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Purification and Partial Characterization of a *Fucus vesiculosus* Agglutinin

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Fucus vesiculosus agglutinin has been purified to homogeneity by conventional chromatographic procedures and characterized as a mucopolysaccharide with 90 % carbohydrate content. Estimated molecular weight is about 2×10^6 daltons. It has no sub-unit structure and its isoelectric point is 3.2. It contains 1.23 % S, 0.24 % Ca and 0.06 % P.

Agglutinin mediated sheep red blood cell agglutination was inhibited only by glycoproteins with complex lateral oligosaccharide chains resembling some of the oligosaccharide chains found in the erythrocyte membrane glycoproteins. Metaperiodate treatment of the sheep red cells rendered them non-agglutinable. Sequential degradation of the oligosaccharide chains with glycosidases suggests that inner mannose residues are implicated in the receptor binding-sites for the agglutinin. Consequently we think that this agglutinin can be a lectin or a lectin-like molecule with complex saccharide specificity.

Tropical marine algae, British seaweeds and brown algae extracts have been found to contain substances with agglutinating activity (3, 4, 26, 35). As far as we know, previous publications on *Fucus vesiculosus* agglutinins deal with their agglutinating properties but pay very little attention to the chemical nature of the molecule(s) responsible for such activity. In view of the increasing importance of agglutinins, specially lectins, in several fields, as well as the immunomodulatory properties found in *Fucus vesiculosus* extracts in an as yet unpublished work, we considered a chemical characterization of the above mentioned molecule(s) to be of interest (22).

Up to day, the only exhaustively studied algal agglutinin is that obtained from the red alga *Agardhiella tenera* (31).

This paper reports the purification of an agglutinin extracted from the brown alga *Fucus vesiculosus* as a first step for an indepth study of its biological significance.

Materials and Methods

Specimens of the brown alga *Fucus vesiculosus* were collected from Ría de

Vigo (Pontevedra, Spain) and processed within a few hours.

All chemicals were from Sigma (U.S.A.).

Sheep red blood cells were supplied by «Materiales y Reactivos» (Spain).

Meningococcal type B polysaccharide (MBP) was donated by Dr. J. Faro, Departamento de Microbiología, Universidad de Santiago (Spain).

Isolation of the active fraction. Whole algae (10 kg) were carefully cleaned, cut in small pieces and homogenized in a Waring-blendor in 0.15 M NaCl. The resulting suspension, after standing overnight at 4° C, was filtered through a double gauze and centrifuged at 1.000 × *g* for 20 min at 4° C. The pellet was discarded and solid ammonium sulphate was added to the supernatant to a 35 % saturation and, after standing overnight at 4° C was centrifuged as above. The supernatant was collected and saturated to 55 % with more ammonium sulphate. The precipitate was centrifuged as above, dissolved in 0.14 M NaCl - 0.01 M phosphate buffer (pH 7.2) (phosphate-buffered saline), exhaustively dialysed against distilled water and lyophilized.

Purification of the agglutinin. The active fraction was applied to a Sephadex G-25 column (4 × 90 cm) equilibrated with 0.05 M Tris-CHl buffer (pH 7.2) (Tris buffer) and eluted with the same buffer. Fractions showing agglutinating activity were pooled, dialysed and lyophilized. This material was then chromatographed in a Sepharose CL-4B column (1.5 × 100 cm) in Tris buffer. The active fractions were pooled, dialysed and lyophilized. This material is considered as the purified *Fucus vesiculosus* agglutinin (FVA).

Purity criteria. Polyacrylamide gel electrophoresis was done according to DAVIS (8). Agarose gel electrophoresis was done by the method of VAN ARKEL *et al.* (34). Isoelectric focusing in agarose gels was performed as described by BURNETT *et al.* (6). Gels were stained with Coomassie blue (13) for proteins with toluidine 0 (19) or Alcian blue (18) for mucopolysaccharides, and by the periodic acid - Schiff method (16) for glycoproteins. Rate-zonal gradient centrifugation was done in 5 % - 20 % sucrose gradients in Tris buffer in 17 ml polypropylene tubes in a Sorvall OTD-2 ultracentrifuge equipped with a SW 27.1 rotor. The experiment was run for 6 hours at 120.000 × *g* at 4° C. At the end of the run fractions of 0.5 ml were collected and analyzed for hemagglutination and density. Analytical gel-filtration was performed in a Sepharose CL-4B column (0.9 × 100 cm) in Tris buffer.

