

Characterization of a major allergen (cod)

Observations on effect of denaturation on the allergenic activity

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An allergenically active protein isolated from cod white muscle albumin and designated DS 22 was subjected to denaturation experiments by 8M urea/ β -mercaptoethanol and high concentrations of guanidine hydrochloride. The allergenic activity and antigenicity of DS 22 were maintained after reduction and alkylation by iodoacetamide. The randomly folded material apparently recovered most of its native conformation by spontaneous reoxidation through dialysis against water. This was indicated by the recovery of the molecular immunopotency, ultraviolet spectral profiles, and electrophoretic characteristics. The findings suggest that the allergenic activity of DS 22 is not directly determined by the stereochemical configuration of the protein molecules but by the sequence of certain amino acids.

The isolation of an allergenically active protein fraction, DS 22, from the white muscle tissue of cod has been reported.¹⁻⁴ DS 22 was further separated into allergenic and non-allergenic protein constituents by the isoelectric focusing technique with the use of 8M urea as solvent.⁵ Previous studies¹ showed that the allergenic activity of cod myogen was maintained after precipitation with concentrated acids and denaturation by heat. In the present investigations the correlation between the allergenic activity of DS 22 in its native, denaturated, and renaturated molecular forms is examined.

The effect of denaturation was studied with the use of the reduction of pancreatic ribonuclease (RNase),⁷ elaborated by Anfinsen and Haber,⁶ as a model. In Anfinsen and Haber's experiments the RNase molecule was unfolded by the reduction of its four disulfide bonds by means of β -mercaptoethanol in 8M urea. Reoxidation of the molecule under proper conditions of concentration, temperature, and pH resulted in a major recovery of the native conformation.

MATERIAL AND METHODS

Fraction DS 22 was prepared from the white muscle tissue of cod by procedures described in detail elsewhere.^{1, 2} Chemical reagents were of A.R. grade or the highest quality commercially available. Guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), urea (BDH, Ltd., England), β -mercaptoethanol, monoethyl amine, and iodoacetamide (Fluka, Buchs SG, Switzerland) were used. Other equipment and chemicals were obtained and used as described in preceding papers.¹⁻³

Denaturation of DS 22

Preliminary experiments were performed on two 2 mg. samples. One was dissolved in 2 ml. of freshly prepared 8M urea (Sample 1) and the other in 2 ml. of saturated guanidine

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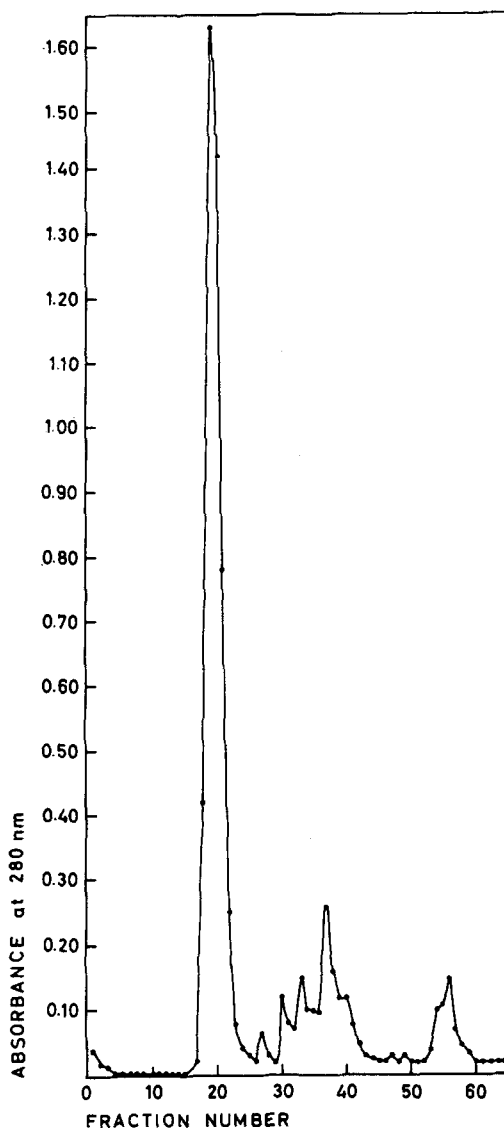


