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ASSAY-DEPENDENT SPECIFICITY OF A MONOCLONAL ANTIBODY WITH ALGINATE

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ABSTRACT

Monoclonal antibody 2-8.7, prepared to carbohydrates extracted from *Fucus*, labelled cell-wall antigen with greater intensity after calcium alginate cross-links were disrupted. The suspected alginate specificity of this antibody was confirmed by modified enzyme-immunoassay methods. Incubation conditions were found in which antibody 2-8.7 was specific for the polyguluronate sequences of alginate. Immunofluorescence labelling by antibody 2-8.7 on *Fucus* germling sections confirmed specificity changes related to the antigen–antibody incubation conditions.

INTRODUCTION

Monoclonal antibodies have been prepared to carbohydrate extracts containing alginate and fucans from the brown alga *Fucus distichus*[1]. Antibody 2-8.7 was unique among these antibodies in exhibiting[1] greatly enhanced immunofluorescence labelling following pretreatment of algal sections with ethylenediaminetetra-acetic acid (EDTA). This finding suggested an alginate specificity for antibody 2-8.7, since calcium cross-linking of polyguluronate sequences causes[2] molecular interactions in alginate. Enzyme-immunoassay screening during the production of antibody was positive for antibody 2-8.7 against several crude carbohydrate fractions. Therefore, the specificity of this antibody was investigated with defined carbohydrate fractions and modified enzyme-immunoassay conditions. Immunofluorescence-labelling studies were carried out to confirm changes in specificity related to antigen–antibody incubation conditions. An abstract which includes this work has been reported[3].

Alginate is an unbranched copolymer of D-mannuronate (M) and L-guluronate (G), the units having a block-wise distribution[4]. With alginate as antigen, a specific antigen–antibody interaction may depend upon local sequences in the molecule. For the present study, alginate fragments were prepared which represented the parts of the original molecule where the two uronates occur in contiguous sequences (M- and G-blocks)[5]. For comparison, a sample of an intact

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alginate was included where these homopolymeric blocks occur interspersed with sequences characterised by a high transition frequency (MG-blocks). Thus, the dependence of antigen–antibody interaction on sequence should be detected as differences in the quantitative enzyme-immunoassay (EIA) reactions of these preparations.

EXPERIMENTAL

Enzyme immunoassay (EIA). — Alginates[6] and alginate block types[5] were prepared from the brown algae *Stilophora rhizoides* (UUS, G-blocks), *Ascophyllum nodosum* fruiting bodies (CaL, M-blocks), and *Laminaria digitata* (L_{20}, MG-blocks). The *Macrocystis* preparation was subjected to mild, homogeneous hydrolysis before use (HCl, pH 3.5–4, 2 h, 100°). Alginate blocks and pectate were 20–30 units long, as determined by ^{1}H-n.m.r. spectroscopy. The following buffers were used for enzyme immunoassay: *A*, 50mM tris(hydroxymethyl)aminomethane-HCl (Tris, pH 7.6) containing 0.4mM $CaCl_2$ and 0.02% of NaN_3; *B*, buffer *A* containing 0.05% of Tween-20 (Sigma); *C*, M diethanolamine-HCl (pH 9.8) containing 0.05mM $MgCl_2$ and 0.02% of NaN_3.

The assay was carried out in polystyrene 96-well microtitre plates (Dynatech Immulon 1 or Linbro) with a volume of 0.1 or 0.2 mL per well. Antigen preparations were made in distilled water at 2–5 mg/mL and diluted to the appropriate concentration in buffer *A*. Buffer *A* was used as the reagent blank. Antigen solutions were incubated for 1 h at room temperature, and each well was then emptied by aspiration and washed 3 times with buffer *A*, individually or with a Dynatech Miniwash. In order to avoid non-specific interaction of immunoglobulins with plastic wells, 1% bovine serum albumin (Kohn fraction V, Sigma) in buffer *B* was then used to block remaining plastic binding-sites overnight at 4°. Following 3 washes with buffer *B*, monoclonal antibodies in the hybridoma supernatant were diluted in that buffer 20–50 times, then added and incubated for 1 h at room temperature. Hybridoma supernatant containing a monoclonal antibody of unrelated specificity was used as a control. After 3 washes with buffer *B*, alkaline phosphatase-conjugated goat or sheep anti-mouse IgG (light and heavy chain specific; Sigma, Tago) diluted 1,000–3,000 times in buffer *B* was added and incubated for 1 h at room temperature. After 3 washes in buffer *B*, enzyme substrate (*p*-nitrophenyl phosphate, 1 mg/mL) in buffer *C* was added. The reaction was stopped, after sufficient development of colour, by adding 0.1 mL of 100mM cysteine in distilled water[7]. Absorbance was read at 405 nm in a Titertek Multiscan or a Dynatech Minireader, with buffer *C* as the blank.

