

Studies of Hypersensitivity to Fish

Partial Purification and Crystallization of a Major Allergenic Component of Cod*

K. AAS and J. W. JEBSEN

Paediatric Department and Paediatric Research Institute, Rikshospitalet University Hospital, Oslo, and Norwegian Fisheries Research Institute, Bergen

Fish is an important cause of hypersensitivity reactions such as asthma and urticaria in some populations (2). Hypersensitivity to fish is well suited as basis for studies of different aspects of human immediate allergy (2) and has been investigated in some detail by one of us (2-8, 13). The isolation of more purified allergens is, however, needed both for diagnostic and therapeutic purposes and for studies of the basic mechanisms of allergy (1, 31, 33). In a previous paper (8), the isolation of an allergenically active** protein fraction from cod white muscle extract was reported. The active component was separated from the sarcoplasmic proteins (myogens) of the fish by fractionation of the myogen extract on DEAE-cellulose columns. In this way a Fraction A showing minimal activity and a Fraction D showing very high activity were obtained. (These fractions were originally called DEAE I and DEAE III respectively.) Disc electrophoresis and immunodiffusion experiments showed that Fraction D consisted of a heterogeneous group of proteins. The active components of particular interest for the present investigation were found in two distinct and electrophoretically fast moving zones P 3 and P 4 (previously designated D III PAE 3 and D III PAE 4). These zones contained the same, or closely related cross-reacting allergen(s) as assessed by passive transfer cross neutralization tests.

* This study was supported by grants from the Norwegian Research Council for Science and the Humanities (Norges almenvitenskapelige forskningsråd) and from the Norwegian Fisheries Research Fund.

** Subsequently 'active' and 'activity' refer to the allergenic activity as assessed by biological tests.

In the present study attempts were made to purify the major allergenic component present in Fraction D P 3 and D P 4. The active fractions isolated were compared with the active components of cod and of steam emanations from cod in order to check whether the allergen(s) isolated was (were) the same as those usually eliciting hypersensitivity reactions in the clinical sense (2). To throw light on the nature of the allergen(s), fractions corresponding to the P 3 and P 4 proteins were also prepared by different methods such as ammonium-sulphate precipitation and fractionation by heat coagulation.

Material and Methods

Myogen extract. A preparation of sarcoplasmic proteins (myogen) was prepared from cod (*Gadus callarias* L.) as described previously for the reference extract (3) with the exception that distilled water was used as extraction fluid instead of 0.9% NaCl solution containing 0.5% phenol. The extract was dialysed for 72 h at +4°C against large volumes of distilled water, and the precipitates formed were discarded after centrifugation.

Allergen extracts. Reference extracts from cod and salmon (*Salmo Salar*) were prepared as described before (3). Allergen extract from horse dander was purchased from Allergologisk Laboratorium, Copenhagen (2).

Specific rabbit antisera to cod and salmon antigens. These were produced, collected and stored as described before (3).

Cod hypersensitive individuals and control individuals. These were selected among 825 children suffering from allergic urticaria and/or asthma as described in a previous paper (2).

Serum for passive transfer experiments. Serum was collected from a fish allergic donor 'D. E.' as described in a previous communication (4). Serum was also collected from a child [patient No. 83, see (2)] who had previously been shown to possess the capacity to discriminate between cod and salmon with respect to reaginic activity (7). All hypersensitive serum donors selected also showed marked positive skin reactivity to horse dander extracts making them well suited as controls for passive transfer tests. For control purposes, serum was collected also from a non-allergic donor and from an allergic donor with no clinical or skin reactivity to fish (4).

Sephadex gel G 75, bead form, was purchased from Pharmacia Ltd, Stockholm (Sweden). The particle size was stated to be 40–120 μm .

Reference proteins for molecular weight or size estimation. For the estimation of molecular weight (or size) by the molecular sieve method, the following reference proteins were purchased in a 'Molecular Weight Markers (protein)' kit from Mann Research Laboratories, New York (USA) (molecular weights indicated in brackets): Cytochrome C from horse heart (12,400), crystalline ribonuclease A from bovine pancreas (13,680), myoglobin from horse heart, twice crystallized (17,800), beef pancreas chymotrypsin (22,500), beef pancreas trypsin (23,800), pepsin from hog stomach, 3 times crystallized (35,500), human haemoglobin, twice crystallized (68,000).

Fractionation by ammonium sulphate precipitation. This was carried out as described before (16). During rapid stirring a mixture of solid ammonium sulphate and disodium phosphate [100 G $(\text{NH}_4)_2\text{SO}_4 + 0.71\text{ G Na}_2\text{HPO}_4 \cdot 12\text{ H}_2\text{O}$] was added to the protein extract. Precipitates formed were removed immediately after centrifugation and samples of the remaining solution collected for further study. The last fraction precipitated at a

concentration of ammonium sulphate between 90 and 100% saturation at 0°C (4.55–5.35 Mol/l solution) was left overnight in a cold room (+4°C) before centrifugation.