FIG. 1. Elution diagram of Aliquot 3a. Native DS 22 was reduced by β -mercaptoethanol in 8M urea. The pH of the reaction mixture was adjusted to 3.5 by glacial acetic acid, and its was applied on a (2 X 30 cm.) column of Sephadex G-25 pre-equilibrated with 0.1M acetic acid.

hydrochloride (Sample 2). The mixtures were allowed to stand at +4° C. for 24 hours, and then subjected to ultraviolet spectral characterization and allergenic activity tests. Parts of the preparations were dialyzed against distilled water.

Reduction of DS 22

In subsequent experiments the procedure of Anfinsen and Haber⁶ for the reduction of RNase was followed. A sample of 30 mg. of DS 22 was dissolved in 3 ml. of freshly prepared 8M urea. The pH of the solution was adjusted to 8.6 with 5 per cent monomethyl amine.

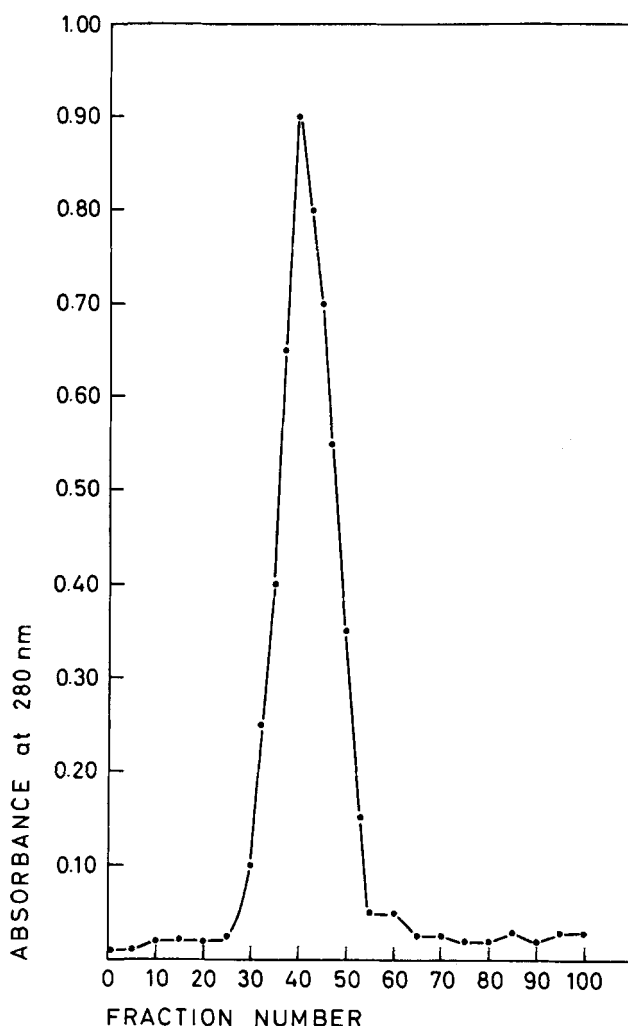


FIG. 2. Refractionation of the urea/mercaptoethanol-treated DS 22. The reduced DS 22 peak eluted from Sephadex G-25 column was subjected to refiltration on a Sephadex G-50 column.

β -Mercaptoethanol (10 mM) was then added, and the tube was flushed with nitrogen gas and allowed to stand at room temperature for 4½ hours. After denaturation, the sample was divided into 3 aliquots (3a, 3b, and 3c) of 1 ml. each. Aliquot 3a was adjusted to pH 3.5 by glacial acetic acid and applied on a Sephadex G-25 column (20 × 300 mm.), which was equilibrated and eluted with 0.1M acetic acid. The three peaks eluted (Fig. 1) were dialyzed against water, and the first one, containing the protein, was lyophilized, dissolved in a minimum volume of ammonium bicarbonate (0.05M, pH 8.2), and applied on a Sephadex G-50 column (30 × 400 mm.) pre-equilibrated with the same buffer (Fig. 2).

