

Characterization of a major allergen (cod)

Effect of enzymic hydrolysis on the allergenic activity

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A previously isolated major allergenic fraction from cod was hydrolyzed with proteolytic enzymes and subjected to in vivo digestion experiments. Effects of these procedures were noted on its allergenic activity measured by ability to induce direct and indirect skin test reactivity and antigenic capacity to react with specific rabbit antiserum. Extensive hydrolysis with trypsin, pepsin, subtilisin, and pronase resulted in complete destruction of both properties. Partial hydrolysis and extensive clastase treatment partially reduced both of these activities to the same extent.

Hypersensitivity to fish is frequently encountered in some populations.¹ The frequency and nature of allergy to fish lend it as a suitable model for study of both the immunologic aspects of hypersensitivity in man and the character of the responsible allergenic components. It is of special interest that fish may act either as a food or inhalant allergen, or both.¹⁻¹⁰

In previous papers,⁷⁻⁹ the isolation of a highly active allergenic fraction (DS 22) from cod and its purification by crystallization were reported. Scarcity of this purified material makes its use prohibitive for extensive experimentation. However, it was found that the mother fraction DS 22 is adequately representative of the allergen for study of the effects of enzymes. If the activity of the mother fraction is destroyed by enzymic hydrolysis, this indicates that the pure allergen as well is affected by such digestion. DS 22 and its derivative crystallized preparation were both shown to contain the major allergen(s) present in cod as eaten by the patients, in steam collected from cooking cod, and in the allergen extract from cod. This was substantiated by the demonstration of allergic symptoms in patients following oral ingestion of the material concealed in beef as well as by means of direct skin testing and passive transfer cross-neutralization experiments.⁷⁻⁹ With several antisera from rabbits injected

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with cod extracts, only one precipitation line was obtained in immunodiffusion experiments; but with the aid of one specific antiserum, it was shown that both the major allergenic fraction DS 22 and the crystals contained 4 antigenic determinants. DS 22 was homogenous in ultracentrifugation, molecular sieve experiments, and in polyacrylamide gel disc electrophoresis at acid pH. By disc electrophoresis at alkaline pH, however, both DS 22 and the crystallized allergenic fraction were separated into 2 subcomponents (P3 and P4) that were antigenically and allergenically identical.⁷⁻⁹ In some experiments, the fastest zone (P3) was split into 2 subcomponent zones, probably due to some degradation effect. The molecular weight was originally estimated at 17,000, but subsequent ultracentrifugal analyses have indicated a molecular weight (M.W.) of the active material to be 14,000 to 16,000.^{9,11} In further experiments, the subcomponents have been separated by electrofocusing on ampholine columns, and the chemical composition of DS 22 and the active subcomponents has been analyzed.¹¹ The major allergenic protein appears to be rather peculiar among fish myogens, and the cod myogen fraction isolated differs from myogens of other species with respect to the ultraviolet (UV) spectrum and amino acid composition; it contains one residue per molecule of tryptophan and one residue of methionine, together with the usual high number of phenylalanine residues. DS 22 contains also small amounts of glucose (0.4 to 1.0 per cent), ribose (0.4 per cent), galactose (0.1 per cent), and mannose (0.1 per cent) but no sialic acid.^{9,11}

The present paper reports on attempts to assess the effect of enzymic digestion on the allergenic activity of the major allergenic fraction, DS 22.

METHODS

The reference allergen extracts from cod (*Gadus callarias* L.) and salmon (*Salmo salar*) were prepared as described previously.² Allergen extracts from horse dander were purchased from Nyegaard and Company, Oslo.¹ The purified myogen fraction DS 22 containing the major allergens from cod was isolated by extraction with distilled water followed by chromatography on DEAE-cellulose columns and repeated gel filtration on Sephadex G-75 as described elsewhere.^{7,8} Cod-hypersensitive and control subjects were selected from among 825 children suffering from allergic urticaria and/or asthma as described in a previous paper.¹ Children with clinical and skin hypersensitivity to allergens other than fish were used as controls. Serum for passive transfer experiments was collected from a fish-allergic adult, D. E., and a patient (No. 83) who had previously been shown to possess the capacity to discriminate between cod and salmon with respect to reaginic activity.⁶ The hypersensitive serum donors also showed markedly positive skin reactivity to horse dander extracts, which made them well suited for passive transfer tests as outlined later. For control purposes, serum was collected also from a nonallergic and an allergic donor with no skin or clinical allergic reactions to fish.⁶ None of the serum donors was allergic to beef. The allergenic activity of the different materials under study was controlled by skin testing in 10 children who were allergic to cod and in an equal number of allergic control individuals tolerating fish. The test materials

