

Carbohydrate structure of human pancreatic elastase 1

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Human pancreatic elastase 1 (E1) is a glycoprotein containing two potential *N*-glycosylation sites, one of which carries a carbohydrate moiety [Wendorf, Geyer, Sziegoleit & Linder (1989) FEBS Lett. 249, 275–278]. In order to study its glycosylation, glycoprotein isolated from post-mortem pancreas tissue of 75 donors was digested with trypsin. Oligosaccharides were liberated from resulting glycopeptides by treatment with peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)-asparagine amidase F, radiolabelled by reduction with KB³H₄ and separated by h.p.l.c. and gel filtration. Major oligosaccharide alditol fractions, representing 67.8 mol% of total glycans, were characterized by methylation analysis and sequential degradation with exoglycosidases. The results revealed that about two-fifths of the partially truncated, mainly biantennary, complex-type glycans found comprised blood group A, B, Le^a (or X), difucosyl A or difucosyl B determinants, which could be assigned to lactosamine antennae linked to Man(α 1–3)- residues of the sugar chains.

INTRODUCTION

About 15 enzymes and proenzymes are synthesized, stored and secreted by the mammalian exocrine pancreas (Scheele *et al.*, 1981). Many of these enzymes, including elastases, are serine proteinases.

Two elastolytic enzymes, elastase 1 and elastase 2, have been isolated from human pancreas (Largman *et al.*, 1976). Elastase 2 corresponds to rat and pig pancreatic elastase 2 (Fletcher *et al.*, 1987; MacDonald *et al.*, 1982; Kawashima *et al.*, 1987), whereas elastase 1 is not the human counterpart to rat and pig pancreatic elastase 1 (Tani *et al.*, 1987). Human elastase 1 (E1) seems rather to belong to a different enzyme family.

Recently, pancreatic enzymes from a variety of animal species have been shown to be immunologically related to E1, and the corresponding pig and bovine enzymes have been identified as proteinase E and subunit III of the procarboxypeptidase complex respectively (Sziegoleit & Linder, 1986; Sziegoleit *et al.*, 1987; Venot *et al.*, 1986). Structural studies (Wendorf *et al.*, 1989) also confirmed that E1 is basically identical with elastase IIIb investigated by Tani *et al.* (1988). E1 is further characterized by its capability to combine with bile acids and cholesterol during intestinal passage, generating protein–sterol complexes (Sziegoleit, 1982; Sziegoleit & Linder, 1991).

Previous studies demonstrated that E1 is a glycoprotein containing two potential *N*-glycosylation sites, only one of which (at Asn-86) was found to be glycosylated (Wendorf *et al.*, 1989). Monosaccharide constituent analyses revealed the presence of fucose, mannose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine in molar proportions of about 1.5:3.0:2.0:5.0:0.4. Since carbohydrate moieties can influence the solubility, stability, proteinase-resistance and also the antigenicity of a glycoprotein (Berger *et al.*, 1982; Feizi & Childs, 1987), we have now carried out a detailed carbohydrate structure analysis of the oligosaccharides bound to Asn-86 of E1.

MATERIALS AND METHODS

Materials

The mixture of α -1,2-fucosidase and β -galactosidase from

Aspergillus niger was generously provided by Dr. R. Nuck (University of Berlin, Berlin, Germany). β -Mannosidase from *Polyporus sulfureus* was a gift from Dr. Y.-T. Li (Tulane University, New Orleans, LA, U.S.A.). Sialylated biantennary oligosaccharides containing one or two sialic acid residues were kindly supplied by Dr. G. Strecker (University of Lille, Villeneuve d'Ascq, France) and radiolabelled by reduction with KB³H₄. Similarly radiolabelled 2,4-branched triantennary glycans and tetra-antennary oligosaccharides with three and four sialic acid residues respectively, as well as corresponding asialo-oligosaccharide alditols, were obtained from α_1 -acid glycoprotein and kindly provided by Dr. H. Geyer (University of Giessen, Giessen, Germany). Glucose oligomers were prepared by partial hydrolysis of dextran as described by Yamashita *et al.* (1982).

Isolation and purification of E1

Human pancreases were collected within 24 h *post mortem* during autopsy of 75 individuals and stored at –20 °C. Two pools of 35–40 organs, gathered within 8–10 weeks, were used as starting material for the isolation of E1.

The purification procedure followed essentially the protocol described previously (Sziegoleit, 1984). Briefly, organs were macroscopically freed from adjacent and fatty tissue, homogenized in ice-cooled water in a commercial Waring Blendor and centrifuged at 7000 *g* for 60 min at 4 °C, resulting in the formation of three layers of material. The intermediate layer was recovered and delipidated by extraction with Triton X-100 as detailed elsewhere (Sziegoleit, 1982). The aqueous phase obtained was concentrated by tangential flow ultrafiltration (Pellicon PTGC 05; Millipore, Eschborn, Germany) and simultaneously dialysed against water and finally against 25 mM-sodium phosphate buffer, pH 5.8. The resulting solution was applied to a 1.7-litre column of DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden), equilibrated with the sodium phosphate buffer described above. Proteins were eluted with 4 litres of a linear 100–800 mM-NaCl gradient in the same buffer at a flow rate of 94 ml/h. Fractions (22 ml) containing E1 were identified by fused rocket immunoelectrophoresis with a specific antiserum, pooled, concentrated and dialysed against 25 mM-sodium phosphate buffer, pH 6.8. After addition of solid (NH₄)₂SO₄ to a final

Abbreviations used: E1, human pancreatic elastase 1; GlcNAcOH, *N*-acetylglucosaminol; PNGase F, peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)-asparagine amidase F (EC 3.5.1.52) from *Flavobacterium meningosepticum*.

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concentration of 1 M, the protein solution was chromatographed through an octyl-Sepharose CL-6B column (5 cm × 25 cm; Pharmacia), on which E1 did not adsorb under these buffer conditions. The flow-through was then applied to a phenyl-Sepharose CL-6B column (5 cm × 25 cm; Pharmacia), equilibrated with the same buffer. Elution of E1 was achieved by linearly decreasing the concentration of $(\text{NH}_4)_2\text{SO}_4$ from 1 M to zero. E1-containing fractions were identified as described above and concentrated by pressure dialysis. The protein was finally purified by gel filtration on a TSK HW 50 column (2.6 cm × 50 cm; Merck, Darmstadt, Germany), equilibrated with 25 mM-ammonium acetate buffer, pH 6.0. All chromatographic steps were carried out at 4 °C.

