CHARACTERIZATION OF THE SULFATED GLYCOPEPTIDE OF CHICKEN PEPSINOGEN

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S-sulfonated chicken pepsinogen was digested with TPCK-trypsin; large tryptic peptides, separated on Sephadex G-25 fine, were subjected to additional cleavage with α-chymotrypsin. The hold-up fraction of the chymotryptic digest from the Sephadex G-25 column, was resolved by high voltage electrophoresis. The three most acidic zones contained glycopeptides of identical amino acid sequence Val-Ser-Thr-Asn-Glu-Thr-Val-Tyr, yet differed in the composition of the sugar moiety. These glycopeptides, moreover, bear different numbers of sulfate groups which enabled the resolution of the peptides. The most acidic glycopeptide contains 7 glucosamine residues, 3 mannose residues and 5 sulfate groups, the second one 6 glucosamine residues, 3 mannose residues and 4 sulfate groups and the slowest, minority glycopeptide, 5 glucosamine residues, 2 mannose residues and 2 sulfate groups. The entire sugar moiety is attached to one site of the chain *via* asparagine. In other experiments the glycopeptides were also isolated from the thermolytic digest of chicken pepsin; their C-terminal sequence was shorter by two amino acid residues. The tentative assignment of the glycopeptides to the amino acid sequence of pepsinogen resulted from the analysis of the limited tryptic digest of the whole protein molecule. Chicken pepsinogen is glycosylated at the site of the chain occupied by a phosphoserine residue in hog pepsinogen A.

Chicken pepsinogen is a glycoprotein with a relatively small sugar moiety[1-3] localized in the pepsin part of the chain. Systematic sequential studies on this protein which have been carried out in this Laboratory, required this sugar moiety to be also characterized. Glycosylation as one of the possibilities of posttranslational modification has so far been observed and studied in more detail (except for chicken pepsinogen) with two carboxyl proteinases only. The glycopeptides isolated from the pepsinogens of monkey gastric mucosa[4] had the same primary structure yet slightly differed in the composition of the carbohydrate side chain. With respect to the existing homologies with other carboxyl proteinases the sugar moiety is attached to the polypeptide chain in the immediate neighborhood of the first reactive aspartic acid residue (Asp 32, ref.[5]). The sugar moiety of hog spleen cathepsin D (ref.[6]) most likely plays an important role in the transport of the enzyme to the lysozomes[7]. The sugar component was detected both in the light and in the heavy chain of the two chain form of the enzyme; hence, cathepsin D is modified by the carbohydrate side chains at more than one site of the molecule. The amino acid sequence of the N-terminal portion of the cathepsin D molecule has been determined up to the first reactive aspartic acid residue (Asp 32) yet the position corresponding to the site of carbohydrate attachment in monkey pepsinogen is not glycosylated. The sugar moiety of the light chain of cathepsin D is therefore most likely attached to some other part of the molecule.

This study was aimed at the isolation of the glycopeptide from chicken pepsinogen,

the determination of its primary structure, the assay of the content of the individual sugars, the determination of the nature of the bond between the protein and the sugar, and the assignment of the glycopeptide to the primary structure of the protein.

EXPERIMENTAL

Material

Chicken S-sulfo-pepsinogen (SSCPG) and chicken pepsin were prepared as described elsewhere[1]. TPCK-trypsin and α-chymotrypsin were supplied by Worthington Biochem. Corp., Freehold, New Jersey, U.S.A. Thermolysin and pronase CB were preparations of Calbiochem, San Diego, Calif., U.S.A. Sephadex G-25 fine, Sephadex G-50 fine, DEAE-Sephacel, Con-A-Sepharose, and Lentyl-Sepharose were products of Pharmacia Fine Chemicals AB, Uppsala, Sweden. The chemicals employed for the sequencing experiments were supplied by Beckman, Palo Alto, Calif., U.S.A. Polyamide layer sheets were purchased from BDH Chemicals, Poole, England, and Silufol, silica gel layer sheets from Kavalier, Czechoslovakia. The remaining chemicals were of analytical purity grade.