were used in protein concentrations of approximately 20 mg. per 100 ml. unless otherwise stated.¹ For assessment of relative activity of different materials, scratch and passive transfer tests were performed with serial dilutions from the standard concentration. Passive transfer experiments and cross-neutralization tests were performed as described previously²⁻⁵ and as outlined for the *in vivo* experiments in the Results section in this paper.

Specific rabbit antisera to cod antigens were produced, collected, and stored as described elsewhere.³ DEAE-cellulose fractionation, gel filtration, polyacrylamide gel disc electrophoresis, protein estimation, UV spectrophotometry, sterile filtration, dialysis, double diffusion-in-gel experiments, and photographic recording were performed as outlined in previous communications.^{3, 4, 7, 8}

The enzymes pronase, carboxypeptidase A, leucine aminopeptidase, elastase, neuraminidase, beta-glucosidase (from sweet almonds), and beta-galactosidase (from yeast) were obtained from Koch-Light Laboratories, Colnbrook, Bucks, England. Pepsin was obtained from Difeo Laboratories, Detroit, Mich.; trypsin was procured from British Drug Houses Ltd., Chemical Division, Poole, England, and subtilisin was obtained from Sigma Chemical Company, St. Louis, Mo.

The carbohydrate content was estimated by gas-liquid partition chromatography as described by Clamp and associates¹² on a gas chromatograph model 402, F & M Corp., Avondale, Pa.

Enzyme hydrolysis

All enzymes were used at one per cent of the weight of the protein. The digestions were performed at 37° C. for 48 hours or shorter periods for partial hydrolysis. Optimal pH and hydrolysis conditions were selected as advocated for each enzyme.¹³⁻¹⁸ To stop the digestions, the material was heated to 80° C. for 10 minutes, previous experiments having shown that the allergenic activity of cod is resistant to such treatment.³⁻⁵ For each experiment, 4 aliquots were prepared. One aliquot consisted of the enzyme solution without any allergenic material added. One aliquot contained the allergen (DS 22) solution mixed with an enzyme preparation which had been inactivated by heating to 80° C. for 10 minutes. These preparations served as controls. Two aliquots contained a mixture of the allergenic solution and the active enzyme preparation. The allergen (DS 22) was used in a concentration of 1 mg. per milliliter and was mixed with the enzyme preparation after sterilization by Millipore filtration. Samples were allowed to react for 10, 30, and 60 minutes and 2, 4, 6, and 48 hours, and then the reactions were stopped. One sample containing DS 22 and active enzyme was dialyzed against large volumes of distilled water at 4° C., whereas one similar sample was left undialyzed. Sterile filtration was carried out before biological tests were done.

RESULTS

Digestion *in vivo*

Sites on the volar surfaces of the forearms of a nonallergic volunteer were sensitized with serum from the cod-allergic donor, D. E., the patient (No. 83)

Table I. Passive transfer cross-neutralization (*in vivo*) tests.

Site	Serum used (1/10)	Challenges of sites					
		No. 1 (after 24 hr.): oral ingestion of DS 22, pure (20 mg./100 ml.)	No. 2 (+ 6 hr.): I.C.* injection of DS 22, 0.1 ml. (0.02 mg./100 ml.)	No. 3 (+ 6 hr.): oral ingestion of DS 22 mixed with beef (20 mg./200 Gm.)	No. 4 (+ 24 hr.): I.C. injection of DS 22, 0.1 ml. (20 mg./100 ml.)	No. 5 (+ 1 hr.): I.C. injection of salmon extract (20 mg./100 ml.)	No. 6 (+ 24 hr.): I.C. injection of horse dander extract 1/10,000 (w/v)
1	D. E.	0		++++	0	0	+++
2	Control	0		0	0	0	0
3	D. E.	0	+++	?	0	0	+++
4	D. E.	0		++++	0	0	++++
5	No. 83	0		++++	0	++++	+++
6	Control	0	0	0	0	0	0
7	No. 83	0		++++	0	++++	+++
8	D. E.	0		++++	0	0	++++

*I.C., intracutaneous.