Solutions of purified E1 were concentrated to about 2 mg of E1/ml, supplemented with solid $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 10 mM and dialysed against distilled water at 4 °C. Under these conditions spontaneous crystallization of the protein occurred within 24–48 h.

Analytical gel electrophoresis

Analytical SDS/PAGE was carried out by the procedure of Laemmli (1970) in slab gels containing 12.5% polyacrylamide. Gels were stained for protein with Coomassie Brilliant Blue (Serva, Heidelberg, Germany).

Isolation of tryptic-digest glycopeptides

E1 was carboxymethylated and fragmented with trypsin (Serva) as described elsewhere (Wendorf *et al.*, 1989). Resulting (glyco)peptides were separated by gel filtration and reverse-phase h.p.l.c. and characterized by amino acid and hexosamine analysis as well as by N-terminal amino acid sequencing, as detailed elsewhere (Wendorf *et al.*, 1989).

Isolation of oligosaccharides

E1 glycans were released by treatment of tryptic glycopeptides with PNGase F from *Flavobacterium meningosepticum* (Boehringer Mannheim, Mannheim, Germany), separated from residual peptides by reverse-phase h.p.l.c., radiolabelled by reduction with KB^3H_4 and desalted as described previously (Geyer *et al.*, 1983; Strube *et al.*, 1988).

Chromatographic procedures

(a) Desalting of glycopeptides and oligosaccharide alditols, (b) separation of oligosaccharide alditols by Bio-Gel P-4 chromatography or h.p.l.c. on a column of LiChrosorb- NH_2 (Merck), (c) reverse-phase h.p.l.c. for separation of oligosaccharides from peptides and (d) ion-exchange h.p.l.c. on a column of Mikropak AX-5 (Varian, Walnut Creek, CA, U.S.A.) for fractionation of charged oligosaccharides were carried out as described in detail previously (Schlüter *et al.*, 1985; Geyer *et al.*, 1987; Strube *et al.*, 1988; Strube & Geyer, 1989).

For chromatographic characterization of oligosaccharide alditol fragments obtained after enzymic digestion, a Bio-Gel P-4 column (–400 mesh; 1.6 cm × 180 cm; Bio-Rad, Munich, Germany) equipped with an injection valve (U6K) and a model 6000 A pump (both from Waters, Milford, MA, U.S.A.) was used at 55 °C with aq. 0.02% (w/v) NaN_3 as eluent. Fractions (1.8 ml) were collected at a flow rate of 0.3 ml/min. Glucose oligomers used as standard were monitored by refractive-index measurement with a model R401 detector (Waters).

High-pH anion-exchange chromatography was carried out on a Dionex (Sunnyvale, CA, U.S.A.) BioLC system as described previously (Pfeiffer *et al.*, 1990). Oligosaccharide alditols were

separated by a linear gradient from 100% (v/v) eluent A (0.1 M-NaOH) to 24% (v/v) eluent B (0.1 M-NaOH/0.25 M-sodium acetate) in 72 min at a flow rate of 1 ml/min. The effluent was fractionated (250 μl) into tubes containing 50 μl of 0.5 M-acetic acid.

After addition of scintillation cocktail (Roth, Karlsruhe, Germany), portions of all chromatographic fractions were monitored for radioactivity with a model 4450 (Packard, Downers Grove, IL, U.S.A.) liquid-scintillation counter. Radiolabelled oligosaccharide alditol fractions obtained were desalted by ion-exchange chromatography on Amberlite AG MB-3 mixed-bed resin (Serva) as described elsewhere (Strube *et al.*, 1988).

Enzymic digestions

Treatment of oligosaccharide alditols with α -galactosidase from green coffee (*Coffea*) beans, β -galactosidase, β -N-acetylhexosaminidase and α -mannosidase from jack beans (*Canavalia ensiformis*; all from Sigma, Deisenhofen, Germany) and α -fucosidase from ox kidney (Boehringer Mannheim) was performed as described elsewhere (Geyer *et al.*, 1984; Strube *et al.*, 1988). For digestion with α -N-acetylglucosaminidase from chicken liver (Sigma) the oligosaccharide alditols were dissolved in 50 μl of 50 mM-sodium citrate buffer, pH 4.7, containing 0.1–0.5 mg of BSA/ml. After addition of 0.08–0.24 nkat of enzyme, the mixture was incubated for 24 h at 37 °C.

Degradation of oligosaccharide alditols with a mixture of α -1,2-fucosidase and β -galactosidase from *Aspergillus niger* was carried out in 50 μl of 50 mM-sodium acetate buffer, pH 3.9, with 0.01 nkat of the total enzyme activity for 24 h at 37 °C.

For treatment with β -mannosidase from *Polyporus sulfureus*, oligosaccharide alditols were dissolved in 100 μl of 50 mM-glycine/HCl buffer, pH 2.6, and incubated with 1.7 nkat of enzyme for 24 h at 37 °C.

Methylation analysis

Oligosaccharides were permethylated (Paz-Parente *et al.*, 1985) and hydrolysed. Partially methylated alditol acetates obtained after NaBH_4 reduction and acetylation were analysed by capillary g.l.c.–m.s. with the use of the instrumentation and micro-techniques described previously (Geyer *et al.*, 1983).

RESULTS

Liberation and fractionation of E1 glycans

Human elastase 1 (E1) was isolated from a pool (75 samples) of post-mortem pancreas tissue and purified as described in the Materials and methods section. Enzyme preparations obtained were analysed by SDS/PAGE. Subsequent staining for protein with Coomassie Brilliant Blue revealed only one protein band with an apparent molecular mass of about 28 kDa (Fig. 1a). The purified glycoprotein spontaneously crystallized as shown in Fig. 1(b).

The protein was carboxymethylated and digested with trypsin as described previously (Wendorf *et al.*, 1989). Resulting glycopeptides were isolated by gel filtration and reverse-phase h.p.l.c. As shown previously (Wendorf *et al.*, 1989), two glycopeptides are thus obtained, both containing the N-glycosylation site at Asn-86 of the E1 molecule. Since both glycopeptides were shown to contain the same monosaccharide constituents in similar molar proportions (Wendorf *et al.*, 1989), the two glycopeptide fractions were combined. Starting from 73 mg of glycoprotein, 1.8 mg of pooled glycopeptides was subjected to carbohydrate structure analysis.

