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Glycosylation of the thrombin-like serine protease ancrod from *Agkistrodon rhodostoma* venom. Oligosaccharide substitution pattern at each *N*-glycosylation site

GÜNTER PFEIFFER[1], DIETMAR LINDER[1], KARL-HERMANN STRUBE[2] and RUDOLF GEYER[1]*

[1] *Biochemisches Institut am Klinikum der Universität, Friedrichstr. 24, D-6300 Giessen, Germany*

[2] *BASF AG, Hauptlabor, D-6700 Ludwigshafen, Germany*

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In a previous study, we determined the structures of the glycans present in ancrod, a thrombin-like serine protease from the venom of the Malayan pit viper *Agkistrodon rhodostoma* (Pfeiffer *et al.* (1992) *Eur J Biochem* **205**:961–78). In order to allocate the various carbohydrate chains to distinct *N*-glycosylation sites of the molecule, we have now isolated individual glycopeptides. Peptide moieties were identified after deglycosylation with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F by amino acid analysis and sequencing. Liberated oligosaccharides were assigned to the previously deduced carbohydrate structures by high performance liquid chromatography. Although only quantitative differences were observed, the results indicate that each glycosylation site of ancrod carries its characteristic oligosaccharide pattern. Furthermore, all potential sites were shown to be substituted by carbohydrates.

Keywords: glycoprotein, glycopeptides, *N*-linked oligosaccharides, snake venom

Abbreviations: HPAE-HPLC, high pH anion exchange HPLC; RP-HPLC, reversed phase HPLC; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; PAD, pulsed amperometric detection.

Introduction

The venom of the Malayan pit viper *Agkistrodon rhodostoma* contains a thrombin-like serine protease, termed ancrod, which acts specifically on fibrinogen. In contrast to thrombin, however, this enzyme cleaves only fibrinopeptide A, leaving the Bβ-chain of the molecule intact. Furthermore, it does not activate Factor XIII [1, 2]. Consequently, ancrod-generated clots are not cross-linked, and are more susceptible to plasmin degradation, resulting in a rapid clearance from circulation. When administered to humans, the enzyme acts as an anticoagulant and lowers the plasma viscosity by reducing the fibrinogen concentration. Therefore, purified ancrod is used for therapeutic defibrinogenation in patients suffering from various vascular diseases. Repeated administration of this agent, however, results in a decrease of its pharmacological activity due to the induction of an antibody response, thus limiting its clinical applicability.

Detailed structural studies on the total *N*-glycans present in this glycoprotein have been carrried out [3], demonstrating that its oligosaccharides belong to the class of complex *N*-glycans and carry an α-fucosyl residue at C-6 of GlcNAc-1 and solely α3-linked *N*-acetylneuraminic acid. Mono-, di-, tri-, and tetrasialylated glycans were found in relative amounts of about 1.8, 34.3, 48.8, and 13.5%, respectively. As a striking feature, about 80% of the sugar chains comprised one to four type-1 lactosamine antennae containing a Galβ3GlcNAc unit. Only a small portion was found to carry a type-2 chain, characterized by the monosaccharide sequence Galβ4GlaNAc, an incomplete antenna, terminating in an *N*-acetylglucosamine residue, or a GalNAcβ4GlcNAc branch which was, in part, further substituted by NeuAcα3 residues.

In order to understand the contribution of the polypeptide chain to the heterogeneity of ancrod glycosylation, we have now analysed the substitution pattern of its five *N*-glycosylation sites [4].

Materials and methods

Materials

The structures of ancrod-derived oligosaccharide alditols used for column calibration were described in detail

* To whom correspondence should be addressed.

previously [3]. Purified ancrod was a generous gift from Knoll AG (Ludwigshaften, Germany).

Analytical gel electrophoresis

Analytical SDS-PAGE was carried out as described by Laemmli [5] in slab gels containing 15% polyacrylamide at an acrylamide/bisacrylamide ratio of 174:1 (by mass). Gels were stained for protein with Coomassie brilliant blue (Serva, Heidelberg, Germany).

Isolation of glycopeptides

Ancrod (2 mg) was carboxymethylated, digested with trypsin (treated with *N*-tosly-L-phenylalanylchloromethane; Serva, Heidelberg, Germany) and separated by reversed phase (RP) HPLC at pH 6 as described elsewhere [6]. The mixture of glycopeptides obtained in the case of fraction P3 (see the Results section) was desalted and subjected to further cleavage by endoproteinase Glu-C from *Staphylococcus aureus* V8 (Boehringer, Mannheim, Germany) according to [7].

