

Characterization of a Major Allergen (Cod). Chemical Composition and Immunological Properties

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Introduction

Allergy to fish is well suited for model studies of the chemical and immunological nature of the allergen(s) [1-5]. A very active allergen (DS 22) has been isolated from cod and crystallized [4]. Fraction DS 22 appears to be one of the purest food allergens reported and the only one that has been crystallized. The present work reports some studies concerning the chemical, physical and immunological nature of this major allergen.

Material and Methods

Allergen, the major allergen (DS 22) was prepared from cod (*Gadus callarias* L.) white muscle tissue as described in previous papers [3, 4], with slight modifications as outlined in the preparation scheme (fig. 1). Chemical reagents and equipment used were as described previously [1-5]. Besides the following equipment were utilised: ratio recording spectrophotometer, Beckman Dk. 2A. Quantitative amino acid analyzer, Beckman Unichrome. Analytical ultra centrifuge, model E, manufactured by Spinco Division, Beckman Instrument Inc. Cellulose 300 MN, Macherey-Nagel and Co. D-516, Düren, Germany. Amino acids standards, Fluka AG, Buchs SG, Switzerland.

Determination of the C-terminal and N-terminal amino acids. Carboxypeptidase 'A' was activated according to HIRS, MOORE and STEIN [19]. Leucine aminopeptidase was activated as described previously [18], avoiding inhibitors [17].

Enzymic incubation. Incubation of the antigen with the enzymes was performed as outlined elsewhere [5].

Identification of the C-terminal and N-terminal amino acids by thin layer chromatography. Cellulose 300 MN was used as coating material. N-butanol, acetic acid and water 80:20:20 V/V as solvent. 4-8 sets of analyses were made for each material investigated.

UV absorption spectra. These were performed for 1 mg/ml solution of the material in phosphate buffer pH 7.4 and in 0.1 N NaOH solution at pH 10. Scanning from 360 to 220 nm was done in 1 cm lightpath cuvettes.

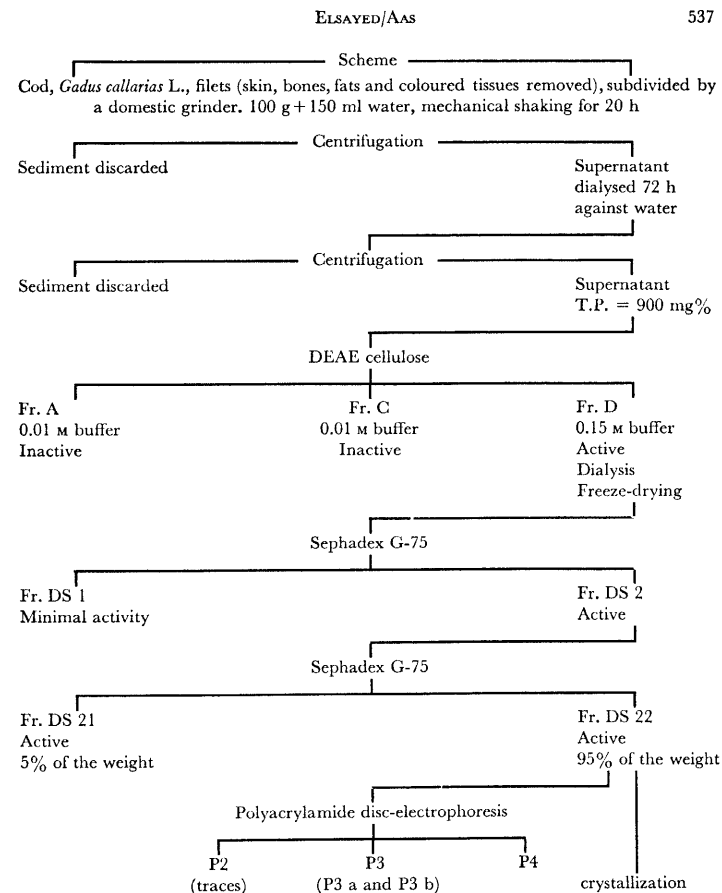


Fig. 1. Preparation scheme of (DS 22).

Hydrolysis of the protein for sugar estimation. This was performed in 4 mg aliquots as described in detail elsewhere [12]. For acetyl neuraminic acid release, acid hydrolysis was performed according to GOTTSCHALK [14], and enzymic hydrolysis was done by subtilisin for 48 h [5].

Gas-liquid chromatography. The method described by CLAMP *et al.* [8] was used. 5-10 mg aliquots of the material in question were hydrolysed. Samples of 0.1-5 µl of the silyl derivatives were injected to one of the two columns of the gas chromatograph. The temperature

of the column was adjusted to 180, 220 or 250°C according to the desired experimental conditions [12]. The amount of monosaccharides was calculated according to LEHNHARDT and WINZLER [24].