Part of Aliquot 3b was rapidly diluted with the 8M urea-mercaptoethanol reagent and used for allergenic activity tests and ultraviolet spectral scanning. The rest of this aliquot was dialyzed against distilled water.

Reoxidation of all the reduced unalkylated DS 22 samples was avoided by nitrogen flushing, sealing the tubes, and storing at -20° C. until the tests were performed.

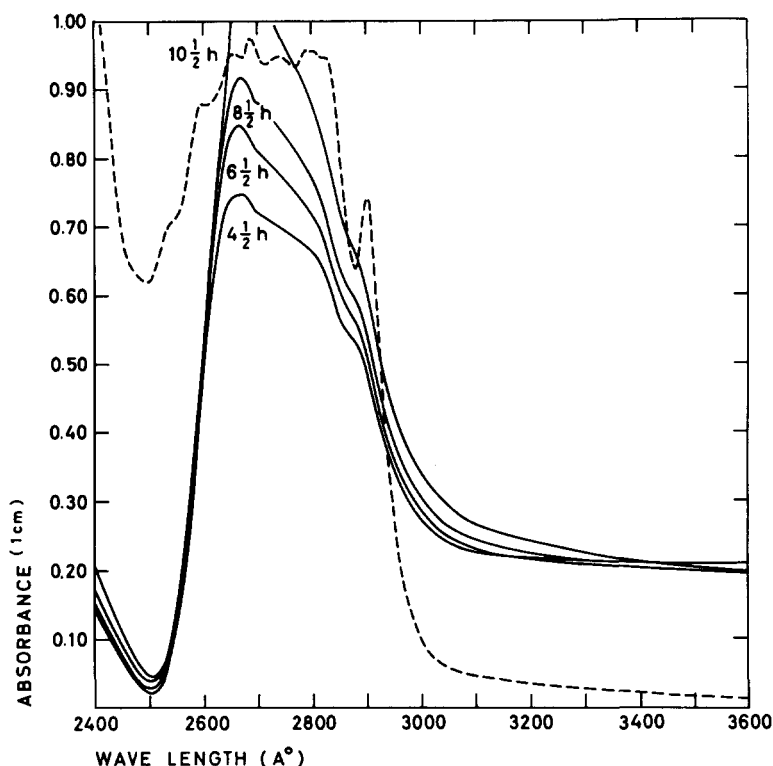


FIG. 3. Ultraviolet spectra of Aliquot 3b. Reduction of DS 22 was performed by mercapto-ethanol in 8M urea for $4\frac{1}{2}$, $6\frac{1}{2}$, $8\frac{1}{2}$, and $10\frac{1}{2}$ hour intervals. The line-dot profile represents the ultraviolet spectrum of the native DS 22 at neutral pH.

Stabilization of the reduced DS 22 by iodoacetamide alkylation

Aliquot 3c was stabilized against spontaneous reoxidation of the sulfhydryl groups by blocking them with 0.5 ml. of 0.5M iodoacetamide. The tube was flushed with nitrogen, and the mixture was allowed to stand for 2 hours at room temperature. The resulting carboxyamido-methyl derivative was diluted with the same reducing-stabilizing reagent to be used in allergenic activity tests, immunodiffusion, and spectral scanning.

Assessment of the allergenic activity

All samples used for biological tests (Samples 1 and 2, Aliquots 3b and 3c) were of 0.2 mg. per milliliter protein concentration and were sterilized by Millipore filtration.² The allergenic activity of the denaturated and the dialyzed samples was compared with that of the native DS 22 by means of passive transfer cross-neutralization tests.⁸ Passive-transfer testing was restricted to the use of two fish-allergic sera and one normal control because of the inherent danger of transferring serum hepatitis to the recipient by this method. Two series of experiments were performed on the volar surface of the forearms of a normal nonallergic volunteer who was sensitized with 0.05 ml. aliquots of the fish-allergic sera and the normal control serum. The sensitized sites were challenged 24 hours later. A volume of 0.1 ml. of sterile, blank denaturing solvent was applied to the skin test sites a few minutes before the challenges. These were performed through the drops of the blank in attempts to avoid reoxidation of the nonalkylated reduced DS 22 (Aliquot 3b) during the biological tests. In order to reduce the effect of irritants contained in some of the samples, only quantities sufficient to leave a bleb with a diameter of approximately 1 mm. were injected

