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Purification to homogeneity of *Charonia lampas* α-fucosidase by using sequential ligand-affinity chromatography

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An α-fucosidase from the liver of the marine gastropod *Charonia lampas* was purified to homogeneity using a procedure that included cation-exchange and gel-filtration chromatography, chromatofocusing and a final series of affinity-chromatography steps which involved the following gel-immobilized ligands: *N*-(5-carboxy-1-pentyl)-1,5-dideoxy-1,5-imino-L-fucitol, *N*-(5-carboxy-1-pentyl)-2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol and thio-β-D-galactoside. The enzyme was found to be a tetrameric glycoprotein with a native M_r of 208000, and to exist in a number of isoforms displaying pI values in the range 6.0–6.4. Substrate-specificity studies using a number of fucosylated oligosaccharides of the lacto-*N* and lacto-*N*-*neo* series and a synthetic disaccharide confirmed that the enzyme catalyses the hydrolysis of a broad range of fucosidic linkages, and established the following hierarchy of susceptibility: Fucα2Galβ4Glc ⋙ Fucα6GlcNAc > Fucα2Galβ4GlcNAc > Galβ3(Fucα4)GlcNAc ⋙ Galβ4(Fucα3)GlcNAc. Similar relative rates of hydrolysis were also demonstrated using biantennary oligosaccharide alditols as substrates which contained fucose linked either α3 or α6 to the *N*-acetylglucosaminitol residue of the chitobiosyl core.

INTRODUCTION

The liver of the marine gastropod, *Charonia lampas*, is a rich source of an α-fucosidase (EC 3.2.1.51) which has a broad aglycon specificity and is therefore a valuable reagent for the identification and structural characterization of a diverse range of fucosylated oligosaccharides [1]. Efforts aimed at the purification of this enzyme utilizing conventional chromatographic procedures [2] were unsuccessful in removing other contaminant exoglycosidases, which can seriously compromise the use of the fucosidase as an analytical reagent. Similar problems have been encountered in isolating α-fucosidases from mammalian [3], bacterial [4] and plant [5] sources and only affinity chromatography, primarily using immobilized 1-amino-fucose [6] or a fucosylated oligosaccharide [7] as a ligand, has been successful in providing near-homogeneous enzyme preparations [6,7].

The demonstration that imino sugars are potent inhibitors of exoglycosidases [8] (K_i values typically in the micromolar range) suggests that these monosaccharide analogues may have considerable value as affinity ligands, especially since the imino function provides a means for attachment to a suitable matrix and can sometimes be manipulated chemically to create selective binding of a particular glycosidase. For example, attachment of the *N*-carboxypentyl form of deoxynojirimycin to Sepharose generates an affinity gel which can discriminate between α-glucosidase I and α-glucosidase II [9], and the mannose analogue, deoxymannojirimycin, similarly immobilized has been used to isolate a processing α-mannosidase from pig liver [10]. The importance of this class of ligand was further demonstrated recently when *N*-(5-carboxy-1-pentyl)-1,5-dideoxy-1,5-imino-L-fucitol was used to purify to homogeneity a novel form of α3/4-fucosidase from almond [11]. Here we describe the use of a sequential affinity-chromatographic scheme which utilizes the same fucose analogue as well as thio-β-D-galactoside and an imino sugar analogue of *N*-acetylglucosamine to provide a homogeneous preparation of *C. lampas* α-fucosidase that is free from contaminating exoglycosidases.

MATERIALS AND METHODS

Materials

A freeze-dried liver extract of *C. lampas* was purchased from Seikagaku Kogyo Co., Tokyo, Japan. *p*-Nitrophenyl (PNP) glycosides, *p*-aminobenzyl-1-thio-β-D-galactopyranoside–agarose and other biochemicals were from Sigma or Koch-Light. Affi-Gel 102 was obtained from Bio-Rad Laboratories and f.p.l.c. materials were from Pharmacia. *N*-Glycosidase F (EC 3.2.2.18) was purchased from Boehringer Corp. (London) Ltd. Linear oligosaccharides, LNT, LNNT, LNFP I, LNFP II, LNFP III, 2'FL, 6FN, M_1N_1, M_2N_1, M_3N_1, used in this study (see the Abbreviations used footnote for structures) were purified from human milk or mannosidosis urine [11] or purchased from BioCarb Chemicals, Lund, Sweden. Tritium-labelled oligosaccharide alditols, Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAcol, Manα6(Manα3)(Xylβ2)Manβ4GlcNAcβ4(Fucα3)GlcNAcol and Galβ4(Fucα3)GlcNAcβ2Manα6[Galβ4(Fucα3)-

