

References

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Partial Characterization of Homogeneous Allergens (Cod)

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The isolation and crystallization of a protein fraction (DS 22) containing the major allergen(s) in cod white muscle myogen has been reported [1, 2]. This fraction was found to contain small amounts of monosaccharides. The amino acid analysis of this fraction revealed peculiar composition of the aromatic amino acids with a high proportion of phenyl alanine residues and a low proportion of tryptophan and tyrosine residues. The molecular weight of DS 22 was estimated to be 14,500 by sedimentation and diffusion methods [10]. The effect of enzymatic degradation on the biological allergenic activity was also reported [3]. Further purification of DS 22 by isoelectric focusing on carrier ampholytes in a sucrose density gradient resulted in the isolation of one major and 5 minor constituents which were designated according to their estimated isoelectric points. The allergenic activity and antigenicity of the subcomponents were investigated and an allergenic identity was demonstrated between Fr. pI 4.75 and DS 22 [11]. Fr. pI 4.75 was shown to contain the major allergenic determinant(s) of cod and was considered a suitable representative for model studies of the allergenic proteins.

The results given below present the chemical composition, the UV absorption characteristics and the sedimentation analysis for Fr. pI 4.75 and for some of the other polypeptides separated by the isoelectric fractionation technique.

Material and Methods

Fr. pI 4.75 was isolated from the white muscle myogen of cod (*Gadus callarias*) as outlined elsewhere [1, 2, 11]. A ratio-recording spectrophotometer, Beckman DK. 2A was used for UV spectra scanning. Quantitative amino acid analyzer, Beckman Unichrome was utilized

for the amino acid composition. For sedimentation analysis an analytical ultracentrifuge Spinco Model E, equipped with an electronic speed control was used. The rest of the equipment and chemicals were purchased as described elsewhere [1, 2, 3, 10].

Ultraviolet absorption spectra. Scanning was performed from 360 to 220 nm on 0.1% aliquots in phosphate buffer at pH 7.4, in 10-mm light-path length quartz cuvetts.

Carbohydrate analysis. 2–5 mg of the samples were mixed with 2 ml 0.5 M HCl in dry methanol. Methanolysis (at 70°C for 24 h) and acetylation were performed according to the procedure described by CLAMP *et al.* [6]. For sialic acid release, acid hydrolysis was employed according to GOTTSCALK [12]. The dry residues were dissolved in 250 μ l of a mixture of pyridine: trimethyl silyl chloride: hexamethyl disilazane in the ratio of 5:1:1 v/v. The suspensions were allowed to settle down at room temperature and 0.1–2 μ l of the clear supernatants were injected onto one of the columns. The temperature of the column was adjusted to 180 or 220°C for the hexoses and pentoses or the acetyl neuraminic acid analyses respectively. D-Mannitol was chosen as standard reference both with regards to quantitative and qualitative analyses since it is stable and gives only one peak well separated from the other monosaccharides. The amount of monosaccharides was estimated according to the relation used by LEHNHARDT and WINZLER [15].

Amino acid composition. Constricted 15-ml-glass tubes were soaked in conc. HNO₃ for one week before the analyses were run, then thoroughly washed and dried. Aliquots of 2 mg of the material in 2 ml 6 N HCl were allowed to freeze, the tubes were filled with purified nitrogen gas and sealed under vacuum. Hydrolysis took place at 110°C for 24 h. The acid was removed *in vacuo* over NaOH pellets and the dried residues were stored at –20°C until the analyses were performed. The procedure of HIRS *et al.* [13] for performic acid oxidation was used, and the material was subsequently treated with 6 N HCl hydrolysis as described before. Enzymatic hydrolysis was done by means of subtilopeptidase 'A' as outlined elsewhere [3].

The one column system of DUS *et al.* [7, 8] was utilised. Tryptophan estimation was done as outlined before [17]. The amount of each amino acid in μ M was estimated relative to a standard calibration mixture of amino acids of 0.25 μ M of each component. The latter was applied to the amino acid analyzer column before the samples were analyzed. Nor-leucine was included in amounts of 0.25 μ M as an internal marker in both calibration mixtures and samples. The number of residues of Fr. pI 4.75 were calculated relative to arginine which was assumed to form one residue per mole. For the other constituents the number of residues were calculated relative to threonine. The values obtained were corrected to the nearest integer.