Isolation of oligosaccharides and peptide residues

Ancrod glycans were released from individual glycopeptides by treatment with peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* (Boehringer, Mannheim, Germany), separated from residual peptides by RP-HPLC at pH 6, reduced with sodium borohydride, and desalted by gel filtration using a Bio-Gel P-2 column as described earlier [8, 9].

Chromatographic procedures

Desalting of glycopeptides or sialylated oligosaccharides with columns (1 cm × 25 cm) filled with Bio-Gel P-2 (Bio-Rad, Munich, Germany) and of asialo-oligosaccharides with columns (0.5 cm × 5 cm) filled with mixed-bed ion-exchange resin (MB-3, Serva, Heidelberg, Germany) was performed as reported previously [6].

For RP-HLC, a column (0.46 cm × 25 cm) filled with ODS-Hypersil (3 µm; Shandon, Astmoor, UK) was employed; either a gradient of 0–25% acetonitrile in 25 mM sodium acetate, pH 6.0, or 0.01% trifluoroacetic acid, pH 2, was applied at 60 °C and a flow rate of 1 ml min^{-1} [10]. Separation of monosaccharides and oligosaccharide alditols by high pH anion exchange HPLC (HPAE-HPLC) and their monitoring by pulsed amperometric detection (PAD) was carried out using a CarboPac PA-1 column and a Dionex (Sunnyvale, CA, USA) BioLC system described in detail previously [11]. For the separation of sialylated and neutral oligosaccharides, a linear gradient of 0.01–0.19M and 0–0.005M sodium acetate in 0.1M sodium hydroxide for 140 and 150 min, respectively, were employed. Separation of neutral monosaccharides was achieved under isocratic conditions with 0.1M sodium hydroxide as eluent within 10 min; after about 4–6 runs, the column was washed with 0.1M sodium hydroxide:0.5M sodium acetate for 10 min and re-equilibrated for 10 min under starting conditions. For fractionation of sialylated oligosaccharide alditols, a column (0.46 cm × 25 cm) filled with LiChrosorb NH_2 (5 µm; Merck, Darmstadt, Germany) was additionally used. A gradient of buffer (25 mM potassium phosphate, pH 5.3; 40–60% for 90 min) in acetonitrile was applied, and glycans were monitored by UV absorbance at 205 nm.

Digestion with sialidase

Treatment of oligosaccharide alditols with sialidase from *Vibrio cholerae* (Behringwerke, Marburg, Germany) was carried out as described [3].

Carbohydrate analysis

Samples were taken up in 50 µl 1M trifluoroacetic acid, heating to 80 °C for 16 h, dried under vacuum, and analysed for monosaccharides by HPAE-HPLC.

Amino acid analysis

Determination of amino acid composition was performed on a Biotronik (Frankfurt, Germany) model LC 6001 analyser, using *o*-phthalaldehyde as post-column colouring reagent. Prior to analysis, samples (0.3–1 nmol) were hydrolysed with 6M aqueous HCl, containing 0.02% by vol 2-mercaptoethanol, for 24 h at 110 °C in evacuated sealed tubes [7].

Peptide sequencing

Peptides (about 0.1–1 nmol) were sequenced by Edman degradation on an Applied Biosystems (Foster City, CA, USA) pulsed-liquid-phase sequencer, model 477 A, using the standard protocol recommended by the manufacturer (normal-1). Phenylthiohydantoin derivatives of amino acids were identified by an on-line analyser (model 120 A; Applied Biosystems) [7].

Results and discussion

Glycosylation status of ancrod

In order to examine whether ancrod is glycosylated at each of its five potential *N*-glycosylation sites [4], the protein and partially deglycosylated products obtained from it by incubation with PNGase-F were analysed by SDS-PAGE (Fig. 1). Whereas untreated ancrod gave a homogeneous band, a set of five distinct degradation products with higher electrophoretic mobility was obtained after enzymatic treatment, indicating that the protein is substituted by five *N*-linked oligosaccharides.

