Radioimmunochemical evidence for a role of carbohydrate in antigenic determinant(s) on human liver α -L-fucosidase

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A competitive-binding radioimmunoassay method was employed to investigate the role of carbohydrate in antigenic determinant(s) of human liver α -L-fucosidase. Competition curves were used to quantify the concentrations of competitors needed to cause 30% inhibition of the precipitation of ^{125}I -labelled α -L-fucosidase. The isoelectric forms of α -L-fucosidase, which are related by sialic acid residues, were separated preparatively and used as competitors in the radioimmunoassay. A pattern of increasing effectiveness as competitors with increasing acidity of the forms was found, suggesting that sialic acid may be involved in the antigenic determinant(s) of α -L-fucosidase. Specificity was exhibited when sugar and sugar derivatives were used as competitors in the radioimmunoassay: a 51-fold range of competitive ability was found, and sialic acids (*N*-acetylneuraminic acid and *N*-glycolylneuraminic acid) and colominic acid (a polymer of *N*-acetylneuraminic acid) were the best competitors. The results of our studies suggest that carbohydrate contributes to antigenic determinant(s) of α -L-fucosidase and that sialic acid is probably the major sugar involved.

Although numerous physiologically important proteins have been shown to be glycoproteins, the functional significance of the carbohydrate moieties of these macromolecules is not well understood (Spiro, 1973; Kornfeld & Kornfeld, 1976). A recent review (Olden *et al.*, 1982) has indicated that two major functions of the carbohydrate portions of glycoproteins are (1) to act as 'sorting signals' for directing glycoproteins (e.g. lysosomal hydrolases) to specific cellular organelles (e.g. the lysosome) and (2) to protect glycoproteins from proteolytic degradation. In addition, recent investigations have given evidence for a role of carbohydrate in antigenic determinants of several glycoproteins (see the Discussion section).

The importance of the lysosomal glycosidase α -L-fucosidase (fucoside fucosidase, EC 3.2.1.51) in human metabolism is indicated by the fact that deficiency of its activity leads to the genetic disease fucosidosis (Alhadeff, 1981). α -L-Fucosidase has been purified from human liver (Robinson &

Thorpe, 1974; Alhadeff *et al.*, 1975a) and found to be a glycoprotein containing approx. 7% carbohydrate by weight (Alhadeff & Freeze, 1977). The three major sugars in human liver α -L-fucosidase are mannose (3%), glucosamine (2%) and *N*-acetylneuraminic acid (1%). Several isoelectric forms of human liver α -L-fucosidase exist, and these appear to be related, at least in part, by sialic acid residues (Thorpe & Robinson, 1975; Turner *et al.*, 1975; Alhadeff *et al.*, 1975a,b, 1978). These isoelectric forms exhibit subtle but significant differences from one another with regard to pH-optimum curves, thermostability properties and apparent Michaelis constants for the *p*-nitrophenyl and 4-methylumbelliferyl substrates (Alhadeff *et al.*, 1978; Alhadeff & Andrews-Smith, 1980a).

We have recently developed a competitive-binding radioimmunoassay (RIA) method for human liver α -L-fucosidase and used it to demonstrate a significantly decreased amount of fucosidase protein in liver and spleen tissue from a patient with fucosidosis (Andrews-Smith & Alhadeff, 1982). In the present investigation this RIA and various competitors (the separated isoelectric forms of α -L-fucosidase, sugars and sugar deriva-

Abbreviation used: RIA, radioimmunoassay.

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tives) have been employed to give evidence for a role of carbohydrate in antigenic determinant(s) of human liver α -L-fucosidase.