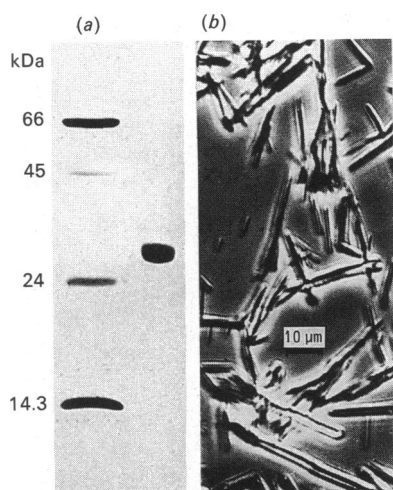


Fig. 1. Characterization of purified E1

(a) Analytical SDS/PAGE of purified E1 (about 7 µg) under non-reducing conditions. Molecular masses of standard proteins are indicated: BSA, 66 kDa; ovalbumin, 45 kDa; trypsinogen, 24 kDa; lysozyme, 14.3 kDa. (b) E1 crystals formed after dialysis of a solution of purified enzyme against distilled water.

Glycopeptides were treated with PNGase F. Oligosaccharides released were separated from residual peptides by reverse-phase h.p.l.c. Carbohydrate constituent analysis of the remaining peptides demonstrated that all glycans were liberated by the enzyme (results not shown). Oligosaccharides obtained were desalted, radiolabelled by reduction with KB^3H_4 and desalted again. The resulting oligosaccharide alditols contained 5×10^6 c.p.m.

Analytical ion-exchange h.p.l.c. (results not shown) revealed that none of the oligosaccharide alditols obtained was negatively charged, indicating that this preparation of E1 did not carry any sialylated glycans. For further fractionation, oligosaccharide alditols were therefore subjected to h.p.l.c. on a LiChrosorb- NH_2 column. As shown in Fig. 2, E1 glycans represented a highly diverse mixture of different sugar chains. Oligosaccharides were subsequently pooled as indicated by horizontal brackets. Fractions 1–4 (comprising 32.9% of total radioactivity) were subfractionated by Bio-Gel P-4 chromatography (Fig. 3), whereas fractions 5–8 (60.4% of total radioactivity) were further separated by high-pH anion-exchange chromatography (Fig. 4). The results demonstrated that each fraction obtained after h.p.l.c. on the LiChrosorb- NH_2 column still represented a mixture of glycans. Oligosaccharide alditol subfractions obtained in amounts sufficient for carbohydrate analysis were pooled as indicated by horizontal brackets. Fractions given in parentheses were not further studied. Since subfractions 54, 61 and 83 were found still to contain a mixture of glycans (see below), they were, in part, further separated by Bio-Gel P-4 chromatography, yielding the major subfractions 541, 611, 831 and 832 (results not shown). In the case of fraction 61, subfractionation was only possible after treatment of the glycans with β -N-acetylhexosaminidase before gel filtration, which resulted in a partial degradation of minor constituents, whereas major oligosaccharide constituents remained unaffected.

Analysis of oligosaccharide alditols

Oligosaccharide alditol fractions 12, 21, 31–33, 41, 51, 52, 541, 611, 62–64, 72, 74, 75, 78, 83 and 84 obtained by gel filtration (cf. Fig. 3), high-pH anion-exchange chromatography (Fig. 4) or

subfractionation of fractions 54 and 61 as described above were subjected to methylation analysis. The results are summarized in Table 1. For structural assignment of outer substituents, portions of fractions 21, 32, 41, 51 and 52 were preparatively degraded with exoglycosidases as detailed below. Oligosaccharide alditol fragments obtained after sequential treatment with α -mannosidase (21' and 32'), β -N-acetylhexosaminidase and α -mannosidase (41' and 51') or β -N-acetylhexosaminidase/ α -N-acetylglucosaminidase/ α -1,2-fucosidase and β -galactosidase (from *A. niger*) and α -mannosidase (52') were again studied by methylation analysis. The results are also included in Table 1.

For determination of the anomeric configurations of monosaccharide constituents as well as for enzymic sequencing of the glycans, oligosaccharide alditols obtained were degraded by successive treatment with the exoglycosidases listed in Table 2. Untreated glycans and reaction products were analysed by Bio-Gel P-4 chromatography (see, e.g., Fig. 5), and their sizes were determined by co-chromatography with glucose oligomers (Yamashita *et al.*, 1982; Kobata *et al.*, 1987). Oligosaccharide fragments obtained from fractions 12, 21, 31 and 32 eluted at about 6.5 glucose units were pooled before digestion with α -fucosidase, β -mannosidase and β -N-acetylhexosaminidase.

Structural conclusions

The results obtained after methylation analysis (Table 1) and sequential degradation with exoglycosidases (Table 2) allow the following conclusions on the structures of the major oligosaccharides from the E1 preparation studied (cf. Table 3). Fraction 12 glycans (1.3 mol% of total oligosaccharides) contained about one terminal fucose residue (2,3,4-FucOH; see Table 1), two terminal (2,3,4,6-ManOH) and one 3,6-disubstituted (2,4-ManOH) mannose residues, one 4-substituted *N*-acetylglucosamine residue [3,6-GlcN(Me)AcOH] and one 4,6-disubstituted *N*-acetylglucosaminitol residue [1,3,5-GlcN(Me)-AcOH]. Sequential degradation with α -mannosidase, α -fucosidase, β -mannosidase and β -N-acetylhexosaminidase resulted in the release of two mannose residues, one fucose residue, one mannose residue and one *N*-acetylglucosamine residue respectively. Thus it may be concluded that the glycans of this fraction represented the pentasaccharide core of mammalian glycoprotein *N*-glycans carrying an additional fucose residue at the innermost *N*-acetylglucosaminitol residue. The linkage position of this fucose residue is proposed in accordance to literature data (Montreuil, 1982; Kornfeld & Kornfeld, 1985).

Fraction 21 oligosaccharides (3.4 mol%) were found to contain one terminal *N*-acetylglucosamine residue linked to C-2 of a subterminal mannose residue of the oligosaccharide core, in addition to one terminal mannose residue [see values for 2,3,4,6-ManOH, 3,4,6-ManOH and 3,4,6-GlcN(Me)AcOH in Table 1]. In order to assign the terminal *N*-acetylglucosamine residue to a distinct mannose residue, fraction 21 glycans were preparatively degraded with α -mannosidase. A sample of the fragment obtained (designated 21') was again subjected to methylation analysis. The results revealed the disappearance of the terminal mannose residue as well as the complete conversion of 3,6-disubstituted mannose residues into 6-substituted derivatives (cf. values for 2,3,4,6-, 2,3,4- and 2,4-ManOH in Table 1). Therefore it can be assumed that the terminal *N*-acetylglucosamine residue was exclusively linked to C-2 of Man-4' (for numbering of sugar residues see structure of fraction 64 glycans in Table 3). Anomeric configurations were again substantiated by exoglycosidase studies.

