PREPARATION OF *N*-ACETYLGLUCOSAMINE DERIVATIVES OF PROTEINS

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1. Introduction

The preparation of protein hexose conjugates, by coupling glycosylamines (1-amino-1-deoxy-sugars) to the carbodiimide activated carboxyl groups of the polypeptide chains was described recently [1]. Thus the neutral sugar containing mono- and disaccharides were attached by glycosylamine linkage to the protein. The products obtained were used as substrates for glycosyl transferases [2] as well as in immunological studies [3].

However, in naturally occurring glycoproteins *N*-acetyl-glucosamine, but not neutral sugars are attached to the polypeptides by means of a glycosylamine linkage [4].

In this paper we describe a method for the preparation of *N*-acetyl-glucosamine derivatives of proteins, linked at least partially by an aspartamido-glucosaminidic linkage to the polypeptide chain.

2. Methods

2.1. *Lysozyme-N-acetylglucosamine conjugate*

400 mg of 2-acetamido-1-amino-3,4,6 tri-*O*-acetyl-1,2 dideoxy-β-D-glycose [5] was dissolved in 1.5 ml dimethylsulfoxyde and mixed with a solution of 30 mg lysozyme (Sigma Chemical Co, Saint-Louis, Mo, USA) in 1 M of 5 M aqueous guanidinium HCl. The reaction was initiated by the addition of 60 mg 1-ethyl-1,3(3-diaminopropyl) carbodiimide hydrochloride (Ott Chemical Co., Muskegon, Mi, USA) in 1.0 ml 5 M guanidinium hydrochloride. The pH of the reaction mixture was maintained at 4.75 by automatic titration in a pH-stat (Radiometer, Copenhagen) with 4.0 M HCl. At the end of the acid uptake (about 2 hr) the reaction mixture was dialysed against 0.001 M hydrochloric acid and against distilled water and lyophilised.

100 mg of the product was dissolved in 25 ml 0.3 N NaOH. The mixture was left 1 hr at room temperature, neutralized with 2 N acetic acid, and dialysed.

2.2. *Tryptic digestion*

The desacetylated product was carboxymethylated [6] and digested with trypsine (Worthington, 2 X cryst.) according to Canfield and Anfinsen [7]. The tryptic peptides were separated on Aminex A5 ion exchange resin (Bio-Rad Laboratories, Richmond, Ca) column (0.9 X 20 cm) using pyridine-acetate gradient for the elution [8]. The elution was monitored at 280 nm, and the peptides were detected : a) as spots on filter paper by toluidine after chlorination [9], and b) on silica thin layers by the ninhydrine reaction [10]. Peptide maps were obtained on silica thin layers as described [10].

2.3. *Analytical methods*

The glucosamine content of the peptides was detected by the Elson-Morgan reaction [11] and glucosamine was identified in the hydrolysates by thin-layer chromatography [12].

The desacetylation of the *O*-acetylated derivative of the lysozyme-*N*-acetyl-glucosamine conjugate was controlled by the colorimetric determination of the *O*-acetyl group by the method of Hestrin [13] modified by Abrams [14].

2.4. *Detection of the aspartamido–glucosaminic linkage*

The three main glucosamine containing glycopeptide fractions [1, 3, 4] were pooled and hydrolysed with pronase (Sigma Chemical Co, Saint-Louis, Mo) at pH

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8.8 and 49°C (enzyme–substrate ratio 1:25). The pH was readjusted after 48 hr and the digestion was continued by addition of pronase (enzyme–substrate ratio 1:25) for further 48 hr.

The pronase digest was submitted to high voltage paper electrophoresis in pyridic-acetate water 89:10:1 (v/v/v) at pH 3.8. The region corresponding to the aspartic acid was eluted, and the eluted mixture was chromatographed on cellulose thin layer (Schleicher et Schüll, Selecta 1440) using *n*-butanol–acetic acid–water (12:3:5, v/v/v) and *n*-butanol–pyridine–water (6:4:3, v/v/v) as solvents. The chromatograms were devoloped in a continuous flow chamber, and the spots were detected with ninhydrine [15].

The crude synthetic 2-acetamido-1 (L-β-aspartamido)-1,2 dideoxy-β-D-glucose [5] was used as reference substance.

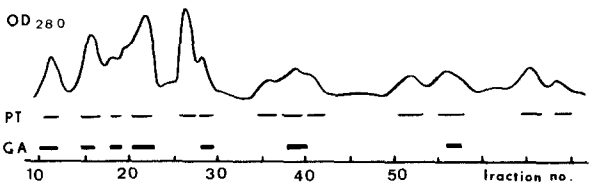

Fig. 1. Chromatography of peptides obtained after 3 hr of tryptic digestion, of reduced carboxymethylated lysozyme *N*-acetyl-glucosamine conjugate on Aminex A5 column (0.9 × 20 cm). Elution : linear gradient from pH 3.1 : 0.2 M to pH 5.0 2.0 M pyridine acetate. Optical densities were monitored at 280 nm ; the deviation of the base line is corrected on the diagram. TP = fractions reacting with toluidine after chlorination. GA = glucosamine containing fractions.

