

THE CHEMISTRY OF ALLERGENS. XII. PROTEOLYSIS OF THE COTTONSEED ALLERGEN

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THE isolation and properties of the principal allergen of cottonseed, CS-1A, have been described in a series of articles from this laboratory starting in 1939. CS-1A was immunologically distinct from other allergens and antigens in cottonseed and was classified as a natural protease. The protein nature of CS-1A was established by chemical properties and isolation procedures,¹⁻³ amino acid composition,^{4, 5} and antigenic properties.⁶⁻¹⁰ The present study was undertaken to answer several questions concerning the effect of digestive proteolytic enzymes on the cottonseed allergen as well as to obtain general fundamental information on this subject. The questions were: (1) is allergenic activity due to structural peculiarities which make the allergen resistant to proteolysis; (2) can partial enzymic hydrolysis take place without loss of allergenic properties; (3) is allergenic activity due to an unrecognized non-protein contaminant present in trace proportions.

The role of proteolysis in the body's defense against ingested allergenic proteins and the immunologic significance of protein hydrolytic products have been subjects of much investigation and speculation. Complete review of the literature on this subject is outside the scope of this article, only a few references particularly pertinent to the present problem being cited.

Opinion of Zinsser and Bayne-Jones¹¹ and of Hartley,¹² based on their evaluation of then existing evidence, was that the antigenic property of native proteins is destroyed early on hydrolysis, probably before formation of such relatively high-molecular-weight, cleavage products as proteoses. Landsteiner¹³ stated that amino acids and low-molecular weight peptides are inactive as antigens and that attempts to produce antibodies for relatively high-molecular weight proteoses have been, in general, unsuccessful. However, instances of anaphylaxis and formation of antibodies for peptic heteroproteins absorbed on charcoal have been reported.¹³ Loss of capacity to react with precipitins for unaltered protein by peptic digests has been observed.¹³

Ratner and Gruehl¹⁴ and Walzer¹⁵ reviewed the earlier history of the immunologic aspects of the absorption of ingested proteins. They considered the positive passive transfer reactions obtained on ingestion of allergens to be caused by absorption of unaltered allergenic proteins.

Stull and Hampton¹⁶ determined the antigenicity of primary and secondary proteoses prepared by peptic digestion of proteins from several sources using the Schultz-Dale technique. New specificities were developed in some cases

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and in other cases the products were not antigenic. The degrees of antigenicity and whether or not new specificities developed depended on the source of the protein and the experimental technique employed.

Urbach and associates^{17, 18} studied the immunologic behavior of the propeptans, which are the degradation products obtained by peptic digestion of foods with subsequent mild tryptic digestion. Urbach¹⁹ stated that pollen propeptans stripped of all native protein still possessed ability to react in passively sensitized sites.

Much difficulty in this field is caused by the indefinite nature of protein hydrolytic products, the possible presence of immunologically significant traces of undigested, native protein in enzyme digests, the use of complex foods or pollens as substrates, the lack of determination of the degree of enzyme digestion, and other difficultly controlled factors. Availability of the principal allergen of cottonseed as a well-defined protein fraction, CS-1A, prompted this study.

The present article is a progress report describing results of proteolysis of CS-13-Endo, a purified fraction of CS-1A. The crystalline enzymes used were trypsin, chymotrypsin, pepsin, and carboxypeptidase.

EXPERIMENTAL

CS-1A.—Fraction CS-1A was isolated from depigmented, defatted cottonseed as previously described.^{1, 2, 5}

Preparation of Substrate, CS-13-Endo.—An unimportant polysaccharide and some denatured protein were eliminated from CS-1A by precipitation of the active fraction with picric acid and recovery of the fraction, CS-13, by removal of the picric acid. CS-13 is separable into fractions by dialysis. The smaller molecules in CS-13 which pass through the membrane possess the same antigenic specificity as the larger molecules retained by the membrane.²⁰ Therefore, the fraction remaining inside the membrane, after prolonged dialysis of CS-13, CS-13-Endo, is an ideal substrate because any increase in dialyzability after proteolysis of CS-13-Endo can be used as a measure of proteolysis and also dialysis can be used to fractionate the enzyme hydrolysates. Details of the preparation of the substrate, CS-13-Endo, from CS-1A are described below.