Monosaccharides splitting. This was performed by enzymic methods [5] and by sodium metaperiodate oxidation [21].

Amino acid composition. Acid hydrolysis [11] took place at 110°C for periods of 18, 24 and 48 h. Enzymic hydrolysis was done according to Dus *et al.* [11]. Hirs' method [19] for per-formic acid oxidation was employed and was followed by acid hydrolysis [11]. Amino acid analysis was done as described before [9, 10]. Tryptophan was estimated as outlined previously [25]. By assuming that arginine has one residue, the numbers of residues of the other amino acids were calculated and corrected to the nearest integer.

Ultracentrifugation and partial specific volume determination. These were performed according to SCHACHMAN [27].

Results

C-terminal and N-terminal amino acids. By thin layer chromatography it was shown that carboxypeptidase 'A', had liberated aspartic acid, glutamic acid and arginine. A fourth faint spot was shown in some plates and could not be identified as amino acid.

The incubation with leucine aminopeptidase did not result in any liberation of the N-terminal amino acids in the first 30 min of incubation with the substrate. After incubation of 3 h a spot having R_f comparable to arginine was observed.

Effect of liberating the C-terminal and N-terminal amino acids on antigenicity as assessed by double-diffusion-in-gel. The antigenic composition of DS 22 was not affected by the splitting of the C- and N-terminal amino acids and by the subsequent removal of Arg, Glu and Asp from the former and Arg from the latter, as demonstrated in the precipitation system used.

Allergenic activity test. The allergenic activity was not significantly altered by the removal of Arg, Asp and Glu from the C-terminal, and of Arg from the N-terminal or both.

UV spectrophotometry and fluorescence investigations. A standard curve was plotted for the protein in effect. OD at 260, 280 and 290 nm were registered for samples of increasing concentrations (fig. 2).

The spectra differed from the usual pattern observed with other proteins (fig. 3). A nearly constant absorption was found between 259 and 283 nm. Several small peaks at 258, 264, 268 and 280 nm were considered to be caused mainly by phenyl alanine and tyrosine, and a bigger peak at 290 nm was attributed to tryptophan, whereas the

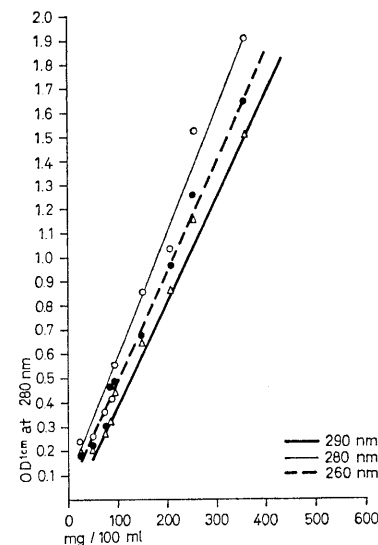


Fig. 2. Standard curves of (DS 22) as obtained by UV absorption.

augment in absorption occurring at 245 nm could be caused by histidine, methionine, cysteine together with the absorption caused by peptide bonds. The shift of the spectrum towards longer wave lengths at pH 10, was considered a result of the ionization of the (OH) group of the tyrosine in highly alkaline medium. DS 22 showed no fluorescence when seen under UV light of wave length 253.7 and 366 nm at a distance of 6 in from the UV lamp.

Estimation of the carbohydrates by gas chromatography. The value obtained for the total monosaccharides, present in DS 22 (table II) was 0.882 mg% (fig. 4). This corresponds to one half or one residue per mole of the allergen (cf. estimated molecular weight). No peaks were detected at the expected relative retention time of sialic acid as assessed by standard runs.

Attempts to split of the monosaccharide moiety. This was performed both by enzymic degradation and by sodium metaperiodate oxidation. Preliminary trials with metaperiodate oxidation were performed for

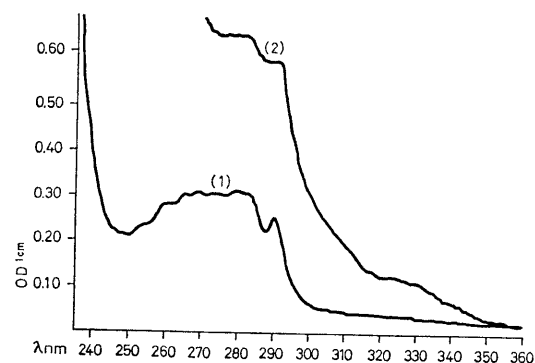


Fig. 3. UV spectra of 0.1% solutions of neutral DS 22 (1) and alkaline DS 22 (2).