Methods

High voltage electrophoresis at pH 5·6 and paper chromatography were carried out as described before[8,9]. The amino acid analyses[10,11] were performed on 20 h hydrolysates of the samples. The N-terminal amino acids of the glycopeptides were determined by dansylation[12,13] with chromatographic identification of the derivatives on polyamide layers[14]. The stepwise degradation of the peptides was carried out according to Edman[15] and the phenylthiohydantoins were identified on Silufol[16] or by gas chromatography either without or after silylation[17]. Alternatively, the primary structures of the glycopeptides were determined by the dansyl-Edman procedure[18]. The N-terminal part of the primary structure of the high molecular weight fragment resulting from limited tryptic digestion of SSCPG was determined in Beckman Model 890 C Amino Acid Sequenator according to the modified Beckman No 102 974 program.

Sugar analyses: The colorimetric assay of neutral sugars was effected by the phenol-sulfuric acid method[19] using mannose as a standard. The samples for gas chromatography of sugars were hydrolyzed by two different procedures. Methanolysis under nitrogen (1·5M-HCl in absolute methanol) at 80°C for 24 h was used for the determination of neutral sugars. For the analysis of hexosamines the samples were hydrolyzed in sealed evacuated tubes in 4M-HCl at 100°C for 4 h and then acetylated in the mixture acetic anhydride–pyridine (2 : 3) at 55°C for 1 h. Both hydrolysates were silylated in the mixture N,O-bis(trimethylsilyl) acetamide–pyridine (1 : 1) at 55°C for 30 min. The chromatography of the derivatized sugars was carried out in Beckman Model GC-65 Gas Chromatograph on a 200 cm column packed with Gaschrom Q (100—200 mesh), coated with a liquid phase containing 5% of SE-30. The hexosamines were also determined in the amino acid analyzer[20]. The acetyl groups of the glycopeptides were identified by hydrazinolysis with subsequent dansylation[21].

Before the determination of bound sulfate the samples were hydrolyzed (1M-HCl at 100°C for 6 h) and the sulfate liberated was determined colorimetrically[22]. The chemical desulfation of the glycopeptide was carried out in dimethyl sulfoxide containing 2% of pyridine (100°C, 9 h, ref.[23]). The glycopeptide-containing fraction was freed of organic solvents on Sephadex G-25 and lyophilized.

Chymotryptic hydrolysate of the high molecular weight fraction of the tryptic digest of SSCP: The trypsinolysis of SSCPG and the fractionation of the digest on a column of Sephadex G-25 fine have been described before[16]. The hold-up fraction and the adjacent fraction were pooled, dissolved in 15 ml of 0·5% ammonium carbonate, and the pH adjusted to 8·25 by glacial acetic acid. Subsequently 1·5 mg of α-chymotrypsin (enzyme to substrate ratio 1 : 100, w/w) was added and the material was digested 2 h at 37°C. After acidification to pH 4 and removal of a small quantity of precipitate by centrifugation the supernatant was resolved on a column of Sephadex G-25 fine (4·4 × 200 cm) equilibrated with dilute acetic acid (pH 3·5) (Fig. 1). The flow rate was 60 ml per hour and the volume of the individual fractions 10 ml. The absorbance of the latter was measured at 230 nm and their neutral sugar content determined by the phenol — sulfuric acid assay and subsequent absorbance measurement at 490 nm. The hold-up fraction only gave a positive test for the presence of saccharides; the glycopeptides resolved into three zones (CT-1 to —3) of highest anodic mobility were isolated from this fraction by high voltage electrophoresis.

Thermolytic digest of chicken pepsin: The digestion and the fractionation of the digest on Sephadex G-25 fine have been described before[24]; the glycopeptide contained in the hold-up fraction was resolved into three fractions (TL-1 to -3) by the procedure described above.