Abbreviations used: LNT, Galβ3GlcNAcβ3Galβ4Glc (lacto-*N*-tetraose); LNNT, Galβ4GlcNAcβ3Galβ4Glc (lacto-*N*-*neo*tetraose); $N_1G_1Glc_1$, GlcNAcβ3Galβ4Glc; LNFP I, Fucα2Galβ3GlcNAcβ3Galβ4Glc (lacto-*N*-fucopentaose I); LNFP II, Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (lacto-*N*-fucopentaose II); LNFP III, Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (lacto-*N*-fucopentaose III); 2'FL, Fucα2Galβ4Glc (2'-fucosyl-lactose); 6FN, Fucα6GlcNAc (6-fucosyl-*N*-acetylglucosamine); M_1N_1, Manβ4GlcNAc; M_2N_1, Manα3Manβ4GlcNAc; M_3N_1, Manα2Manα3Manβ4GlcNAc; DFJ, deoxyfuconojirimycin (1,5-dideoxy-1,5-imino-L-fucitol); CPDFJ, carboxypentyldeoxyfuconojirimycin [*N*-(5-carboxy-1-pentyl)-1,5-dideoxy-1,5-imino-L-fucitol]; CPAG, *N*-(5-carboxy-1-pentyl)-2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol; h.p.a.e.c., high-performance anion-exchange chromatography; PNP, *p*-nitrophenyl.

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GlcNAcβ2Manα3]Manβ4GlcNAcβ4(Fucα6)GlcNAcol were prepared from human serotransferrin, horseradish peroxidase and human parotid gland glycan respectively by hydrazinolysis, sodium boro[^{3}H]hydride reduction and purification using Bio-Gel P-4 gel-filtration chromatography [12] and Dionex high-performance anion-exchange chromatography (h.p.a.e.c.) (see below). GlcNAcβ2Manα6(GlcNAcβ2Manα3)Manβ4GlcNAcβ4-GlcNAc was isolated by h.p.a.e.c. after digestion of the transferrin oligosaccharide with jack bean β-galactosidase. The structure of each oligosaccharide was confirmed using 500 MHz proton n.m.r. apart from the parotid glycan which was characterized by exoglycosidase sequencing [13]. Almond meal α3/4-fucosidase was prepared according to a published method [11]. *N*-(5-Carboxy-1-pentyl)-1,5-dideoxy-1,5-imino-L-fucitol (CPDFJ) was provided by G. D. Searle & Co., St. Louis, MO, U.S.A. *N*-(5-Carboxymethyl-1-pentyl)-2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol (MeCPAG) was synthesized from 2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol [14] in a manner identical with that described for a similar derivatization of deoxyfuconojirimycin [11]. MeCPAG was converted into the free carboxylic acid form [*N*-(5-carboxy-1-pentyl)-2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol; CPAG] by treatment for 20 min with 0.1 M-NaOH.

H.p.a.e.c.

Oligosaccharides and imino sugars were chromatographed on a CarboPac PA-1 column (5 mm × 250 mm) (Dionex BioLC System) eluted with 150 mM-NaOH/30 mM-sodium acetate at a flow rate of 1 ml/min, and monitored by triple-pulsed amperometric detection using the following pulse potentials and durations: $E_1 = 0.01$ V ($t_1 = 120$ ms), $E_2 = 0.6$ V ($t_2 = 120$ ms), $E_3 = -0.93$ V ($t_3 = 180$ ms). Oligosaccharide alditols were chromatographed using similar conditions but using an eluent of 25 mM-NaOH. Radioactivity was detected using a Berthold radioactivity monitor model LB507A which was calibrated with an ^{3}H-labelled biantennary oligosaccharide alditol isolated from human immunoglobulin G.

Determination of M_r

Native M_r determinations were made using a Superose 12 HR 10/30 column which was calibrated with the following proteins (supplied by Sigma): lactate dehydrogenase ($M_r = 140000$), conalbumin (77000), ovalbumin (45000) and myoglobin (17700). Chromatography was performed at room temperature using an eluent of 50 mM-sodium acetate buffer, pH 5.0, containing 0.1 M-NaCl at a flow rate of 0.3 ml/min.

Enzyme assays

Routinely, the activity of α-fucosidase was measured at 37 °C by incubating an appropriate amount of enzyme (5–50 μl, depending on the degree of purification) in a total volume of 1.0 ml of 50 mM-sodium acetate buffer, pH 4.5, containing 150 mM-NaCl and 3 mM-PNP-α-L-fucoside. After 15 min, the reaction was stopped by the addition of 2 ml of 0.5 M-Na_2CO_3, and the absorbance at 400 nm measured. One unit of enzyme activity is defined as that amount which releases 1 μmol of phenolate anion ($\epsilon = 17700$ M^{-1}·cm^{-1} at 400 nm) per min. Activity towards native oligosaccharides was assayed by incubation of the substrate (1.2 mM) and enzyme in 20 μl of the above buffer for between 1 and 48 h. The reaction mixture was desalted using Dowex AG3-X4 (OH^- form, 100–200 mesh) and AG50W-X12 (H^+ form, 100–200 mesh) and the products were separated and detected by h.p.a.e.c.-pulsed amperometry. The amount of enzyme-released fucose was calculated from peak areas by applying the following experimentally determined detector response factors for fucose relative to starting substrate: fucose:2′FL, 1.00:1.10; fucose:6FN, 1.00:1.08; fucose:LNFP I, 1.00:2.00; fucose:LNFP II, 1.00:1.72; fucose:LNFP III, 1.00:1.60. Hydrolysis of ^{3}H-labelled oligosaccharide alditols was measured by radioactive detection of the reaction products separated by h.p.a.e.c. after incubation of the substrate (10^5 c.p.m., approx. 20 pmol) in 10 μl of reaction buffer for various times at 37 °C with purified α-fucosidase.