Analytical methods. Protein was estimated by the KJELDAHL (33), LOWRY *et al.* (23) and dye-fixation (5) methods. The aminoacid composition was determined in a Liquimat III analyzer (Kontron) after hydrolysis of 10 mg of FVA in 6 N HCl, under nitrogen, in sealed ampoules, et 110° C for 24 hours. Norleucine was used as an internal standard.

Neutral sugars were estimated with reference to the glucose by phenol - sulphuric acid (11) and the anthrone (25) procedures. Fucose content was calculated by the method of DISCHE and SETTLES (10) with reference to L-fucose. The aminosugar content was determined by the ELSON-MORGAN (12) method after hydrolysis of 10 mg of FVA in 4 NHCl for 4 hours at 110° C in tubes sealed under nitrogen. Amino sugars were separated from neutral sugars in a Dowex 50X4 column. N-acetyl-glucosamine was used as standard.

Uronic acids were determined by the carbazol - sulphuric acid method (9) using glucuronic acid - lactone as standard.

Sialic acids were estimated according to WARREN as described by KENNEDY (17).

Identification of the component sugars

of the FVA molecule was done by continuous thin-layer chromatography (1) on Silicagel G-60 plates (Merck). Ethyl-acetate/pyridine/acetic acid/water (60:30:5:10, by vol.) was used as developer solvent, and 0.1 M ammonium monovanadate in 1 M sulphuric acid as the locating reagent.

The inorganic content in FVA was quantified by energy-dispersive X-ray fluorescence spectroscopy in a Kevex 7075 Microanalyzer (Kevex Corp.). The sample was fixed on the support with colloidal graphite and shadowed with gold-palladium. Excitation was produced with an electronic gun at 30 kV. Data were processed according to a Quantex program (Kevex Corp.). Concanavalin A and human alpha-globulin were used for internal calibration of the apparatus.

Molecular weight was determined according to ANDREWS (2) in a Sepharose CL-4B column (0.9 × 100 cm) in Tris buffer. Blue dextran, thyroglobulin, bovine serum albumin and vitamin B_{12} were used for calibration of the column.

Sub-unit composition analysis was done by gel-filtration in a Sepharose CL-4B column (0.9 × 100 cm) in Tris buffer with 6 M urea. Also, SDS-polyacrylamide gel electrophoresis according to LAEMLI (20) was used for the same purpose.

The isoelectric point was estimated by agarose gel electrofocusing by the method indicated above. Because of the low isoelectric point of the FVA, it had to be calculated according to CATSIMPOOLAS (7).

Hemagglutination assays. Were done by serial twofold dilutions of the solutions in Microtiter plates (32).

Hemagglutination inhibition assays. Serial twofold dilutions of the potential inhibitors were made in Microtiter plates in a final volume of 25 µl in phosphate-buffered saline. To each well were added 25 µl of an agglutinin solution (titre 1/4) and mixtures were incubated for two hours at room temperature. Finally, 50 µl of 1 % sheep red cells (SRC) were added to each well and titres were read two hours later.

Erythrocyte treatments. SRC were treated with sodium metaperiodate as described by OFEK *et al.* (24). Papain treatment was done according to GILBOA-GARBER (15), and trypsin, pronase and glycosidases treatments were done by the procedures described by SALIT and GOTSCHLICH (27).

Results

Purification procedures. The course of the two chromatographic steps of the purification is shown in figure 1. In the Sephadex G-25 column activity was eluted in the first peak, which corresponds to the column void volume. In the Sepharose column active fractions are those corresponding to the first major peak, which was eluted in the same volume as the blue dextran marker. Sugar analysis of the fractions by the phenol — sulphuric acid method gave a pattern similar to that of the 280 nm absorbance curves (not shown in figure). Yields, hemagglutinating activity and recoveries in each purification step are summarized in table I. Final yield was 181.9 mg of purified FVA, corresponding to a 43.8 % recovery of the total activity in the initial NaCl extract. Final activity was 204.8 units per mg of FVA.