Fractionation by heat coagulation. In the heat coagulation experiments a crude undialysed extract made from equal volumes of minced fresh cod white muscle and distilled water was used. Aliquots of 25 ml each were heated separately in glass tubes for 1 min to the temperature desired and then kept at this temperature in a water bath for approximately 20 min. The extract was then cooled to approximately +5°C, centrifuged at 12,000 rpm for 20 min, and the supernate was collected for study. Fractions obtained after coagulation at temperature increments of 10°C starting at +20°C were used.

Steam distillation of cod muscle components. Approximately 1000 g of cod white muscle tissue was steam-distilled for 3 h. The volume of the distillate obtained was approximately 100 ml. This was stored at +4°C after sterile filtration had been carried out.

Skin testing, passive transfer experiments and passive transfer cross neutralization experiments. These were performed as described previously (4) after the allergenic material in question had been adjusted to total protein concentrations of approximately 20 mg/100 ml. Different series of passive transfer experiments were performed at intervals of more than 5 weeks to avoid the interference by previously injected serum. In each new series one material previously tested was included to check that the reactivity of the recipient's skin had not changed.

Ultracentrifugation.* The ultracentrifugation was carried out with a Spinco analytical centrifuge Model E at the Biochemical Institute of the University in Bergen*.

The measurements was carried out in phosphate buffer, pH 7.3 and ionic strength $I = 0.05$.

The molecular weight was calculated after the method of SCHACKMAN (27) from the values of sedimentation and diffusion made at 20°C corrected to zero concentration.

DEAE-cellulose fractionation, polyacrylamide gel disc electrophoresis, protein determination, sterile filtration, concentration of dilute fractions, dialysis, double diffusion-in gel experiments, and photographic recording were performed as described in previous papers (3–8).

Experiments and Results

Production of the Major Allergen Fraction D

The cod myogen extract was separated on DEAE-cellulose columns 28 × 900 mm with the use of phosphate buffers at pH 5.4 as described before (8). The active Fraction D was eluted with a 0.15 M buffer and collected for further studies after less active proteins had been eluted with 0.01 M buffer at +4°C.

Examination of Fraction D by Disc Electrophoresis Using Different pH

It was attempted to separate Fraction D at different pH. At pH 4.3 most of the protein was precipitated and was unfit for electrophoresis. At pH 6.8 poor separation was obtained. The main protein group was found as one broad and undistinct zone in place of the

* We sincerely thank cand. real. KAREN HELLE, Biochemical Institute, The University in Bergen, for carrying out the molecular weight determination by means of analytical ultracentrifuge, and Professor AGNAR NYGAARD, head of the Biochemical Institute, for facilities put at our disposal.

P 3 and P 4 region. At pH 8.3 Fraction D was separated into 2 distinct and dominant zones corresponding to P 3 and P 4 and 3–6 additional but faintly stained zones as described in a previous paper (8). A similar result was obtained also at pH 9.5. For all subsequent studies buffer pH 8.3 was used in the disc electrophoresis experiments.

Purification of the Major Allergen(s) by Means of Repeated Gel Filtration

Freeze-dried and dialysed Fraction D material was filtered through Sephadex G 75 (column 28×800 mm) with a 0.05 M NH_4HCO_3 buffer pH 8.2. This buffer was also used by JOHNSON and MARSH (17) for the separation of allergens in rye pollen. Two fractions were obtained, D S 1 and D S 2 (Fig. 1). Fraction D S 1

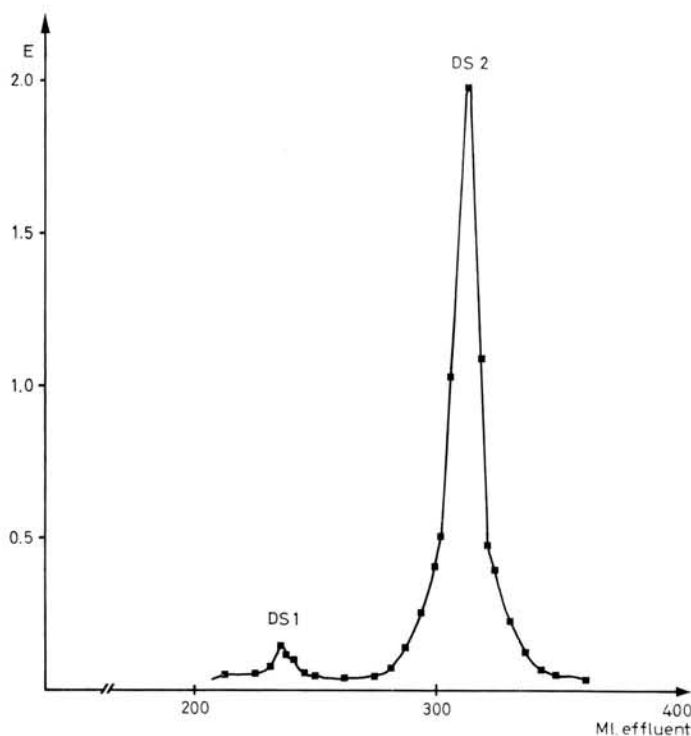


Fig. 1. Elution diagram for first gel filtration of the allergenic material (Fraction D). — Column: 28×800 mm. Buffer: 0.05 M NH_4HCO_3 , pH 8.3. Material: 100 mg dialysed and freeze-dried Fraction D, solved in 2 ml of the buffer. — Flow rate: 18 ml/h. — Yield: D S 1: 8.5 mg, D S 2: 70.2 mg.

contained minimal allergenic activity. This fraction was shown to contain 7 components after separation by disc electrophoresis (Fig. 3). The fastest migrating component was located to the P 3 zone region, but was relatively narrow and faintly stained. Fraction D S 2 was very active. By disc electrophoresis D S 2 was separated into two distinct and dominant zones corresponding to P 3 and P 4 and a third minor zone in front of P 3 [corresponding to P 2 (8)].