Detection of contaminating glycosidases in preparations of α-fucosidase

Hydrolysis of PNP-glycosides (3 mM) of α- and β-galactose, α- and β-*N*-acetylglucosamine, α- and β-mannose, α- and β-*N*-acetylgalactosamine, α- and β-glucose and β-xylose were measured (see above) after incubation with α-fucosidase (6 units/ml of 50 mM-sodium acetate buffer, pH 4.5, containing 0.15 M-NaCl and 0.02% sodium azide) for 2–16 h at 37 °C. The activities of β-galactosidase, β-*N*-acetylglucosaminidase and α- and β-mannosidase were also assayed using the oligosaccharide substrates LNT, LNNT, $N_1G_1Glc_1$, M_2N_1, M_3N_1 and M_1N_1 respectively. These measurements were made at an α-fucosidase concentration of 9–14 units/ml and a substrate concentration of 1.2 mM. After 16–40 h at 37 °C, the reactants were desalted and a portion (equivalent to 10 nmol of substrate) was analysed by h.p.a.e.c. as described above.

Determination of kinetic constants

Enzyme, 0.33 unit, was incubated for 15 min in 0.5 ml of 50 mM-sodium acetate buffer, pH 4.5, containing 0.15 M-NaCl and PNP-α-L-fucoside at concentrations ranging from 0.2 to 5.0 mM. At intervals of 2.5 min the reaction was stopped by the addition of 1 ml of 0.5 M-Na_2CO_3, and the absorbance read at 400 nm. Initial rates were determined at each substrate concentration and these values were then used to calculate values for K_m and $V_{max.}$ using a weighted non-linear regression analysis (Multifit 2.0, Day Computing, Cambridge, U.K.).

Protein assay

Protein was determined by a micro-modification of the Pierce BCA method (Pierce Europe B.V.). Samples (2–10 μl) were applied in duplicate to flat-bottomed microtitre plates (Flow Laboratories) and 90 μl of reagent (made according to the manufacturer's instructions) was added. After incubation for between 10 and 30 min at 37 °C or room temperature, the absorbance at 570 nm was measured using a Dynatech MR600 microplate reader using BSA (0.2–20.0 μg) as a standard.

Isoelectric focusing and SDS/PAGE

Isoelectric focusing was performed using a Pharmacia Phast Gel apparatus with manufacturer-supplied, pre-cast pH gradients. For SDS/PAGE (10% gel), samples were prepared by dissociation in 2% SDS and 5% 2-mercaptoethanol, at 100 °C for 5 min. Electrophoresis was performed using a discontinuous buffer system [15] and high-M_r protein standards (29000–205000) provided by Sigma for the determination of subunit M_r values. Gels were silver-stained as described by Morrissey [16].

Preparation of affinity gels

The imino sugars, CPDFJ and CPAG, were covalently attached to Affi-Gel 102 using the carbodi-imide method. Carbodiimide (1 mmol) and Affi-Gel 102 (65 μmol of free amino groups) were added to an aqueous solution containing 250 μmol of the required imino sugar and 250 μmol of L-fucose (an internal non-reacting standard). The pH was maintained at 4.8 for 30 min by the addition of 0.1 M-HCl and the gel gently mixed overnight at 4 °C. The derivatized gel was washed sequentially with 50 mM-sodium acetate buffer, pH 5.0, containing 0.5 M-NaCl and 50 mM-

Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl and then packed in a Pharmacia SR 5/5 column, which was equilibrated with 50 mM-sodium acetate buffer, pH 4.5, containing 0.15 M-NaCl and 1% CHAPS. The amount of bound ligand was determined by h.p.a.e.c-pulsed amperometric analysis of the supernatant at the beginning and end of the coupling reaction. From the change in the ratio of the peak areas of the imino sugar and fucose it was calculated that, in each case, there was 20 μmol of covalently bound ligand/ml.

Purification of α-L-fucosidase

Buffer extraction and $(NH_4)_2SO_4$ fractionation. This and all other steps were performed at 4 °C unless otherwise stated. Freeze-dried liver extract (75 g) from *C. lampas* was suspended in 2 l of 0.1 M-sodium acetate buffer, pH 4.0, containing 1 mM-phenylmethanesulphonyl fluoride, stirred for 2 h and then centrifuged at 14300 *g* for 30 min. The supernatant was retained for further purification and the pellet re-extracted for 30 min as before. The combined supernatants were made 75% saturated by the addition of solid $(NH_4)_2SO_4$ over a period of 30 min. After centrifugation at 14300 *g* for 30 min, the precipitated material was dissolved in 200 ml of 50 mM-sodium acetate buffer, pH 4.5 (buffer A), and exhaustively dialysed against the same buffer.

CM-Sepharose chromatography. The dialysed material was chromatographed as two separate samples. Each was applied to a CM-Sepharose FF column (5 cm × 46 cm), equilibrated with buffer A, which was then washed with a further 2 l of buffer A at a flow rate of 200 ml/h. The column was eluted with a 4.5 l linear gradient of 0–500 mM-NaCl in buffer A. Fractions (10 ml) that contained α-fucosidase were pooled and concentrated by an 80%-$(NH_4)_2SO_4$-precipitation step. The resulting precipitate was recovered by centrifugation, as above, dissolved in 150 ml of 0.1 M-sodium acetate buffer, pH 4.5, containing 0.1 M-NaCl (buffer B), and dialysed against the same buffer.