Analytical ultracentrifugation. The allergens were dissolved in 0.1 M phosphate buffer at pH 7.2. Diffusion coefficients were determined by the 'maximum ordinate-area' method [9] and the observed sedimentation and diffusion coefficients were corrected to standard conditions ($S_{20, w}$ and $D_{20, w}$) according to the relation given by SCHACHMAN [20]. The partial specific volume was evaluated from the amino acid composition according to the procedure described by SCHACHMAN [20]. The sedimentation coefficient was determined at a rotor speed of 68,000 rpm at 20°C using a 12-mm aluminium, double sector centerpiece. The photographs were taken with the interference optics. The diffusion coefficient was measured in the centrifuge at 14,000 rpm at 20.5°C using a charcoal filled epon, double sector, capillary type, synthetic boundary cell. The photographs were taken with the Schlieren optics.

Results

UV-Absorption Spectra

The spectra of the subcomponents (fig. 1) showed some similarity to the spectrum of the original material DS 22 [10]. The constant

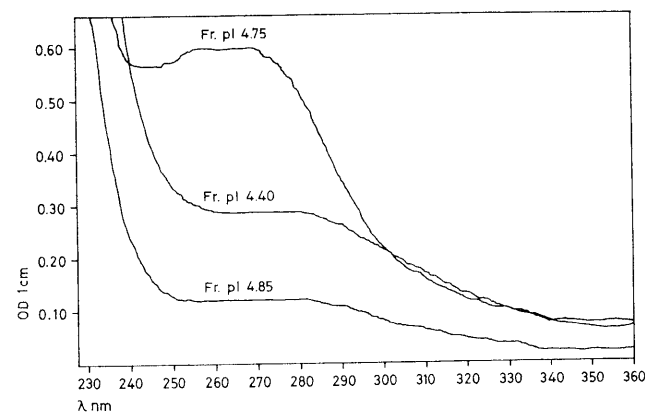


Fig. 1. UV-absorption spectra of 0.1% samples of the subcomponents and the parent material DS 22.

absorption zone (between 258 and 280 nm) which was found in DS 22 was also shown in the subunits with a shorter range (255–270 nm) in case of Fr. pI 4.75. The augment at 290 nm for Fr. pI 4.75 was probably due to tryptophan and the one between 240 and 250 nm for all the subunits was likely due to the absorption caused by the peptide bonds together with other factors.

Gas-liquid chromatography. The highest sugar content (1.5%) was found for Fr. pI 4.75 (table I) which was quantitatively the most important constituent and also contained most of the allergenic activity.

Fraction pI 4.85 contained no sugars as no peaks were observed under the same experimental conditions as for the other constituents.

Fraction pI 9.60 and fraction pI < 3.00 were not investigated as they were separated close to the electrodes of the isoelectric focusing column [11] and were allergenically almost inactive fractions.

Amino acid composition. Fraction pI 4.75 contained a much higher proportion of amino acids residues than the other fractions (table II). As in the case of fraction DS 22 the neutral amino acids form the major part of the chain(s) (table III). Lysine was represented in 12 residues responsible for the positive charge of the material. Other basic amino

Table I. Gas-liquid partition chromatography of the sugar contents of the separated fractions arranged according to their successive isolation from the isoelectric focusing column [11]
(1) Relative retention time, (2) monosaccharide, (3) amount (% of weight)

Fr. pI 5.00			Fr. pI 4.85			Fr. pI 4.75			Fr. pI 4.40		
(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
0.23	Ribose	0.037				0.24	Ribose	0.206	0.24	Ribose	0.134
0.42	not identified	—	Carbohydrate			0.44	not identified	—	0.44	not identified	—
—	—	—	free, no			—	—	—	0.51	not identified	—
0.56	Galactose/ Mannose	0.001	peaks			0.55	Galactose/ Mannose	Traces	0.58	Galactose/ Mannose	Traces
—	—	—	observed.			0.65	not identified	—	—	—	—
0.80	Glucose	1.005				0.74	Glucose	1.253	0.73	Glucose	0.154
Total		1.043				Total		1.459	Total		0.288