One hundred grams of CS-1A (ash- and water-free basis) was dissolved in 1,900 ml. of water, and 2,080 ml. of a saturated solution of picric acid was added with stirring. The viscous suspension was cooled to 2 to 3° C. overnight. The suspension was centrifuged in the batch bowl of the Sharples supercentrifuge at 45,000 r.p.m. for twenty minutes. The clear supernatant solution was discarded. The picrate was washed by dispersing in 400 ml. of water followed by supercentrifuging. For removal of picric acid the dried picrate (82.7 grams) was suspended in 4,000 ml. of water, and 15 grams of sodium bicarbonate was added slowly with vigorous stirring. The pH of the solution was 7.0. The pH was adjusted to 9.0 with dilute sodium hydroxide, and 4,000 ml. of cold absolute ethanol was added. The pH of the clear solution was adjusted immediately to 6.0 with 50 per cent acetic acid. To the resulting suspension was

added 12,300 ml. of ethanol. The suspension was kept at 5° C. for four days. The supernatant solution was siphoned off and discarded. The solid was washed successively with 1,500 ml. and 400 ml. portions of cold ethanol. The solid was stirred mechanically with 2,000 ml. of water until dispersed or dissolved. The insoluble matter was removed by centrifugation and discarded. To the solution was added 100 ml. of 1N sodium acetate, buffered at pH 5.6, and 8,400 ml. of ethanol. The resulting suspension was cooled and the pH adjusted to 6.0-6.2 with acetic acid. After standing at 5° C. overnight the slightly cloudy supernatant solution was separated and discarded. The solid was washed successively with 1,000 ml. and 400 ml. portions of cold ethanol. The solid was dissolved in 750 ml. of water and the solution was clarified by centrifugation. The clear solution was filtered into 3,000 ml. of cold ethanol. The heavy suspension was cooled and flocculated by adding dilute acetic acid until the pH was 6.0-6.2. After standing at 5° C. for two days the precipitate was separated by centrifuging at 5° C. The dried precipitate (41.1 grams) was dissolved in 700 ml. of water and the solution was clarified by centrifugation. The solution was filtered into 2,800 ml. of cold ethanol and the suspension was flocculated by adjustment of the pH to 6.0-6.2 with acetic acid and sodium hydroxide; 35 ml. of 1N sodium acetate buffer, pH 5.6, also being added. The solid, separated from the cold suspension by centrifugation, was washed once with 1,000 ml. of cold ethanol and dried in a vacuum over calcium chloride. The solid, designated CS-13, was ground to pass a 40 mesh sieve and equilibrated with air. The yield was 34.7 grams. CS-13 contained 18.83 per cent nitrogen and 4.0 per cent carbohydrate (ash- and water-free basis).

For dialysis, a solution containing 33.2 grams of CS-13 in 330 ml. of water was placed inside a leak-tested, Visking cellulose casing. Toluene was used as preservative. The casing was suspended in a 4 l. beaker with the knotted ends of the casing hanging over the edge of the breaker. The solution was dialyzed against 1,000 ml. portions of water. To the clarified dialysate and endo solutions were added 0.1 volumes of 1N sodium acetate buffer, pH 5.6. The solutions were poured into four volumes of cold ethanol, and if flocculation did not occur, it was induced by adjustment of the pH to 6.0-6.2 with 50 per cent acetic acid and sodium hydroxide solution. The suspensions were stored at 5° C. overnight and the precipitates isolated by centrifugation. The solids were washed once with ethanol and dried in vacuum over calcium chloride. Yields and analyses of the fractions are shown in Table I. Six dialysate fractions were obtained which accounted for 50.2 per cent of the starting CS-13. The dialyzed residue, CS-13-Endo, weighed 9.7 grams or 29.2 per cent of the CS-13. CS-13-Endo contained 17.8 per cent nitrogen and 7.7 per cent carbohydrate (ash- and water-free basis).