TSK-gel-filtration chromatography. Dialysed material was further chromatographed as three separate samples (12 mg/ml of protein) on a TSK HW-55(S) column (5 cm × 90 cm) equilibrated in buffer B at a flow rate of 75 ml/h. Enzyme-active fractions were pooled, concentrated by ultrafiltration using an Amicon YM-10 membrane and dialysed against 50 mM-sodium acetate buffer, pH 4.5, containing 0.15 M-NaCl and 1% CHAPS (buffer C).

Affinity chromatography. Dialysed enzyme was applied to a 3 ml Affi-Gel–CPDFJ column at a flow rate of 10 ml/h. After being washed with 500 ml of buffer C to remove all non-bound material, the column was eluted with buffer C containing 10 mM-CPDFJ. Fractions (1 ml each) were collected, and 5 μl aliquots were removed, diluted 50-fold with buffer C to reduce the concentration of inhibitor, and assayed for enzyme activity. Fractions that contained α-fucosidase were pooled, dialysed against 50 mM-sodium acetate buffer, pH 4.5, containing 0.15 M-NaCl (buffer D). Subsequent purification was restricted to a portion (17.4 units) of the enzyme preparation; the remainder (279 units) was stored at −70 °C until needed.

F.p.l.c. chromatofocusing. Affinity-purified α-fucosidase was dialysed against 25 mM-Bistris buffer, pH 7.1, and applied (flow rate 1 ml/min) at room temperature to a Pharmacia Mono P column (5/20) equilibrated with 25 mM-Bistris buffer, pH 7.1, containing 4% taurine. The enzyme was eluted using pH 4.0 Polybuffer 74 plus 4% taurine, and fucosidase-active fractions (1 ml) were pooled and dialysed against buffer C.

Serial-recycling affinity chromatography. The α-fucosidase from the chromatofocusing step was passed through two affinity columns arranged in series; the first, Affi-Gel coupled to *N*-(5-carboxy-1-pentyl)-2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol (an imino sugar analogue of *N*-acetylglucosamine) and the second, *p*-aminobenzyl β-D-thiogalactopyranoside–agarose. Material eluted from the second column was automatically cycled back to the first column and the chromatography repeated. In this way the sample was cycled four times, at which point the columns were eluted with 10 column volumes of buffer C to remove all non-bound material. The pooled eluate was then rechromatographed on Affi-Gel/CPDFJ as described above. The α-fucosidase that was recovered was dialysed against buffer C containing 0.02% NaN_3, concentrated to 1 ml by ultrafiltration, and stored at 4 °C.

RESULTS AND DISCUSSION

Isolation of *C. lampas* α-fucosidase

Table 1 summarizes the eight-step procedure used to obtain an apparently homogeneous preparation of α-fucosidase. After buffer extraction and $(NH_4)_2SO_4$ precipitation, CM-Sepharose chromatography (results not shown) successfully removed 75% of the contaminating protein in the non-adsorbed fraction. However, most of the exoglycosidases originally present were also bound to the cation exchanger and were co-eluted with the α-fucosidase. The next step, TSK gel filtration (Fig. 1), removed essentially all of the α-*N*-acetylgalactosaminidase, β-xylosidase and α-mannosidase activity but the resulting α-fucosidase preparation still contained substantial amounts of β-galactosidase (EC 3.2.1.23) and β-*N*-acetylglucosaminidase (EC 3.2.1.30). The first chromatography step on CPDFJ–Affi-Gel removed a further 90% of the total protein (Fig. 2), but the α-fucosidase which was eluted specifically using 10 mM-CPDFJ was still contaminated with trace levels of β-galactosidase and β-*N*-acetylglucosaminidase activity (0.005% and 0.1% respectively relative to α-fucosidase activity). Two subsequent steps were therefore designed to eliminate these additional enzymes.

Chromatofocusing using a pH gradient of 4.0–7.1 removed a major isoform of β-*N*-acetylglucosaminidase which had a pI of 4.7 (Fig. 3). However, this step was unable to completely eliminate two remaining isoforms (pI values estimated to be 5.7 and 5.9) from the preparation of α-fucosidase (present as several isoforms with estimated pI values ranging from 6.0 to 6.4).

Initial experiments showed that CPAG, an analogue of *N*-acetylglucosamine, coupled to Affi-Gel was only effective at removing approx. 50% of the β-*N*-acetylglucosaminidase activity, despite being a reasonable inhibitor of the enzyme (50% inhibition at 22 μM). However, a recycling step on the same column successfully removed all of this activity as assessed by assaying the resulting preparation against the trisaccharide, GlcNAcβ3Galβ4Glc and the biantennary substrate, GlcNAcβ2Manα6(GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc. Interestingly, affinity columns composed of *N*-acetylglucosamine (or the 1-thio derivative) coupled to epoxy-activated or nitrophenyl chloroformate-activated agarose were also tested but did not bind the β-*N*-acetylglucosaminidase at pH 4.0 or 7.0. This was an unexpected result since several other plant and bacterial glycosidases were bound by these matrices ([17] and T. D. Butters, unpublished work). Removal of the β-galactosidase activity to provide a homogeneous preparation of α-fucosidase proved to be simpler and was achieved by chromatography on a column of thio-β-D-galactoside–agarose.