Freeze-dried, dialysed Fraction D S 2 was filtered a second time on the same Sephadex G 75 column with the same buffer. Again two fractions were obtained, D S 21 and D S 22 (Fig. 2). The quantity of D S 21 was small compared to that of D S 22. Both these fractions were separated by disc electrophoresis into distinct P 3 and P 4 protein zones. A faint zone corresponding to P 2 could also be distinguished for both preparations when the freeze dried ma-

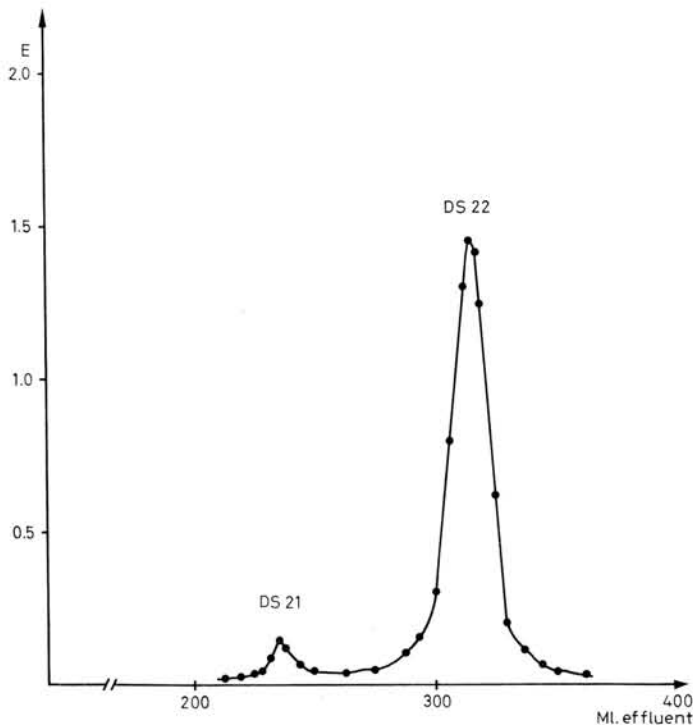


Fig. 2. Elution diagram for second gel filtration of the allergenic material (Fraction DS 2). Column: 28×800 mm. Buffer: $0.05 \text{ M NH}_4\text{HCO}_3$, pH 8.3. Material: 80 mg dialysed and freeze-dried Fraction D S 2 in 5 ml of the buffer. — Flow rate: 16–18 ml/h. — Yield: D S 21: 3.2 mg, D S 22: 58.5 mg.

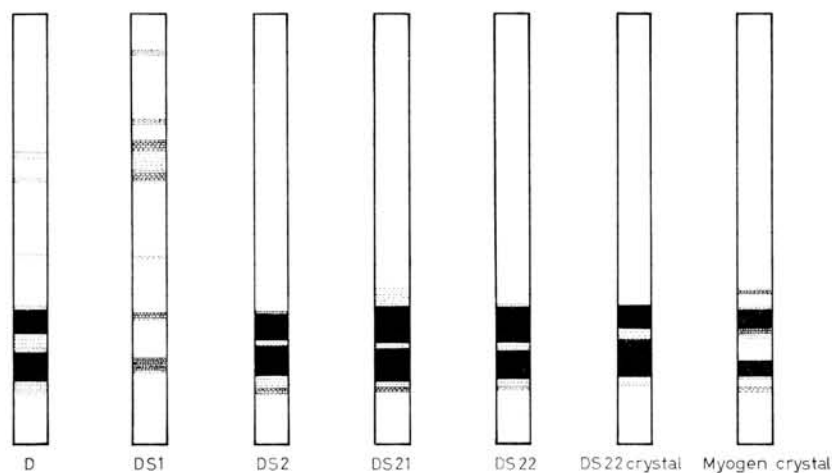


Fig. 3. Schematic illustration of results of polyacrylamide gel disc electrophoresis separation of Fraction D, D S 1, D S 2, D S 21, D S 22, D S 22 crystal and the myogen fraction crystallized at a concentration of 4.55–5.35 mol/l of ammonium sulphate.

terial was applied in concentrations of 100 mg/100 ml or more (Fig. 3). On repeated Sephadex filtration of the main fraction D S 22 small peaks corresponding in elution volumes to D S 21 occurred in addition to those corresponding to D S 22, indicating that D S 21 may at least partly represent a denaturation product of D S 22. As shall be detailed later both D S 21 and D S 22 were very active.

Disc Electrophoresis of Fractions obtained by Ammonium Sulphate Precipitation

Disc electrophoresis of the protein fractions obtained by ammonium sulphate precipitation of myogen at various degrees of salt saturation, showed that the highest proportion of P 3 and P 4 proteins was achieved at 90–100% saturation and in a crystalline fraction obtained at this saturation. However, all these fractions contained more impurities detectable in the disc electrophoresis preparations than D S 22 (Fig. 3).