Purity criteria. Purified FVA is homogeneous as judged by electrophoresis in either 5 % polyacrylamide or 1 % agarose gels, by rate-zonal gradient centrifugation, by isoelectric focusing and by analytical gel-filtration. In polyacrylamide gels, FVA does not enter the stacking gel (2.5 % polyacrylamide), and no bands could be detected along the gel after loading 200 µg of FVA and staining by

Table I. *Yields, activities and recoveries obtained in each step of the* Fucus vesiculosus *agglutinin* (FVA) *purification.*

Purification step	Dry weight (mg)	Activity (units/mg)*	Total activity (units)	Recovery (%)
NaCl extract	32,707.1 **	2.6	85,038.6	100.0
Ammonium sulphate active fraction	3,333.5	19.1	63,669.9	74.9
Sephadex G-25 active fraction	404.2	102.4	41,385.4	48.7
Sepharose CL-4B active fraction	181.9	204.8	37,246.9	43.8

* One unit is defined as the minimum concentration (mg/ml) that produces agglutination.
** Determined on the basis of the sugar concentration in the extract.

the four methods described. FVA stains very faintly with Coomassie blue, but it can be well visualized by the carbohydrate-staining techniques. In sucrose gradients, FVA sediments at the bottom of the tubes, and no material is detected along the gradient by 205 nm spectroscopical examination. Analytical isoelectric focusing shows a single band, and only one peak is obtained by analytical gel-filtration. This peak corresponds to the active fraction. Figure 2 shows a diagram of the electrophoretic patterns of FVA solutions in each purification step.

Chemical analysis. Protein, carbohydrate, aminosugar, uronic acids and sialic acids contents are shown in table II. As can be seen, carbohydrates are present in tenfold higher concentration than protein. LOWRY'S method tends to overestimate protein concentration as compared with KJELDAHL and dye-fixation methods. No sialic acids were detected.

Aminoacid composition analysis is shown in table III. Lysine is the most abundant and cysteine, arginine and proline are absent.

Thin-layer chromatography analysis of the component sugars of the FVA resulted in two spots which did not correspond to any of the sugars usually found in glycoproteins or mucopolysaccharides.

Table II. *Chemical analysis of the* Fucus vesiculosus *agglutinin* (FVA).
Analysis were done with FVA solutions (1 mg/ml) in distilled water. Aminosugars and sialic acids were determined after hydrolysis of the FVA molecule (see text).

Substance analyzed	Analytical method *	Value (μg/ml FVA)
Protein	Kjeldahl	50
	Dye-fixation	45
	Folin-Lowry	864
Neutral sugars	Phenol-sulphuric	512
	Anthrone	452
Fucose	Dische	200
Sialic acids	Warren	0
Aminosugars	Elson-Morgan	25
Uronic acids	Carbazol-sulphuric	76

* For references, see text.

Molecular weight estimation. Molecular weight of the FVA was found to be arround 2×10^6 daltons. Figure 3 shows the plot of the Mr versus elution volume obtained in the Sepharose CL-4B column.

Inorganic content estimation. Inorganic content analysis shows a high content of sulphur (1.23 %) and calcium (0.24). Phosphorus is present in appreciable amounts (0.06 %).

Isoelectric point determination. The isoelectric point was found to be 3.2 ± 0.12. It was calculated on the basis of the migration distance of FVA from different application points, and referred to the migration distance of human alpha-1-antitrypsin.

Sub-unit analysis. Neither gel filtration with denaturating buffer nor SDS-polyacrylamide gel electrophoresis showed sub-unit structure in the FVA molecule.