Materials and methods

General

All procedures were carried out at 2–4°C unless otherwise stated. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Enzyme solutions were concentrated by vacuum-dialysis in a collodion-bag apparatus with 25000- M_r -retention collodion bags (Schleicher and Schuell). Agarose- ϵ -amino-hexanoyl-fucopyranosylamine was purchased from Miles-Yeda and used as the affinity resin for purifying α -L-fucosidase. α -L-Fucosidase activity was assayed with 4-methylumbelliferyl α -L-fucopyranoside (Koch-Light Laboratories) as previously described (Alhadeff & O'Brien, 1977). A unit of α -L-fucosidase is the amount of enzyme that hydrolyses 1 nmol of substrate/min at 37°C. Absorbance was read on a Beckman model 24 spectrophotometer and fluorescence on a Turner model 111 fluorimeter. Radioactivity was counted with a Searle model 1197 automatic γ -radiation system with 90% efficiency. Reagents for iodination of α -L-fucosidase included 99.8%-pure carrier-free Na^{125}I of specific radioactivity 14.8 mCi/ μg of iodine (Amersham International), chloramine-T and dimethyl sulphoxide (grade I) (Sigma Chemical Co.) and sodium metabisulphite. Human serum albumin (fraction V) was from Sigma Chemical Co., rabbit anti-goat immunoglobulin G was from Research Products International Corp. and ampholytes were from LKB Produkter.

Sugar and sugar derivatives to be used as potential competitors in the RIA were the highest grade available and prepared as saturated solutions at 23°C in 0.1 M-sodium citrate buffer, pH 5.0, containing human serum albumin (10 mg/ml). These included *N*-acetylneuraminic acid (type VII, isolated from human urine), *N*-glycolylneuraminic acid, colominic acid, D(+)-mannose, D-mannosamine hydrochloride, D(+)-galactose, D(+)-galactosamine hydrochloride, *N*-acetyl-D-galactosamine, α -D(+)-glucose, β -D(+)-glucose, D(+)-glucosamine hydrochloride, *N*-acetyl-D-glucosamine, D-glucuronic acid, L(+)-arabinose, α -L(-)-fucose, D(-)-ribose, 2-deoxy-D-ribose and D(+)-xylose (purchased from Sigma Chemical Co), *N*-acetylneuraminic acid (hydrolysed from *Escherichia coli* colominic acid) (from P-L Biochemicals), *N*-acetyl-D(+)-mannosamine (purchased from Pfanstiehl) and the β -methyl glycoside of neuraminic acid (methoxyneuraminic acid) (purchased from Boehringer-Mannheim).

Purification of α -L-fucosidase

α -L-Fucosidase was purified to apparent homogeneity (according to standard criteria) from 500 g portions of two autopsied human livers (which appeared normal on gross pathological examination) by affinity chromatography on agarose- ϵ -amino-hexanoyl-fucopyranosylamine essentially as previously described (Alhadeff *et al.*, 1975a). The enzyme was purified in 60 and 69% yields with final specific activities of approx. 30000 units/mg of protein. (Procedures involving human tissues were approved by an Institutional Committee on Investigations/Activities Involving Human Subjects.) A portion of homogeneous α -L-fucosidase was used previously for preparation of antisera (in a female yearling goat) from which the immunoglobulin G fraction was purified (Andrews-Smith & Alhadeff, 1982). This immunoglobulin G fraction is designated antibody I in the RIA. A second portion of homogeneous α -L-fucosidase was radioiodinated to a final specific radioactivity of 2.6×10^6 d.p.m./ μg by using the chloramine-T procedure as previously described (Andrews-Smith & Alhadeff, 1982).