Fraction 31 glycans (2.1 mol%) contained two terminal *N*-acetylglucosamine residues linked to C-2 of the subterminal mannose residues [see values for 3,4,6-ManOH and 3,4,6-GlcN(Me)AcOH in Table 1]. By the same line of reasoning

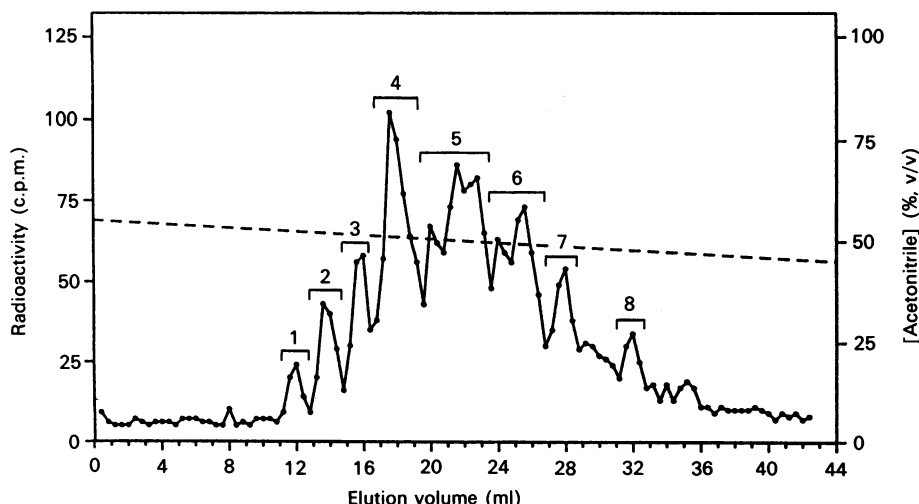


Fig. 2. Fractionation of oligosaccharide alditols from E1

Radiolabelled oligosaccharide alditols, obtained from E1 glycopeptides after treatment with PNGase F and reduction with $\text{KB}^3\text{H}_4/\text{NaBH}_4$, were fractionated by h.p.l.c. on a LiChrosorb- NH_2 column (5 μm particle size; 4.6 mm $\times$ 250 mm) with a linear gradient of acetonitrile (55–45%, v/v; 45 min) in 15 mM-potassium phosphate buffer, pH 5.3. Fractions (0.4 ml) were collected at 1 ml/min and monitored for radioactivity.

detailed above it may therefore be concluded that fraction 31 glycans represented fucosylated core oligosaccharides extended by two *N*-acetylglucosamine residues, as shown in Table 3.

Fraction 32 glycans (2.6 mol%) contained terminal galactose (2,3,4,6-GalOH) in addition to terminal mannose residues (2,3,4,6-ManOH). In order to allocate outer substituents, these glycans were again preparatively degraded by α -mannosidase. Subsequent methylation analysis of the resulting oligosaccharide fragment (32') revealed that the lactosamine antenna present was almost exclusively linked to Man-4' (cf. values for 2,3,4- and 2,4-ManOH in Table 1). Anomeric configurations proposed in Table 3 were again deduced from the results obtained by exoglycosidase studies (see Table 2).

Fraction 33 was still a mixture of glycans, the major component of which was found to contain terminal galactose as well as terminal *N*-acetylglucosamine residues, in addition to 2-substituted and 3,6-disubstituted mannose, 4-substituted *N*-acetylglucosamine and 4-substituted *N*-acetylglucosaminitol [1,3,5,6-GlcN(Me)AcOH; see Table 1] residues. Sequential degradation with β -galactosidase, β -*N*-acetylhexosaminidase and α -mannosidase resulted in the release of one galactose, two *N*-acetylglucosamine and two mannose residues respectively, yielding a fragment that was co-eluted with the authentic Man β 1-4GlcNAcOH standard at an elution volume equivalent to about 3 glucose units (Yamashita *et al.*, 1982). Thus it may be concluded that the majority of fraction 33 glycans (1.0 mol%) represented monogalactosylated biantennary glycans lacking GlcNAc-1. Since the enzyme preparation used in this study for liberation of E1 sugar chains also contained trace activity of endo- β -*N*-acetylglucosaminidase F, it can be assumed that these oligosaccharides are artificially produced from fraction 41 glycans (see below) by this enzyme. Although not studied in detail here, it may therefore be concluded that terminal galactose residues are similarly distributed to GlcNAc-5 and -5' in both cases.

Fraction 41 glycans (comprising 12.9 mol% of total oligosaccharides) were found to represent monogalactosylated biantennary species carrying at GlcNAc-1 (α 1-6)-linked fucose. For allocation of terminal galactose residues, oligosaccharides were preparatively degraded with β -*N*-acetylhexosaminidase and α -mannosidase. Resulting fragments (41') were again subjected to methylation analysis. The results revealed the presence of 3- and 6-substituted mannose residues (see values for 2,4,6-ManOH

and 2,3,4-ManOH in Table 1), indicating that terminal galactose residues were linked to GlcNAc-5 or 5'. Remaining terminal and 3,6-disubstituted mannose residues are obviously due to incomplete degradation with α -mannosidase. Since the enzyme from jack beans (*Canavalia ensiformis*) cleaves Man(α 1-6)Man linkages at a higher rate than Man(α 1-3)Man linkages (Kobata, 1979), it may be assumed that galactose residues were bound to GlcNAc-5 and -5' in almost equal proportions.

Fraction 51 glycans (3.1 mol%) comprised 3,4-disubstituted *N*-acetylglucosamine [6-GlcN(Me)AcOH in Table 1] as well as terminal fucose, galactose and *N*-acetylglucosamine residues, in addition to the typical sugar constituents of the fucosylated pentasaccharide core. Sequential degradation with β -*N*-acetylhexosaminidase and α -mannosidase resulted in the loss of one *N*-acetylglucosamine and one mannose residue (see Table 2) respectively, and a simultaneous conversion of 3,6-disubstituted mannoses into 3-substituted sugar constituents, indicating that branched *N*-acetylglucosamine residues were linked to Man-4 (see value for 2,4,6-ManOH obtained after methylation analysis of fragment 51' in Table 1). Remaining terminal and 3,6-disubstituted mannose residues are probably again due to incomplete degradation with α -mannosidase. Attempts to remove terminal galactose by use of β -galactosidase from jack beans (*Canavalia ensiformis*) or *Diplococcus pneumoniae* failed, presumably because of sterical hindrance. Therefore the question as to whether the Gal(Fuc)GlcNAc element represented an Le^a or X determinant cannot be answered here (for structural assignment of the blood group determinants mentioned see Watkins *et al.*, 1988).