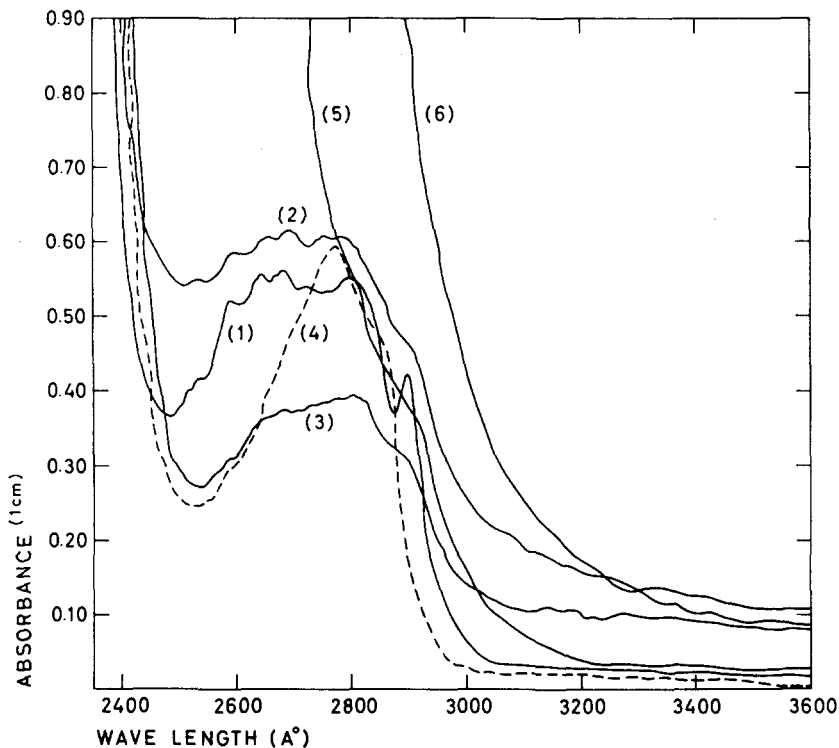


FIG. 4. Ultraviolet spectra of native, denaturated, and renaturated DS 22 and bovine pancreatic RNase as a model. The first 4 samples were in phosphate buffer (0.1M, pH 7.3), while the last 2 were in their corresponding denaturing solvents. (1) Native DS 22 1.5 mg. per milliliter; (2) DS 22 after reduction and fractionation on Sephadex G-25; (3) reduced DS 22 (Sample 1) after spontaneous reoxidation by dialysis against distilled water; (4) RNase 1 mg. per milliliter as a protein spectrum model; (5) 8M urea-denaturated DS 22 for 24 hours at +4° C. (Sample 1); (6) guanidine hydrochloride-denaturated DS 22 for 24 hours at +4° C. (Sample 2).

into the test sites. The reactions were read, registered, and photographed after 20 minutes (Fig. 5). For cross-neutralization tests, the sites were repeatedly challenged at 8 to 12 hour intervals with the native DS 22, Aliquot 3b or 3c, respectively, until the specific reactivity had been completely exhausted. Following this, the site was again challenged with the other material in question. Finally, the cross-neutralization test sites were challenged with an unrelated allergen (cow dander extract, 1:100 w/v), as outlined before,⁹ to assure that the sites still possessed the ability to react. Sensitized sites were also challenged with serial tenfold dilutions of DS 22, Aliquots 3b and 3c, respectively, in order to find the minimal doses capable of eliciting a positive wheal and flare reaction.

