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Localization and characterization of the glycosylation site of human pancreatic elastase 1

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Crystalline elastase 1 from human pancreas was digested with trypsin. Two peptides, containing the potential *N*-glycosylation sites at Asn-86 and Asn-125, were isolated and analyzed by amino acid analysis, sequencing and carbohydrate component analysis. The results demonstrate that only Asn-86 is glycosylated.

Elastase 1; Glycoprotein; Glycosylation site; Oligosaccharide composition; (Human pancreas)

1. INTRODUCTION

Human pancreatic elastase 1 (E1), a member of an acidic elastase family [1], was found to associate with bile acids and cholesterol [2], a unique function which may be significant in cholesterol metabolism. To gain insight into this function and to compare E1 with elastase isoenzymes, which have recently been sequenced after gene cloning [3,4], we are studying the structure of E1, a glycoprotein [2,5]. We report here the partial sequence analysis of E1, confirming the two potential *N*-glycosylation sites which are also present in the sequence of protease E [3] and elastase III [4], the assignment of the oligosaccharide to a specific peptide sequence and analysis of the components of the carbohydrate moiety.

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Abbreviations: E1, human pancreatic elastase 1; PTH, phenylthiohydantoin; rHPLC, reversed-phase high-performance liquid chromatography; S, tryptic peptide containing the sequence ...Asn-X-Ser/Thr...; TPCK, tosylnitrophenyl chloromethyl ketone

2. MATERIALS AND METHODS

2.1. *Preparation and crystallization of E1*

As detailed [2], E1 was purified to homogeneity from human necrobiotic pancreas tissue and crystallized by dialyzing the enzyme vs bidistilled water at 4°C.

2.2. *Proteolysis*

To prevent autodigestion, E1 (2–5 mg) was incubated for 5 min at 95°C in 0.1 M Tris-HCl (pH 8.3), containing 1 mM EDTA and 6 M guanidine hydrochloride, being deaerated with N_2. Dithiothreitol was added to a final concentration of 2 mM and the solution was incubated for 90 min at 37°C in the dark. Iodoacetic acid, neutralized with NaOH, was added to a final concentration of 5 mM. After incubation for 1 h at 37°C in the dark, mercaptoethanol (1%, v/v) was added and the solution dialyzed exhaustively against distilled water. Reduced and carboxymethylated E1 was digested with TPCK-treated trypsin (Serva, Heidelberg) (1:50, w/w) for 3 h at 37°C in 0.1 M NH_4HCO_3 buffer (pH 8.2). For subfractionation, tryptic peptides (0.5–2 mg) were further digested with endoproteinase Asp-N (Boehringer, Mannheim) (1:200, w/w, 18 h, 37°C, 50 mM sodium phosphate buffer; pH 8.0).

2.3. *Fractionation of (glyco)peptides*

Tryptic digests were fractionated using gel-permeation chromatography followed by rHPLC [6]. Peak fractions were collected as recorded by continuous flow monitoring of the absorbance at 220 nm.

2.4. *Protein analytical methods*

The amino acid (or hexosamine) composition of (glyco)peptides was determined using a Biotronik (Frankfurt) model LC

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6001 analyzer after 24 h (or 2 h) of hydrolysis at 110°C (or 100°C) in 6 N (or 4 N) aqueous HCl using *o*-phthaldialdehyde as colouring reagent. Amino acid sequence analysis of the (glyco)peptides (each 30–50 nmol) was performed on a model Mini 15 (Sequemat; Watertown, MA) solid-phase sequencer resulting in repetitive yields of 75 ± 10% after coupling of peptides to aminopropyl glass with 1-ethyl-3-dimethylaminopropylcarbodiimide (both Pierce, Rockford, IL) [7]. PTH-amino acids were identified by rHPLC [8] using Gynkotek (München) equipment (including a model SP-6A absorbance detector) coupled to a Shimadzu CR-3A integrator.

2.5. *Carbohydrate analytical methods*

The mixture of alditol acetates obtained after hydrolysis of glycopeptides was analyzed by capillary gas liquid chromatography-mass fragmentography as in [9,10].

2.6. *Deglycosylation of glycopeptides*

Glycopeptides were deglycosylated by treatment with peptide:*N*-glycosidase F (Boehringer) as described [11].

3. RESULTS

Gel-permeation chromatography of the tryptic digest of E1 is depicted in fig.1. Fractions were analyzed for glycopeptides by determination of hexosamines after hydrolysis, combined as indicated (A,B) and subjected to rHPLC (fig.2). Glycopeptides were monitored as described above, each peak fraction being subjected to amino acid analysis (table 1). Those having identical amino acid composition were combined (fig.2, hatched peaks) and further characterized by end-group determination and partial solid-phase peptide sequencing.