Some points regarding the CPDFJ–Affi-Gel affinity chromatography step are worth noting. Since the affinity of CPDFJ for α-fucosidase is high (K_i = 5.6 μM; T. D. Butters, unpublished work), a relatively large excess of the ligand was needed to elute the bound enzyme. Although this process appeared specific for α-fucosidase, and CPDFJ showed no inhibitory action against β-galactosidase or β-hexosaminidase, these contaminant activities were still found in the post-affinity

Table 1. Purification of *C. lampas* α-fucosidase

A detailed description of each step is given in the Materials and methods section.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
Buffer extract	740	11400	0.065	100	1
75%-Satd. $(NH_4)_2SO_4$ precipitate	648	7880	0.082	88	1.3
CM-Sepharose chromatography	383	1970	0.194	52	3.0
TSK chromatography	370	318	1.176	50	18.1
Affinity chromatography	296 (17.4)*	32.6 (1.9)	9.080	40	139.7
F.p.l.c. chromatofocusing	(5.5)	(0.6)	(9.48)	(13)	(145.9)
Serial-recycling affinity chromatography	(3.3)	(0.3)	(11.1)	(8)	(170.8)

* Values in parentheses refer to a sample of α-fucosidase from the affinity-chromatography step which was further purified as shown.

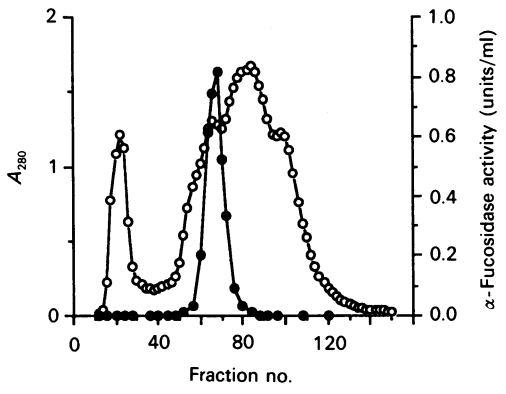

Fig. 1. TSK-gel-filtration chromatography of *C. lampas* α-fucosidase

The column eluate was monitored for protein by absorbance at 280 nm (○) and fractions were assayed for enzyme (●). Full details are given in the Materials and methods section.

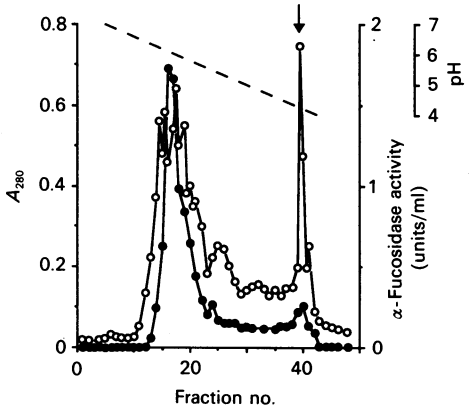

Fig. 3. F.p.l.c.-chromatofocusing of *C. lampas* α-fucosidase

The column was developed using Polybuffer 74, pH 4.0. The eluate was monitored for protein by absorbance at 280 nm (○) and fractions were assayed for enzyme activity (●); the broken line shows the pH gradient. The elution position of β-*N*-acetylglucosaminidase activity is indicated by the arrow. Full details are given in the Materials and methods section.

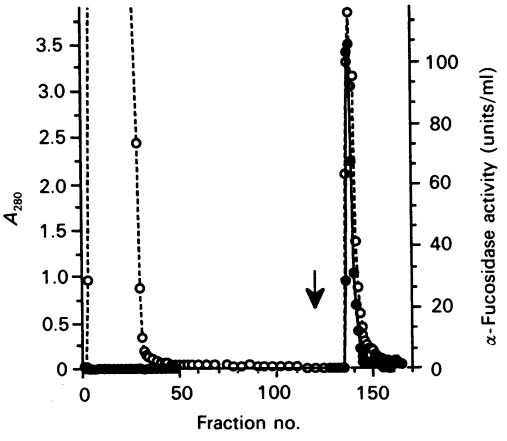

Fig. 2. CPDFJ affinity chromatography of *C. lampas* α-fucosidase

After sample loading, the column was washed with buffer C until the absorbance at 280 nm (○) had returned to baseline. The arrow indicates the position where elution was begun with buffer containing 10 mM-CPDFJ. Fractions were assayed for α-fucosidase activity (●) after first being diluted to reduce the concentration of the inhibitor. Full details are given in the Materials and methods section.