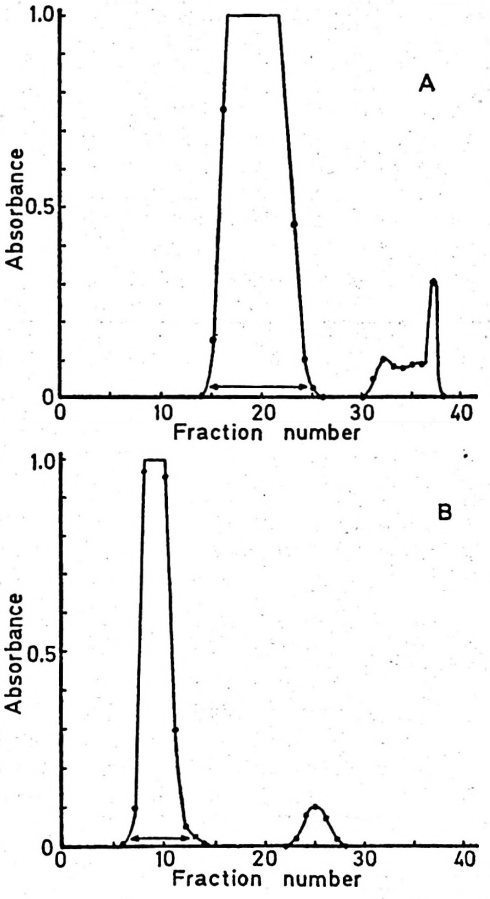

Fig. 1. *Gel-filtration purification steps.* *A*) Sephadex G-25 chromatography of the ammonium sulphate precipitated fraction. *B*) Sepharose CL-4B chromatography of the active fractions of the Sephadex G-25 colum. (●—●) absorbance at 280 nm. (↔) active fractions.

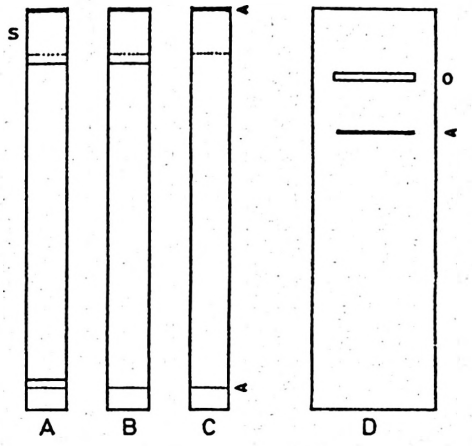

Fig. 2. *Diagram of the electrophoretic pattern of the material obtained in each purification step.* *A*) ammonium sulphate fraction. *B*) Sephadex G-25 fraction. *C*) Sepharose CL-4B fraction. All three gels are 5 % polyacrylamide and stained with the periodic acid-Schiff technique. *D*) Sepharose CL-4B fraction in 1 % agarose stained with toluidine 0. Black arrow indicates the FVA position. White arrow is the bromphenol blue marker. *O*) application well.

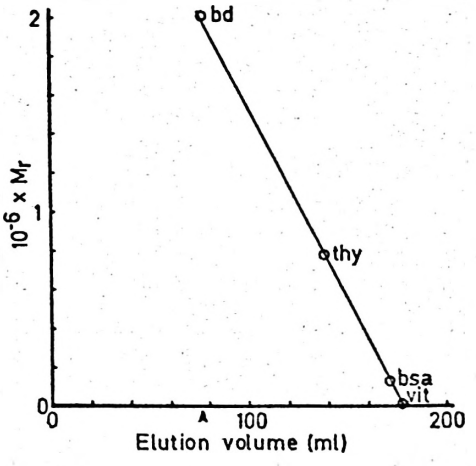

Fig. 3. *Molecular weight determination of the* Fucus vesiculosos *agglutinin* (FVA) *in a Sepharose CL-4B column.* Arrow indicates the elution volume of the FVA. (bd) blue dextran, (thy) thyroglobulin, (bsa) bovine serum albumin, and (vit) vitamin B_{12}.

Table III. Fucus vesiculosus *agglutinin* (FVA) *aminoacid analysis.*
FVA was hydrolysed and aminoacids were estimated in an automatic analyzer. Values are expressed as percentages of the total aminoacid content.

Aminoacid	Value * (%)	Aminoacid	Value * (%)
Trp	N.D.**	Val	5.4
Lys	13.5	Ala	5.3
Ser	9.7	Orn	5.0
Asp	9.4	Gly	4.1
Tyr	9.2	Phe	3.9
Glu	8.7	Leu	2.6
Met	7.0	Ile	2.1
His	6.0	Phosphoserine	0.3

* Not corrected for destruction during hydrolysis.
** Not determined.