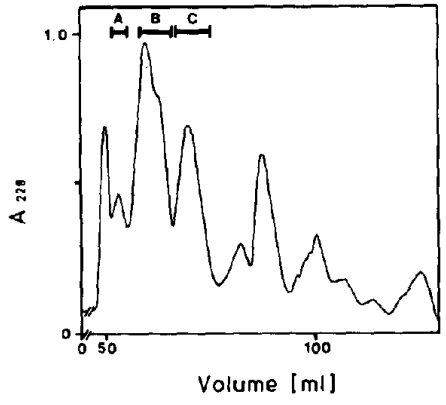

Fig.1. Gel permeation chromatography of carboxymethylated E1 after digestion with trypsin. Column, TSK HW-40 S (Merck, Darmstadt, FRG), 15 × 900 mm; buffer, 25 mM ammonium acetate (pH 6.0) containing 0.2 g/l NaN_3; flow rate, 18 ml/h at 25°C. The elution profile was recorded by continuous flow monitoring of the absorbance at 228 nm. Glycopeptide fractions according to their hexosamine content were combined as indicated (A,B). Pool C contains peptide S2 (M_r = 3600) with the potential glycosylation site at Asn-125.

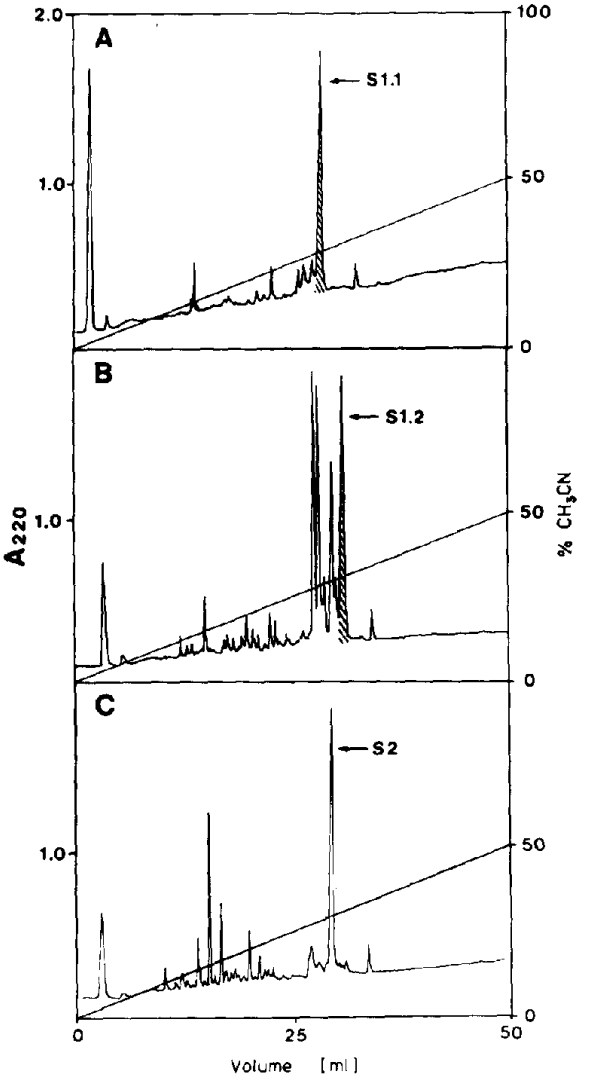

Fig.2. Isolation of E1 (glyco)peptides from fractions A–C (see fig.1) by rHPLC on a 3 μm ODS column (Shandon, Astmoor, UK) (0.46 × 25 cm). A linear gradient of acetonitrile in 25 mM ammonium acetate buffer (pH 6) was applied. Only the hatched fractions contain glucosamine. The pure (glyco)peptides were identified by amino acid analysis and solid-phase sequencing before and after subfractionation by endoproteinase Asp-N (see fig.3).

Comparison of our analytical data with the amino acid sequence of elastase IIIb published by Tani et al. [4] allowed identification and assign-

Table 1

Amino acid analysis of tryptic (glyco)peptides from elastase 1 containing potential *N*-glycosylation sites (s)

Amino acid	Composition[a]					
	S1.1	(Glu)[b]	S1.2	(Glu)[b]	S2	(Ser)[b]
Cys[c]	(2)	2.2	(–)	–	(1)	1.3
Asx	(5)	4.9	(3)	2.7	(3)	2.7
Thr[d]	–	–	(–)	–	(2)	1.6
Ser[d]	(2)	1.7	(1)	0.8	(2)	1.9
Glx	(3)	3.8	(3)	2.7	(3)	3.5
Gly	(3)	2.9	(2)	2.3	(4)	3.6
Ala	(2)	2	(–)	–	(4)	3.8
Val	(3)	2.5	(2)	1.1	(1)	1.0
Met	(–)	–	(–)	–	(–)	–
Ile	(4)	3.2	(2)	1.1	(2)	1.9
Leu	(3)	3	(2)	1.8	(4)	5.2
Tyr	(–)	–	(–)	–	(1)	1.1
Phe	(1)	0.9	(1)	0.8	(–)	–
His	(1)	0.8	(1)	1	(–)	–
Lys	(1)	0.8	(–)	–	(–)	–
Arg	(1)	1.0[e]	(1)	1.0[e]	(1)	1.0[e]
Trp[f]	(1)	+	(1)	+	(1)	+
Pro[g]	(3)		(3)		(4)	
GlcNAc[f]		+		+		–