Pronase digest of glycopeptide CT-1: Glycopeptide CT-1 in 0·1M Tris-HCl (pH 8·0) containing 15 mM $CaCl_2$ was digested with pronase (enzyme–substrate 1 : 100, w/w). The cleavage was allowed to proceed 7 days and the addition of an identical aliquot of pronase was repeated twice daily. The resulting shorter glycopeptide was desalted and purified by high voltage electrophoresis.

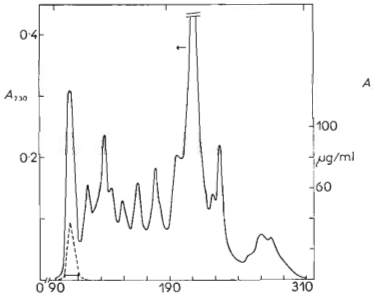

FIG. 1

Chromatography of chymotryptic digest of large tryptic peptides from SSCPG on Sephadex G-25 fine. Full line absorbance at 230 nm, broken line sugar content in mannose equivalents (µg/ml), *n* fraction number. The pooled fraction is marked by a horizontal bar

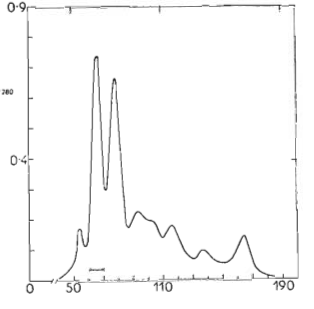

FIG. 2

Chromatography of limited tryptic digest of phthalylated SSCPG on Sephadex G-50 fine. A_{280}, absorbance at 280 nm, *n* fraction number. Pooled fraction FT-1 is marked by a horizontal bar

Limited tryptic digest of phthalylated SSCPG: The phthalylation of SSCPG dissolved in 1% NH_4HCO_3 containing 8M urea was effected by stepwise addition of aliquots of an phthalic anhydride solution in dioxane during 1 h with intensive stirring[25]. The pH of the solution was maintained at 8·5 by titration with 2M-NaOH. Subsequently $NH_2OH.HCl$ (12 mg per 1 mg of protein) was added to the reaction mixture, the pH was adjusted by 6M-NaOH to 9·0, and the mixture was allowed to stand 20 h at room temperature. The phthalylated protein was desalted and digested with TPCK-trypsin at an enzyme to substrate weight ratio of 1 : 150, 2 h at 37°C. The lyophilized digest was fractionated on a column of Sephadex G-50 fine (3·0 × 70 cm), equilibrated with 0·5% NH_4HCO_3 (pH 8·5) (Fig. 2). The flow rate was 35 ml per hour and the volume of the individual fractions 2·8 ml. From the pooled fractions of the effluent aliquots were removed, digested with chymotrypsin, and the glycopeptides located by the technique of peptide maps in the first, high molecular weight fraction only (FT-1, Fig. 2). The latter was purified by ion exchange chromatography on a column of DEAE-Sephacel (1 × 20 cm), equilibrated with 0·05M phosphate buffer at pH 7·1; the glycopeptide-containing fragment was eluted by 6M guanidine hydrochloride only, desalted, and lyophilized.

RESULTS

The glycopeptide isolated from the chymotryptic digest of the tryptic fragments was purified and resolved into three zones by electrophoresis at pH 5·6 (CT-1 to -3). The amino acid analyses of the individual fractions yielded identical results (Table I). Neither were any differences detected by sequential analysis of the fractions with direct identification of the phenylthiohydantoins. Aspartic acid was found in the fourth step by dansyl-Edman degradation and the amino acid sequence of the glycopeptide is therefore the following: Val-Ser-Thr-Asx(CHO)-Glu-Thr-Val-Tyr. This shows that the sugar moiety of the glycopeptide is attached to the peptide *via* aspara-