Table II. Amino acid composition of the constituents isolated by isoelectric focusing of fraction DS 22 [11]

Material	Fr. pI 5.00	Fr. pI 4.85	Fr. pI 4.75	Fr. pI 4.40
Amino acid	Assumed No. of residues per mole			
Lysine	(1)	(1)	(12)	(1)
Histidine	(1)	(0-1)	(0-1)	(1)
Ammonia	—	—	(10)	—
Arginine	—	—	(1)	—
Aspartic acid	(3)	(3)	(13)	(2)
Threonine	(0-1)	(0-1)	(1)	(0-1)
Serine	(5)	(3)	(6)	—
Glutamic acid	(5)	(4)	(10)	(3)
Glycine	(8)	(4)	(10)	(5)
Alanine	(3)	(3)	(20)	(1)
Valine	(2)	(2)	(7)	(2)
Methionine	(1)	—	(0-1)	(1)
Isoleucine	(1)	(1)	(5)	(1)
Leucine	(1)	(1)	(4)	(1)
Tyrosine	(0-1)	(0-1)	(1)	(0-1)
Phenyl alanine	—	(3)	(10)	(1)
Cysteic acid	—	—	(1)	(0-1)
Tryptophan	—	—	(1)	—
Total	33	28	114	22

Table III. Comparison between the amino acid analyses of DS 22 and Fr. pI 4.75 under the same experimental conditions

Material	DS 22	Fr. pI 4.75
Amino acid	Assumed No. of residues per mole	
Lysine	(12)	(12)
Histidine	(0-1)	(0-1)
Ammonia	(6)	(10)
Arginine	(1)	(1)
Aspartic acid	(12)	(13)
Threonine	(2)	(1)
Serine	(5)	(6)
Glutamic acid	(8)	(10)
Proline	(0-1)	—
Glycine	(9)	(10)
Alanine	(19)	(20)
Valine	(5)	(7)
Methionine	(1)	(0-1)
Isoleucine	(5)	(5)
Leucine	(5)	(4)
Tyrosine	(1)	(1)
Phenyl alanine	(10)	(10)
Cysteic acid	(1)	(1)
Tryptophan	(1)	(1)
Total	(105)	(114)

acids, histidine and arginine were represented in (0-1) residue each. Sulphur containing amino acids were represented in one residue for methionine and one residue for cysteic acid. Tryptophan was investigated on both enzymatic hydrolyzates and non-hydrolysed samples and an average value of 0.79% for Fr. pI 4.75 was found. Taking the estimated molecular weight of Fr. pI 4.75 into consideration, it was found that tryptophan forms one residue/mole. The dicarboxylic amino acids form 23 residues for both aspartic acid and glutamic acid which contribute to the acidic nature of Fr. pI 4.75.

The partial specific volume was evaluated from the amino acid composition and a value of 0.725 ml/g was obtained.

Analytical Ultracentrifuge

The sedimentation coefficient, $S_{20, w}$ was determined to be 2.11 S at a protein concentration of 0.15%.

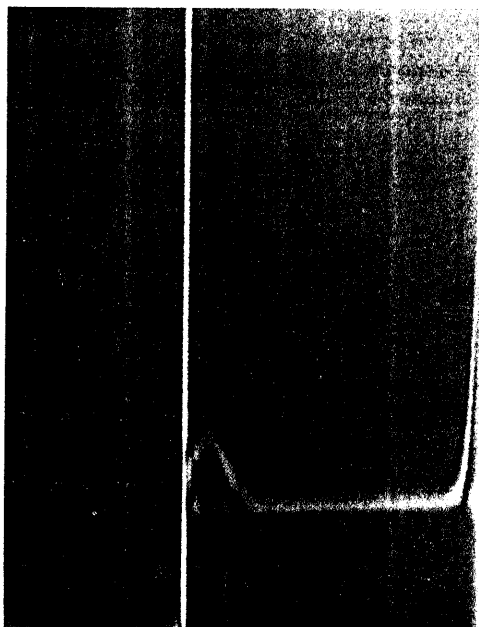


Fig. 2. Sedimentation pattern of Fr. pI 4.75. The photograph was taken at a speed of 68,000 rpm, temperature +20° C.