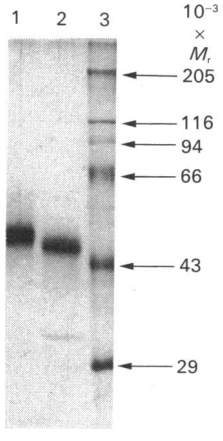

Fig. 4. SDS/PAGE of purified *C. lampas* α-fucosidase

Electrophoresis was performed under reducing conditions as described in the Materials and methods section. Protein was detected by using silver stain. The arrows indicate the positions of M_r markers. Lane 1, α-fucosidase; lane 2, α-fucosidase after digestion with *N*-glycosidase F (N.B. the minor band at M_r 35000 is *N*-glycosidase F); lane 3, protein standards.

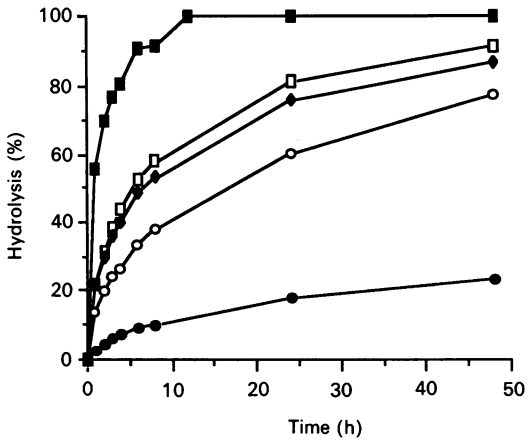

Fig. 5. Hydrolysis of oligosaccharides by α-fucosidase from *C. lampas*

Oligosaccharides (1 mM) were digested with enzyme at 0.45 unit/ml (except 2'FL, where the concentration was 0.045 unit/ml). At the times shown, portions (10 nmol of substrate) were removed for assay by h.p.a.e.c. of enzyme-released fucose. Each data point is the mean from two duplicate runs. Full details are given in the Materials and methods section. □, 6FN; ◆, LNFP I; ■, 2'FL; ○, LNFP II; ●, LNFP III.

preparation of fucosidase. This result could argue for a close hydrophobic association between these enzymes and the fucosidase, which was supported by the fact that inclusion of detergent in the affinity chromatography step significantly reduced the amount of each contaminant in the eluted material.

Overall, the method achieved a purification factor of 171 with a yield of 8 %, similar to values reported by Iijima and Egami [2] who used gel-filtration and hydroxyapatite chromatography to purify this enzyme. The failure of affinity chromatography to increase dramatically the purification factor probably results from the relative abundance of the enzyme, accounting for almost 0.3 % of the total protein present in the original liver extract, and the fact that only small amounts, by weight, of contaminating exoglycosidases are present at this stage of the purification.

Purity and properties of α-fucosidase

The purified enzyme displayed a native M_r of 208 500 by Superose 12 gel-permeation chromatography and migrated as a single band with an M_r of 49 500 by SDS/PAGE (Fig. 4). Hence, the enzyme appears to be a tetramer composed of essentially identical subunits. Similar M_r characteristics have been described for α-fucosidases from other sources [18–20].

When assayed against PNP-glycosides, the preparation of α-fucosidase was found to be free of detectable α- and β-*N*-acetylgalactosaminidase, α- and β-glucosidase, α- and β-mannosidase, α- and β-galactosidase, β-xylosidase and α-*N*-acetylglucosaminidase activity. However, β-*N*-acetylglucosaminidase activity was detected at a level of 0.01 % relative to that of the α-fucosidase. This activity against PNP-β-D-GlcNAc is in contrast with measurements made against several natural sugar substrates under more stringent conditions, i.e. higher concentration of α-fucosidase and prolonged incubation time (see the Materials and methods section). No β-*N*-acetylglucosaminidase activity was detected by the h.p.a.e.c.-pulsed amperometric assay using oligosaccharide substrates of GlcNAcβ3Galβ4Glc or GlcNAcβ2Manα6(GlcNAcβ2Manα3)-Manβ4GlcNAcβ4GlcNAc (the sensitivity of the h.p.a.e.c.-pulsed amperometric method is such that a contaminant activity can readily be detected at a level of 0.001 % relative to that of the α-

Table 2. Hydrolysis of radiolabelled oligosaccharide alditols by α-fucosidases

After incubation of the appropriate alditol substrate with the indicated concentrations of *C. lampas* α-fucosidase and almond α-fucosidase III, the reaction products were desalted, separated by h.p.a.e.c. and their peak areas quantified as described in the text.

Substrate	Site of hydrolysis	Hydrolysis (%)		
		C. lampas		Almond
		0.0045 units/ml	9.0 units/ml	0.017 units/ml
IgG glycan Galβ1-4GlcNAcβ1-2Manα1,6 Fucα 1,6 ← Site a Manβ1-4GlcNAcβ1-4GlcNAc.ol Galβ1-4GlcNAcβ1-2Manα1,3	a	100	100	0
Parotid gland glycan Fucα1,3 ← Site b Galβ1-4GlcNAcβ1-2Manα1,6 Fucα 1,6 ← Site a Manβ1-4GlcNAcβ1-4GlcNAc.ol Galβ1-4GlcNAcβ1-2Manα1,3	a	100	100	0
	b	0	65	100
Fucα1,3 ← Site b				
Horseradish peroxidase glycan Manα1,6 Manβ1-4GlcNAcβ1-4GlcNAc.ol Manα1,3 Xylβ1,2 Fucα1,3 ← Site c	c	0	100	0