†Rubor and edema persisting after the I.C. injection 6 hours before the oral ingestion.

allergic to both cod and salmon, and the control individuals sensitive to allergens other than fish. Approximately 24 hours later, the recipient ate 20 mg. of DS 22 after fasting approximately 24 hours. No reaction occurred. One of the sites sensitized with serum of Donor D. E. was tested with the injection of 0.1 ml. of DS 22 (0.02 mg. per 100 ml.) and showed a markedly positive reaction, while the other sites remained unaffected. The recipient ate 20 mg. of DS 22 6 hours later, this time mixed with approximately 200 Gm. of pure beef. Within the next 30 minutes, urticarial wheals occurred at all sites sensitized with the fish-allergic sera except in the one injected with DS 22 6 hours previously. The control sites remained negative. All sites were challenged 24 hr. later (when all reactions had disappeared) with the intracutaneous injection of the reference extract from cod, which contained approximately 20 mg. per 100 ml. of protein. No reactions were elicited, which indicated that the cod-reacting reagin in the sensitized sites had been completely exhausted by the former tests. One hour later, the sites were challenged with the reference extract from salmon. This time, the sites sensitized with serum of Patient No. 83 reacted with a marked 4 plus (++++) wheal, whereas the other sites remained negative. A final challenge approximately 24 hours later with horse extracts in all sites elicited markedly positive reactions in all allergic sera sites but none in the control sites, which showed that the skin still possessed the ability to react on specific challenge (Table I).

Extensive proteolytic hydrolysis *in vitro*

We studied the effect of hydrolysis on antigenicity, allergenic activity, and results of disc electrophoresis. After extensive hydrolysis with trypsin, pepsin, subtilisin, and pronase, respectively, the protein was degraded to the extent that both the allergenic activity and the ability to react with the specific anti-serum were lost. Similar treatment with elastase for 48 hours resulted in loss of

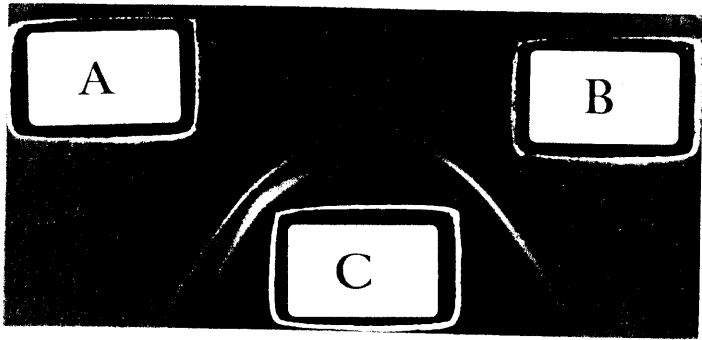


Fig. 1
Result of hydrolysis with elastase as shown in a double diffusion-in-gel experiment. *A*, Original material, DS 22; *B*, material treated with elastase for 48 hours, and *C*, antiserum.

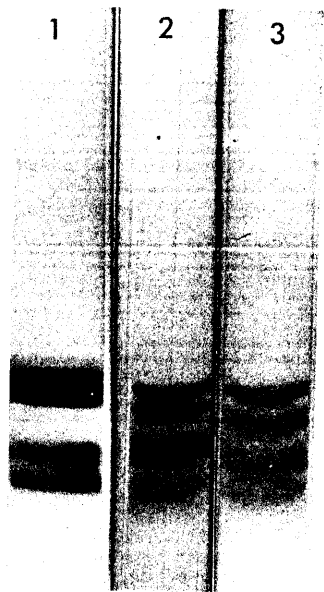


Fig. 2
1, Polyacrylamide gel disc electrophoresis of original material, DS 22; 2, material treated with leucine aminopeptidase; 3, material treated with carboxypeptidase.

approximately 50 per cent of the allergenic activity. The elastase-treated material elicited only one or possibly two overlapping precipitation lines, probably representing the fusion of the two almost overlapping precipitation lines of the original DS 22 material which were adjacent to the antigen well. The antigenic determinants responsible for the two close precipitation lines adjacent to the antiserum well apparently were lost (Fig. 1). The materials treated with these enzymes all passed the polyacrylamide gel column without giving any visible zones, whereas the original DS 22 material, mixed with inactivated enzymes, was separated into the distinct fast-migrating zones (P3 and P4) typical for DS 22 as such (Fig. 2).