Table IV. *Inhibition of FVA-mediated hemagglutination.*
All substances were tested at 100 mg/ml initial concentration except mucin, which was at 3 mg/ml.

Substance	M.I.C.* (μg/ml)	Substance	M.I.C. (μg/ml)
Arabinose	—**	N-ac.-galactosamine	—
Alpha-met.-mannoside	—	N-ac.-glucosamine	—
Mannitol	—	N-ac.-neuraminic	—
Xylose	—	Cellobiose	—
Glucose	—	Trehalose	—
Sorbitol	—	Melezitose	—
Fructose	—	MBP	5
Maltose	—	Fetuin	10
Raffinose	—	Mucin	40
Inositol	—	Thyroglobulin	200
Fucose	—	Gangliosides	500
Mannose	—	Melibiose	—

* Minimum inhibitory concentration.
** No inhibition at the highest concentration tested.

Inhibition of FVA-mediated agglutination. As seen in table IV none of the simple sugars tested were able to inhibit the FVA-mediated agglutination of SRC at the concentrations tested. Only more complex substances were inhibitory. The minimum inhibiting concentrations were as follows: MBP, 5 μg/ml; fetuin, 10 μg/ml; thyroglobulin, 200 μg/ml; mucin, 40 μg/ml and gangliosides (type III) 500 μg/ml.

Agglutination of treated SRC. Metaperiodate treatment of SRC completely prevented their agglutination by FVA, whereas papain or trypsin treated SRC were more agglutinable than the controls (table V).

Neuraminidase treated SRC were more agglutinable than controls (titre 1/65,536; six twofold dilutions more than control SRC) and later treatment of these erythrocytes with beta-galactosidase, follow-

Table V. *Effect of treatment with sodium metaperiodate and proteolytic enzymes on sheep red cells (SRC) agglutinability.*
SRC were treated with sodium metaperiodate or proteolytic enzimes (see text). After treatment, agglutinations were done as usually with serial twofold dilutions of a 10 mg/ml FVA solution.

Treatment	M.A.C.* (μg/ml)
Sodium metaperiodate	—**
Trypsin	9.8
Papain	4.9
Pronase	2.4

* Minimum agglutinating concentration.
** Negative agglutination.

Table VI. *Effect of treatment with different glycosidases on sheep red cells (SRC) agglutinability.*
SRC were treated sequentially with different glycosidases (see text). After treatment agglutinations were done as usually with serial twofold dilutions of a 10 mg/ml FVA solution.

Treatment	Relative agglutinability (%)
Neuraminidase	100
Neuraminidase + galactosidase	100
Neuraminidase + mannosidase	100
Neuraminidase + mannosidase + galactosidase	100
Neuraminidase + galactosidase + mannosidase	25

ed by alpha-mannosidase reduced its agglutinability by 75 %. Treatment with beta-N-acetyl-glucosaminidase did not alter the agglutinability of neuraminidase - beta-galactosidase - alpha-mannosidase pretreated SRC (table VI).

Discussion

Previous publications on *Fucus vesiculosus* agglutinin(s) report only the agglutinating properties of the algal extracts and the failure to inhibit the agglutination with simple sugars. Data reported show that *Fucus vesiculosus* extracts agglutinate erythrocytes regarding the species (3, 4, 26, 35).

Purification of FVA to homogenity was achieved by a three-step conventional procedure. Electrophoretic analysis of the material obtained in each purification step indicates that FVA (even in the first step) is the maior constituent. Another interesting point is the almost total absence of bands in the gels. Only two bands appear when the ammonium sulphate fraction is analyzed, which could be the minor peaks that appear in the gel-filtration columms. These results suggest that *F. vesiculosus* proteins have a low solubility in the NaCl buffer used for the initial extraction, since the saturation range with ammonium sulphate is intermediate and wide enough to expect the presence of a higher number of proteins. This view is reinforced by the fact that there is a low increase in the FVA specific activity throughout the purification process, indicating that FVA is a major constituent in the extract, and also agrees with the relatively high yield and simplicity of the process.