Table I. Reaction to skin tests in 6 fish hypersensitive patients

Material	No. ¹ 13	29	66	77	80	88
0.1% histamine	+++	+++	+++	+++	+++	+++
Control solution	-ve	-ve	-ve	-ve	-ve	-ve
Fr. DS 22	+++++	++++	+++++	+++++	+++++	+++++
Fr. DS 22/carboxypeptidase, 10 min	+++++	++++	○	+++++	+++++	○
Fr. DS 22/LAP 10 min	+++++	○	++++	+++++	○	+++++
Fr. DS 22/LAP/carboxypeptidase, 10 min	+++++	++++	+++++	+++++	○	○
Leucine aminopeptidase	-ve	-ve	-ve	-ve	-ve	-ve
Carboxypeptidase 'A'	-ve	-ve	-ve	-ve	-ve	-ve
Fr. DS 22 incubated for 10 min	++++	++++	++++	+++++	++++	○

¹ Numbers referring to patient's number in the clinical material. ○ Not done.

6 and 24 h respectively. The uptake was measured spectrophotometrically. Analysis of the sugar content of the dialysed products was performed by gas chromatographic method (fig. 5). All the mono-

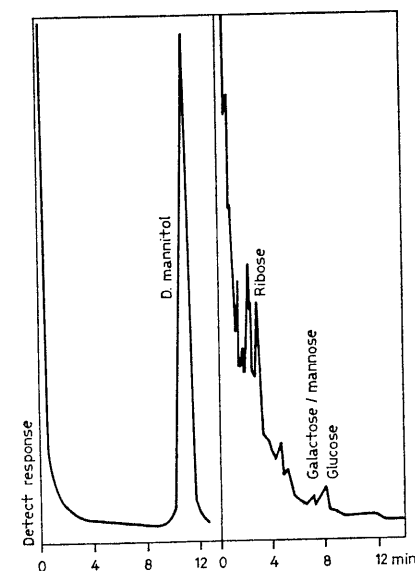


Fig. 4. Gas chromatograms of D-mannitol and (DS 22) at 180°C.

Table II. Relative retention time and amounts of identified monosaccharides in (DS 22)

Relative retention time	Identification	Amount mg/100 mg
0.11	not identified	-
0.18	not identified	-
0.25	ribose	0.44
0.39	not identified	-
0.62	galactose/mannose	0.102
0.71	glucose	0.340
1.02	Mannitol	-
	contamination	-
Total amount of sugar in Fr. DS 22		0.882%

saccharides present in DS 22 were detected in both the enzymic and metaperiodate oxidation products (table III). No peaks were obtained with lactose/metaperiodate oxidation system, indicating that the

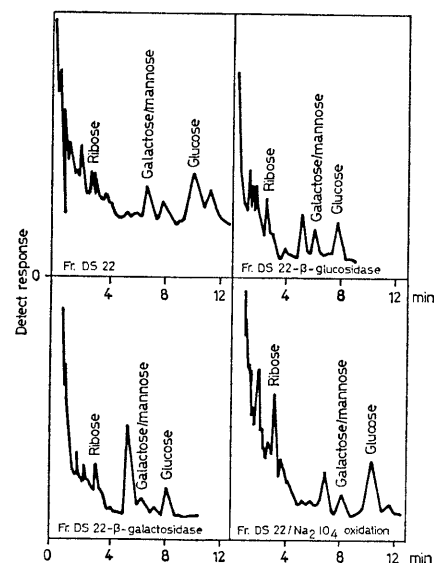


Fig. 5. Gas chromatograms of the products obtained after attempts to split the sugar moieties from (DS 22).

metaperiodate had oxidised lactose and resulted in formaldehyde and formic acid which do not give peaks on gas chromatographic registrations. The sugars were not splitted from DS 22 protein chain(s) by the methods used.

Allergenic activity and antigenicity of the products of enzymic degradation. No remarkable differences were observed in the skin reactions when the enzyme-treated allergen was compared to the control samples in the skin testing. The antigenic determinants were not affected as judged by results obtained when materials were allowed to diffuse in gel against the specific rabbit antiserum.

