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ENZYME INHIBITION

The Trypsin Inhibitor of Alfalfa

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A trypsin inhibitor was isolated from alfalfa meal by extraction with water, dialysis, and precipitation with ethyl alcohol. A kinetic study indicated it to be a noncompetitive inhibitor. It was inactivated slowly by heat, and amino acids were liberated from it by acid hydrolysis. Its properties indicate a polypeptide or a noncoagulable protein.

KENDALL (*3*) reported that in vitro hydrolysis of casein by trypsin is inhibited by aqueous extracts of the fresh forage of alfalfa, ladino clover, and soybeans. Beauchene and Mitchell (*1*) detected a similar inhibition of trypsin by extracts of commercial dehydrated alfalfa, indicating that the inhibitory substance withstands the heat of the dehydration process.

A trypsin inhibitor in alfalfa meal conceivably could be partially responsible for the growth depression which has been observed when alfalfa meal is added to broiler rations at levels of 10% or more. Investigation of this possibility will require isolation of the inhibitor in sufficient quantity for in vivo studies. This report is concerned with a method of isolating the trypsin inhibitor and attempts to determine its chemical nature.

Experimental

The inhibitory activity of extracts of alfalfa meal, and of the fractions obtained from them, was determined by measuring the decrease in the amount of amino acids and peptides released during the in vitro digestion of casein by trypsin when the extracts or fractions were present. Amino acids and peptides were determined by a modification of the method of Spies and Chambers (*6*), which depends on the reaction of these substances 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 (*1*) was used.

A 3% solution of casein in pH 8.4 phosphate buffer was prepared and adjusted to pH 8.4 with dilute sodium hydroxide. Five milliliters of the casein solution were placed in each of four 25-ml. beakers, 2 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 was adjusted to 8.4, if necessary, by means of a Beckman pH meter equipped with microelectrodes. To beakers 1 and 3 was added 1 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	Mixture
1	Casein, trypsin
2	Casein, inactivated trypsin
3	Casein, trypsin, inhibitor
4	Casein, inactivated trypsin, inhibitor

The beakers were covered with small watch glasses and placed in a water bath maintained at 37° C. After 4 hours, 5 ml. of the solutions were placed in test tubes and the undigested casein was precipitated by adding 5 ml. of a 10% trichloroacetic acid solution. The samples were filtered and the filtrates were adjusted to pH 7 with 40% sodium hydroxide. Five milliliters of each filtrate were placed in 15-ml. graduated centrifuge tubes and 3 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 5 minutes, and centrifuged at 2000 r.p.m. to sediment the excess copper phosphate. The intensity of the blue color of the supernatant solutions was measured at 620 mμ with a Beckman DU spectrophotometer. A standard curve was prepared by carrying known quantities of alanine through the procedure.

Isolation of Trypsin Inhibitor. Preliminary extraction studies were performed by extracting 25-gram portions of commercial dehydrated alfalfa 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 Büchner 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 dialyzates so obtained were combined and concentrated to the same volume as the original extract.

The inhibitory activities of the various extracts are shown in Table I, from which it is apparent that the inhibitor was soluble in water but was not extracted by acetone or 95% ethyl alcohol. However, aqueous ethyl alcohol extracted the substance, with a progressive increase in inhibition as the alcohol concentration was decreased. The dialyzed extract was as inhibitory as the undialyzed extract, while the dialyzate was noninhibitory. These properties were utilized in the procedure which was adopted for isolation of the inhibitor.

Concentrates of the inhibitor were prepared from both commercial dehydrated alfalfa meal and alfalfa dried at 50° C. in a laboratory oven. Twenty-

Table I. 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 to Casein Solution	Amino Nitrogen, Mg. per Tube after Incubation	Inhibition, %
None	1.09	0
Acetone extract	1.07	2
Ethyl alcohol extract		
95%	1.08	2
60%	0.71	35
40%	0.68	38
Water extract	0.62	43
Dialyzed water extract	0.63	42
Dialyzate	1.06	2
Supernatant after alcohol precipitation of water extract	1.08	2

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 Büchner funnel, and the residue was washed several times with water. When low temperature meal was used, the filtrate was heated almost to boiling and the coagulated protein was removed by centrifugation. This step was not necessary when commercial meal was used, as it does not contain water-soluble protein (*2*). However, extracts of the latter usually were turbid, and filtration under vacuum through Whatman No. 50 paper covered with a layer of Supercel was used to clarify them. The protein-free amber colored filtrate 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.