Immunofluorescence. — *Fucus* embryos were grown to the 2-week, carrot-shaped, germling stage[8]. Germlings were aldehyde-fixed and embedded in glycol methacrylate[8]. Below the apical meristem, the germling thallus has columnar epidermal cells at the surface and developing cortical and medullary cells within. Extracellular matrix is forming between medullary cells at this stage of

development. Subapical cross-sections 2-μm thick were labelled for indirect immunofluorescence.

IgM antibody 2-8.7 and an unrelated IgM monoclonal antibody as a control were used for labelling experiments in hybridoma medium[1]. This medium, Dulbecco's Modified Eagle's Medium with high glucose (M.A. Bioproducts), contained 15% of newborn calf serum, 150mM sodium chloride, and 3–4mM Ca^{2+} in addition to other salts and organic nutrients. In addition, antibody 2-8.7 in the supernatant of hybridoma cells grown in HB-101 serum-free medium (Hana Biologics) was concentrated 11.2-fold in a Minicon 125 (Amicon) and diluted 10-fold in buffer *B* or buffer *B* with mM EDTA for labelling at enzyme immunoassay conditions. Sections were incubated for 1 h at room temperature in antibody 2-8.7 or control antibody, and then rinsed for 5–15 min in several changes of buffer *B*. Fluorescein-labelled goat anti-mouse IgM (heavy chain specific) affinity-purified antibody (Cappel) was diluted 40 times with Dulbecco's medium containing 15% of foetal calf serum (Irvine Scientific; inactivated at 56°) and incubated on all sections for 30 min. After washing in buffer *B* for at least 5 min, sections were mounted in an antioxidant mountant, viewed with a Zeiss epifluorescence microscope, and photographed as described previously[1]. A Zeiss KP560 interference filter was used to reduce intracellular autofluorescence.

RESULTS AND DISCUSSION

Enzyme immunoassay. — The compositions of the antigen preparations and their responses in enzyme immunoassay analysis are given in Table I. Uronate compositions are given as the relative occurrence of mannuronate (F_M) and guluronate (F_G), together with the nearest-neighbour frequencies as determined by n.m.r. spectroscopy[9]. Antigens UUS, CaL, and L_{20} were selected to represent the three types of blocks constituting the intact alginate molecule, namely, guluronate, mannuronate, and mixed types, respectively. This is clearly reflected in the occurrence of the GG (F_{GG}), MM (F_{MM}), and MG + GM ($F_{MG} + F_{GM}$) doublets in the respective preparations. It should be noted that none of these materials was homogeneous with respect to sequence. The remaining alginate was a whole alginate preparation from *Macrocystis pyrifera*. As seen from the diad frequencies of Table I, this preparation contained all three block types.

The standard Tris buffer used in the present enzyme immunoassay procedure contained 0.4mM Ca^{2+}, which was optimal for rocket immunoelectrophoresis of alginate antigens with rabbit antisera[10]. Calcium levels an order of magnitude higher caused alginate aggregation and concomitant loss of electrophoretic mobility, but removal of the available Ca^{2+} by chelation with EDTA eliminated the alginate (but not the fucan) precipitin arcs. The guluronate blocks of alginate have a very high affinity for, and will remove, Ca^{2+} from the buffer in the enzyme immunoassay. Since calcium alginate is insoluble, it is necessary to control the concentration of Ca^{2+} during the antigen-coating step in order to keep the antigen in solution. All the antigens listed in Tables I and II were 0.5 mg/mL.