Ultraviolet absorption spectra

Recordings were made on a ratio-recording spectrophotometer (Beckman DK 2A) with 1 cm. path length, stoppered quartz microcuvettes for: (1) Aliquot 3b (0.75 mg. protein per milliliter) after 4½, 6½, 8½, 10½, and 24 hours at room temperature (Fig. 3); (2) native DS 22, urea-denaturated DS 22 (Sample 1) before and after dialysis, guanidine hydrochloride-denaturated DS 22 (Sample 2), the protein peak obtained from the Sephadex G-50 column of Aliquot 3a, and RNase (Fig. 4).

Spectral analyses of Samples 1 and 2 and Aliquots 3b and 3c were performed with the

denaturated protein placed in the sample beam of the spectrophotometer and the denaturing agent placed in the reference beam.

Disc electrophoresis was performed in 7.5 per cent acrylamide gel at pH 8.3 by the method of Davis¹⁰ as described in detail previously.¹

Immunodiffusion was performed for: (1) DS 22, the protein peak isolated by gel filtration of Aliquot 3a, and Aliquot 3b following dialysis of these three substances against distilled water; (2) Aliquot 3c in 8M urea/mercaptoethanol/iodoacetamide mixture.

The method¹¹ of Ouchterlony was employed with an antiserum against cod albumins raised in rabbits by the procedure described earlier.¹²

RESULTS

Gel filtration

Sephadex G-25 gel filtration of the reduced DS 22 (Aliquot 3a) resulted in one large protein peak and two small ones, the latter corresponding to mercaptoethanol, urea, and low-molecular weight contaminants (Fig. 1). Refractionation of the protein peak on Sephadex G-50 yielded one single peak (Fig. 2).

Ultraviolet spectra

The absorbance of DS 22 material denaturated by 8M urea or guanidine hydrochloride for 24 hours at +4° C. (Samples 1 and 2) increased steadily toward short wave lengths. A similar spectral profile was found for Aliquot 3c, except that it showed higher absorption at 320 to 360 m μ . The spectral pattern of the dialysis-renaturated DS 22 was similar to that of the native one except for the peak at 290 m μ , which appeared as a shoulder in the renaturated molecular form (Fig. 4). The time course of the denaturation caused by reduction by 8M urea/ β -mercaptoethanol depicted in Fig. 3 showed that the maximum absorbance of the reduced protein (0.75 mg. per milliliter) at 267 m μ increased with time. Masking of the peak at 259 m μ and much lower absorbance at 250 m μ were also observed.

Allergenic activity

The content of the reducing substances present in the solvents for the reduced material used in the biological tests resulted in a transient redness and a burning sensation in all challenged sites. No wheal or flare reaction occurred in the sites sensitized with the control serum, whereas both native and denaturated DS 22 (Aliquots 3b and 3c) elicited large passive-transfer reactions in sites sensitized with the fish-allergic serum (Fig. 5). The reactions to the denaturated material were approximately 25 to 50 per cent larger than those produced by the native material used in comparable protein concentrations. However, comparison of the activity of the two preparations was probably subject to error, since the irritating effect of the solvent for the denaturated material may have enhanced the reactivity of the sites tested with this material. The end dilutions still capable of eliciting positive wheal and flare reactions in allergic serum sites were 2.10⁻¹⁰ mg. per milliliter for native DS 22 and 2.10⁻¹⁰ mg. per milliliter and 2.10⁻¹² mg. per milliliter for Aliquots 3b and 3c, respectively, for both of the two sera used. Each material rendered the sites unreactive to both of the two others, indicating a complete or very closely related identity of the allergenic



FIG. 5. Results of passive-transfer tests on the volar surface of the forearm of a normal volunteer. Reactions elicited in skin sites sensitized with cod-allergic serum by the native allergenic fraction DS 22 (**DS 22**) and Aliquot 3c (**DEN**) in concentrations of 0.02 mg. per milliliter.

activities. The results were reproducible in two repeated experiments with the same materials.

Acrylamide disc electrophoresis at pH 8.3 showed no difference in the number or in the mobility of the bands of the renaturated and native DS 22.

Immunodiffusion

The native DS 22, the protein peak eluted from Sephadex G-50, and the dialyzed Aliquots 3b and 3c were immunologically indistinguishable when allowed to diffuse against the antiserum used. Immunodiffusion of Aliquot 3c in the denaturing mixture also showed reactions of identity with the native DS 22 against the antiserum being employed.