TABLE I

Amino acid composition of glycopeptides. The values are not corrected. Symbols CT, TL, and FT stand for the combined tryptic and chymotryptic, thermolytic, and limited tryptic digest, respectively

Designation of glycopeptide	Number of amino acid residues
CT-1 to -3	$Asp_{1.0}$, $Thr_{1.9}$, $Ser_{0.9}$, $Glu_{1.0}$, $Val_{1.9}$, $Tyr_{0.9}$
TL-1 to -3	$Asp_{1.0}$, $Thr_{1.9}$, $Ser_{0.9}$, $Glu_{1.0}$, $Val_{1.0}$
FT-1[a]	$Cys_{1.2}$[b], $Asp_{14.0}$, $Thr_{11.6}$, $Ser_{15.3}$, $Glu_{10.3}$, $Pro_{6.1}$, $Gly_{13.9}$, $Ala_{7.0}$, $Val_{10.8}$, $Met_{3.6}$[b], $Ile_{8.1}$, $Leu_{9.3}$, $Tyr_{8.4}$, $Phe_{9.3}$, $Lys_{3.1}$, $His_{1.2}$, $Arg_{1.2}$

[a] Tryptophan was not determined; [b] determined as cysteic acid and methionine sulfone.

gine. The quantitative sugar analyses of the individual fractions (Table II) were different. The entire sugar moiety is represented by mannose and glucosamine. No other monosaccharide was detected. The most acidic fraction CT-1 contains 7 glucosamine residues and 3 mannose residues, the second main fraction CT-2 6 glucosamine residues and 3 mannose residues, and the least acidic, minority fraction CT-3 5 glucosamine residues and 2 mannose residues. The molar ratio of the two main fractions was roughly 2 : 1. The minority fraction represented 10% only of the material contained in the most acidic fraction. One mol of glycopeptide contains on the average approximately 6·5 mol of glucosamine and 3 mol of mannose; these data are in agreement with the sugar content of intact pepsinogen and pepsin determined earlier[1]. The entire sugar moiety of the molecule is therefore attached to one site of the polypeptide chain only. The analyses of the thermolytic digest (glycopeptides TL-1 to -3, Table I) gave similar results.*

The glycopeptide in spite of its relatively high molecular weight (around 3 000 daltons) showed an electrophoretic mobility on the peptide map comparable with the mobility of glutamic acid; its R_F was practically zero. This indicated that there was

TABLE II

Sugar composition and sulfate content of the individual glycopeptide fractions of the combined tryptic-chymotryptic digest (CT-1 to -3)

Method	CT-1			CT-2			CT-3		
	$glcNH_2$	man	SO_4^{2-}	$glcNH_2$	man	SO_4^{2-}	$glcNH_2$	man	SO_4^{2-}
Gas chromatography	7·3	2·9	—	5·8	2·8	—	—	2·1	—
Amino acid analysis	6·7	—	—	5·9	—	—	5·0	—	—
Phenol sulfuric acid method	—	3·4	—	—	3·3	—	—	—	—
Benzidine method	—	—	5·3	—	—	3·9	—	—	—
Mobility after desulfatization	—	—	5	—	—	4	—	—	2
Nearest integer	7	3	5	6	3	4	5	2	2

* Unlike the peptide isolated from the chymotryptic digest the thermolytic peptide was shorter by 2 amino acid residues at the C-terminus: Val-Ser-Thr-Asx(CHO)-Glu-Thr.