Enzymes.—The enzyme preparations were obtained from Worthington Biochemical Laboratory. Pepsin was twice crystallized from alcohol. Trypsin was "salt free," twice crystallized. Carboxypeptidase was a thrice crystallized water suspension. The Worthington chymotrypsin was recrystallized four times by E. F. Jansen, Western Regional Research Laboratory.

TABLE I. DIALYSIS OF COTTONSEED ALLERGEN, CS-13

FRACTION	TIME OF DIALYSIS DAYS	NITROGEN IN DIALYSATE % OF TOTAL	YIELD OF SOLID* GRAM	YIELD OF SOLID % OF TOTAL	ANALYSIS OF FRACTION	
					NITROGEN† %	CARBOHY- DRATE‡ %
Dialysate No. 1	7	10.5	2.5	7.5	19.6	0.75
Dialysate No. 2	14	12.1	3.4	10.2	19.5	0.85
Dialysate No. 3	21	12.1	3.4	10.2	19.5	0.87
Dialysate No. 4	20	9.5	2.8	8.4	19.8	0.85
Dialysate No. 5	28	9.1	2.4	7.2	19.3	0.86
Dialysate No. 6	42	8.8	2.3	6.7	19.5	0.86
CS-13-Endo	132§	—	9.7	29.2	17.8	7.70

*Air-dried basis.

†Ash- and water-free basis.

‡Determined by the method described in Ref. 30.

§Total time of dialysis.

Proteolysis.—The substrate was a solution containing 50 mg./ml. of CS-13-Endo. The solvent for the trypsin-chymotrypsin and the chymotrypsin-trypsin proteolysis was 0.2M boric acid-potassium chloride buffer, pH 8.5; the pH of the substrate solution being 8.33. The solvent for carboxypeptidase digestion was 0.2M phosphate buffer, pH 7.4; the pH of the substrate solution being 7.27. For digestion with pepsin, the pH of a water solution of CS-13-Endo was adjusted to 1.73 with 6N hydrochloric acid. After thirty hours' digestion with pepsin the pH was 2.10. The pH was readjusted to 1.79 with hydrochloric acid. The pH was 1.8 at the end of digestion. Toluene was used as preservative.

Proteolysis was carried out at $35^{\circ} \pm 0.1$ C. The proportion of enzyme to CS-13-Endo was 1 to 50, a second equal amount of enzyme being added after forty-eight hours. Following forty-eight hours' treatment with trypsin and also with chymotrypsin the solutions were halved. To one half was added a second quantity of the starting enzyme and to the other half was added, appropriately, either trypsin or chymotrypsin. Aliquots of each substrate solution were removed before the first addition of enzyme to serve as controls. Control solutions were kept at 35° during the corresponding proteolysis.

Measurement of Proteolysis.—The recently described ultraviolet spectrophotometric method for analysis of amino acids and peptides with their copper salts²¹ was used to determine the rate and time of completion of proteolysis. Analyses were run on 5 ml. aliquots of solution prepared by diluting 0.25 ml. of substrate solution into 50 or 100 ml. of water.

Comparative Rates of Dialysis.—Five milliliters of solution was placed in an 8-inch length of leak-tested, 18/32 inch Visking sausage casing. The dialyzing tubing was suspended in 100 ml. of water in a 250 ml. beaker by fastening the tied ends of the casing over the edge of the beaker. Toluene was used as preservative. After twenty-four hours, without agitation, the outer solution was removed and nitrogen content was determined by the Kjeldahl micro-method. From the nitrogen content of the solution and its volume the percentage of nitrogen dialyzed in twenty-four hours was determined. Results are shown in Table II.

Cutaneous Tests.—A scratch which just penetrated the epidermis was made on the inner forearm of a Type II¹ cottonseed-sensitive subject. The test solu-

tion was placed on the scratch and worked in with a clean toothpick. The intensity of the reaction was determined by the size of the wheal produced in fifteen or thirty minutes. Simultaneous control tests were conducted to provide a comparison of potency.