Fraction 52 glycans (7.3 mol%) were found to contain 2,3-disubstituted galactose as well as terminal *N*-acetylglucosamine and fucose residues, in addition to terminal *N*-acetylglucosamine (see Table 1). As demonstrated in the case of fraction 75 carrying a similar structural element (see below), treatment with α -*N*-acetylglucosaminidase resulted in a complete conversion of 2,3-disubstituted galactose residues into 2-substituted compounds, which is consistent with the assumption that fraction 52 glycans carried a blood group A (type 2) determinant. In order to assign this structural element to a distinct lactosamine antenna, oligosaccharides were sequentially degraded with exoglycosidases as indicated in Table 2. Methylation analysis of the oligosaccharide fragment (52') obtained after treatment with α -mannosidase

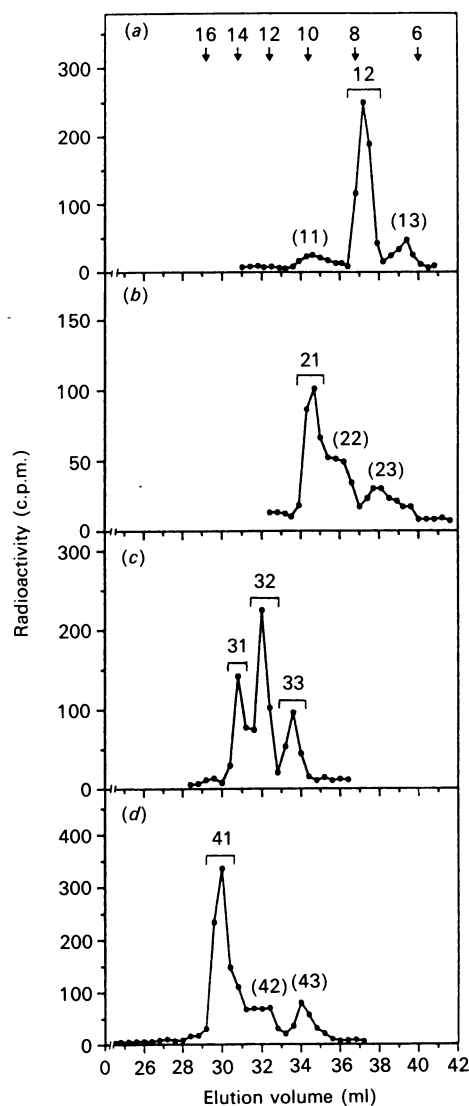


Fig. 3. Subfractionation of E1 oligosaccharide alditols by gel filtration

Oligosaccharide alditol fractions 1–4 (see Fig. 2) were chromatographed on a Bio-Gel P-4 column (–400 mesh; 6 mm × 2000 mm) at room temperature and hydrostatic pressure with aq. 0.02% (w/v) NaN_3 as eluent. Fractions (0.4 ml) were collected at 1 ml/h and monitored for radioactivity. (a)–(d) Subfractionation of glycan fractions 1–4 respectively. Fractions subjected to further structural analysis were pooled as indicated by horizontal brackets. Numbers (6–16) with arrows indicate elution volumes of glucose oligomers (with 6–16 glucose units). Fractions 12 in (a), 21 in (b), 31–33 in (c) and 41 in (d), E1 oligosaccharide alditol subfractions, preparatively isolated.

revealed that the blood group A determinant included exclusively Gal-6 (cf. values for 2,4-ManOH and 2,4,6-ManOH in Table 2). Owing probably to sterical hindrance, Man-4' could not be directly removed by α -mannosidase after release of GlcNAc-5'.

Analytical data obtained in the case of fraction 541 glycans (11.0 mol%) demonstrate that corresponding oligosaccharides represented biantennary complex-type glycans, as shown in Table 3.

Structures of fraction 611 and 62 glycans (3.2 and 4.7 mol%) were found to be identical with those of oligosaccharides from fractions 51 and 52 respectively, except that GlcNAc-5' was now substituted by additional Gal(β 1-4)- residues [cf. values for

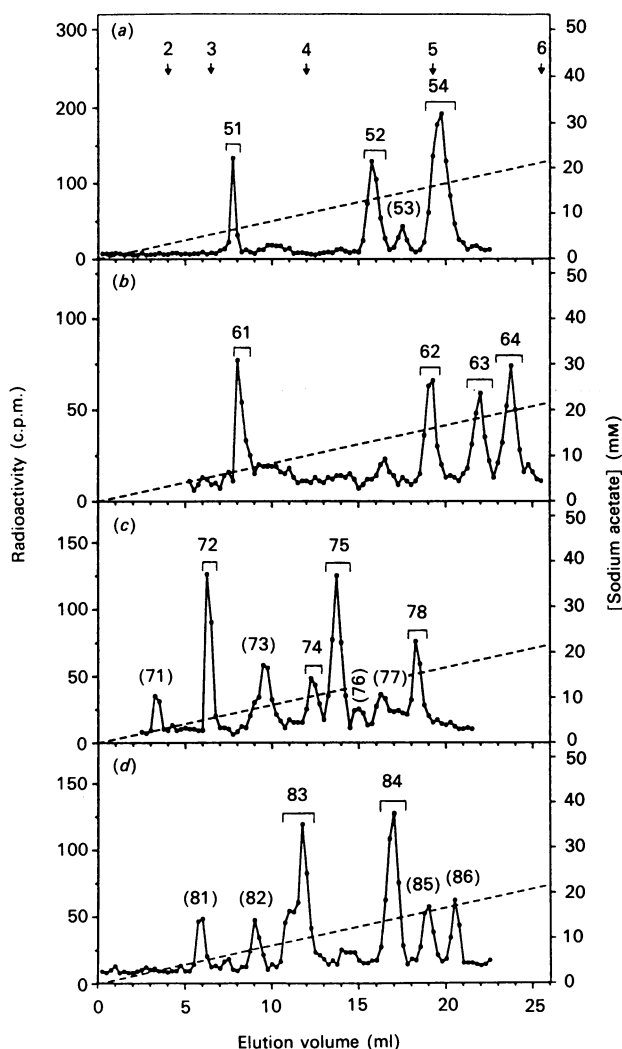


Fig. 4. Subfractionation of E1 oligosaccharide alditols by high-pH anion-exchange chromatography