Fractionation of peptides and glycopeptides

Peptides and glycopeptides obtained from reduced and carboxymethylated ancrod by treatment with trypsin were fractionated by RP-HPLC at pH 6 as shown in Fig. 2a. Four well separated clusters of glycopeptide peaks (P1, P2,

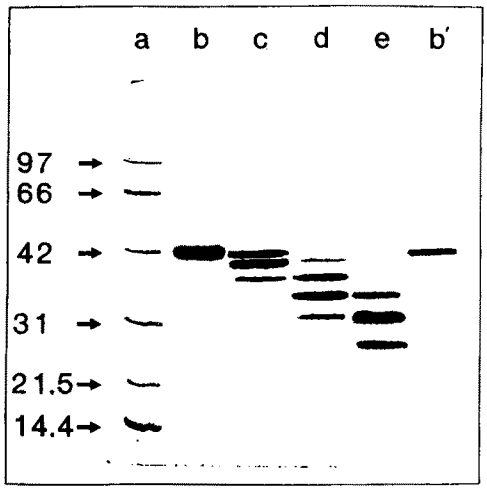

Figure 1. Analytical SDS-PAGE of ancrod and products obtained by deglycosylation. Ancrod (lanes b and b') and degradation products obtained by treatment with 0.64 pkat (c, d) or 3.2 pkat (e) PNGase F per µl for 6 h (c) or 48 h (d, e) were subjected to analytical SDS-PAGE under reducing conditions. Molecular masses of standard proteins (lane a) are indicated: phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 42 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

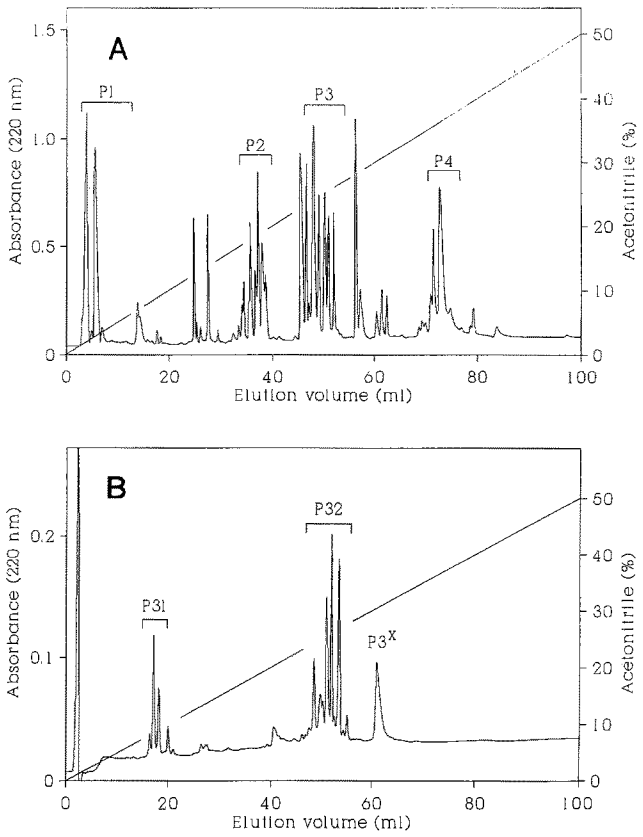

Figure 2. Fractionation of glycopeptides derived from ancrod. (a) Ancrod was reduced, carboxymethylated, digested with trypsin and subjected to RP-HPLC at pH 6. P1, P2, P3 and P4, glycopeptide fractions isolated as indicated by brackets. (b) Subfractionation of glycopeptide fraction P3 (see above) after treatment with endoproteinase Glu-C from *S. aureus* V8 by RP-HPLC at pH 6. P31 and P32, glycopeptide subfractions isolated as indicated by brackets; $P3^x$, nonglycosylated fragment.

P3 and P4 in Fig. 2a) were identified by monosaccharide analysis of aliquots from each fraction (data not shown) and pooled as indicated. Since fraction P3 comprised a mixture of two glycopeptides, containing Asn-99 as well as Asn-148 (see below), it was further incubated with endoproteinase Glu-C from *S. aureus* V8 and again subjected to RP-HPLC at pH 6, yielding two glycopeptide fraction (P31 and P32) in addition to an unglycosylated fragment ($P3^x$; see Fig. 2b).