TABLE II. SUMMARY OF THE EFFECT OF ENZYMES ON COTTONSEED ALLERGIC FRACTION, CS-13-ENDO

ENZYME OR ENZYME COMBINATION	TIME OF ACTION HR.	COPPER- REACTING NITROGEN % OF TOTAL	NITROGEN DIALYZED IN 24 HR. % OF TOTAL	CUTANEOUS TEST*	
				1:50,000	1:500,000
Trypsin	71	23.8	40.2	0	±
Trypsin-Chymo- trypsin†	48-24	25.0	50.6	0	0
Control	71	6.3	0.8	4+	3+
Chymotrypsin	72	19.7	39.1 ± 0.0§	2+	±
Chymotrypsin- trypsin‡	48-24	27.7	56.5 ± 0.0§	0	0
Control	72	6.2	0.7	4+	3+
Pepsin	72	11.9	28.2 ± 0.2§		3+
Control	72	6.0	10.3 ± 0.2§		3+
Carboxypeptidase	72	10.3	19.8		2+
Carboxypeptidase	240	12.7	23.1 ± 0.1§		4+
Control	72	7.2			4+
Control	240	6.3	3.6		3+

*Diluted on a nitrogen basis.

†Trypsin forty-eight hours followed by chymotrypsin for twenty-four hours.

‡Chymotrypsin forty-eight hours followed by trypsin for twenty-four hours.

§Average of duplicate determinations.

||The relatively high percentage of nitrogen dialyzed from this control solution is undoubtedly due to dialysis from the strongly acid solution used for peptic digestion. In a different experiment in which the acid control solution was neutralized before dialysis, the per cent nitrogen dialyzed was almost the same as that which dialyzed from a similar solution which had never been acidified.

RESULTS AND DISCUSSION

The relative rates and amounts of proteolysis of CS-13-Endo by trypsin, chymotrypsin, pepsin, and carboxypeptidase are shown in Fig. 1. The percentage of copper-reacting nitrogen in enzyme hydrolysates and control solutions is plotted as ordinates against time in days as abscissas. Enzymic hydrolysis is proportional to the percentage of copper-reacting nitrogen in the hydrolysates. The percentage copper-reacting nitrogen divided by 0.54 gives an approximate percentage-degree of completion of hydrolysis. This factor is based on the amino acid composition of CS-1A fractionation products.²²

Results, summarized in Table II, show the percentages of copper-reacting nitrogen, the dialyzability, and the skin-reactivity of the final enzyme hydrolysates compared to control solutions.

CS-13-Endo was digested by all four enzymes. Of the enzymes used alone, trypsin caused the most rapid and complete hydrolysis in seventy-two hours (curve T); the copper-reacting nitrogen was 23.8 per cent and the nitrogen dialyzed in twenty-four hours was 40.2 per cent. Digestion with trypsin for forty-eight hours followed by digestion with chymotrypsin for twenty-four hours or digestion in reverse order with these enzymes caused more hydrolysis than did hydrolysis for seventy-two hours with either enzyme used alone (curves *T-Ch* and *Ch-T*). The most hydrolysis was caused by digestion with chymotrypsin for forty-eight hours followed by digestion with trypsin for

twenty-four hours. After this treatment the copper-reacting nitrogen was 27.8 per cent and the nitrogen dialyzed was 56.5 per cent as compared to 25 per cent copper-reacting nitrogen and 50.6 per cent nitrogen dialyzed when the digestion was carried out in reverse order.

Skin-reacting capacity of CS-13-Endo was destroyed by digestion with trypsin but slight activity remained after chymotrypsin digestion. The skin-reacting capacity of CS-13-Endo was destroyed by the combined action of trypsin and chymotrypsin regardless of the order of their use. These results unequivocally confirm the conclusions from previous work that allergenic activity of CS-1A is caused by protein and not by an unrecognized nonprotein contaminant.