Oligosaccharide alditol fractions 5–8 (see Fig. 2) were separated by high-pH anion-exchange chromatography on a CarboPac PA1 column (4.6 mm × 250 mm) with a gradient of sodium acetate (0–60 mM; 72 min) in 0.1 M-NaOH. Fractions (0.25 ml) were collected into tubes containing 50 μ l of 0.5 M-acetic acid at 1 ml/min and monitored for radioactivity. (a)–(d) Subfractionation of glycan fractions 5–8 respectively. Fractions subjected to further structural analysis were pooled as indicated by horizontal brackets. Numbers (2–6) with arrows indicate elution volumes of glucose oligomers (with 2–6 glucose units). Fractions 51, 52 and 54 in (a), 61–64 in (b), 72, 74, 75 and 78 in (c) and 83 and 84 in (d), E1 oligosaccharide alditol subfractions, preparatively isolated.

2,3,4,6-GalOH and 3,6-GlcN(Me)AcOH in Table 1 as well as results summarized in Table 2].

Fraction 63 glycans (3.5 mol%) contained 2,3-disubstituted galactose as well as terminal galactose and fucose residues, in addition to terminal *N*-acetylglucosamine (see Table 1). In analogy to the results obtained in the case of fraction 832 glycans (see below) after treatment with α -galactosidase and methylation analysis, it may be concluded that fraction 63 species similarly carried a blood group B (type 2) determinant. Degradation with exoglycosidases yielded a fragment with the same elution volume as the one produced in the case of glycans carrying blood group A determinants (see Fig. 4 and Table 2). Owing to the availability of only small amounts of material, a precise allocation of the Gal α 1-3(Fuc α 1-2)Gal element was not possible. In accordance

Table 1. Methylation analysis of oligosaccharides from E1

Oligosaccharide alditol fractions obtained by gel filtration (fractions 12–41; cf. Fig. 2), high-pH anion-exchange chromatography (fractions 51–84; Fig. 4) as well as major subfractions or fragments obtained thereof by gel filtration (fractions 541 and 611) or sequential degradation with exoglycosidases (fractions 21', 32', 41', 51' and 52') were permethylated and hydrolysed. The partially methylated alditol acetates obtained after reduction and peracetylation were analysed by capillary g.l.c.-m.s. Results are expressed in peak ratios of the alditol derivatives found. Abbreviations: 2,3,4-FucOH, 2,3,4-tri-*O*-methylfucitol; 1,3,5,6-GlcN(Me)AcOH, 2-deoxy-2-(*N*-methyl)acetamido-1,3,5,6-tetra-*O*-methylglucitol; etc. The values for fractions 12 or 33 are based on the sum of mannose residues = 3.0 or 2.4-ManOH = 1.0 respectively, whereas those for the remaining fractions are normalized either on 3,4,6-ManOH = 1.0 (fractions 21, 21', 32, 32', 41', 51' and 52') or on 3,4,6-ManOH = 2.0 (fractions 31, 41, 51, 52, 541, 611, 62–64, 72, 74, 75, 78, 83 and 84). Methyl sugar derivatives originating from minor constituents are given in parentheses. (+) indicates trace.

Alditol derivative	Peak ratio of alditol derivative														
	Fraction ...	12	21	21'	31	32	32'	33	41	41'	51	51'	52	52'	541
2,3,4-FucOH		0.6	0.7	0.4*	0.8	0.9	0.2*	(0.25)	0.7	1.0	0.55*	3.8	0.25	1.0	1.0
2,3,4,6-ManOH		1.85	0.75	–	–	1.15	–	(0.35)	(+)	(0.15)	–	(0.3)	–	–	–
2,3,4-ManOH		–	–	1.25	–	–	1.15	–	–	0.3	–	–	–	–	–
2,4,6-ManOH		–	–	–	–	–	(+)	–	–	0.45	–	0.65	–	0.75	–
3,4,6-ManOH		(+)	1.0	1.0	2.0	1.0	1.0	1.65	2.0	1.0	2.0	1.0	2.0	2.0	2.0
2,4-ManOH		1.15	1.0	–	1.0	0.9	–	1.0	0.8	(0.15)	1.05	(0.25)	1.05	–	0.7
2-ManOH		–	–	–	–	(+)	–	–	(+)	–	–	–	–	–	–
2,3,4,6-GalOH		–	–	–	–	0.85	0.7	0.6	0.8	1.05	0.65	1.3	–	–	1.2
3,4,6-GalOH		–	–	–	–	–	–	–	–	–	–	–	0.75	–	2.2
4,6-GalOH		–	–	–	–	–	–	–	–	–	–	–	–	–	0.8
1,3,5,6-GlcN(Me)AcOH		–	–	–	–	–	–	0.4	(+)	–	–	–	–	–	–
1,3,5-GlcN(Me)AcOH		1.5†	1.0	1.25	0.8	1.15	1.7†	(0.15)	1.6†	1.0	2.0†	1.2	1.9†	1.2	0.9
3,4,6-GlcN(Me)AcOH		–	0.9	1.1	2.6	(+)	–	0.7	1.15	(+)	1.15	–	1.5	1.1	0.8†
3,6-GlcN(Me)AcOH		1.1	0.8	1.0	1.0	1.25	2.2	0.75	2.1	2.05	1.05	1.0	2.1	0.75	2.3
6-GlcN(Me)AcOH		–	–	–	–	–	–	–	–	–	0.7	0.7	–	–	0.7
3,4,6-GalN(Me)AcOH		–	–	–	–	–	–	–	–	–	–	–	0.6	–	–

* Values sometimes too low probably due to evaporation losses.

† Unexplained high values for 4,6-disubstituted *N*-acetylglucosaminol.

‡ Presence of 3,4,6-GlcN(Me)AcOH not consistent with exoglycosidase studies (see Table 2).