Isolation of oligosaccharides and identification of corresponding peptide moieties

The individual glycopeptide fractions obtained were treated with PNGase-F in order to liberate the oligosaccharides present. Resulting products were subjected to RP-HPLC at pH 6 (cf. Fig. 3). As verified by monosaccharide constituent analysis (data not shown), oligosaccharides could be completely released from each glycopeptide fraction and were eluted from the column within the range of 4–18 ml. Deglycosylated peptides P2* and P31* corresponding to glycopeptide fractions P2 and P31 were easily identified as single peaks eluting at later retention times due to the loss of oligosaccharides (see, for example, Fig. 3a). As shown for P2*, this chromatographic shift often resulted in a further purification of the peptide. Amino acid sequencing (Table 1) as well as composition analysis (Table 2) of P2* and P31* proved that they correspond to glycopeptides comprising Asn-229 and Asn-99, respectively (Fig. 4). The data for P2* further demonstrated that Pro-234 is indeed the C-terminal amino acid of ancrod, as predicted by cDNA sequencing [4]. In the case of fraction P32, however, two peptides (P321* and P322*, cf. Fig. 3b) were obtained. Both of them were found to represent amino acid residues 141–157 corresponding to the glycopeptide with Asn-148. Their different retention times on RP-HPLC could not be explained on the basis of amino acid analysis and sequencing (see Tables 1 and 2). Perhaps amino acids other than Cys have been modified in part under the conditions used for carboxymethylation, thus resulting in two different peptide peaks [12]. For isolation of peptide P1* (see Fig. 2c), aliquots of fractions in the range of 4–16 ml were subjected to amino acid analysis (data not shown). Subsequent peptide analysis (Tables 1 and 2) revealed that P1* comprised amino acid residues 78–80 (cf. Fig. 4). The deglycosylated peptide P4* was recovered in very low yields only by RP-HPLC at pH 6. In order to obtain sufficient amounts for peptide analysis, RP-HPLC had to be carried out at acidic pH by employing a gradient of acetonitrile in 0.01% trifluoroacetic acid (Fig. 3d). For carbohydrate analysis,