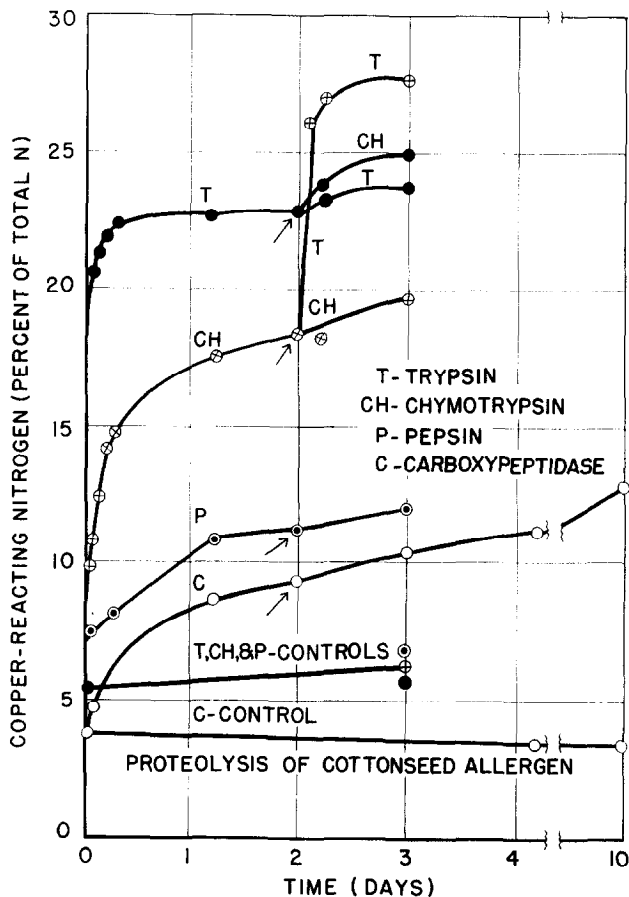


Fig. 1.—Relative rates and amounts of proteolysis of CS-13-Endo.

Both pepsin and carboxypeptidase hydrolyzed CS-13-Endo to a lesser degree than did trypsin or chymotrypsin. The skin-reacting capacity was not destroyed nor apparently even diminished by either pepsin or carboxypeptidase. A peptic digest was fractionated by dialysis. Fast- and slow-dialyzing fractions were obtained. The slow-dialyzing fraction was more skin-reactive than the fraction remaining inside the dialyzing membrane. This indicated

that the intact protein is not necessary for allergenic activity but that peptide bonds may be split by pepsin without destroying allergenic activity. However, this point must be investigated further before an unequivocal conclusion can be reached.

In contrast, on dialysis of the carboxypeptidase digest, the fraction remaining inside the membrane was more skin-reactive than the fractions which dialyzed. This is to be expected because in general carboxypeptidase splits amino acids from the ends of polypeptide chains whereas pepsin splits peptide bonds deeper within the peptide chain.

The precipitinogenic capacities of the enzyme digests of CS-13-Endo were determined with the agar plate method of Ouchterlony,^{23, 24} using rabbit antiserum from rabbits sensitized to CS-1A. Solutions containing 0.1 mg. of nitrogen per milliliter were used for precipitin tests. Trypsin, chymotrypsin, and carboxypeptidase caused a decrease in the precipitinogenic capacity of the allergen but did not destroy it completely. Pepsin completely destroyed precipitinogenic capacity* as shown by the agar plate method, but the peptic digest gave a slightly positive precipitin reaction by the ring test. Reason for this discrepancy is not known. These results indicated the complexity of enzymic action in relation to precipitinogenic and skin-reactive properties of substrates and conditions of the experiment. It should be pointed out, as did Stull and Hampton,¹⁶ that varying experimental conditions or substrate preparations may give varying results because of the large number of possible split products that can result from substances as complex as proteins. Further fundamental studies along these lines are needed.

The peptic digest of CS-13-Endo was antigenic as shown by its capacity to sensitize guinea pigs. The peptic digest retained 25 per cent of its capacity to induce contraction in sensitized guinea pig uteri as shown by the Schultz-Dale technique. No new specificity was detected in the peptic digest as shown by cross-neutralization tests with CS-13-Endo using Schultz-Dale tests.