The clear amber solution again was concentrated to about 30 ml. and 250 ml. of ethyl alcohol 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 and had a glassy appearance. About 0.6 gram was obtained per 100 grams of alfalfa meal. It was readily soluble in water and could be purified further by repeating the centrifugation, dialysis, and alcohol precipitation. The amber color was never completely eliminated, however.

The material which was obtained by this procedure strongly inhibited the in vitro digestion of casein by trypsin.

Table II. Activity of Trypsin Inhibitor of Alfalfa

Substances Added to Casein Solution	Absorbance at 620 Mμ	Amino Nitrogen, Mg. per Tube after Incubation	Net	Inhibition, %
Trypsin	0.215	1.10	1.09	0
Boiled trypsin	0.003	0.01	...	..
Trypsin, 2 ml. of extract[a]	0.190	0.9	0.62	43
Boiled trypsin, 2 ml. of extract[a]	0.070	0.34	...	..
Trypsin, 4 mg. of isolated inhibitor	0.114	0.58	0.56	49
Boiled trypsin, 4 mg. of isolated inhibitor	0.005	0.02	...	..

[a] 25 grams of meal per 200 ml. of water.

The activity of a typical preparation is shown in Table II.

Heat Stability. Although extracts of commercial dehydrated alfalfa meal are inhibitory (*1*), it should not be assumed that the inhibitor is heat stable. It is possible that the duration of heating during dehydration is too short to have an appreciable effect on the inhibitor.

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 (Table III) show that inhibitory activity had decreased markedly after 0.5 hour, and was almost completely absent after 3 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.

Electrophoretic Behavior. One hundred milligrams of the inhibitor were dissolved in pH 8.6 borate buffer, and the solution was subjected to a current of 20 ma. for 30 minutes in an Aminco portable electrophoresis apparatus. Two components were detected under these conditions, indicating a lack of homogeneity of the inhibitor as isolated by the procedure employed.

Nature of Inhibition. The nature of the inhibition was studied by measuring the effect of a given concentration of inhibitor with varying concentrations of substrate. A 5% casein solution was prepared by dissolving 15 grams of casein in 300 ml. of pH 8.4 phosphate buffer and adjusting the pH to 8.4 with a few drops of 40% sodium hydroxide solution. Aliquots of this solution were diluted with buffer to yield solutions of 0.5, 1, 2, 3, and 4% 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 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 concentration:

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

Table III. Effect of Heating at 98° C. on Activity of Inhibitor

Time of Heating, Hr.	Inhibition, %
0	35
0.5	13
1	11
3	2
7	0

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

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

The reaction flasks were placed in a water bath maintained at 37° C. The enzyme solution was added at zero time and a 5-ml. aliquot was withdrawn and added to 5 ml. of 10% trichloroacetic acid to stop the reaction. Additional aliquots were treated similarly after 2 hours. Each sample so obtained was allowed to stand 15 minutes and was filtered through dry filter paper. The pH was adjusted to 7, and 5 ml. of the resulting filtrate were used for amino acid determination. Flask C was used as a blank to correct for small quantities of free amino acids in the casein and enzyme.