TABLE I

URONIC ACID COMPOSITION OF ANTIGEN PREPARATIONS AND THEIR ENZYME IMMUNOASSAY RESPONSE IN BUFFER *B*

Antigen preparation	*EIA response* A_{405}	*Antigen composition*					
		F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
UUS	0 86	0.10	0.90	0.05	0.05	0.05	0.85
Macrocystis	0.61	0.62	0.38	0.39	0.23	0.23	0.15
CaL	0.31	0.96	0.04	0.95	0 01	0.01	0.03
L_{20}	0 08	0 52	0.48	0.14	0.38	0.38	0 10
Pectate	0.83	—	—	—	—	—	—

TABLE II

ENZYME IMMUNOASSAY RESPONSE OF ANTIGEN PREPARATIONS AT VARIOUS CONCENTRATIONS OF EDTA IN BUFFER *B*

Antigen preparation	*No EDTA*	*mM EDTA*	*5mM EDTA*
UUS	0.86	0 96	0 48
Macrocystis	0.61	0.59	0.42
CaL	0.31	0.23	0.15
L_{20}	0.08	0.10	0.04
Pectate	0.83	0 26	0.08

The three block preparations behaved very differently in enzyme immunoassay analysis in buffer *B* with antigen dilutions of 0.5 mg/mL and antibody 2-8.7 hybridoma supernatant diluted 1:20 (Table II). There was maximum response for guluronate blocks, considerably less for mannuronate blocks, and no response for mixed blocks. As expected from its intermediate composition, *Macrocystis* whole alginate gave a response between those of the two homopolymeric block preparations. The results suggest that antibody binding depends on uronate sequence, with stronger affinity for guluronate blocks. Pectate, a (1→4)-linked polymer of α-D-galacturonate, was included for comparison, and the results in Table II demonstrate binding of the anti-alginate antibody to pectate similar to that for guluronate blocks. Since a preferential specificity was indicated among the block types of alginate, this observation was unexpected. Polyguluronate and polygalacturonate have a common feature in that they bind[11] Ca^{2+}. It is conceivable that the specificity under these experimental conditions is directed towards the calcium salt of a glycuronan and does not necessarily depend on the chemical identity of the uronate involved. The unrelated monoclonal antibody serving as a control did not react above background levels with these antigens.

Addition of EDTA to buffer *B* introduced an agent competing with the glycuronan for the available Ca^{2+}. At the low ionic strength and low concentration of Ca^{2+} (0.4mM) used, addition of mM EDTA led to a drastic decrease in the

response of pectate, whereas that of polyguluronate was not influenced. At 5mM EDTA, there was no detectable interaction of antibody and sodium pectate. The interaction of antibody with alginate was also significantly reduced at this concentration, most probably due to a lack of Ca^{2+}. This observation suggests that Ca^{2+} is necessary for antigen–antibody binding in this system.

Addition of EDTA to the buffer system thus changes the apparent specificity of the enzyme immunoassay methods toward guluronate blocks as the only active antigen. At EDTA concentrations of 2–5mM, the method may be used for analysing alginate preparations for their content of this block type. The results shown in Fig. 1 demonstrate that there is a linear relationship between EIA response and the relative concentration of guluronate blocks in mixtures of the two homopolymeric blocks with a total alginate concentration of 0.2 mg/mL. The results in Fig. 1 represent the upper part of the useful range of antigen concentrations. By adjusting antibody concentrations and reaction time, the method is useful for antigen concentrations one-tenth those given in Fig. 1.

Immunofluorescence labelling. — Control antibody in medium did not label *Fucus* germlings and only diffuse intracellular autofluorescence was seen. Antibody 2-8.7 in medium labelled intracellular inclusions in epidermal cells, and intracellular and peripheral cell antigen in cortical and medullary cells (Fig. 2A). There was little extracellular labelling on most subapical sections. Antibody 2-8.7 and control antibody in buffer *B*, with or without mM EDTA, non-specifically labelled the cuticle at the plant surface (Fig. 2B and C), which contains concentrated sulfated polymer as indicated by Alcian staining[8]. Specific labelling by antibody 2-8.7 in buffer *B* was minimal in a thin, inner cell-wall layer of interior cells