Preparation of isoelectric forms of α -L-fucosidase

Portions of homogeneous α -L-fucosidase (1200–1300 units) were subjected to preparative isoelectric focusing in an LKB 8101 (110 ml) column essentially as previously described (Alhadeff *et al.*, 1978). The starting current was approx. 2 mA at 400 V. Electrofocusing was conducted with 1% (v/v) ampholytes (pH 5–8) in a sucrose gradient (0–67%, w/v) for 60–62 h. The column was eluted, the pH of each fraction was determined at 0–2°C with a Beckman digital pH-meter, and 50 μl portions of each fraction were assayed for α -L-fucosidase activity for 15 min. The fractions containing each form were pooled separately and concentrated in collodion bags by vacuum-dialysis, with the outer reservoir containing 100 ml of 0.01 M-L-fucose (to stabilize α -L-fucosidase) in 0.01 M- NaH_2PO_4 , pH 5.5, containing 0.02% NaN_3 . After concentration, each form was diluted 3:4 with human serum albumin (10 mg/ml) in 0.1 M-sodium citrate buffer, pH 5.0. These separated isoelectric forms were used as competitors in the RIA. The weight of each form used was calculated from the enzymic activity employed divided by the measured specific activity.

RIA procedures

Preliminary direct-binding curves and competition curves with purified human liver α -L-fucosidase were derived as previously described (Andrews-Smith & Alhadeff, 1982). In the competition studies, various potential competitors (the isoelec-

tric forms of α -L-fucosidase, sugars and sugar derivatives) were substituted for unlabelled α -L-fucosidase and incubated with antibody I (the immunoglobulin G fraction of antiserum to purified α -L-fucosidase) at a final dilution of 5×10^5 for 20h at 4°C. (This amount of antibody I led to precipitation of 50% of the radioactivity associated with 20ng of ^{125}I -labelled α -L-fucosidase.) Addition of ^{125}I -labelled α -L-fucosidase (20ng; 5.2×10^4 d.p.m.) was followed by a 20h incubation at 4°C, after which 5 μl of antibody II (rabbit anti-goat immunoglobulin G) was added with a final 20h incubation at 4°C. (The amount of antibody II added was determined in a separate experiment to obtain maximum antibody-I-antibody-II-complex formation and precipitation.) After centrifugation at 48000g for 30min, 95 μl supernatants were immediately removed, pellets were washed with 100 μl of 0.1M-sodium citrate buffer, pH 5.0, containing human serum albumin (10mg/ml) and re-centrifuged, and the 100 μl washes were removed. Radioactivities of pellets (10 μl), supernatants (95 μl) and washes were counted in an automatic γ -radiation counter. Percentage inhibition (%) of precipitation of ^{125}I -labelled α -L-fucosidase was calculated according to the equation:

$$\%I = \frac{[\% \text{ c.p.m. precipitated (no competitor)} - \% \text{ c.p.m. precipitated (with competitor)}]}{[\% \text{ c.p.m. precipitated (no competitor)}]} \times 100$$

Data were plotted on semi-logarithmic graphs with percentage inhibition of radioactivity precipitated on the ordinate and concentration of competitor on the abscissa. K_i -30% values (which are defined as the concentration of competitor necessary to cause a 30% inhibition of radioactivity precipitated) were determined from these plots. The intra-assay variability was approx. 3–4% and the inter-assay variability was approx. 10–15%.

Results

Effectiveness of isoelectric forms of α -L-fucosidase as competitors in the RIA

Human liver α -L-fucosidase was purified by affinity chromatography to apparent homogeneity (Alhadeff *et al.*, 1975a), and portions of purified enzyme were subjected to preparative isoelectric focusing. The resulting isoelectric profile was similar to that previously described (Alhadeff & Andrews-Smith, 1980a) and included eight forms with isoelectric points (pI values) ranging from 4.8 to 6.8. These eight forms were independently combined, dialysed, concentrated and used as competitors in the RIA for α -L-fucosidase. The two most neutral forms (I and II) were the poorest