3. Results and discussion

The hexosamine content of the lysozyme *N*-acetyl-glucosamine reaction product determined by the Morgan-Elson reaction was about 2–3% in the different preparations. This hexosamine content corresponds to 1.8–2.5 hexosamine residues per molecule of lysozyme. The coupling of the 1-amino-1-deoxy derivatives of the hexoses (β-glucosyl-, α and β -galactosyl- and β-mannosylamine) to lysozyme gives products with similar carbohydrate content (2–2.5%) [15]. Thus about 2 to 3 out of the 11 free carboxyl groups of lysozyme (the C-terminal leucine, 2 glutamic and 8 aspartic acid residues) were substituted.

To obtain information on the distribution of the *N*-acetyl-glucosamine residues on the lysozyme molecules and on the nature of the sugar–protein linkage, the *O*-desacetylated condensation product was submitted to tryptic digestion. As it was reported by Canfield and Anfinsen [7] lysozyme gives 17 fragments after trypsine digestion. Nine of them contain free carboxyl groups (T3, T5, T7, T8, T9, T11, T13, T16 and T18) [7]. In this experiment seven hexosamine containing peptide peaks were eluted from Aminex A5 column (fig. 1). Eight carbohydrate containing spots were identified on the thin layer peptide maps (fig. 2). The peptide maps after 3 and 7 hr of trypsin treatment were not significantly different. The presence of the C-terminal leucine in the tryptic digest (fig. 2) indicates that the carboxyl end-group was not quantitatively blocked by the *N*-acetylglucosamine. This result, as well as the number of glycopeptide fractions found in the tryptic digest (seven or eight, see above) and the hexosamine content of the crude reaction product (about 2 mol hexosamine/mol protein) suggest that there is no priviledged substitution of the different free carboxyl groups of lysozyme.

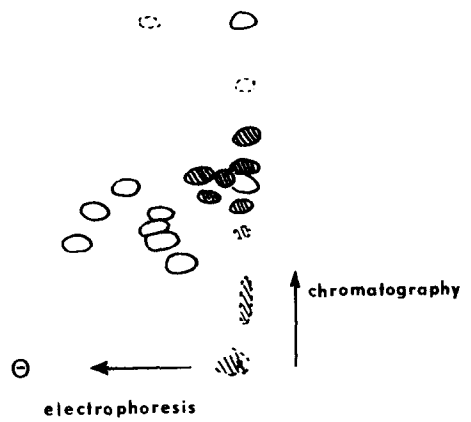

Fig. 2. Peptide map of a tryptic digest of carboxymethylated lysozyme *N*-acetylglucosamine conjugate on silica thin layers (Selecta 20 × 20 of Schleicher et Schüll, Dassel, Germany). Electrophoresis pyridine–acetic acid–water 10:1:89, 2 hr, 300 V. Chromatography : pyridine–*n*–butanol–acetic acid–water 40:68:14:25 (v/v/v/v)

ninhydrine positive spots

ninhydrine and orcinol-sulfuric acid positive spots

Thus the formation of several closely related substitution products can be assumed as it was found in the case of the galactosylamine–lysozyme conjugate [16].

The formation of glycosamine containing peptides on tryptic digestion indicates that the carbohydrate is covalently bound, and not adsorbed to the protein.

Two glutamic acid (T3, T7) and six aspartic acid (T5, T8, T9) containing peptides were isolated from the tryptic digest of the unmodified lysozyme [7]. Thus the presence of the eight hexosamine containing peptides in the tryptic digest of the *N*-acetylglucosamine conjugate suggests that at least a part of the *N*-acetylhexosamine residues is linked to an aspartic acid containing peptide.

As the carbodiimide activates only the free carboxyl groups of a polypeptide chain [17], we can reasonably assume that the 1-acetamido-1-amino-1,2-dideoxy-β-D-glucose is linked to the β-carboxyl group of an aspartic acid residue.

On the other hand, the partial substitution of the free carboxyl-groups of two glutamic acid residues (Glu 7 and 35) as well as that of the C-terminal leucine [1] cannot be excluded.

The identical chromatographic and electrophoretic mobilities of one of the proteolytic degradation products of the glucosamine containing glycopeptides (1, 2, and 3, fig.1) and of the 2-acetamido-1-L-β-aspartamido)-1,2-dideoxy-β-D-glucose can be considered as a further indication for the aspartamido–glucosaminic linkage in the lysozyme *N*-acetylglucosamine conjugate.

From the results presented, it appears that the described method enables the preparation of covalently linked *N*-acetylglucosamine derivatives of proteins. The *N*-acetyl-glucosamine linkage involves at least partially the amido-nitrogen of asparagine, as it was found in natural glycoproteins [4].

Acknowledgements

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