Amino acid composition. The amino acid analysis data depicted in table IV show that the neutral amino acids form the major proportion of the molecule (Gly 10, Ala 23, Val 6, Thr 2, Leu 7 and Ile 6). The sulphur containing amino acids were represented in 3 residues. From 0-1 residues were found for histidine, arginine, proline, tyrosine and

Table III. Identification of sugars in the enzymic and oxidative degradation dialyzates of (DS 22). *a* and *β*-p indicate *a* and *β* pyranose isomers respectively

Fr. DS 22/ <i>β</i> -glucosidase Relative retention time	Identification	Fr. DS 22/ <i>β</i> -galactosidase Relative retention time	Identification	Fr. DS 22/ Na ₂ IO ₄ Relative retention time	Identifi- cation
0.26	ribose	0.26	ribose	0.252	ribose
0.63	galactose/ mannose	0.605	gal./mann.	0.630	gal./mann.
0.70	glucose <i>a</i> -p	0.69	Glu <i>a</i> -p	0.693	Glu <i>a</i> -p
1.19	glucose <i>β</i> -p	1.20	Glu <i>β</i> -p	—	—

Table IV. Amino acid composition of (DS 22)

Amino acid	Moles/mole protein	Nearest integer
Lysine	13.86	(14)
Histidine	0.30	(0)
Ammonia	5.61	(6)
Arginine	1.00	(1)
Aspartic acid	14.88	(15)
Threonine	1.76	(2)
Serine	5.16	(5)
Glutamic acid	9.70	(10)
Proline	0.15	(0)
Glycine	10.29	(10)
Alanine	23.11	(23)
Valine	6.36	(6)
Methionine	0.70	(1)
Isoleucine	5.56	(6)
Leucine	7.18	(7)
Tyrosine	0.53	(1)
Phenyl alanine	10.28	(10)
Cysteic acid	1.60	(2)
Tryptophan (25)	0.78	(1)
Total	—	120

tryptophan. The 14 residues of lysine were the ones responsible for the positive charge of the allergen. Whereas the dicarboxylic amino acids causing the acidic nature of the allergen had 15 residues of Asp and 10 residues of Glu. A high number [10] was found for Phe as compared to the other aromatic amino acids.

Tryptophan was estimated to 0.77 and 0.80% in both the enzymic hydrolysed and the native allergen respectively. This was found to form 0.78 residue/mole, which was corrected to nearest integer 1 residue/mole (table IV).

Ultracentrifugation analysis. The sedimentation coefficient was determined by ultracentrifugation at 68,000 rpm and 21°C, in 0.1 M phosphate buffer at pH 7.2.

$$S_{\text{obs}} = 1.89 \cdot 10^{-13}, s = 1.89 S,$$

$$S_{20, w} = 1.91 \cdot 10^{-13}, s = 1.91 S.$$

The diffusion coefficient was performed at 8,000 rpm at 20°C in a synthetic boundary cell.

$$D_{\text{app}} = 11.5 \cdot 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$$

$$D_{20, w} = 11.75 \cdot 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$$

The partial specific volume was calculated from the amino acid composition [27] and a value of 0.73 was obtained. By using this value the molecular weight of the sedimentary matter was estimated to be 14,500.

Discussion

The basal mechanism of hypersensitivity reaction is still an enigma. Many problems have to be solved before a fair explanation is attained. Among these problems are the chemical nature and the specific immunogenic properties of the allergens responsible for induction of the pathological immune-reactions. Many authors have studied the chemistry of different allergens by aid of various methods of separation and purification. Most of these studies were concerned with inhalant allergens. Food allergens have received less attention, in spite of being also important causes of different allergic diseases.

UV absorption spectra of cod allergens are comparable with those of myogens of other fish species. They show characteristics similar to

those of a myogen fraction (component 3) isolated from carp [23]. The peaks at 253, 259 and 265 nm which were found in the carp component 3 are found also in cod white muscle allergen studied. Close similarity was found between the UV spectrum of DS 22 and the fast component of the myogen fraction reported by RAO *et al.* [26].

The estimation of the small amount of sugars in DS 22 was considered necessary in view of the possible importance of the glycosidic bond in allergic activity [6, 28]. The major allergenic component of cod (DS 22) have been found to be glycoprotein in nature, containing small amounts of monosaccharides. The sugar-peptide bonding was highly resistant to carbohydrase degradation as well as to metaperiodate oxidation. This suggests the existence of a firm kind of bonding between this peptide and the monosaccharides. A similar rigidity was also reported by JOHNSON and MARSH [20] to be characteristic for grass pollen allergens.

Studies by BERRENS and BLEUMINK [7] of the UV absorption spectra of a number of partially purified allergenic fractions, have shown some very specific characteristic features departing from the usual absorption curves of proteins or polysaccharides. They supposed that this disparity was due to glycosidically bonded chromophores incorporated in the molecular structure, and responsible for the specific absorption maxima in the spectra.

