AMINO ACID SEQUENCES OF TWO GLYCOPEPTIDES ISOLATED FROM TRYPTIC AND CHYMOTRYPTIC HYDROLYSATES OF HUMAN LACTOTRANSFERRIN

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1. Introduction

In a previous paper [1], we reported that human lactotransferrin [2–4] (also termed lactoferrin [5]) contains an asparaginyl-*N*-acetylglucosaminide linkage. In this paper, we shall deal with the amino acid sequences of two tryptic and two chymotryptic glycopeptides of lactotransferrin in which this type of linkage is involved.

2. Material and methods

Lactotransferrin isolated with the highest degree of purity from human milk [6] was first subjected to reduction and alkylation [7]. It is then submitted to tryptic (EC 3.4.4.4) or chymotryptic (EC 3.4.4.5) digestion (E/S: 1/50, pH 8.2, at 37°C for 8 hr [8]. The enzymic digests were submitted to gel filtration (Biogel P-30) and the glycopeptide subfractions were further repurified by a free flow electrophoretic separation (Elphor Vap-II) which was carried out at pH 2.4 in 0.5 M acetic acid, at a potential gradient of 1700 V. The glycopeptide subfractions obtained by this method were digested with neuraminidase (EC 3.2.1.18) [9] and the desialyzed glycopeptides were isolated by preparative paper electrophoresis (pH 6.5 in pyridine–acetic acid–water 90:3:1400, 7 V/cm for 16 hr).

The amino acid composition of glycopeptides was determined after acid hydrolysis (5.6 N HCl, 24 hr at 105°C under N_2) by using an Autoanalyzer (Beckman Multichrom) [10]. The tryptophan was identified after hydrolysis with *p*-toluence sulfonic acid [11].

Neutral sugars and hexosamines were determined by classical colorimetric methods [12]. Neutral monosaccharides were identified and estimated by paper chromatography [13] and by gas–liquid chromatography of the trimethylsilyl derivatives of methylglycosides [14]. Hexosamines were estimated by gas–liquid chromatography after methanolysis, *N*-reacetylation and trimethylsilylation [14].

The detection of O-glycosidic linkages was performed by treatment with 0.3 M $NaBH_4$ in 0.1 N NaOH at 20°C for 48 hr [15]. The samples were dried. hydrolyzed with 5.6 N HCl and submitted directly to amino acid analysis.

The N-terminal amino acids were identified by dansylation [16] and C-terminal by hydrazinolysis [17] and by carboxypeptidase A (EC 3.4.2.1) and B (EC 3.4.2.2) digestions [18]. Edman degradation was carried out under the conditions reported by Han et al. [19].

The PTH-amino acids were identified by thin layer

chromatography in the modified system E [19] and the systems D and H reported by Edman [20]. The PTH-asparaginyl-glycans were isolated and identified according to the procedure described by Monsigny et al. [21].

The thermolysin and pronase digestions of glycopeptides were perfomed at 37°C for 24 hr at pH 8.5.

3. Results

3.1. Preparation of the glycopeptides

Tryptic or chymotryptic digestions of the reduced and alkylated human lactotransferrin lead in each case to the isolation of four glycopeptidic subfractions after free continuous electrophoresis. However, after neuraminidase digestion of these four subfractions and further separation by paper electrophoresis, only two glycopeptides were isolated.

3.2. Amino acid and carbohydrate composition

The amino acid compositions of the tryptic glycopeptides I_T and II_T and chymotryptic glycopeptides I_C and II_C are shown in table 1. The amino acid compositions of glycopeptides I and II are significantly different. On the other hand, the glycopeptides seem very homogeneous because, in the amino acid compositions, each residue reaches the nearest molar ratio and shows very low contents of contamination.

Centesimal and molar carbohydrate compositions of tryptic and chymotryptic sialic acid-free glycopeptides I and II are closely similar as reported in table 2.