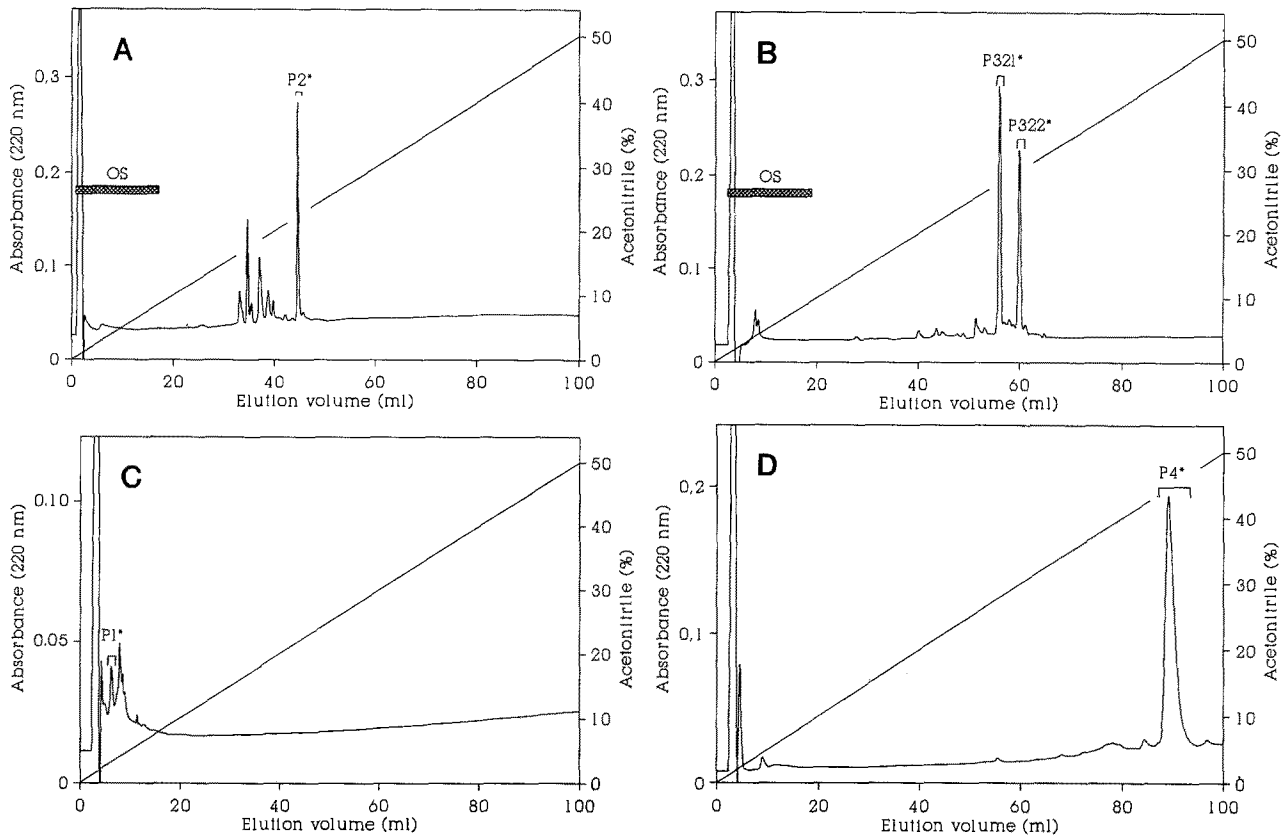

Figure 3. Isolation of deglycosylated peptides and corresponding oligosaccharides. Glycopeptide fractions P2 (a), P32 (b), P1 (c) and P4 (d) obtained from ancrod (see Fig. 2) were desalted, incubated with PNGase-F and separated by RP-HPLC at pH 6 (a–c) or pH 2 (d). P2* (a), P321* and P322* (b), P1* (c) and P4* (d), peptides derived from glycopeptide fractions P2, P32, P1 and P4 isolated as indicated by brackets. Liberated oligosaccharides (OS) were recovered in the first 4–18 ml of the column effluent. For carbohydrate analysis, P4 glycans were separated from peptide material by RP-HPLC at pH 6 (not shown).

P4
VIGGDECNINEHRFLVAVYEGTNWTFICGGVLIHPEWVIT
P1
AEHCARRRMNLVFGMHRKSEKFDDEQERYPKKRYFIRCNK
P31 ⇓ P3^{X}
TRTSWDEDIMLIRLNKPVNNSE*HIAPLSLPSNPPIVGSDC*
P32
RVMGWGSINRRIHVLSDEPRCANINLHNFTMCHGLFRKMP
KKGRVLCAGDLRGRRDSCNSDSGGPLICNEELHGIVARGP
P2
NPCAQPNKPALYTSVYDYRDWVNNVIAGNATCSP

Figure 4. Amino acid sequence of anchrod as deduced from the cDNA sequence reported by Bach *et al.* [4]. Potential *N*-glycosylation sites are underlined. Expected tryptic glycopeptides are given in bold letters. The C-terminal half clipped from one of the P3 glycopeptides (cf. Fig. 2) by endoproteinase Glu-C from *S. aureus* V8 (arrow) is given in italics and indicated as P3^{x}. Glycopeptide fractions P1, P2, P31, P32 and P4 (see Fig. 2) are assigned on the basis of amino acid analysis and sequencing data (cf. Tables 1 and 2).

however, corresponding oligosaccharides were isolated by RP-HPLC at pH 6. Amino acid analysis and sequencing (cf. Tables 1 and 2) demonstrated that P4* comprised amino acid residues 14–46 (Fig. 4). In all cases, analysis demonstrated that homogeneous peptides can be obtained by this approach.

Analysis of oligosaccharides alditols derived from Asn-23, -79, -99, -148 and -229

Glycans were liberated from individual glycopeptides by treatment with PNGase-F. Except for those from glycopeptide fraction P1 (cf. Fig. 3), they were separated from residual peptides by RP-HPLC at pH 6, reduced with sodium borohydride and subjected to HPAE-HPLC (cf. Fig. 5). Under the conditions used, oligosaccharide alditols are separated primarily on the basis of their sialic acid content. Previous studies [3], however, demonstrated that structural features like branching and substitution by different antennae [e.g. NeuAcα3Galβ3GlcNAc, NeuAcα3Galβ4GlcNAc, NeuAcα3GalNAcβ4 (GlcNAc or GlcNAc)] also contribute to the retention times of the oligosaccharide alditols present. Hence, most of the glycans derived from ancrod could be separated in one run. As examples, the separations of glycans derived from glycopeptide fractions P4 (Asn-23) and P2 (Asn-229) are shown in parts a and d of Fig. 5, respectively. Based on the structural studies described recently [3], resulting oligosaccharide fractions

Table 1. Amino acid sequence analysis of purified peptides obtained after deglycosylation of ancrod glycopeptides and RP-HPLC (cf. Figs 2 and 3). The major amino acid liberated at each cycle is indicated in one letter code along with its recovery in picomoles. The Asp residues resulting from cleavage of glycosyl-Asn bonds by PNGase-F are given in bold letters. Cys was identified as the carboxymethyl derivative.