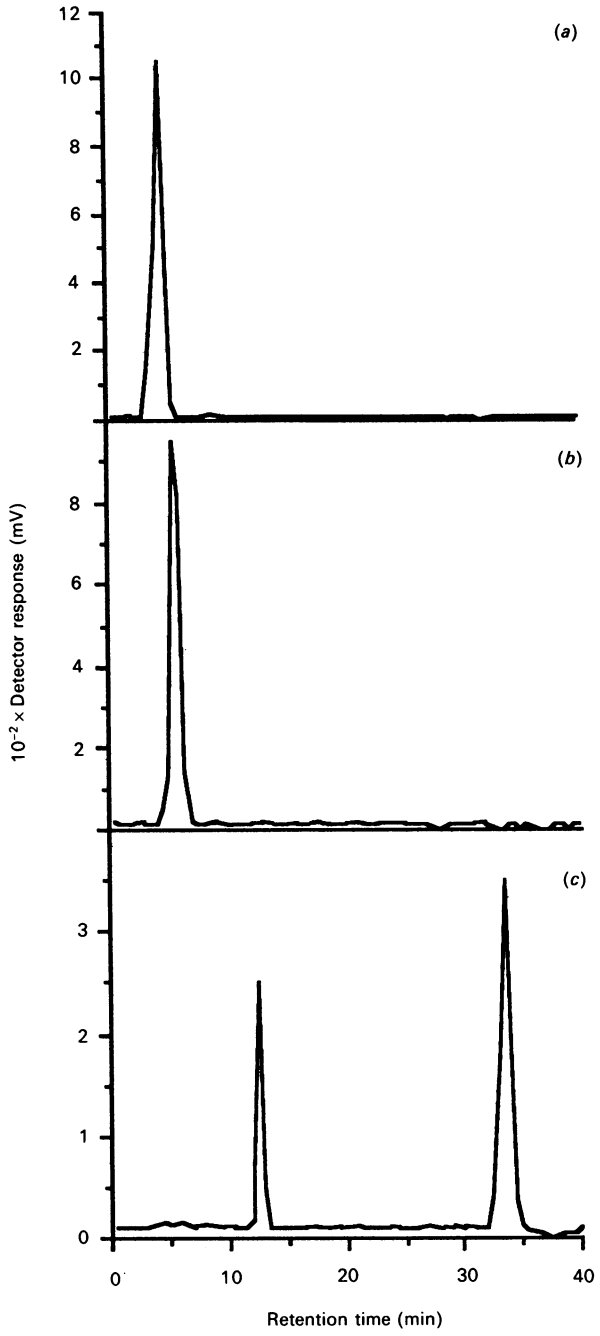

Fig. 6. H.p.a.e.c. of parotid gland glycan after digestion with *C. lampas* α-fucosidase

The ^{3}H-labelled alditol (10^5 c.p.m.) isolated from parotid gland was analysed by chromatography using a CarboPac PA-1 column eluted with 25 mM-NaOH, as described in the text, before (*a*) and after a 16 h incubation with affinity purified *C. lampas* α-fucosidase at 0.009 unit/ml (*b*) or at 9 units/ml (*c*). Radioactively labelled reaction products were detected using an in-line radioactivity monitor.

fucosidase [11]). In addition, the purified enzyme demonstrated no β-galactosidase or α- or β-mannosidase activity when assayed against LNT, LNNT, M_3N_1, M_2N_1 or M_1N_1.

Isoelectric focusing showed that the purified fucosidase existed in a number of isoforms which exhibited pI values ranging from 6.0 to 6.4 (results not shown). This feature is shared by the human liver [21] and edible-clam enzymes [22]. The ability of our affinity-purified enzyme to bind to concanavalin A–Sepharose (results not shown) suggests that it contains *N*-linked oligosaccharides with core mannose structures. This was confirmed by SDS/PAGE (Fig. 4) of α-fucosidase that had been treated with *N*-glycosidase F (40 units/ml for 16 h at 37 °C) which showed that the M_r of the monomer had been reduced from 49500 to 46800 [consistent with the enzyme being approximately 4.5% (w/w) carbohydrate].

The fucosidase was extremely stable and no loss of activity was detected even after storage for 3 months at 4 °C. However, freeze-drying caused inactivation of the enzyme unless 2.5 mg of BSA/ml was present. In this case, at least 80% of enzyme activity was retained. The enzyme exhibited a pH optimum of 3.0 and demonstrated a similar broad pH profile to that reported by Iijima & Egami [2]. Its activity was unaffected by a number of reagents including Zn^{2+}, EDTA, Mg^{2+} and cysteine (each at 10 mM), but 1 mM-Cu^{2+} and 0.1 mM-*p*-chloromercuriphenylsulphonic acid caused 41 and 50% inhibition of activity respectively.