The observation that a skin-reactivity is retained by a dialyzable fragment of the pepsin digest of CS-13-Endo is of possible significance in relation to the absorption of ingested allergens. Freeman,²⁵ Lewis and Grant,²⁶ and, independently, M. Walzer^{27, 28} first observed that ingested egg and fish allergens were absorbed into the circulatory system and were capable of inciting passive transfer reactions in sensitized sites. It has been generally regarded that specific reaction of allergen in sensitized sites was a property of the unaltered allergen. The possibility was suggested by Spies, Chambers, Bernton, and Stevens²⁹ that digestive enzymes might cause alteration of the cottonseed allergen without destroying its specific property of reacting with reagins. Positive passive transfer reactions were obtained with a fraction of the peptic digest of CS-13-Endo which had passed through a dialyzing membrane. But compari-

*The precipitinogenic capacities of a peptic digest of CS-13-Endo and of CS-13-Endo were determined using the method of Ouchterlony as follows: The pH of each solution was adjusted to 6.9. Rabbit antiserum was prepared using CS-1A as antigen. Twofold serial dilutions were used in each series of tests. CS-13-Endo gave precipitin reaction in dilutions from 0.1 to 0.0007 mg./ml. of nitrogen. The peptic digest gave no precipitin reaction in dilutions from 3.2 to 0.0015 mg./ml. of nitrogen.

son of the passive transfer potency* of this fraction with that of CS-13-Endo was not sufficiently precise to eliminate conclusively the possibility that the reaction obtained with the dialyzable fraction was not due to some unchanged allergen in the peptic digest. That skin-reactivity is retained by a dialyzable fraction of the peptic digest supports the view that more highly diffusible, active fragments of allergen are produced in the stomach by pepsin or pepsin and gastric acidity in the first stages of digestion than existed in the original allergen. This may contribute to increasing the speed of absorption as shown by the rapidity with which passively sensitized sites are incited to reaction when allergen is ingested. It should be pointed out, however, that undialyzed CS-1A contains some allergen which is readily dialyzable. CS-13-Endo was used as substrate for the present studies to eliminate, as far as possible, this dialyzable portion of CS-1A.

SUMMARY

The effects of four crystalline enzymes were studied on CS-13-Endo, a purified fraction of CS-1A, the principal allergen of cottonseed. The enzymes were trypsin, chymotrypsin, pepsin, and carboxypeptidase. CS-13-Endo was digested by all of these enzymes. Skin-reacting capacity was destroyed by trypsin. Decreased activity remained after digestion with chymotrypsin. These results unequivocally show that allergenic activity is caused by protein and not by an unrecognized nonprotein contaminant in CS-13-Endo. Both pepsin and carboxypeptidase digested CS-13-Endo but to a lesser degree than did trypsin or chymotrypsin. The skin-reacting capacity was not destroyed by pepsin or carboxypeptidase. A dialyzable fraction of the pepsin digest was slightly more potent in skin-reactivity than the nondialyzable fraction. This indicated that the intact protein was not essential for allergenic activity. The peptic digest of CS-13-Endo was antigenic. No new specificity developed in the peptic digest.

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*The threshold quantities of a fraction of the peptic digest of CS-13-Endo that had passed through a membrane and of CS-13-Endo required to produce positive passive transfer reactions were determined. Three sites were sensitized on each upper arm of the recipient using 0.05 ml. of serum from a cottonseed-sensitive subject in each site. Three days later equal quantities of nitrogen of the dialysis fraction and of CS-13-Endo were introduced into corresponding sites on opposite arms. The wheals which formed were measured after fifteen to thirty minutes. The threshold quantities of nitrogen required to produce positive reactions were from 1 to 10 μmg for each fraction. In the three recipients tested, CS-13-Endo appeared slightly more potent than the peptic digest dialysate but the latter fraction produced wheals with pseudopods and heat in the same concentrations of nitrogen as did CS-13-Endo.

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