competitors and exhibited significant competition ($\geq 10\%$ inhibition) only at high concentration ($> 20 \text{ ng}/100 \mu\text{l}$). Competition curves for the other six forms, as well as for purified unlabelled α -L-fucosidase (the whole enzyme), are depicted in Fig. 1. The percentage inhibition of precipitation is approximately linear for the whole enzyme from 0.2 to 6.0 ng of competing protein. From this curve the concentration of unlabelled α -L-fucosidase necessary to cause a 30% inhibition of radioactivity precipitated (K_i -30% value) was determined to be 1.4 ng/100 μl . The graphically determined K_i -30% values for each of the isoelectric forms for a typical experiment are summarized in Table 1. This Table shows that the most acidic form (VIII) is the best competitor and is more than 6 times as effective a competitor as the two most neutral forms (I and II). A relationship of increasing effectiveness as competitor with increasing acidity appears to exist for the isoelectric forms of α -L-fucosidase. Although the absolute K_i -30% values varied somewhat from experiment to experiment, the above relationship of relative values was found in five separate experiments with three different preparations of α -L-fucosidase isoelectric forms from two different human livers.

Effectiveness of sugars and sugar derivatives as competitors in the RIA

Various sugars and sugar derivatives were used as competitors in the RIA for α -L-fucosidase.

Fig. 1. Inhibition of precipitation of ^{125}I -labelled α -L-fucosidase by unlabelled α -L-fucosidase and its isoelectric forms

III (●), IV (□), V (▲), VI (△), VII (▽) and VIII (○) are isoelectric forms with pI values of 6.0, 5.8, 5.6, 5.3, 5.0 and 4.8 respectively; ■, whole enzyme. See the Materials and methods section for details.

Table 1. Effectiveness of isoelectric forms of α -L-fucosidase as competitors in the RIA for α -L-fucosidase
For details see the text.

Isoelectric form of α -L-fucosidase	pI	K_i -30% (ng/100 μ l)
I	6.8	> 20
II	6.4	> 20
III	6.0	11.0
IV	5.8	9.0
V	5.6	6.2
VI	5.3	4.8
VII	5.0	3.6
VIII	4.8	3.0

Fig. 2. Inhibition of precipitation of 125 I-labelled α -L-fucosidase by sugars and sugar derivatives
○, *N*-Glycolylneuraminic acid; ●, *N*-acetylneuraminic acid; □, mannosamine; ■, mannose.
See the Materials and Methods section for details.

Representative competition curves for four of these sugars (*N*-glycolylneuraminic acid, *N*-acetylneuraminic acid, mannosamine and mannose) are illustrated in Fig. 2, and the K_i -30% values (means $\pm$ S.D.) for these and several other sugars are summarized in Table 2. Although it appears that colominic acid is the single best competitor employed, its K_i -30% value ($4.3 \mu\text{mol}/100 \mu\text{l}$) must be interpreted with caution, since limited amounts of this polymer of *N*-acetylneuraminic acid allowed for only one competition curve to be run. The best sugar competitors were *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid, with K_i -30% values of 10.9 ± 1.9 and $10.7 \pm 2.0 \mu\text{mol}/100 \mu\text{l}$ respectively. The curves and K_i -30% values for *N*-acetylneuraminic acid from two sources (human urine and colominic acid

Table 2. Effectiveness of sugars and sugar derivatives as competitors in the RIA for α -L-fucosidase
For details see the text. K_i -30% values are given as means $\pm$ S.D.

Competitor	No. of trials	K_i -30% ($\mu\text{mol}/100 \mu\text{l}$)
Colominic acid	1	4.3
<i>N</i> -Acetylneuraminic acid*	2	10.0 ± 0.1
<i>N</i> -Acetylneuraminic acid†	6	10.9 ± 1.9
<i>N</i> -Glycolylneuraminic acid	4	10.7 ± 2.0
D-Mannosamine	4	68.4 ± 7.7
D-Glucosamine	5	77.1 ± 6.6
D-Galactosamine	3	85.8 ± 8.4
<i>N</i> -Acetyl-D-mannosamine	3	95.0 ± 11.0
<i>N</i> -Acetyl-D-galactosamine	4	102 ± 4.1
<i>N</i> -Acetyl-D-glucosamine	4	118 ± 11.5
L-Fucose	4	156 ± 13
L-Arabinose	2	158 ± 13
2-Deoxy-D-ribose	2	159 ± 1
D-Mannose	5	165 ± 21
D-Ribose	2	188 ± 12
D-Glucose (α)	5	195 ± 15
D-Xylose	2	200 ± 4.5
D-Glucose (β)	4	202 ± 18.5
D-Galactose	3	219 ± 14.6

* From hydrolysed *E. coli* colominic acid (P-L Biochemicals).