a large number of strongly acidic, polar groups bound to the sugar residues of the molecule. The quantitative determination of inorganic phosphate was negative. Elemental analysis revealed in the unresolved glycopeptide fraction 4·4% of sulfur corresponding roughly to 4 to 5 sulfate groups. The sulfate content of the individual fractions (Table II) is 5 groups in fraction CT-1 and 4 groups in fraction CT-2; the sulfate content of the minority fraction CT-3 was not determined because of lack of material. Four spots, designated CT-11 to CT-14, were observed on the electropherogram of fraction CT-1 after its desulfation in 2% pyridine in dimethyl sulfoxide. The most acidic spot showed a mobility identical with that of intact glycopeptide CT-1. Using Offord's equation[26] for electrophoretic mobility on paper electrophoresis the ratio of net charges e_1/e_2 for two selected spots was established (Table III) from the electrophoretic mobilities determined and the molecular weights calculated (on the assumption that all amino groups of glucosamine are acetylated and that two neighboring spots differ by one sulfate group only). In addition to the values found the nearest integers of the e_1/e_2 ratio are also given (Table III). The Table shows that the net charge of intact peptide CT-1 equals -6 (anodic mobility); this is in agreement with the value of 5 sulfate groups and one glutamic acid residue found in glycopeptide CT-1. The second most acidic of the pattern of four spots obtained after desulfatation corresponds in mobility to fraction CT-2 (net charge -5). Glycopeptide CT-3, which considerably differs in molecular weight from glycopeptide CT-1, probably contains two sulfate groups.

We were able to decrease the length of the peptide chain both at the N- and the C-terminus by pronase to the peptide Asn(CHO)-Glu-$(Thr)_{0.5}$. This glycopeptide contains the entire sugar moiety of the molecule; this is another piece of indirect evidence showing that the entire sugar moiety is attached to the asparagine residue.

TABLE III

Ratio of net charge of individual zones (CT-11 to -14) obtained by electrophoresis (at pH 5·6) of partially desulfated glycopeptide CT-1

Zone	CT-11/CT-12	CT-11/CT-13	CT-11/CT-14	CT-12/CT-13	CT-12/CT-14	CT-13/CT-14
Net charge ratio	1·16	1·51	2·03	1·27	1·70	1·36
Nearest integer	6/5	6/4	6/3	5/4	5/3	4/3

The possibility of attachment *via* serine or threonine has been eliminated by incubation of glycopeptide CT-1 in 0·5M-NaOH at 25°C for 24 h. We did not observe any significant decrease of the values of serine or threonine after such treatment of the peptide; in the positive case this decrease should be at least 50%.

The position of the glycopeptide in the polypeptide chain emerged from sequential analysis of fragment FT-1 (Fig. 3). The N- and C-terminal part of the determined N-terminal sequence of the fragment shows marked homologies with the structures of other carboxyl proteinases[27,28]; moreover the peptide contains the amino acid sequence characterizing the neighborhood of the asparagine residue with the attached sugar moiety. It follows from a summary of all the data that this asparagine residue occupies the same position in the molecule of chicken pepsinogen as the phosphoserine residue in the molecule of hog pepsinogen, *i.e.* position No 114 according to the numbering system of Foltmann[27].

DISCUSSION

It has unambiguously been shown that chicken pepsinogen contains a sugar moiety which is covalently attached to the polypeptide chain *via* an asparagine residue. On the basis of the present knowledge of the structure of globular glycoproteins[28,29] it is obvious that the bond between the protein and the sugar moiety is effected *via* the structural unit asparagine-N-acetylglucosamine by an N-glycosidic bond in β-configuration. An interesting feature of the sugar component of chicken pepsinogen is the high content of glucosamine with respect to mannose (roughly 2 : 1). According