3.3. Peptide structure

The amino acid sequences of the following glycopeptides: glycoheptadecapeptide I_{T-17}, glyco-tetradecapeptide I_{C-14}, glyco-decapeptide II_{T-10} and glycononapeptide II_{C-9} are specified in the fig. 1. The covalent structure of these peptides were established;

(i) by Edman degradation;

(ii) by the determination of N and C-terminal amino acids;

(iii) by the determination of the structure of glycooctapeptide I_{Th-8} obtained after thermolysic digestion of glycopeptide I_{C-14} and of the structure of the glyco-tripeptides I_{P-3} and II_{P-3} resulted from pronase digestion of glycopeptides I_{C-14} and II_{C-9};

(iv) carboxypeptidase A liberates tryptophan cysteine from glycopeptide II_{C-9}; carboxypeptidase B removes respectively arginine and lysine from glycopeptides I_{T-17} and II_{T-10}.

Table 1
Amino acid composition (moles/mole glycopeptide) of the desialyzed tryptic and chymotryptic glycopeptides isolated from reduced and alkylated human lactotransferrin.

Amino acids	Tryptic glycopeptides		Chymotryptic glycopeptides	
	I_T	II_T	I_C	II_C
CysCM	–	0.4	–	0.4
Asp	1.2	0.9	0.9	1.0
Thr	0.8	0.8	0.9	0.8
Ser	0.4	0.9	–	0.8
Glu	1.6	0.8	1.8	1.1
Pro	3.6	–	2.7	–
Gly	1.0	1.1	0.9	0.8
Ala	1.9	0.1	1.9	0.2
Val	0.6	–	0.6	–
Ile	0.6	–	0.6	–
Leu	1.0	1.0	1.0	1.0
Phe	0.8	0.9	–	0.5
Lys	0.2	0.8	–	–
Arg	0.6	–	–	–
Try	+	+	+	+

Table 2
Carbohydrate composition of the desialyzed tryptic and chymotryptic glycopeptides isolated from reduced and alkylated human lactotransferrin.

	Tryptic glycopeptides		Chymotryptic glycopeptides	
	I_T	II_T	I_C	II_C
Centesimal composition				
Neutral monosaccharides	27.0	32.7	28.4	37.0
N-Acetylhexosamines	19.7	23.5	23.0	28.8
Molar composition				
Galactose	1.8	2.0	1.8	2.0
Mannose	1.9	1.9	2.0	2.2
Fucose	1.1	1.2	1.2	1.2
N-Acetylglucosamine	3.5	3.7	3.8	4.2

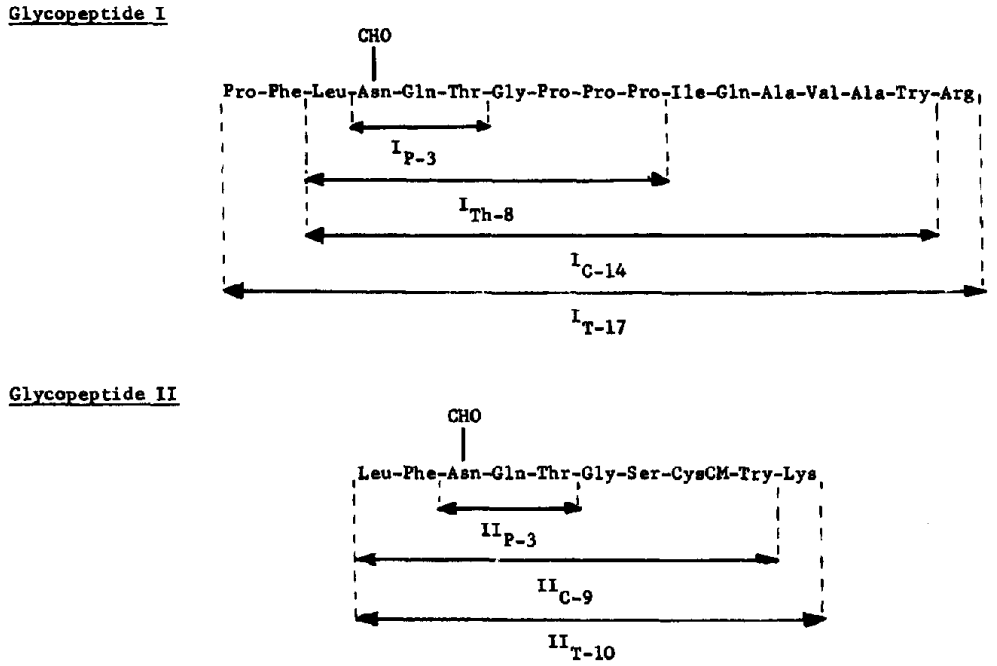

Fig. 1. Amino acid sequences of the glycopeptides obtained from chymotryptic and tryptic hydrolysis of human reduced and alkylated lactotransferrin.