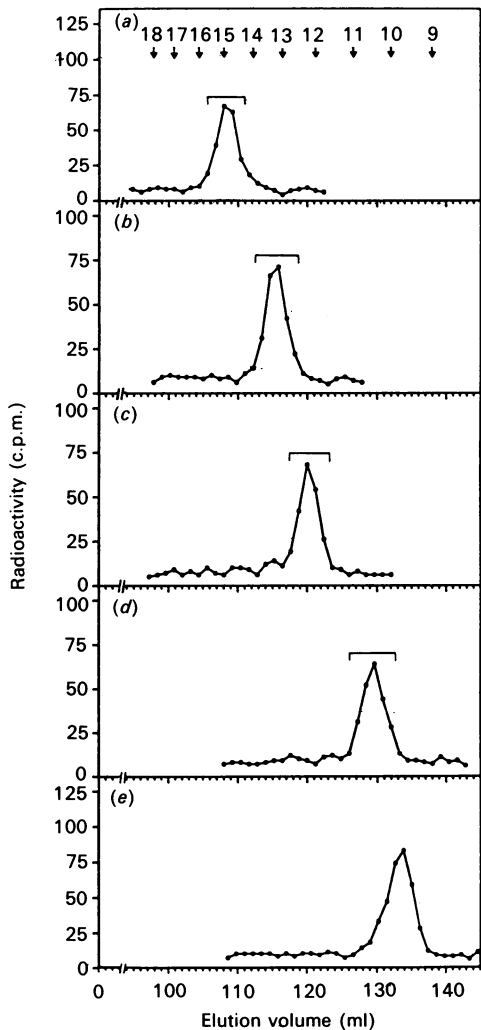


Fig. 5. Sequential degradation of E1 oligosaccharide alditol fraction 63 with exoglycosidases

After analysis of starting material by gel filtration (a), oligosaccharide alditol fraction 63 (see Fig. 4) was successively digested with β -N-acetylhexosaminidase (b), α -galactosidase (c), α -1,2-fucosidase/ β -galactosidase from *A. niger* (d) and α -mannosidase (e). Reaction products were chromatographed on the same Bio-Gel P-4 column (–400 mesh; 1.6 cm $\times$ 180 cm) at 55 °C with aq. 0.02 % (w/v) NaN_3 as eluent. Fractions (1.8 ml) were collected at 0.3 ml/min and monitored for radioactivity. Column calibration with glucose oligomers (with 9–18 glucose units) is shown by arrows. Horizontal brackets indicate fractions pooled for further digestion.

with the results obtained from the analyses of fraction 52 and 62 glycans, it may be assumed, however, that the blood group determinants again included Gal-6.

Fraction 64 glycans (4.6 mol%) were found to represent exclusively biantennary complex-type oligosaccharides carrying a bisecting N-acetylglucosamine residue, as shown in Table 3 (cf. value for 2-ManOH in Table 1). Owing probably to the presence of GlcNAc-9, degradation with exoglycosidases was incomplete (see Table 2).

Fraction 72 (2.2 mol%) was found still to comprise a heterogeneous mixture of glycans, as revealed by methylation analysis (see Table 1) and exoglycosidase studies (results not shown). From methylation data it may be concluded that the oligosaccharides carried, in part, difucosyl blood group A, B or Le^b (or Y) determinants [see values for 2,3,4,6-, 3,4,6- and 4,6-GalOH as well as those for 6-GlcN(Me)AcOH and 3,4,6-

Table 2. Degradation of oligosaccharides from E1 with exoglycosidases

Main fractions of the oligosaccharide alditols obtained by gel filtration (fractions 12–41; cf. Fig. 3), high-pH anion-exchange chromatography (fractions 51–84; Fig. 4) or major subfractions obtained thereof by gel filtration (fractions 541, 611, 831 and 832) were digested with enzymes in the sequence indicated below. Reaction products were analysed by gel filtration on a calibrated Bio-Gel P-4 column (cf. Fig. 5). The results are expressed in glucose units (Yamashita *et al.*, 1982; Kobata *et al.*, 1987) of the oligosaccharide fragments obtained. Abbreviation: N.D., no degradation with corresponding exoglycosidase possible.

Enzyme used	No. of glucose units of fragments obtained																			
	Fraction ...	12	21	31	32	33	41	51	52	541	611	62	63	64	74	75	78	831	832	84
None		8.2																		
α -Mannosidase			10.1	12.2	11.3	10.1	13.4/13.4	14.5	16.2	14.4	15.0	17.0	14.9	15.1	16.5	15.6	16.0	17.6	15.5	17.5
β -Galactosidase			9.2		10.3															
β -N-Acetylhexosaminidase					9.2	9.0*	/12.3	N.D.		12.6	14.2	16.0		12.9			14.8	16.5		16.6
α -N-Acetylgalactosaminidase			7.3	8.3	7.4	4.9	11.4/8.2	12.5	14.5	8.3	12.4	14.3	13.1	11.5†	15.2†		13.2	14.7	13.7	15.3†
α -Galactosidase									12.6			12.5			N.D.		13.8	12.9		N.D.
α -1,2-Fucosidase/ β -galactosidase†													12.5				12.4		13.0	
α -Mannosidase		6.6	6.5	6.5	6.5	3.0	10.8†/6.4	11.5†	10.4	6.5	11.5†	9.6	10.4	9.7	N.D.		12.5†	10.4		N.D.
α -Fucosidase																				
β -Mannosidase																				
β -N-Acetylhexosaminidase																				
* Main component of this fraction.																				
† Degradation incomplete.																				
‡ From <i>A. niger</i> .																				

Table 3. Structures proposed for the main oligosaccharide alditol fractions obtained from the E1 preparation investigated in this study

The amount (mol%) of each glycan was calculated from the distribution of radioactivity incorporated after reduction with KB^3H_4 . In the structure of fraction 64 the numbering of sugar residues in complex-type glycans (Vliegthart *et al.*, 1983) is shown.

Fraction	Structure	Amount (mol%)	Fraction	Structure	Amount (mol%)
12		1.3	611		3.2
21		3.4	62		4.7
31		2.1	63		3.5
32		2.6	64		4.6
33 (major component)		1.0	74		0.9
41		12.9	75		1.9
51		3.1	78		1.1
52		7.3	831		0.5
541		11.0	832		1.1
			84		1.6

GalN(Me)AcOH in Table 1]. Detailed structures, however, cannot be proposed in this case.

Fraction 74 glycans (0.9 mol%) represented predominantly biantennary bisected oligosaccharide species (without Gal-6') carrying a blood group A (type 2) determinant (see Table 1). In analogy to the results described above, this determinant is tentatively assigned to the lactosamine antenna linked to Man-4. Similarly to fraction 64 glycans, oligosaccharide alditols of this fraction could not be degraded by sequential treatment with β -N-acetylhexosaminidase and α -mannosidase (results not shown).