† From human urine (Sigma Chemical Co.).

hydrolysate) were comparable and not significantly different from the curves and K_i -30% values for *N*-glycolylneuraminic acid. No competition was seen for the β -methyl glycoside of neuraminic acid (methoxynuraminic acid) when up to $25 \mu\text{mol}/100 \mu\text{l}$ were used.

The hexosamines and their *N*-acetylated derivatives were significantly less effective than the sialic acids as competitors, with K_i -30% values ranging from 68 to $118 \mu\text{mol}/100 \mu\text{l}$. Each hexosamine appeared to be a slightly better competitor than its *N*-acetylated derivative. The pentoses and the hexoses were the poorest sugar competitors, with K_i -30% values ranging from 156 to $219 \mu\text{mol}/100 \mu\text{l}$. No competition was seen for D-glucuronic acid when up to $220 \mu\text{mol}/100 \mu\text{l}$ of this negatively charged sugar was used in the RIA. In all, specificity was found for the sugar competitors with a 51-fold range of competitive ability exhibited.

Discussion

Many recent investigations have addressed the question of whether the carbohydrate portions of specific glycoproteins are involved in antigenic determinants of these macromolecules (reviewed in Horowitz, 1978). One experimental approach to answer this question has been to destroy the carbo-

hydrate moieties (e.g. by periodate oxidation) and/or to remove certain sugars selectively by treatment with specific glycosidases (e.g. neuraminidase) and to assess immunochemically what effect the treatment has had on the antigenicity of the glycoprotein. Although this approach has been useful in studying several glycoproteins, including bacterial glucoamylase (Pazur *et al.*, 1981), human serum thyroxine-binding globulin (Cheng *et al.*, 1979), a rye-grass pollen glycoprotein (Howlett & Clarke, 1981), mouse tumour plasma-membrane glycoproteins (Prat & Comoglio, 1976) and the C-terminal peptide of the β -subunit of human chorionic gonadotropin (Birhen *et al.*, 1980), it has potential pitfalls, since periodate oxidation (e.g. at concentrations above 0.1 M) and glycosidase preparations (which can contain proteinases) can alter the protein backbone of glycoproteins (Marshall & Neuberger, 1972). Thus these treatments can lead to erroneous conclusions about the role of carbohydrate in antigenic determinants.

Another more sensitive and specific approach to assess the role of carbohydrate in antigenic determinants of glycoproteins has employed the use of sugars, oligosaccharides and other glycoconjugates as inhibitors of the immunoprecipitation of unlabelled or radiolabelled antigen. This approach has been used to give evidence for an involvement of specific sugars and oligosaccharides in the antigenicity of several glycoconjugates: mannose and *N*-acetylneuraminic acid in human serum low-density lipoprotein (Goldstein & Chapman, 1981), chondroitin 6-sulphate tetrasaccharide and hexasaccharide in a chick cartilage proteoglycan (Jenkins *et al.*, 1981) and β -D-glucose 1-phosphate in a streptococcal cell-wall glycan (Pazur, 1982).