3.4. Carbohydrate-peptide linkage

The amino acid compositions of glycopeptide I_C and II_C, before and after alkaline treatment are identical; no threonine or serine residue is destroyed. Therefore, this proves that no seryl or threonyl-*O*-glycosidic linkage is present in these two glycopeptides. On the other hand, Edman sequential degradation cleaves the asparaginyl–glutamine peptide linkage and leads to the PTH-derivative of asparaginyl–glycan [21].

4. Discussion

The two glycopeptides isolated from tryptic and chymotryptic hydrolysates of human lactotransferrin are significantly different in their polypeptide sequences but possess very similar carbohydrate compositions. The *N*-glycosylamine linkage between asparagine and *N*-acetylglucosamine has been confirmed and no *O*-glycosidic linkage can be detected. The amino acid sequences around the two attachement sites are very similar as indicated clearly by the peptide structure: Asn–Gln–Thr–Gly in both of them. These results confirm once more that the first *N*-acetyl-glucosaminyl-transferase requires recognition of an Asn–X–Thr/Ser sequence [22] to synthetise an *N*-glycosylamine linkage and demonstrate also that we have isolated two glycans which are certainly conjugated on two different sites of the peptide chain as the amino acid sequences of glycopeptides I and II are quite different.

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References

[1] Spik, G., Monsigny, M. and Montreuil, J. (1966) Compt. Rend. 263, 893.

[2] Montreuil, J. and Mullet, S. (1959) Compt. Rend. 153, 1364.
[3] Montreuil, J., Tonnelat, J. and Mullet, S. (1960) Biochim. Biophys. Acta 45, 413.
[4] Blanc, B. and Isliker, J. (1961) Bull. Soc. Chim. Biol. 43, 929.
[5] Masson, P.L. and Heremans, J.F. (1967) Protides of the Biological Fluids 14th Colloquium of Brugges, 115.
[6] Descamps, J., Spik, G., Brazier, J. and Montreuil, J. (1971) Ann. Nutr. Alim. 25, A81.
[7] Fleischman, J.B., Pain, R.H. and Porter, R.R. (1962) Arch. Biochem. Biophys. Suppl. 1, 174.
[8] Hirs, C.H.W., Moore, S. and Stein, W.A. (1956) J. Biol. Chem. 221, 151.
[9] Cassidy, J.T., Jourdian, G.W. and Roseman, S.C. (1965) J. Biol. Chem. 240, 3501.
[10] Spackman, D.H., Stein, W.A. and Moore, S. (1958) Anal. Chem. 30, 1190.
[11] Liu, T.Y. and Chang, Y.H. (1971) J. Biol. Chem. 240, 2842.
[12] Montreuil, J. and Spik, G. (1963) Méthodes colorimétriques de dosage des glucides totaux, Lab. Chim. Fac. Sci. Lille ed.,
[13] Montreuil, J. and Spik, G. (1967) Méthodes chromatographiques et électrophorétiques de dosage des oses 'neutres', Lab. Chim. Biol. Fac. Sci. Lille ed.,
[14] Fournet, B. (1973) Sci. Doct. Thesis, Lille.
[15] Tanaka, K., Bertoli, M. and Pigman, W. (1964) Biochem. Biophys. Res. Commun. 16, 404.
[16] Hartley, B.S. (1970) Biochem. J. 119, 805.
[17] Akabori, S., Ohno, K. and Narita, K. (1952) Bull. Chem. Soc. Japan 25, 214.
[18] Fraenkel-Conrat, H., Harris, J.I. and Levy, A.L. (1955) Methods Biochem. Anal. 2, 339.
[19] Han, K.K., Planchon, B., Dautrevaux, M. and Biserte, G. (1973) Ann. Pharmaceutiques Fr., in press.