Substrate specificity

The purified α-fucosidase hydrolysed PNP-α-fucoside with a K_m of 0.26 mM and a $V_{max.}$ of 11.05×10^{-6} mol/min per mg of protein, values that are in good agreement with the only other published kinetic data for this synthetic substrate [2]. Fig. 5 shows the relative rates of hydrolysis (as assessed by h.p.a.e.c.-pulsed amperometric analysis) of Fucα6GlcNAc and a number of fucosylated linear milk oligosaccharides isolated from human milk. The most susceptible linkage was Fucα2Gal, contained in the trisaccharide 2′FL, which was completely hydrolysed in less than 12 h at an enzyme concentration of 45 munits/ml. Despite the use of a 10-fold higher concentration of enzyme, none of the hydrolyses involving the other substrates went to completion, even after incubation for 48 h. The Fucα2Gal linkage present in LNFP I and the Fucα6GlcNAc linkage in the synthetic disaccharide 6FN were hydrolysed at similar rates (50% hydrolysis at 4.6 h and 5.8 h respectively) and were significantly better substrates than the Fucα4GlcNAc linkage of LNFP II which was cleaved by 50% in 16.6 h. LNFP III, which contains a Fucα3GlcNAc linkage, was an extremely poor substrate and after 48 h was only hydrolysed by 20%. Even at 9 units/ml (the highest concentration of α-fucosidase tested), when there was complete hydrolysis of the other oligosaccharides, LNFP III was only hydrolysed by 66% after 36 h. The relative activity of the fucosidase is in general agreement with the results of an earlier study [1] which utilized the corresponding ^{3}H-labelled alditols as substrates. The ability of *C. lampas* α-fucosidase to catalyse the hydrolysis of a broad range of fucosidic linkages is also a property of several mammalian α-fucosidases [6,19] and distinguishes it from the plant and bacterial enzymes, which have a much narrower substrate specificity [4,5,11].

Also tested as substrates were three oligosaccharide alditols: a biantennary oligosaccharide isolated from human immunoglobulin which contains a core GlcNAcβ4(Fucα6)GlcNAcol, a biantennary oligosaccharide isolated from human parotid gland, which contains a core GlcNAcβ4(Fucα6)GlcNAcol and two outer-arm Galβ4(Fucα3)GlcNAc sequences, and an oligosaccharide from horseradish peroxidase, which contains a core GlcNAcβ4(Fucα3)GlcNAcol sequence (for full structures, see Table 2).

Treatment of the trifucosylated parotid oligosaccharide for 16 h with 0.009 unit of the fucosidase/ml (conditions that cause efficient hydrolysis of the core-associated Fucα6GlcNAcol linkage present in the oligosaccharide isolated from human IgG, Table 2) resulted in conversion of the substrate into a single product which, by h.p.a.e.c. (Fig. 6*b*), was eluted with a retention

time of 5.7 min (retention time of the substrate was 4.5 min, see Fig. 6*a*). At 9 units/ml, there were two products: a major one (65% of the total) which had the same retention time (33.8 min) as the nonasaccharide, Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc, and a partially defucosylated product with a retention time of 12.5 min (Fig. 6*c*). Both fucosylated products from the digestions using low and high concentrations of enzyme were susceptible to almond α3/4-fucosidase (17 munits/ml for 16 h [11], Table 2) suggesting that they contained two and one outer-arm Fucα3GlcNAc sequences (site b) respectively. The monofucosylated product could represent a single isomer containing a Fucα3GlcNAc sequence on either the -3 or -6 branch, or a mixture of both. The present data do not allow us to differentiate between these three possibilities. However, the ability of h.p.a.e.c. at high pH to resolve similar branch isomers, Galβ4GlcNAcβ2Manα3(GlcNAcβ2Manα6)-Manβ4GlcNAcβ4GlcNAc and Galβ4GlcNAcβ2Manα6-(GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (T. D. Butters, unpublished work), may argue in favour of a single product. It is clear from these results that digestion with low concentrations of enzyme results in cleavage that is restricted to site a, while at high concentrations of fucosidase both b sites are apparently hydrolysed, but at low and possibly dissimilar rates. The Fucα3-GlcNAcol sequence (site c, Table 2) of a horseradish peroxidase-derived oligosaccharide [23] was completely hydrolysed by *C. lampas* α-fucosidase but only at a concentration of 9 units/ml.

It is evident therefore that this enzyme can be used to discriminate between an *N*-glycan which contains a core Fucα6GlcNAc linkage and one which contains Fucα3GlcNAc. This result may reflect differences in either spatial arrangement of the aglycon moiety or a requirement for an additional, possibly hydrophobic (acetyl), recognition/binding site. In this respect it is interesting to note that the core-associated Fucα3GlcNAcol linkage (site c, Table 2) of the horseradish peroxidase glycan was completely refractory to almond α3-fucosidase using conditions that resulted in the complete hydrolysis of the Fucα3GlcNAc linkage present at both b sites of the parotid oligosaccharide. This apparent resistance is not due to an altered open-ring conformation of the terminal GlcNAc residue, since unreduced oligosaccharides from horseradish peroxidase are equally resistant to hydrolysis (T. D. Butters, unpublished work). This additional information about the almond enzyme will be of value in further optimizing its use for oligosaccharide sequencing [11] and may indicate that the almond enzyme has a substrate-binding site that cannot properly accommodate a hydrophobic *N*-acetylglucosamine residue adjacent to the Fucα3GlcNAc sequence.

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