Cycle	*Amino acid (yield, pmol) for peptide fraction*					
	*P1**	*P2**	*P31**	*P321**	*P322**	*P4**
1	C(893)	D(379)	L(187)	C (278)	C (195)	F(66)
2	**D(911)**	W(390)	N(125)	A (460)	A (306)	L(46)
3	K(519)[a]	V (637)	K(227)	N(257)	N(189)	V(82)
4	–	N(213)	P (99)	I (423)	I (267)	A(61)
5		N(450)	V(120)	N(251)	N(125)	V(46)
6		V (476)	**D (76)**	L (224)	L (207)	Y(34)
7		I (403)	N (74)	H (32)	H (46)	E(21)
8		A (393)	S (40)	**D(112)**	**D(132)**	G(29)
9		G(259)	E (22)	F (371)	F (228)	T(12)
10		**D(201)**		T (210)	T (156)	**D(15)**
11		A (233)		M(289)	M(214)	–[b]
12		T (142)		C (221)	C (157)	T (9)
13		C (140)		H(110)	H (73)	F (4)
14		S (50)		G(183)	G(138)	I (6)
15		P (34)[a]		L (191)	L (152)	C (3)
16		–		F (177)	F (147)	
17				R (45)[a]	R (39)[a]	
18				–	–	

[a] C-terminal amino acid (cf. Fig. 4).
[b] No Pth-Xaa derivative observed.

Table 2. Amino acid composition of purified peptides obtained after deglycosylation of ancrod glycopeptides and RP-HPLC (cf. Figs 2 and 3). Amino acid values are listed as molar ratios normalized either on Lys (P1*, P31*), Ala (P2*) or Arg (P321*, P322* and P4*). Theoretical compositions to be expected from the amino acid sequence of ancrod [4] are given in parentheses. Cys was identified as the carboxymethyl derivative. The presence (+) or absence (−) of Trp was only qualitatively determined. Amino acids are indicated in one letter code.

Amino acid	*Determined amount (theoretical value) for peptide fraction*					
	*P1**	*P2**	*P31**	*P321**	*P322**	*P4**
C	1.2 (1)	1.3 (1)	– (–)	2.1 (2)	1.5 (2)	2.3 (2)
N/D	1.0 (1)	4.2 (4)	2.5 (2)	2.9 (3)	3.1 (3)	0.8 (1)
T	– (–)	0.9 (1)	– (–)	0.8 (1)	0.9 (1)	2.8 (3)
S	– (–)	1.1 (1)	0.7 (1)	– (–)	– (–)	– (–)
Q/E	– (–)	– (–)	0.9 (1)	– (–)	– (–)	3.2 (3)
G	– (–)	1.1 (1)	– (–)	0.9 (1)	1.2 (1)	3.2 (3)
A	– (–)	2.0 (2)	– (–)	0.9 (1)	1.0 (1)	2.9 (3)
V	– (–)	1.6 (2)	0.8 (1)	– (–)	– (–)	3.4 (4)
M	– (–)	– (–)	– (–)	1.2 (1)	1.6 (1)	– (–)
I	– (–)	0.7 (1)	– (–)	1.0 (1)	1.2 (1)	2.3 (3)
L	– (–)	– (–)	0.9 (1)	2.0 (2)	2.3 (2)	2.2 (2)
Y	– (–)	– (–)	– (–)	– (–)	– (–)	1.0 (1)
F	– (–)	– (–)	– (–)	1.7 (2)	2.1 (2)	1.9 (2)
H	– (–)	– (–)	– (–)	1.6 (2)	2.3 (2)	2.3 (2)
W	– (–)	+ (1)	– (–)	– (–)	– (–)	+ (2)
K	1.0 (1)	– (–)	1.0 (1)	– (–)	– (–)	– (–)
R	– (–)	– (–)	– (–)	1.0 (1)	1.0 (1)	1.0 (1)
P	– (–)	– (1)	– (5)	– (–)	– (–)	– (–)