The analytical data are presented in Table IV. From these values a plot of $1/S$ and $1/V$ was prepared (Figure 1) according to the double reciprocal method of Lineweaver and Burk (*4*). Failure of the two lines to intersect

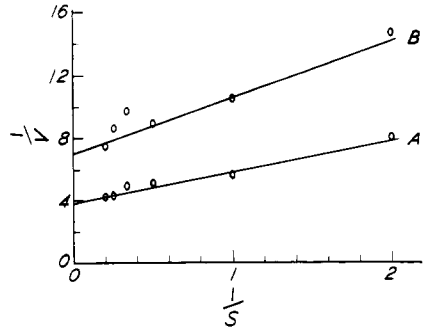

Figure 1. Double reciprocal plot of the data of Table IV

A. Inhibitor absent B. Inhibitor present

Table IV. Effect of Inhibitor and Substrate Concentration on Hydrolysis of Casein by Trypsin

	A. Inhibitor Absent						B. Inhibitor Present					
Casein concentration, S, %	5	4	3	2	1	0.5	5	4	3	2	1	0.5
Absorbance at 620 mμ after 2 hours at 37° C., V	0.238	0.237	0.201	0.196	0.178	0.123	0.133	0.115	0.102	0.112	0.095	0.068
$1/S$	0.20	0.25	0.33	0.5	1	2	0.20	0.25	0.33	0.5	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

indicates that the inhibition was noncompetitive.

Chemical Nature of Inhibitor. Because the active principle was precipitated from water by dilution with a relatively large volume of ethyl alcohol, it was thought that it might be inorganic in nature. However, this was shown not to be true when a portion of the isolated substance was ashed and the residue was found to be noninhibitory.

The inhibitor probably is not a heat-coagulable protein, as coagulable material was eliminated during isolation. However, it could be a polypeptide with a sufficiently low molecular weight not to coagulate with heat. This possibility was investigated by refluxing 5 mg. of the isolated material with 6N hydrochloric acid overnight. The hydrochloric acid was removed by repeated distillation at reduced pressure at 40° C. The final residue was dissolved in 1 ml. of water and 25 μl. were chromatographed on Whatman No. 1 filter paper, using 8 to 2 phenol-water as the developing solvent.

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

Although the data suggest the inhibitor is a polypeptide, it is recognized that the amino acids might have arisen from hydrolysis of a polypeptide impurity. Further investigation of its purity will be needed before it can be characterized unequivocally.

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, extracts of dehydrated plant tissue are amber in color. Often the intensity of this color is sufficient to cause appreciable light absorption at the wave length used to measure the blue color of the amino acid–copper complex.

Inspection of Table II will indicate 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 ethyl alcohol precipitation eliminated the free amino acids and much of the substances responsible for the amber color.

Isolation of the trypsin inhibitor will permit in vivo studies to determine whether it is involved in chick growth depression. If the inhibitor were altered while in the crop or gizzard, it might no longer inhibit trypsin in the intestinal tract. Crude extracts are not suitable for such tests, because other constituents of alfalfa are known to inhibit growth (*5*).

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MEASUREMENT OF URINARY ANDROGENS

The Effect of Interfering Chromogens in Spectrophotometric Determination of Ruminant Urinary Androgens

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As has been demonstrated, androgenic 17-ketosteroids (17-KS) frequently appear in the urine, originating in most mammals as steroid metabolites of either the testes or adrenal glands (*3*). Of these compounds, androsterone [androstane-3 (α)-ol-17-one] and dehydroepiandrosterone [Δ^5-androstene-3(β)-ol-17-one], or androstenolone, as recently suggested (*4*), are not only produced in the greatest amounts, but are also among those which are the most active biologically.

The quantitative determination by various spectrophotometric methods of these and related compounds, all of which are frequently referred to as "urinary androgens," has classically been interpreted as a measure of testicular and/or adrenal cortical function.

Among the spectrophotometric methods are the Zimmerman (*5*), Pincus (*9*), and Allen (*1*) reactions, each of which produces chromogens having characteristic absorption maxima in the visible wave length range. These reactions have been employed by many workers to study the 17-KS content of urines isolated from a variety of subjects (*3*). However, certain nonsteroid ketones as well as non-17-KS present in biological materials probably confound the interpretation of results obtained as a consequence of using the chemical methods of 17-KS assay.

Only relatively recently has the probable nature of at least one group of these compounds been recognized (*6*) as ionone derivatives. In addition, cow urine has been shown to contain a group of chromogens (*7*) which very probably are ionone derivatives arising from dietary carotenoids. These compounds are ex-