[a] Molar ratios of amino acids; data expected from the sequence of elastase IIIb [4] are given in parentheses. [b] NH_2-terminal amino acid determined by dansylation. [c] Determined as carboxymethylcysteine. [d] Data not corrected. [e] Based on Arg = 1. [f] Not quantified. [g] Not determined

ment of the E1 glycopeptides. Using this approach, two homogeneous glycopeptides could be identified. The first (S1.1) represents the peptide Glu-66...Lys-100, which was not cleaved by trypsin after Arg-87, the second (S1.2) corresponding to the peptide Glu-66...Arg-87. For further characterization of these fragments, they were deglycosylated by peptide:*N*-glycosidase F which cleaves *N*-glycosidically bound oligosaccharides leaving Asp residues in the polypeptide chain. Component analysis of the carbohydrate moieties revealed that both glycopeptides of E1 contain Fuc, Man, Gal, GlcNAc and GalNAc in the molar ratio of 1.5:3.0:2.0:5.0:0.4. The deglycosylated peptides were subfragmented by endoproteinase Asp-N to obtain smaller peptides (S1P1–S1P4) because sequencing of S1 stopped at step 20 (Trp-85). Within peptide S2 (see fig.2C), containing the second potential glycosylation site at Asn-125, no hexosamines could be detected on amino acid analysis (table 1) and sequencing of peptide S2P3 shows that no oligosaccharide bound at Asn-125. The sequencing results are summarized in fig.3.

Beside peptides S1 and S2, we partially sequenced other parts of E1, namely Val-1...Ser-11; Ser-104...Arg-136; Lys-137...Tyr-157; Thr-173...Arg-182; Ser-183...Gly-191; Val-228...Ser-241. No difference in these amino acid sequences vs elastase IIIb was detected.

4. DISCUSSION

E1 described here was originally detected in human intestinal fluids through its capacity for binding to cholesterol and deoxycholate [2] and shown to be immunologically identical with elastase 1, isolated by Largman et al. [12]. Our structural studies now confirm that this enzyme is basically identical to elastase IIIb, investigated by

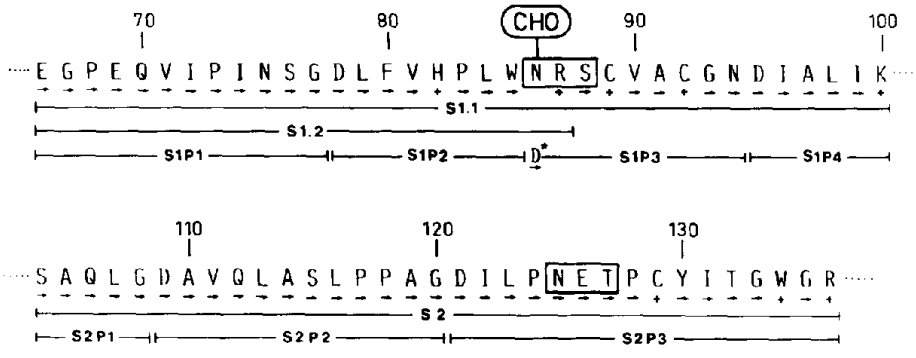

Fig.3. Amino acid sequence of tryptic peptides of E1 containing potential *N*-glycosylation sites (boxes) according to [4]. Isolated tryptic and endoproteinase Asp-N peptides are underlined. Amino acids determined via solid-phase sequencing are designated by (→), and those deduced from amino acid analysis by (+). *N*-glycosidically bound oligosaccharides (CHO). (*) After deglycosylation of peptide S1 with peptide:*N*-glycosidase F, PTH-Asp is found in S1P3.

Tani et al. [4]. To date, no results have been reported about the extent of glycosylation of this enzyme. Here, we have shown that only one of the potential *N*-glycosylation sites of E1 is glycosylated. Apart from the digestive function of E1, the enzyme may play an important role in the intraintestinal transport of cholesterol, a process upon which we now focus further attention. A prerequisite for this function is that E1 survives intestinal passage. Glycosylation could be responsible for protecting E1 against proteolytic attack in the intestine. Structural elucidation of the oligosaccharides may also contribute to a better classification of elastase isoenzymes.

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