Chemical analysis shows that carbohydrates are present in a 10 to 1 ratio with respect to protein in the FVA molecule. Overestimation of protein by LOWRY'S method could be due to FVA-associated xantines that give FVA solutions a greenish colour and interfere with the LOWRY reaction (21). The high content in carbohydrates leads us to consider FVA sugars did not allow their identification, but produced enough information to postulate that the polysaccharide chains in the molecule are composed by only a few distinct monosaccharide units, since only two spots were detected in the chromatogram. This is in agreement with the disaccharide unit repetition showed for the polysaccharide chains of mucopolysaccharides. Unidentification of the spots could be due to either the presence of unusual sugars or a incomplete FVA hydrolysis rendering complex molecules such as aldobiuronic acids (29).

Another finding is that fucose does not appear in the chromatogram but is detected by colorimetric methods. This fact fits with precedent postulate or, alternatively, could be explained by a interference with the colorimetric reaction.

FVA molecular weight, estimated to be about 2×10^6, should be taken with logical reservations because the presence of a high content of sugars in a molecule produces changes in its chromatographic behaviour as compared to a similar Mr pure protein molecule, and so, Mr estimation by gel filtration may produce errors. The same anomalous behaviour takes place when SDS-polyacrylamide gel electrophoresis is used for Mr estimations (28). It should also be mentioned that mucopolysaccharides are polydisperse molecules and heterogeneous in their lateral carbohydrate chains lenght and so their Mr may vary between a wide margin (14). The high Mr, the acidic isoelectric point, the uronic acids content and the high percentage of sulphur in FVA support its characterization as a mucopolysaccharide (14, 28).

Results on the inhibition of the FVA-mediated agglutination did not permit us to categorically affirm that FVA is a lectin. Nevertheless, results obtained from the transformed SRC agglutination exper-

iments gave indirect evidence pointing to a lectin-like nature of the FVA. The mild proteolytic extraction of membrane peripheral proteins increases the agglutinability, indicating that these proteins could be masking the FVA receptors, which must be non-protein molecules or proteins deeply integrated in the membrane. Another indirect indication of the glucidic nature of the FVA binding-sites in the receptors is the loss of agglutinability of the metaperiodate-treated SRC. It is known that sodium metaperiodate destroys the linkages between vincinal carbon atoms with free hydroxyl groups in sugars (30).

The increment in agglutinability observed when the sialic acid residues are extracted could be due to the subsequent reduction of the membrane surface negative net charge. Alternatively, sialic acids could render difficult the access of the FVA to its binding-sites in the receptors.

The reduction of the agglutinability of neuraminidase-treated SRC when the galactose and mannose residues are extracted (but not when only galactose units are extracted) implies that mannose is part of the FVA binding-sites. Experiments are being carried out to elucidate the complete structure of the FVA binding-sites in the SRC surface.

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Resumen

Se purifica hasta la homogeneidad, por métodos cromatográficos convencionales, una aglutinina del alga *Fucus vesiculosus*, y se ha caracterizado como un mucopolisacárido con un 90 % de contenido en carbohidratos. El peso molecular estimado es de alrededor de 2×10^6 daltons. Su estructura no está formada por subunidades, y el punto isoeléctrico de la molécula es de 3,2. El contenido inorgánico de la aglutinina muestra los siguientes porcentajes: 1,23 % S, 0,24 % Ca y 0.06 % P.

La aglutinación de glóbulos rojos de carnero inducida por la aglutinina es inhibida solamente por glicoproteínas con cadenas oligosacarídicas laterales complejas semejantes a las encontradas en las glicoproteínas de membrana de los eritrocitos. El tratamiento de estos últimos con metaperiodato impide por completo su aglutinación. La degradación secuencial de las cadenas oligosacarídicas con glicosidasas sugiere que los residuos internos de manosa están implicados en el lugar de unión del receptor para la aglutinina. En consecuencia, pensamos que esta aglutinina puede ser considerada una lectina o una molécula semejante a las lectinas, con especificidad para azúcares complejos.

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