DISCUSSION

Studies by Anfinsen and Haber on RNase showed that the reduction and unfolding of the protein and the consequent loss of the native conformation resulted in the loss of the biological activity. The activity was mostly recovered by reoxidation of the molecule and the reformation of the secondary and tertiary structure.

In our experiments, the reduced material dissolved in dilute acetic acid, in contrast to the native one which is not soluble at lower pH values. This shows that DS 22 was adequately reduced by the procedure employed, in concordance with previous findings with trypsinogen, chymotrypsinogen, lysozyme, and RNase.⁶ The single peak obtained by fractionation on Sephadex G-50 indicates that the covalent structure of the protein constituents was not changed by the reduction method used.

Differences in the ultraviolet spectra offer a convenient method which has been widely used to follow the unfolding processes of proteins.^{13, 14} In the mixture of protein, mercaptoethanol, and 8M urea without the acetamide alkylation, the liberated sulfhydryl groups are not susceptible to reoxidation as long as mercaptoethanol is present in excess. The changes of the peaks at 290, 276, and 259 $m\mu$ representing the tryptophyl, tyrosyl, and phenylalanyl side chains reflect the effect of denaturation and random folding of the molecule (Fig. 3). These changes are probably due to the exposure of the aromatic side chains which had been buried in the protein hydrophobic interior. The spectrum of Aliquot 3c was similar to that of guanidine hydrochloride denaturation (number 6 in Fig. 4). However, the pH value of the preparation became lower from the liberation of HI due to the reaction of iodoacetamide with the sulfhydryl groups.

The allergenic activity was retained despite the molecular denaturation resulting from the treatment by urea and β -mercaptoethanol. Neither the change of the spatial arrangement of the molecule nor the reduction of the protein seemed to decrease the allergenic potency. The assessment of the activity by means of passive transfer tests introduces the possibility that a part of the preparation may become reoxidized and regain its original folding locally in the tissues during the test and that the positive reaction develops only subsequent to such reoxidation. However, the alkylation-stabilized preparation (Aliquot 3c) showed the same degree of reactivity, confirming allergenic potency of the denaturated preparation. Similar experiments performed by Johnson and Marsh¹⁵ and by King, Norman, and Lichtenstein¹⁶ using purified pollen allergens showed that the material was not oxidized during the biological tests. In contrast to our results, the activity of the pollen allergens was lost following reduction and alkylation.

Dialysis of the reduced DS 22 material (Samples 1 and 2 and Aliquot 3b) allowed spontaneous reoxidation of the molecule after it had been randomly folded by the perturbing agents. It is natural to conclude that the molecule recovered most of its native conformation by dialysis subsequent to treatment with high concentrations of urea or guanidine hydrochloride. This was indicated by the similarity of the ultraviolet spectra of the reoxidized and the native protein (Fig. 4). The conditions for the immunodiffusion experiments were far from ideal with the use of Aliquot 3c in the denaturing solvent as antigen. However, the preparation retained its antigenic determinants despite the loss of the original molecular conformation. Ultraviolet absorption differences in the 300 to 320 $m\mu$ region are apparently caused by an unidentified prosthetic group incorporated in some of the protein constituents of DS 22.¹⁷ Modification of the 290 $m\mu$ peak in the native DS 22 to a shoulder-like profile in the reoxidized form can be caused by a slight change in the hydrophobic structure of the material. The unfolding of a protein molecule will also expose buried ionizing groups, thereby changing the net charge of the molecule. The similarity of the migration of both the native and the reoxidized DS 22 on acrylamide disc electrophoresis columns presents also some indication that the protein has retained its original conformation.

The findings of the present study suggest that the allergenic activity of

the major allergen in cod depends on the sequence of certain amino acid residues and is not determined directly by the secondary, tertiary, or quaternary structure of the molecules. Hypothetically, this may have a connection with the characteristics of allergenicity, this type of molecule probably being able to penetrate the mucosal barrier of the allergic individual without losing its allergenic immunodominance.

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