The diffusion coefficient, $D_{20,w}$ was determined to be $11.74 \cdot 10^{-7} \text{ cm}^2/\text{sec}$ at a protein concentration of 0.3%. Using the value of 0.725 ml/g for the partial specific volume the Svedberg equation yielded 16.200 for the molecular weight of Fr. pI 4.75. One symmetrical peak was obtained (fig. 2) and no heterogeneity was apparent by this method.

Discussion

It has been shown that the purified major allergen Fr. pI 4.75 is representative for the major allergenic activity of cod [11]. This fraction was found to contain small amounts of monosaccharides. BERRENS and co-workers [4, 5] have suggested that the N-glycosidic

linkages represent a universal structural feature among atopic allergens, while others consider the sugar moiety of no influence on the allergenic activity [14, 21]. In previous studies [10] we tried to split the sugar components both by enzymatic and sodium metaperiodate oxidation methods. The carbohydrate components could not be removed from (DS 22) by these methods as shown by gas chromatography. However, one of the constituents isolated by the isoelectric focusing [11], Fr. pI 4.85, possessed high allergenic activity albeit containing no carbohydrate. This fraction was completely identical with the carbohydrate-containing one, Fr. pI 4.75 with respect to the allergenic activity as assessed by cross neutralization tests *in vivo* [11]. Accordingly, we consider the bound sugar moiety of *no direct influence* for the allergenic capacity of fish protein. The determination of small amounts of sugars (ca. 1% or less) in glycoproteins can be disturbed by the interference of large excess of amino acids and polypeptides. These have resulted in some unidentified small peaks of trimethyl silyl derivatives of non-sugar components. The same difficulty has been encountered by other investigators [15].

The UV-absorption spectra of the low molecular weight allergenic albumins, showed unusual patterns differing from the conventional ones of proteins and carbohydrates. High absorption was found for Fr. pI 4.75 and Fr. pI 4.40, between 295–330 nm, which was due to non-identified prosthetic group(s). These albumins are absent in mammalian myogens, do not take any part in glycolysis [18] and their biological function is undetermined as yet. Their amino acid composition was also abnormal. It showed a high number of residues of phenyl alanine (table II) which dominated the other aromatic amino acids. The sulphur containing amino acids were also represented in small numbers (0–1 for methionine and 1 for cysteic acid). Among the basic amino acids lysine had the highest number probably allowing the protein chain(s) to bind to different enzymes or carbohydrates through its ϵ -amino groups.

The amino acid composition of Fr. pI 4.75 showed much similarity to the parent material DS 22 (table III) and to the fast moving fraction isolated by Rao *et al.* [19].

A symmetrical single peak was obtained in analytical ultracentrifugation for Fr. pI 4.75. Small yields were obtained for the other sub-components which did not allow enough material for ultracentrifugation.

Fraction pI 4.75 has shown a high degree of homogeneity as demonstrated by the distinct, intense and fast migrating single band on 7.5% acrylamide gel discs at pH 8.3, by its mono-zone in the isoelectric fractionation, migration in immunoelectrophoresis [11] and by the single peak obtained in ultracentrifugation.

Fraction pI 4.75 contained most of the allergenic determinant(s) of cod low molecular weight albumins. This fraction was, therefore, considered suitable for further studies on the correlation between the protein structure and allergenicity which is presently under investigation.

In subsequent studies Fr. pI 4.75 will be referred to as allergen M.

Acknowledgement

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Summary

Three components with major allergenic activity have previously been isolated from cod white muscle myogen. In the present work partial characterization has been carried out on the components and they have been examined with regard to amino acid composition, carbohydrate composition and ultraviolet-absorption spectrum. The ultraviolet spectra revealed features which are unusual for peptides and believed to arise from unidentified constituents.

The sedimentation and diffusion coefficients were determined for the main component (Fr. pI 4.75) and the molecular weight was accordingly calculated to be about 16,000.

In subsequent studies the main component will be referred to as allergen M.

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