could be assigned to distinct carbohydrate structures. Except for S1 glycans, which represent minor constituents, all identified peaks are labelled exactly as in reference [3]. For further fractionation of oligosaccharide mixtures which were not resolved by HPAE-HPLC [3], fractions eluting at about 60 ml from the HPAE-column were collected as indicated, desalted, and subfractionated by HPLC using a LiChrosorb NH_2 column (see, for example, Fig. 5b, e). Similarly, two fractions eluting shortly after peak S33 were pooled, desalted, treated with sialidase, and subjected again to HPAE-HPLC (Fig. 5c, f). By this approach, about 94–98% of the oligosaccharides present at each glycosylation site could be assigned to the previously deduced structures [3]. Briefly, the sugar chains obtained represent predominantly fucosylated bi-, tri- (2- and 2,6 substituted Manα residues) and tetraantennary glycans (S22, S33 and S42) carrying exclusively NeuAcα3Galβ3GlcNAc (type-1) antennae. Tri- or tetraantennary specifies comprising two or three type-1 antennae in addition to one NeuAcα3Galβ4GlcNAc (type-2) antenna are less prevalent (see S32 and S41). The same holds true for glycans containing one NeuAcα3GalNAcβ4GlcNAc (S211 and S31) or an incomplete antenna terminating in an *N*-acetylglucosamine residue (S23, S352 and S342). Fraction S341 and S351 glycans represents structural isomers of S33 glycans, whereas fraction S212 contains biantennary species lacking Fucα6GlcNAc, due to a contaminant endoglycosidase F activity in the commercial PNGase-F preparation used [3].

For ease of discussion, the diversity of glycan structures was reduced by grouping them according to selected structural features, such as oligosaccharide branching and composition of antennae. Concerning the branching pattern at each glycosylation site, no distinction was made between the different isomeric structures of triantennary glycans of fractions S33, S341 and S351. Furthermore, S1 glycans were treated as biantennary species, although some of them represent partially incomplete structures [3]. As shown in Fig. 6, the extent of glycan branching is almost identical at Asn-79 and Asn-99. Both glycosylation sites carry populations of about 30% bi-, 60% tri- and 10% tetraantennary species. Asn-148 is substituted by increased amounts of biantennary glycans and decreased quantities of tetraantennary species, whereas Asn-229 carries predominantly tri- and tetraantennary oligosaccharides. The glycosylation pattern at Asn-23 is characterized by high amounts of

