated substance was lost gradually when it was dissolved in water and the resulting solution was heated at 98° C. on a steam bath. Inactivation was essentially complete after three hours of heating. It was concluded from a kinetic study of the inhibition caused by the isolate that the substance is a non-competitive inhibitor. Acid hydrolysis of the substance resulted in the liberation of amino acids.

Its solubility characteristics, its ability to form complexes with trypsin, its electrophoretic behavior, and the liberation of amino acids from it by acid hydrolysis indicate it to be a polypeptide or a low molecular weight protein. Thus, it appears to be similar to the inhibitors isolated from the egg white and from soybean seeds.