Crystallization of the Myogen Fraction D S 22

As the crystallization product obtained from myogen at 90–100% saturation with ammonium sulphate had been found to be rich in P 3 and P 4 proteins, but also contained proteins not found in the D S 22 preparations, it was considered worthwhile to attempt a similar crystallization of the purer D S 22 material. Very cautiously,

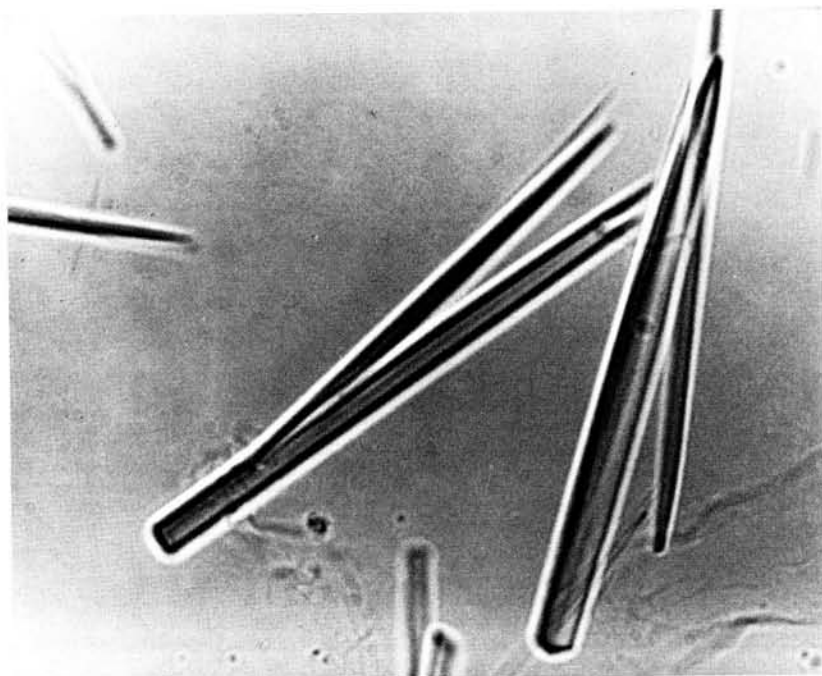


Fig. 4. Photography of crystals obtained from the D S 22 fraction of cod myogen by precipitation with ammonium sulphate at a concentration of 4.55–5.35 mol/l at 0°C (Magnification approximately $\times 4800$).

0.47 g of the phosphate-buffered ammonium sulphate was added stepwise to a solution of 20 mg D S 22 dissolved in 1 ml of distilled water at 0°C. As soon as a faint opalescent turbidity was observed the solution was left at room temperature for approximately 5 h to allow the crystallization to be completed. The mother liquid (the supernatant fluid after centrifugation) was collected for further studies. The crystals obtained are depicted in Fig. 4. They were washed with a saturated ammonium sulphate solution, and were then dissolved in 1 ml of distilled water. This solution and the mother liquid were dialysed for 48 h against large volumes of distilled water at +4°C, and were used in further studies after sterile filtration had been carried out.

Disc Electrophoresis of D S 22 and the D S 22 Crystal Solution

Both D S 22 and the D S 22 crystal solution were separated into two main fractions, P 3 and P 4, on disc electrophoresis. When the



Fig. 5. Results of disc electrophoresis separation of a solution of Fraction D S 22 crystals (total protein content approximately 50 mg/100 ml).

concentration of the material applied exceeded 100 mg/100 ml of total protein, however, trace amounts of stained protein could also be detected corresponding to P 2 (Fig. 3, 5).

Comparison of P 3 and P 4 Eluates from Different Fractions by Immunodiffusion

Fractions P 3 and P 4 obtained after disc electrophoretic separation of the different main fractions were compared in double-diffusion-in gel experiments with the specific reference antiserum. Gel pieces 0.5 cm long were cut corresponding to the P 3 and P 4 zones in question as described before (8) and were eluted with 0.5 ml each of 0.9% NaCl solutions containing 0.5% phenol. P 3 and P 4 eluates from the following fractions were compared: Myogen not coagulated at $+100^{\circ}\text{C}$, the ammonium sulphate fraction (90–100% saturation) from myogen, fractions D, D S 21, D S 22 and the D S crystal solution. Each eluate contained 2–4 antigens. With all solutions, P 3 and P 4 antigens showed identity patterns. Both P 3 and P 4 eluates from Fraction D S 22 and the D S 22 crystal solution contained the same 4 antigens (Fig. 6).

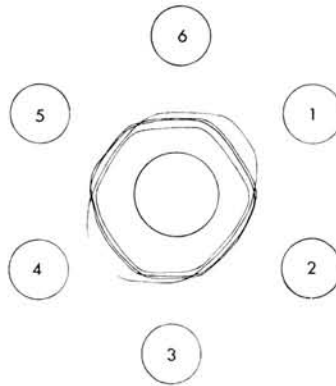


Fig. 6. Immunodiffusion experiment. Patterns obtained when disc electrophoresis gel eluates of P 3 and P 4 from D S 22 and D S 22 crystals are allowed to react with the reference antiserum to cod extract. Materials: D S 22, P 3 (1), D S 22 crystal, P 3 (2), D S 22, crystal, P 4 (3), D S 22, P 4 (4), D S 22 crystal, P 3 (5) and D S 22, P 4 (6). Antiserum in central well.