Neither biologic allergenic activity nor antigenic properties in immunodiffusion were altered by the removal of the N-terminal (arginine) and the C-terminal (aspartic acid, glutamic acid, and arginine) amino acids by aid of leucine aminopeptidase and carboxypeptidase, respectively. Disc electrophoresis of the enzymes themselves resulted in no visible zones in the gel in the concentrations used, but when DS 22 was treated with the carbohydrases mentioned, the resulting material was separated by disc electrophoresis into 4 closely migrating fast-moving zones which apparently occurred as 2 pairs, each representing one of the main zones in the original DS 22 material (Fig. 2). The details of these experiments will be published separately.¹¹ There was no difference in reactivity between dialyzed and nondialyzed material.

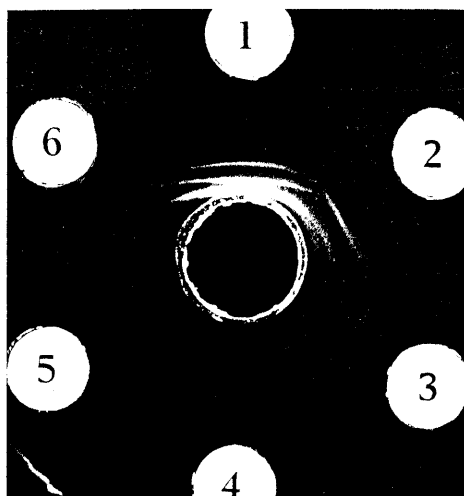


Fig. 3

Results of hydrolysis of DS 22 with pepsin for 30 min. (1), 1 hr. (2), 2 hr. (3), 4 hr. (4), 8 hr. (5), and 24 hr. (6) as shown in double diffusion-in-gel experiment. Antiserum in central well. Antigenicity (pattern) lost after hydrolysis for between 2 and 4 hr.

Effect of partial hydrolysis with pepsin, trypsin, and pronase

With both pepsin and trypsin, the hydrolysis time needed for complete inactivation of the allergen was found to be between 2 and 3 hours in standard experiments. Hydrolysis for shorter time periods resulted in nondialyzable products still possessing the ability to elicit positive reactions in both direct and indirect skin tests. Some loss of allergenic activity occurred, however, even



Fig. 4
Result of treatment of DS 22 with pronase for 24 hr. as shown by polyacrylamide gel disc electrophoresis. Product gives one distinct fast-migrating protein zone.

after short-term hydrolysis, and there was apparently some correlation between reduction of activity as judged by the size of reactions elicited and the extent of hydrolysis. This matter was not investigated in detail in this study however.

Hydrolysis resulted also in a gradual loss of ability to react in immunodiffusion with the specific antiserum (Fig. 3). The complete disappearance of antigenicity in this respect occurred simultaneously with the complete loss of allergenic activity.

Treatment with pronase also resulted in a gradual loss of the allergenic activity, but considerable time was needed for complete inactivation. DS 22 was apparently relatively resistant to degradation by pronase. Even after 24 hours' treatment, the material contained components giving one distinct protein-staining zone in disc electrophoresis experiments (Fig. 4), and this material still possessed some allergenic activity. Treatment for 48 hours destroyed all activity, however.

Effect of *in vitro* digestion with carbohydrases

Treatment of DS 22 with beta-glucosidase, beta-galactosidase, and neuraminidase separately or in combination had no effect on the allergenic activity of the material or on the results of immunodiffusion and disc electrophoresis experiments. However, gas chromatography on the end products showed that the enzymes had not been effective for removal of the sugars present.