Further work in this laboratory has, however, resulted in the isolation of a subcomponent of DS 22 containing no carbohydrates detectable by gas chromatography, but still containing high allergenic activity [13]. The determination of minute amounts of sugars (less than or equal to 1%) in glycoproteins can be disturbed by the interference of the large excess of amino acids and polypeptides. These have resulted in unidentified small peaks of trimethyl silyl derivatives of non-sugar components. The same difficulty has been encountered by others [24].

It was actually difficult to separate galactose and mannose under the conditions of experiment. However, the amount of galactose and mannose appeared negligible, therefore further attempts of their resolution were abandoned.

The three amino acids liberated by carboxypeptidase 'A' from the C-terminal may be representing 3 successive amino acids on one chain, or terminals on different polypeptide chains. The release of arginine, in the first 5 min of the incubation was difficult to interpret as

Table V. Comparison between cod allergen DS 22 and other fish proteins

Amino acid	Carp			Tilapia		Cod
	Comp. 2	Comp. 3	Comp. 5	Comp. 2a	Comp. 2b	Fr. DS 22
Phenyl-alanine	11	8-9	10	8	9	9
Tryptophan	0	0	0	0	0	1
Tyrosine	1	0	0	0	0	1
Methionine	0	0	0	0	0	1
Cysteine	0	1	0	1	2	0-1
Proline	1	0	1	0	0	0-1
Histidine	0	1	1	1	2	0-1
Arginine	1	1	1	1	1	1
Lysine	12	10-11	13	10	11	12

long as there was no available information whether the residues have been released by successive attack on one chain or by simultaneous hydrolysis of more than one chain.

The amino acid composition of fraction DS 22 was rather peculiar as compared to the other proteins. The unbalanced composition in the cyclic amino acids, with a high number of phenylalanine residues and a low number of tryptophan, tyrosine and histidine was comparable with the findings of other investigators for fish sarcoplasmic proteins [15, 16]. The similarity between cod allergen DS 22 and other fish sarcoplasmic proteins, the corresponding data of carp and tilapia is illustrated in table V as modified after HAMOIR [16].

Recently RAO *et al.* [26] have isolated two fractions of the low molecular weight proteins from the white muscle tissue of cod. A close similarity was found between DS 22 and those two fractions in their amino acid composition. Without considering the allergenic activity of DS 22, they claimed DS 22 to be a mixture of the two proteinic fractions. However, the relation between these two fractions and DS 22 can be clarified only after more detailed comparative studies.

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Summary

The characterization of a partially purified and crystallized major allergen from cod white muscle myogen, previously designated DS 22 was reported. By enzymic methods it was shown that its C-terminal amino acids were Arg, Asp and Glu, and its N-terminal amino acid was Arg. The major allergen is glycoprotein in nature, containing small amounts of pentose and hexoses, but no sialic acid. The characteristics of this glycoprotein are different from other proteins. The molecular weight of DS 22 was re-estimated to be 14,500 by sedimentation and diffusion methods.

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Cellular Immunity

Activity and Properties of Human Migration Inhibitory Factor

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The capillary macrophage migration inhibition test with peritoneal exudate cells (consisting of macrophages 50% and lymphocytes 20%) was established as an *in vitro* expression of delayed hypersensitivity of animals by GEORGE and VAUGHAN [1962]. Modification of this test showed that sensitive lymphocytes served as the mediating cells, affecting the activity of macrophages, (bystander) effector cells of delayed hypersensitivity. Lymphocytes were separated from peritoneal exudate [BLOOM and BENNETT, 1967] or peripheral blood [BARTFELD and KELLY, 1968; DUMONDE, HOWSON and WOLSTENCROFT, 1968] of guinea pigs having delayed hypersensitivity. When sensitive lymphocytes were incubated in medium containing specific antigen, they produced migration inhibitory factor (MIF). Supernatant fluid of such cultures inhibited the migration of macrophages present in peritoneal exudate of unsensitized guinea pigs. AL-ASKARI, DAVID, LAWRENCE and THOMAS [1965] found that histocompatibility antigens did not influence this *in vitro* test system.

Similar studies of human *in vitro* delayed hypersensitivity were precluded due to the unavailability of sufficient numbers of human macrophages. THOR and DRAY [1968] reported that human lymph nodes could serve as a source after several days of special processing in culture. We had found that tracheo-alveolar washings of guinea-pig lungs contained many macrophages capable of functioning as the migrating cells for *in vitro* tests of delayed hypersensitivity with guinea-pig peripheral lymphocytes or MIF produced by the latter [BARTFELD and ATOYNATAN, 1969]. We subsequently studied human *in vitro* delayed hypersensitivity utilizing macrophages present in broncho-