Allergenic Activity Assessed by Direct Skin Testing

Preliminarily, scratch tests were performed on 6 cod hypersensitive individuals and on 6 control individuals tolerating fish, with fractions D S 1, D S 2, D S 21 and D S 22. Likewise the myogen not coagulated at $+100^{\circ}\text{C}$, the fraction obtained by ammonium sulphate precipitation of myogen at 90–100% saturation and a solution of the crystals obtained at this $(\text{NH}_4)_2\text{SO}_4$ saturation (after dialysis and sterile filtration) were similarly tested on 2 hypersensitive individuals and 2 controls. The D S 1 material elicited only slight positive (+/++) reactions, but all the other fractions included resulted in strongly positive (++++) reactions. Fraction D S 22 and the D S 22 crystal solution were used in scratch tests on a total of 18 fish hypersensitive individuals and resulted in marked positive reactions (++++) in all 18. All control individuals showed negative reactions. Furthermore, D S 22 and the D S 22 crystal solution were used in 10-fold serial dilutions on 6 cod hypersensitive individuals and compared with the reference extract from cod, a myogen extract and Fraction D. It was found that D S 22 and the D S 22 crystal fraction were 100–10,000 times more active than the original reference extract (Fig. 7), depending on the individual sensitivity of each patient and with reservations taken regarding the limitation of the test method accuracy (18, 31).

Allergenic Activity Assessed by Passive Transfer Testing

Serial ten-fold dilutions were made from the original concentration of approximately 20 mg/100 ml of total protein of the following solutions: The reference extract, myogen extract, the solution of crystals obtained by ammonium sulphate precipitation (90–100% saturation) of myogen, fractions D, D S 21, D S 22, the D S 22 crystal solution and the steam distillate. These were used in passive transfer experiments to determine the lowest concentration of each capable of eliciting a significant three plus (+++) reaction. The most active fractions were compared with each other in a second passive transfer series, but other fractions were tested only once. The area of each wheal elicited was measured also in mm² by transferring the marked outline of the wheal to section paper by translucent plastic tape and counting the number of squares (mm²) covered.

The highest activity was found in the D S 22 crystal solution (lower end-point total protein concentration 2×10^{-10} mg/100ml). The crystals obtained by ammonium sulphate fractionation (90–100% saturation) of myogen, and fractions D S 21 and D S 22 appeared equally active (lower end-point at 2×10^{-9} mg/100 ml). The mother liquid obtained after crystallization of D S 22 elicited three plus (+++) reactions in concentrations of approximately 2×10^{-7} mg/100 ml of total protein. Three plus (+++) reactions were elicited by the steam distillate from cod at a 1/10 dilution of the original concentration obtained. Within the limits of the methods used for assessment of activity, the D S 22 crystal solution appeared 1000–10,000 times more active than the reference extract from cod (Fig. 7 and Table I).

In vivo Cross Neutralization Tests. Challenge of Neutralized Sites by Injection

In vivo cross neutralization (passive transfer inhibition) tests were performed with fractions D S 22, D S 22 crystals and the steam distillate, as well as with the reference extract from cod as described for other cod extract fractions in a previous paper (4). The design and the results of these experiments are outlined in Table II. Both neutralized and not neutralized sites were also tested by the subcutaneous injection of 0.1 ml of the reference extract containing approximately 20 mg/100 ml of total protein, in the upper arm of the recipient but distant to the serum sites. In this way the extract

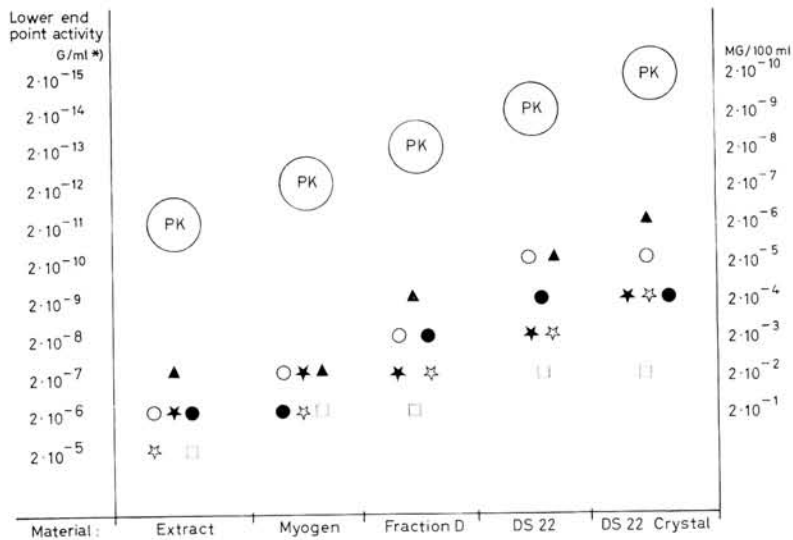


Fig. 7. The results of direct skin (scratch) tests on 6 cod hypersensitive children and of passive transfer tests (PK) with the major allergen fractions from cod compared with the reference and myogen extracts. * Concentration expressed as total protein content in g/ml (left side) and mg/100 ml (right side).

allergens reached the test sites by circulation. These experiments demonstrated that D S 22, D S 22 crystals, the steam distillate and the reference extract contain identical or completely cross-reacting allergen(s) acting as the major allergen(s) for the two cod hypersensitive serum donors in question (Table II).