In the present study a recently developed competitive-binding RIA method for α -L-fucosidase and various competitors has been employed to provide evidence for a role of carbohydrate in antigenic determinant(s) of human liver α -L-fucosidase. The isoelectric forms of α -L-fucosidase were separated preparatively and independently used as competitors in the RIA. A pattern of increasing effectiveness as competitors with increasing acidity of the isoelectric forms was found. Since the isoelectric forms of α -L-fucosidase have been shown to be related by sialic acid residues (Thorpe & Robinson, 1975; Turner *et al.*, 1975; Alhadeff *et al.*, 1975*a,b*, 1978), it is possible that sialic acid (presumably *N*-acetylneuraminic acid) is involved in antigenic determinant(s) of α -L-fucosidase and that the different amounts of this sugar in the forms of α -L-fucosidase are the basis for the different competition curves observed. However, it should be pointed out that there may be other structural differences between the isoelectric forms (besides sialic acid content) that give rise

or contribute to their differing antigenicities. It is also possible that the inhibitory capacity of an isoelectric form may reflect its relative abundance (and hence the relative abundance of antibodies specific for that isoelectric form) in the immunogenic mixture of forms employed to produce the antiserum. This possibility is unlikely, since forms III and IV are most abundant in the immunogenic mixture but have considerably less inhibitory capacity than the less-abundant forms VII and VIII (Alhadeff & Andrews-Smith, 1980*a*).

The finding that the whole (unfractionated) enzyme was a better competitor than any of the isoelectric forms might be explained by the fact that more antigenic sites are present on the whole enzyme than on any individual isoelectric form. This kind of additive effect is particularly plausible if the whole enzyme consists of an aggregation of isoelectric forms, some of which have one or more unique antigenic determinants not possessed by the other forms. Another possible explanation of why the whole enzyme was a better competitor than the individual forms is that isoelectric focusing may have caused slight alterations in the tertiary structure of the forms and decreased their effectiveness as competitors. Still another possibility is that the presence of small amounts of ampholytes in the isoelectric-form preparations (remaining after dialysis) interfered with the competitive-binding RIA.

The idea that carbohydrate might be involved in antigenic determinant(s) of α -L-fucosidase was supported by the results of the sugar competition studies, in which specificity was shown for the effectiveness of the various competitors. The best competitors were sialic acids (*N*-acetylneuraminic acid and *N*-glycollylneuraminic acid) and the polymer of sialic acid, colominic acid. These compounds were effective competitors at concentrations up to 51-fold lower than the other sugars and sugar derivatives studied. No competition was seen for the β -methyl glycoside of neuraminic acid (up to a concentration of 0.25 M) or for D-glucuronic acid (up to a concentration of 2.2 M) (results not shown), suggesting that the effectiveness of *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid as competitors is not due to the acidity or negative charge of these sialic acids. Our findings that relatively high concentrations of even the best sugar competitors are needed for effective competition are similar to the findings made by others investigating the role of carbohydrate in antigenic determinants of other glycoconjugates (Goldstein & Chapman, 1981; Jenkins *et al.*, 1981; Pazur, 1982). This may be due in part to the small molecular sizes of sugars compared with the larger antigenic determinants of which they comprise a part.

The maximum inhibition of immunoprecipitation of α -L-fucosidase caused by the competitors employed in this study was 50–60%. The fact that higher percentage inhibitions were not found suggests that antibody I (the immunoglobulin G fraction of antiserum to purified α -L-fucosidase) is comprised of a heterogeneous population of molecules of which only about half are involved in recognizing carbohydrate-containing antigenic determinants.

The results of the studies reported here, although circumstantial, when taken together suggest that carbohydrate contributes to antigenic determinant(s) of human liver α -L-fucosidase and that sialic acid is probably the major sugar involved. This suggests that the amount of α -L-fucosidase protein in tissues from patients with fucosidosis could be present in higher concentrations than previously reported (Andrews-Smith & Alhadeff, 1982) if α -L-fucosidase from these patients exhibits decreased sialylation (DiMatteo *et al.*, 1976; Alhadeff & Andrews-Smith, 1980b). In addition, the present investigation has shown that the isoelectric forms of α -L-fucosidase have differences in their antigenic determinant(s). This modifies the results of a previous study (Alhadeff *et al.*, 1978) in which a less sensitive immunochemical technique (single-antibody immunoprecipitation) was unable to detect antigenic differences in these isoelectric forms.

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