Fraction 75 glycans (1.9 mol%) were found to contain sugar constituents typical for a blood group A determinant, in addition to about one terminal, one 3-substituted and two 2-substituted mannose residues (see Table 1). As mentioned above, treatment with α -N-acetylgalactosaminidase and subsequent methylation analysis (results not shown) revealed that N-acetylgalactosamine was (α 1-3)-linked. Further degradation with α -mannosidase resulted in the removal of one 2-substituted and the 3-substituted mannose constituent (results not shown), whereas Man-4' was not released, probably owing to sterical hindrance. The glycans of this fraction are therefore concluded to comprise the hybrid type structure depicted in Table 3.

From the data obtained, fraction 78 glycans (1.1 mol%) appeared to be analogues of those of fraction 63 except that GlcNAc-5' was now substituted by an additional Gal(β 1-4)-residue.

In order to save material, methylation analysis of starting material was carried out with pooled fraction 83 glycans (see Table 1). For exoglycosidase studies, individual subfractions obtained by gel filtration as described above were employed (cf. Table 2). The results indicate that subfraction 831 glycans (0.5 mol%) represented biantennary species carrying a difucosyl A (ALe^b or AY) structure, whereas oligosaccharides (1.1 mol%) containing a difucosyl B (BLe^b or BY) unit and lacking Gal-6' prevailed in fraction 832. As mentioned above, the linkage position of terminal galactose was substantiated by treatment of fraction 832 glycans with α -galactosidase and subsequent methylation analysis (results not shown).

Finally, fraction 84 glycans (1.6 mol%) were shown to comprise biantennary complex-type glycans containing bisecting N-acetylglucosamine and a blood group A (type 2) determinant, which is assigned again to GlcNAc-5 according to the results described above.

DISCUSSION

In this study the carbohydrate structure of human elastase 1 (E1) as obtained from post-mortem pancreas tissue of 75 donors was analysed. Oligosaccharides were released from tryptic-digest glycopeptides by treatment with PNGase F and reduced with KB³H₄/NaBH₄. The highly diverse mixture of neutral radio-labelled oligosaccharide alditols thus obtained was separated by a fractionation procedure including conventional h.p.l.c. on an amine-modified silica column, high-pH anion-exchange chromatography and gel filtration. Resulting oligosaccharide alditol subfractions represented mostly individual species. In some cases, however, the fractions still comprised mixtures of different glycans. Owing to the small amounts of material available, only major glycan fractions (67.8 mol% of total oligosaccharides) were subjected to structure analysis.

The results revealed that about 28.9 mol% of the E1 glycans analysed carried blood group A (16.4 mol%), Le^a or X (6.3 mol%), B (4.6 mol%), difucosyl ALe^b or AY (0.5 mol%) and difucosyl BLe^b or BY (1.1 mol%) determinants located at Man(α 1-3)-linked lactosamine antennae of mainly complex-type glycans (cf. structures of fractions 51, 52, 611, 62, 63, 74, 75, 78,

831, 832 and 84 in Table 3; for structural assignment of blood group determinants see Watkins *et al.*, 1988). As described in the Results section, analytical data did not allow a discrimination between Le^a (or Le^b) and X (or Y) type structures. Since remaining glycans were shown to contain exclusively type 2 lactosamine antennae, it may, however, be speculated that fractions 51 and 611 (or 831 and 832) glycans contain X (or AY and BY) rather than Le^a (or ALe^b and BLe^b) determinants respectively. Attempts to substantiate the presence of blood group A and B determinants by use of specific monoclonal antibodies after Western blotting were only successful in the case of A structures (results not shown). Obviously, B determinants occurred in amounts insufficient for unambiguous verification of this epitope. Hence corresponding experiments with monoclonal antibodies to discriminate between Le^a and X determinants were not conducted.

A large proportion of the glycans obtained from this glycoprotein preparation were composed of incomplete complex-type structures, some of which are not compatible with the general route of glycoprotein N-glycan biosynthesis in mammalian cells (Kornfeld & Kornfeld, 1985; Schachter, 1986). Therefore it has to be concluded that the truncated sugar chains of fractions 12, 21 and 32 (see Table 3) reflect significant degradation of E1 glycans by exoglycosidases during isolation and purification of the glycoprotein. Possibly, oligosaccharides lacking outer galactose residues (see, e.g., fractions 31, 41, 51, 52, 63, 74 and 832) similarly represent degradation products. The absence of glycans carrying blood group H determinants as well as the lack of sialylated species might be also due to partial digestion by exoglycosidases.

The most striking result of this study is the observation that E1 glycans contain, in part, blood group determinants. The presence of different blood-group-related carbohydrate structures in this enzyme preparation is clearly due to the fact that post-mortem pancreatic tissue of many donors was pooled for isolation of the glycoprotein. Thus our results resemble that of Childs *et al.* (1986) demonstrating the presence of blood group determinants on human milk galactosyltransferase. These authors also presented evidence for the occurrence of blood-group-specific antibodies in rabbit antisera against purified enzyme, indicating that the corresponding carbohydrate structures are sterically accessible and immunogenic. The question, however, as to whether the antigenicity of human pancreatic E1 is similarly modulated by the presence of antigenic carbohydrates awaits further studies.

Apart from E1, other pancreatic glycoproteins such as carboxylesterase (Guy *et al.*, 1981), lipase (De Caro *et al.*, 1977), glycoproteins P19 and P35 (Guy-Crotte *et al.*, 1988) and chymotrypsin B (A. Sziegoleit, D. Linder, R. Geyer, L. Kanacher & P. Wendorf, unpublished work) have been solely characterized by carbohydrate constituent analyses demonstrating different monosaccharide compositions. Preliminary data obtained by immunoblotting with specific monoclonal antibodies, however, indicate that obviously a number of glycoproteins present in human pancreatic juice carry blood-group-related carbohydrate structures (A. Sziegoleit, unpublished work).

Recent studies on the carbohydrates of glycopeptides from human small-intestine epithelial cells similarly revealed the presence of glycans carrying mainly type 2 blood group determinants (Finne *et al.*, 1989). In agreement with the data presented in this study, the oligosaccharides were devoid of sialic acid. In contrast with E1 sugar chains, however, N-glycans from small intestinal epithelial cells represented predominantly tri- and tetra-antennary structures containing an additional N-acetyl-lactosamine unit, whereas the E1 preparation investigated in this study carried mainly biantennary species without N-acetyl-lactosamine repeats.

The question as to whether the carbohydrates of E1 are

important for maintaining its biological functions or responsible for its extraordinary stability in intestinal fluids and faeces (Sziegoleit *et al.*, 1989) by rendering resistance against proteolytic attack remains to be investigated.

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