Similar *in vivo* cross neutralization tests were also performed with eluates from the P 3 and the P 4 zones obtained after disc electrophoresis of D S 22. These experiments showed that the P 3 and P 4 eluates contained the same or cross-reacting allergen(s), each eluate being capable of complete neutralization of the sites to reactions to the other eluate.

Challenge of Sensitized and Neutralized Sites by Ingestion

The reactivity of both neutralized and not neutralized sites was also tested by the ingestion of cooked cod as such. Four hours after the neutralization of sensitized sites with D S 22, the D S 22 crystal solution and the steam distillate from cod had been completed, the serum recipient ate a meal consisting of approximately 200 g of cooked, freshly drawn cod. A few minutes after the ingestion, whealing reactions occurred in the sensitized, un-neutra-

Table I

Results of passive transfer tests with determination of lower end point concentration capable of eliciting a three plus (+++) reaction for different fractions of cod.

Material	Concentration used (mg/100 ml)*	Wheal area (mm ²)
Histamine (0.1%)		115-162
Control (diluent)		0- 12
Reference extract	2×10^{-6}	135-156
Steam distillate (1/10)		127-143
Fraction D	2×10^{-8}	135
Fraction D S 1	2×10^{-2}	119-134
Fraction D S 2	2×10^{-8}	122-155
Fraction D S 21	2×10^{-9}	102-131
Fraction D S 22	2×10^{-9}	114-160
Fraction D S 22 crystal solution	2×10^{-10}	127-142
Fraction D S 22 supernate**	2×10^{-7}	101-110

* Concentration expressed as total protein.

** Fraction D S 22 supernate: the supernate (or mother liquid) after D S 22 crystals have been sedimented down by centrifugation.

lized sites, and developed into large reactions (+++ + + + +). Sites neutralized with the above mentioned materials showed negative responses. No positive reactions were elicited in the control serum sites.

Control challenges. All sites sensitized with serum from the patient with species (e. g. cod and salmon) specific reagins were next challenged by the injection of approximately 0.02 ml of the reference extract from salmon (3). The testing with this extract of the sites that had been neutralized with respect to cod fractions resulted in positive (+++/+++ +) reactions in all instances. A final challenge of all sites was performed with horse dander extract (1/1000). This resulted in marked positive (+++ + + + +/+++ + + + +) reactions in all sensitized sites, but not in the sites injected with serum from the non-allergic donor. These challenges demonstrated that the sensitized sites still possessed the ability to react with whealing and erythema although the reactivity to the major allergen(s) from cod had been exhausted by specific inhibition.

In vitro Inhibition of the Allergic Activity by Absorption with the Reference Antiserum to Cod Extract

One volume of a solution of D S 22 containing 20 mg/100 ml of total protein was mixed with nine volumes of the reference anti-

Table II
Summary of passive transfer cross neutralization tests.

1st and 2nd antigen injected (mg/100 ml)*	1st injection	2nd injection	Reaction to (area of wheal in mm ²):				Steam distillate**	Horse dander extract 1:1000
			3rd injection reference extract 20 mg/100 ml	D S 22 2×10^{-2} mg/100 ml	D S 22 crystal 2×10^{-2} mg/100 ml	D S 22 2×10^{-2} mg/100 ml		
Reference extract (2×10^{-5})	141	0	0	0	0	0	0	575
D S 22 (2×10^{-7})	260	0	0	0	0	0	0	510
D S 22 crystal sol. (2×10^{-7})	304	0	0	0	0	0	0	582
Cod steam distillate (undiluted)	281	0	0	0	0	0	0	490
Control (diluent)	0	0	1285	1082	1124	231	605	
Histamine 0.1%	115-142							

* Concentration expressed as total protein.

** The cod steam distillate was used undiluted, as directly obtained.

Table III

Results of direct skin (scratch) testing with the allergen fraction D S 22 from cod myogen** used unabsorbed and after absorption with rabbit antisera to cod extract and salmon extract respectively.

Patient*	D S 22**	Scratch test reaction to:		
		D S 22 absorbed with specific antiserum to cod extract**	D S 22 absorbed with (control) antiserum to salmon extract**	The (control) antiserum to salmon extract
13	+++++	—	+++++	—
29	+++++	—	+++++	—
66	+++++	+	+++++	+
77	+++++	—	+++++	—

* Number refers to patient number in the clinical material previously described (2).