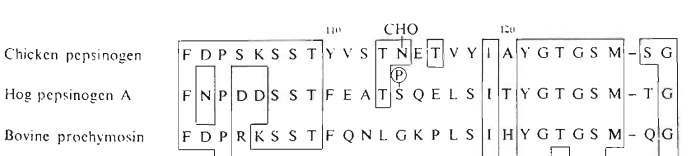

FIG. 3

N-terminal primary structure of limited tryptic fragment FT-1 and its homology with the corresponding parts of other acid proteinase molecules (enboxed). The numbering of the chain, with the exception of *Mucor miehei* pepsin[32] is according to Foltmann and Pedersen[27]. The individual amino acids are given in the one-letter code (IUPAC, ref.[33]). CHO-oligosaccharide chain, P — phosphate residue

to our knowledge such a ratio of monosaccharides is not common[29,30] in simple type oligosaccharides (containing these two monosaccharides) attached *via* asparagine. The sugar moiety of chicken pepsinogen resembles in its composition a complex type oligosaccharide[30] even though it does not contain any other monosaccharide than glucosamine and mannose. It is known that glucosaminoglycanes of a high amino sugar content show an increased resistance to acid hydrolysis. Since pepsin acts at a pH which is extremely low compared to physiological pH this could be one of the reasons of the unusual composition of its sugar moiety. The negative results of the experiments with the binding of pepsin to Con-A-Sepharose and Lentyl-Sepharose carried out in this Laboratory indicate that the mannose residues are "buried" inside the oligosaccharide chain and obviously do not form the non-reducing terminus of the chain.

Very little is known about the sugar composition of other carboxyl proteinases. Cathepsin D, like chicken pepsinogen, also contains glucosamine and mannose, yet at a reversed ratio and this resembles numerous other globular gylcoproteins with a simple oligosaccharide chain attached *via* asparagine. The monkey pepsinogens[4], which contain fucose and galactose in addition to glucosamine and mannose, represent a more complex type of the carbohydrate moiety of the molecule. A comparison of the data available shows that if the carboxyl proteinases are glycosylated then the glycosylation does not proceed according to any general rules valid for the whole family of these proteins.

The microheterogeneity of the sugar moiety of chicken pepsinogen has been demonstrated beyond any doubt. The existence of artifacts is eliminated in view of the fact that identical results were obtained when both pepsinogen and pepsin were used to start with.

The microheterogeneity of the sugar moiety of carboxyl proteinases was first reported for the monkey pepsinogens[4] where genetic variants are probably involved. The problem of chicken pepsinogen remains unsolved since the microheterogeneity could be also due to the ambiguity of the glycosylating synthetic apparatus.

Another interesting feature of the carbohydrate moiety of chicken pepsin represent the sulfate groups. The difference in the number of saccharide residues between the individual glycopeptide fractions obviously corresponds to the difference in the number of sulfate groups. One saccharide residue obviously contains one sulfate group only, most likely bound *via* an O-ester since N-acetyl groups were detected in the glycopeptide molecule. A striking fact is that the sulfated glycoproteins were isolated from gastric mucosa[23,31]. Some of them were shown to possess a high resistance to peptic attack[23]. Since the sugar moiety is localized on the surface of the pepsinogen molecule the presence of sulfate groups could evidence a mechanism preventing pepsin from autolysis. It is also possible, however, that sulfation plays a role in the secretion of pepsinogen by the gastric mucosa.

Fig. 3 shows the primary structure around the glycosylated asparaginyl residue and its homology with the corresponding parts of chains of other carboxyl proteinases. The primary structure -Asn-Glu-Thr- around the glycosylation site corresponds to the general sequence -Asn-X-Thr (Ser)- which is a characteristic "marker sequence" for asparagine glycosylation[34,35]. The position of the asparagine residue in the polypeptide chain is identical to the position of the phosphorylated serine residue in the molecule of hog pepsinogen. This points to a deeper physiological meaning of the posttranslational modification of this part of the molecule. Mucor miehei pepsin contains a topologically identical asparaginyl residue (Fig. 3) yet the "marker sequence" is misssing. Therefore the glycosylated asparagine is the one localized 4 amino acid residues nearer toward the C-terminus where this condition is fulfilled. The inspection of the three-dimensional structure of penicillopepsin[36] shows that this part of the molecule is localized on the surface of the molecule. Steric accessibility of the asparaginyl residue is namely one of the conditions of posttranslational glycosylation.

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