DISCUSSION

Most of the allergen extracts used in clinical diagnosis, specific hyposensitization treatment, and experimental work are crude mixtures containing several antigenic proteins. Each single protein present may represent an antigenic mosaic. Some of the antigens may be of no or only minor importance for clinical hypersensitivity, and some contain the major allergenic activity.⁷⁻⁹ We know but little about the structures responsible for the allergenic activity. This lack of knowledge hampers progress in extract standardization, precise clinical and experimental work, and the development of reliable *in vitro* methods in allergy. Much thorough work has been carried out in different laboratories for the purification of allergens and most of this work has been concerned with inhalant allergens. Allergen fractions have been isolated from various sources.^{9, 10-25} No convincing evidence has been put forward so far that strictly purified, uncontaminated allergens have been isolated. Detailed analyses of the chemical composition of allergenic fractions which are not completely purified appear to be of restricted value, as any component found is as likely to represent non-allergenic impurities as the allergenic, active principle in question. Such analyses may, however, furnish some data mandatory for further purification steps. Within certain limits, the information obtained may be representative for the allergen as such. This applies to the effect of enzymes on allergenic activity of the treated materials, at least where destruction of this property results.

As reported elsewhere, the allergen extract from cod contains minor allergenic components as well as the major allergen.⁷ The present study was concerned with the major allergen only. The biologic allergenic activity of the latter

is very high. Passive transfer reactions may be elicited with concentrations down to 10^{-12} ng. per 100 ml. with some hypersensitive sera.^{8,9} It has previously been shown that the allergenic activity is associated with determinants that are also antigenic to the rabbit.^{3,4} This was substantiated by the results in the present study. The allergenic activity and the antigenicity as assessed by immunodiffusion were completely destroyed by extensive hydrolysis with proteolytic enzymes, whereas partial hydrolysis resulted in gradual loss of both characteristics, reaching complete disappearance of both at approximately the same extent of hydrolysis. The exception found is digestion with elastase, which is an endopeptidase particularly attacking neutral residues. Treatment with elastase leaves a product containing one or two antigenic determinants and some of the allergenic activity as well. These experiments indicate that the allergenic activity is determined by antigenic determinants of a protein or polypeptide chain above a certain critical size (and less than 14,000 to 16,000 M. W.¹¹).

The results of the *in vivo* digestion experiments indicate that the breakdown of allergenic structures contained in food is incomplete if the allergen is introduced with other proteins into the digestive system, as is usually the case. A portion of ingested proteins may be absorbed from the intestinal tract retaining the allergenic activity, as reported by Walzer²⁶ 40 years ago. The presence of nonallergenic proteins may protect the allergen from being completely hydrolyzed *in vitro* and affected by digestive action *in vivo*. This may be an explanation for the divergency of results obtained by different research workers in this field.^{16, 19, 20, 23, 25, 27-31} Some authors find that the activity is destroyed by some proteolytic enzymes and not by others.²⁰ Recent studies by Augustin¹⁹ and by Marsh and colleagues²⁸ demonstrated that the activity of timothy pollen and rye grass pollen is destroyed by pepsin and trypsin. King and Norman²⁷ report that the activity of ragweed pollen is destroyed by pepsin but not by trypsin. Berrens²⁰ found that the house dust allergen(s) is resistant to trypsin and chymotrypsin but not to pepsin. Various allergens may have rather different determinant structures, which allows for differences in results.

Differences are also reported as to the amount of carbohydrates and the significance of the sugars found in the allergenic fractions of various materials. Stanworth,²⁵ studying horse dander allergens, claimed that the activity appeared to be closely connected with the carbohydrate bonds in the allergenic glycoprotein. Berrens and co-workers^{20, 21} hold that N-glycosidic structures are of fundamental importance for several allergens. Other investigators claim that carbohydrates are of no importance.^{23, 32-34} From our experiments, no conclusions can be drawn, as the carbohydrases used were not effective in splitting the glycosidic bonds. More recent experiments with subcomponents harvested by means of electrofocusing on ampholine columns have indicated that much of the allergenic activity is present in a carbohydrate-free component, which makes further experiments with carbohydrases unnecessary.¹¹ The material obtained by partial proteolytic hydrolysis of DS 22 appears to be well suited for further study of the allergen in question, and the methods used for the preparation and control of such fragments may prove valuable for the study of other allergens as well.

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