** Concentration of fish protein approximately 2 mg/100 ml (see text).

serum to cod extract and the reference antiserum to salmon extract respectively, giving a calculated final fish protein content of approximately 2 mg/100 ml. The mixtures were thoroughly shaken and then allowed to stand at $+37^{\circ}\text{C}$ for 2 h. After centrifugation the supernate was allowed to stand at room temperature for another 20 h, and centrifuged again to remove new precipitates. The supernate collected was used in skin (scratch) testing of 4 cod hypersensitive children previously shown not to react to salmon [patients Nos. 13, 29, 66 and 77 (2, 7)]. In all instances the D S 22 material appeared inactive after absorption with the cod extract antiserum, whereas the activity was retained unchanged or only slightly reduced in the D S 22 material treated with the antiserum to salmon extract. This corresponded with the results of similar experiments previously performed with the crude reference extract from cod and the same antiserum (4) (Table III).

Estimation of Molecular Weight (or Size) of D S 22 Proteins by Gel Filtration

Fraction D S 22 was sieved through Sephadex G 75 gel to estimate the molecular weight or size (25, 29, 32) as compared to known proteins using the method described by WHITAKER (32). A standard curve was established for the Sephadex G 75 column (29×800 mm) with the use of seven proteins with known molecular weights, and with the same buffer as used in the previous gel filtrations. The results were plotted on a semilogarithmic scale and the

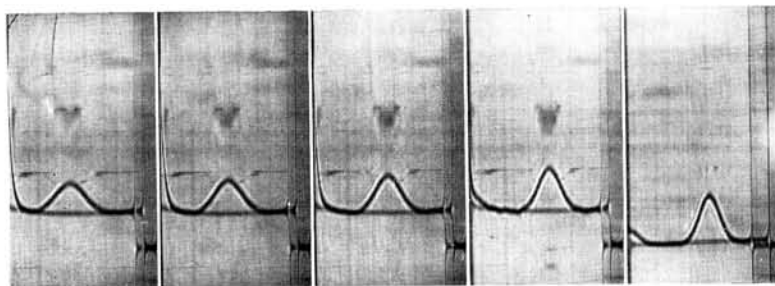


Fig. 8. Ultracentrifugal analysis of Fraction D S 22 (673 mg/100 ml total protein concentration). – Time of centrifugation: 40 min at 10,589 rpm followed by 48 min at 59,780 rpm. Photographs taken at intervals during the centrifugation, direction of migration from left to right. – $S_{20W}^0 = 1.4$, $D_{20W}^0 = 5.8$, Mol. W. = 17,000.

molecular weight (or size) was estimated from the volume of eluant of the D S 22 peak. With this method the molecular weight (or size) of D S 22 appeared to be of an order between that of chymotrypsin (m. w. 22,500) and that of human myoglobin (m. w. 17,800), or approximately 18,000.

Estimation of Molecular Weight of D S 22 Proteins by Ultracentrifugation

Fraction D S 22 gave one single peak (Fig. 8) when analysed in the ultracentrifuge at 59,780 rpm (in phosphate buffer pH 7.3, $I = 0.05$). The sedimentation coefficient was found to be 1.4 and the diffusion coefficient 5.8. The molecular weight of D S 22 was calculated to be 17,000.

Discussion

Highly purified allergens are needed for diagnostic and therapeutic purposes (33). The object of the present study has been to isolate the allergenic component in cod muscle and to elucidate its nature so that it can be used as a tool in further research on human immediate allergy. The isolation of Fraction D S 22 and highly active protein crystals from this fraction appears to be a promising step towards this goal. The simultaneous availability of a principally inactive component, Fraction A, isolated from the same allergen extract (8) provides valuable possibilities for assessment of *in vitro* methods in allergology (6, 8, 13). Fish allergy has given a unique chance, not found with inhalant allergy, to test whether the isolated allergens are identical to the major allergens in the natural source. This study shows that the major allergen(s) of the fish is (are) retain-

ed in the partially purified Fraction D S 22 and in D S 22 crystals. It also shows that the trace amounts of allergen present in the steam from cooking fish, capable of eliciting clinical reactions in susceptible individuals (2), are identical with the major allergen(s) present in the fish itself and isolated in Fraction D S 22.

As reported elsewhere (4), cod extracts also contain minor allergenic components that play a varying role from patient to patient, but this study was only concerned with the major allergenic component(s). The complete inhibition of the allergenic activity obtained by absorption with specific precipitating rabbit antisera substantiates the fact that the major allergen(s) is (are) also antigenic to the rabbit. The specificity of the absorption experiments was borne out by the utilization of fish species discrimination found in allergological respect (7) as a control measure. Our results show that it is most likely that the allergenic determinant for man is identical to one or more of the antigens demonstrated in the immunodiffusion experiments.

The biological activity of the partially purified fish allergen(s) was found to be very high. With the use of the more sensitive intracutaneous skin test method instead of scratch test, positive reactions would have resulted from even more dilute allergen solutions than those stated. The activity of the components have been indicated as the lowest concentration capable of eliciting three plus (+++) reactions, as this degree of reactivity has been found to represent clinical significance in most fish hypersensitive patients, provided that dilute extracts are used (2, 8). Positive reactions of lesser extent were elicited in passive transfer test by ten- to hundred-fold further dilution of the active solutions in question. The lack of uniform and precise methods for assessment of allergenic activity precludes comparisons with the activity stated for other allergens isolated (10, 12, 17, 18, 19, 24, 30).