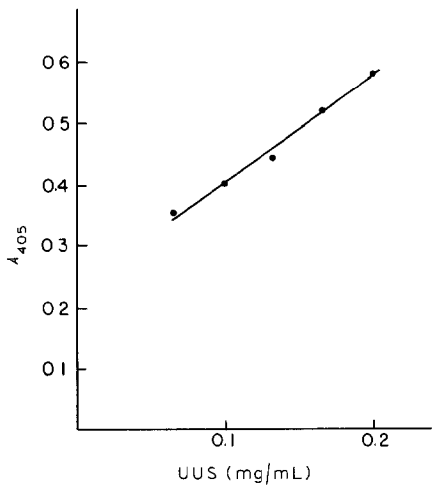

Fig. 1. Enzyme immunoassay response as a function of the block concentration of guluronate (UUS). Total alginate concentration was adjusted to 0.2 mg/mL by the addition of mannuronate blocks (CaL) in buffer *B* containing 5mM EDTA.

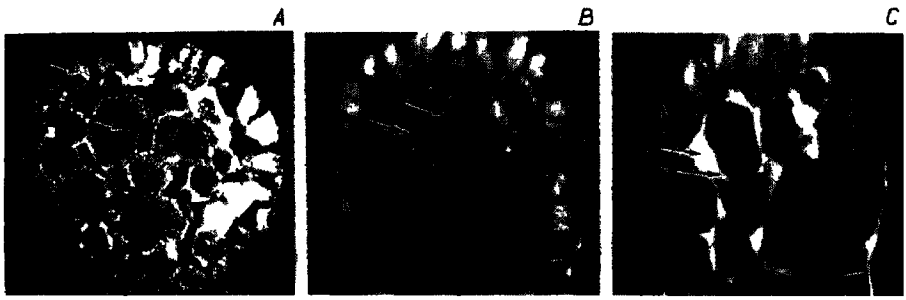

Fig. 2. Indirect immunofluorescence labelling on cross sections of *Fucus* germlings. Intracellular fluorescence is autofluorescence except as noted in *A*. Magnification × 600. Antibody 2-8.7 labelling: *A*, in hybridoma medium [intracellular labelling (arrows) is apparently in small cytoplasmic inclusions of epidermal and interior cells; the periphery of interior cells is also labelled with a punctate appearance, but extracellular matrix is not labelled]; *B*, in buffer *B* [specific peripheral cell labelling is weak (arrows); cuticle labelling (arrowhead) is non-specific]; *C*, in buffer *B* with mM EDTA [specific labelling in cell walls and extracellular matrix (arrows) is strong; cuticle labelling (arrowhead), as in *B*, is non-specific].

(Fig. 2B). In contrast, extracellular matrix in the plant interior was intensely labelled by antibody 2-8.7 in buffer *B* with mM EDTA (Fig. 2C). No intracellular label was seen in low ionic strength buffer *B* with or without mM EDTA.

Antibody 2-8.7 therefore labelled two different antigens on germling sections. An intracellular antigen was primarily labelled in medium, and an extracellular antigen was primarily labelled in low-ionic-strength buffers. Further, the extracellular antigen was largely masked except in buffer *B* with mM EDTA. Alginate antigen may be masked by calcium-mediated self-association or by another organisational state in tissue fibres and gels. These results suggest that polyguluronate in germling tissue is unavailable for antibody binding except at very low levels of calcium. This finding contributes to evidence[1] that antibody 2-8.7 may bind to single glycuronan chains. Further studies are needed to identify the intracellular antigen recognised by antibody 2-8.7 in medium. The change in the pattern of immunofluorescence labelling may depend on factors related to ionic strength and composition, antibody concentration, and other components of the antibody incubation solutions.

Strong ionic interactions with proteins are to be expected for all polymers of high charge density, for example, alginate and pectate. The separation of specific from non-specific antigen–antibody interactions will require extensive investigations of the influence of ionic strength and ion cooperation. It is reasonable to expect that carbohydrate conformation is important in specific interaction with antibody, and conformation is likely to be influenced by such variables as pH, temperature, and the nature of buffer components. Therefore, the results reported here should be considered only as indicating an apparent, but useful, specificity.

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