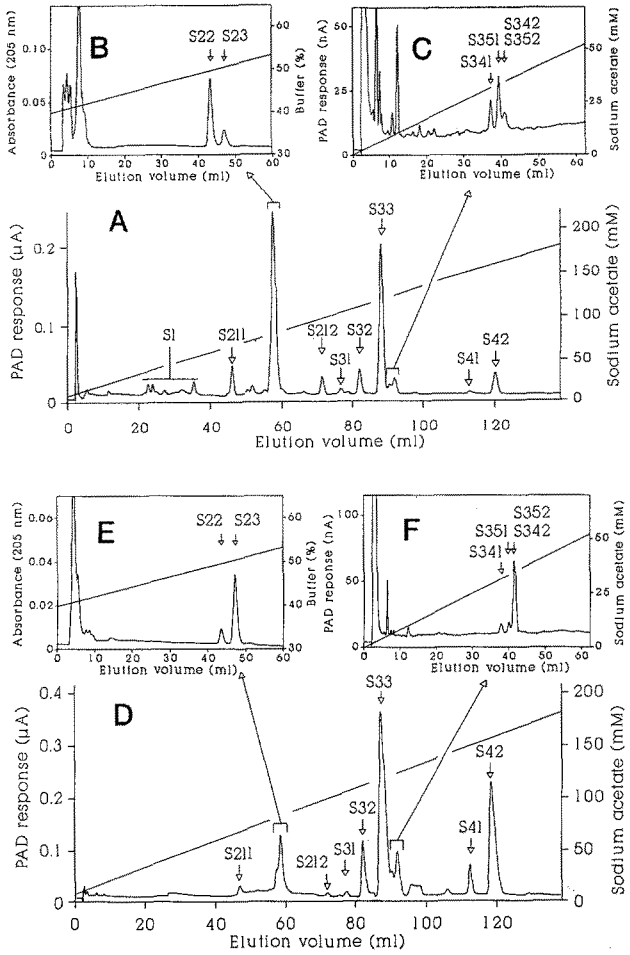

Figure 5. Identification of oligosaccharide alditols derived from individual glycosylation sites. Oligosaccharides liberated from individual glycopeptide fractions by treatment with PNGase-F were separated from residual peptides by RP-HPLC at pH 6 (cf., for example, Fig. 3), reduced and desalted by gel filtration. The resulting oligosaccharide alditols were separated by HPAE-HPLC and monitored by pulsed amperometric detection (PAD), as shown by glycans derived from Asn-23 (a) and Asn-229 (d). Fractions comprising mixtures of glycans were either desalted and subfractionated by HPLC on LiChrosorb NH_2 (b, e) or desalted, treated with sialidase and again applied to HPAE-HPLC (c, f), as indicated by brackets and arrows. S1, S211, S22, S23, S212, S31, S32, S33, S341, S342, S351, S352, S41 and S42 indicate oligosaccharides assigned to previously described structures [3].

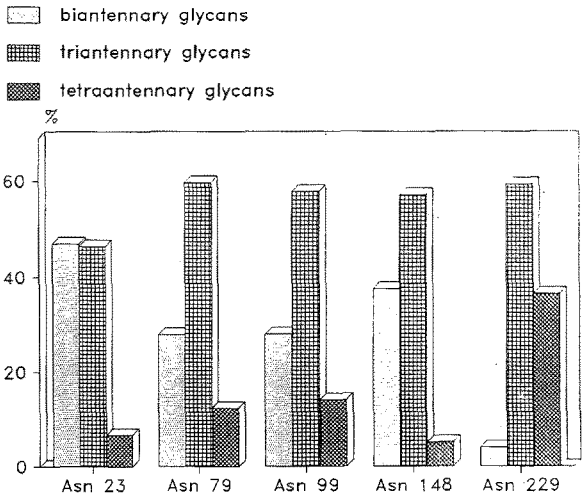

Figure 6. Branching pattern of oligosaccharides at each *N*-glycosylation site of ancrod. Oligosaccharides derived from individual glycosylation sites were identified by HPLC analysis as shown in Fig. 5. The relative proportion of bi-, tri- and tetraantennary species is summarized.

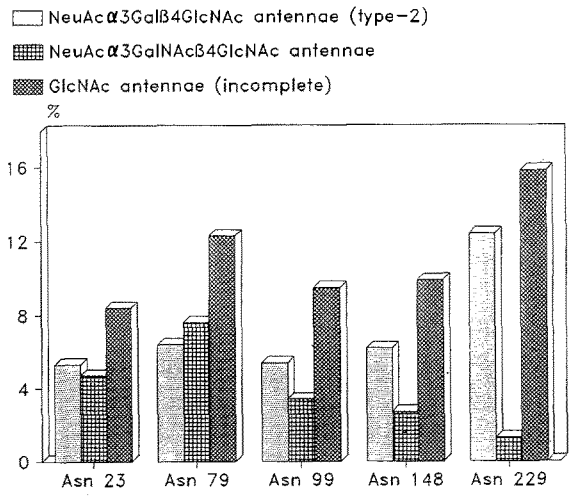

Figure 7. Occurrence of oligosaccharides with branches other than type-1 antennae at each glycosylation site. Oligosaccharides derived from individual glycosylation sites were identified by HPLC analysis as shown in Fig. 5. The relative proportion of glycans carrying one of the antennae is summarized.

biantennary side chains and reduced levels of tri- and tetraantennary species.

The distribution of oligosaccharides carrying branches other than type-1 antennae is shown in Fig. 7. The small amounts of S1 glycans were not taken into account in this case. Similarly, glycans containing only type-1 chains, which dominate at each glycosylation site (70–82%) are not included. The results indicate that highest amounts of glycans with type-2 or incomplete antennae are linked to Asn-229, whereas oligosaccharides containing a sialylated GalNAcβ4GlcNAc antenna are preferentially located at Asn-79. Thus, even though only quantitative differences were observed, each *N*-glycosylation site of ancrod was found to carry its own, characteristic oligosaccharide pattern. The methodological approach described is generally applicable and allows rapid profiling of the oligosaccharide substituents present at individual *N*-glycosylation sites.

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