Although it has been possible to prepare a highly active crystallization product of Fraction D S 22, it must be contended that the purification of the major allergen(s) of cod has not been completed. Fraction D S 22 and the crystal fraction of it were shown by immunodiffusion to contain four different antigens. Fraction D S 22 and the D S 22 crystals were both separated by disc electrophoresis into two fractions (P 3 and P 4), and all 4 antigens were found in both [thus, not only one of the antigens was in common as first suggested (8)]. This limitation in the separation of the antigens is in

many ways comparable with observations made by others. JOHNSON and MARSH (17) have isolated the major allergen of rye pollen. Electrophoretic separation showed two fractions, α and β , but both fractions appeared to have the same molecular weight (32,000) and chemical composition. The antigenic properties and specific activity were stated to be identical for both fractions, but lack of information regarding allergenic activity, as assessed for instance by cross neutralization tests *in vivo*, does not permit conclusions as to whether α and β were allergenically identical or not. KING *et al.* (18, 19) have isolated the major allergens of ragweed pollen. The electrophoretic separation showed two main fractions and two minor fractions. The latter were considered to be artefacts formed during isolation. The molecular weights, chemical composition and antigenic properties were the same for the two major components. MORRIS *et al.* (24) isolated a castor bean allergen. This component was separated into two fractions by electrophoresis, but the fractions had identical antigenic properties. Referring to our own observations regarding the fish fractions D S 22, P 3 and P 4, we must join these authors in concluding that the structural differences in the protein resulting in differences of charge, must be too slight to impart immunological distinctness. Highly purified allergens have been isolated from various sources as reported by different investigators (9, 10, 11, 12, 14, 15, 17, 18, 19, 20, 21, 22, 23, 24, 26, 30), but the results of many of these can not be compared with ours due to differences in methods used. No convincing evidence has been forwarded so far that uncontaminated allergens have been isolated.

At the present stage it is not possible to decide whether all 4 antigens found in the isolated Fraction D S 22 and the D S 22 crystals are present in fresh cod as such. One or more of the antigens may represent degradation products of the naturally occurring ones. The four antigens demonstrated may represent different proteins or molecular variants of the same allergenic protein. The allergenic activity may be caused by haptenic material conjugated to one or more carrier proteins (14, 22). Previous experiments with fish have, however, demonstrated that although some allergenic activity may be found in the dialysate from cod extracts, this activity is minimal in comparison to that of the non-dialysable components, and of significance only in a small proportion of cod allergic children (4). If the major allergen is of haptenic nature, it must be firmly conjugated to the carrier protein.

Since the major allergen component represents only a small proportion of the fish proteins, it is natural to question the cause of its high sensitizing effect. Fraction D S 22 has the principal antigens in common with the fraction obtained by ammonium sulphate precipitation at 90–1000% saturation. This fraction has been shown by JEBSEN and HAMOIR (16) to represent a group of myogens which is not present in mammals. They differ from mammalian myogens in having a low molecular weight, deviating amino acid composition and an acidic character. Hypothetically, this may have a connection with allergenicity, such proteins probably being more easily recognized as 'foreign' by the immune apparatus of disposed individuals. Pollen allergens have also been shown to be acidic (18, 19). Detailed analyses of the chemical composition of allergenic fractions which are only partly purified may appear to be of restricted value. Any protein, peptide or carbohydrate component found in the material is as likely to represent non-allergenic impurities as the allergenic activity in question. Further isolation without degradation of pure allergens is necessary before studies of their chemistry can throw full light on mechanism of their action. However, with the available methods it is questionable whether it is possible to obtain a strictly pure protein without degradation (28).

The quantities yielded by the crystallization procedure and by elution of the disc electrophoresis sub-fractions of D S 22 are too small to be of more than theoretical interest, but they demonstrate representative properties of Fraction D S 22 and indicate that further purification may be achieved. Until that can be completed the highly active Fraction D S 22 from cod furnishes material for renewed investigations concerned with clinical and laboratory work in allergology, such as allergen extract characterization and standardization (3, 5), allergological and immunological fish species discrimination (7), assessment of *in vitro* methods (6, 13), and refined clinical methods for diagnosis and specific treatment (2). These are problems relevant not only to hypersensitivity to fish, but to many types of immediate allergy in human medicine.

Summary

A highly active allergen fraction (D S 22) was isolated from an extract of cod white muscle tissue (myogen) by the combined use of separation on DEAE-cellulose and repeated filtration on Sephadex

G 75 gel. By ammonium sulphate precipitation of this fraction, a crystalline protein was obtained which showed the highest activity. This fraction and the crystal solution obtained were shown to contain the major allergen(s) present in cod, the reference extract from cod, and in steam collected from cooking cod. The major allergen fraction contained 4 antigens. By disc electrophoresis at alkaline pH, it was separated into two components that were antigenically and allergenically identical. Absorption experiments showed that the allergen(s) was (were) also antigenic to the rabbit. The molecular weight of the active fraction (D S 22) was approximately 18,000 as estimated by the molecular sieve method and 17,000 as calculated after ultracentrifugation analyses.

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Authors' addresses: Dr. Kjell Aas, Paediatric Department and Research Institute, Rikshospitalet, Oslo;
J. W. Jebesen, Norwegian Fisheries Research Institute, Bergen (Norway).