THE COMPLETE ENZYMIC DEGRADATION OF GLYCOPEPTIDES CONTAINING O-SERYL AND O-THREONYL LINKED CARBOHYDRATE

V.P.Bhavanandan, E.Buddecke, R.Carubelli* and A.Gottschalk
Max-Planck-Institut für Virusforschung und Physiologisch-Chemisches Institut der Universität, Tübingen

Received May 18, 1964

In recent years a considerable amount of information on the molecular structure of glycoproteins has been published. Little is known, however, on the biosynthesis of these compounds and no information is available on the enzymic breakdown of glycoproteins to their individual constituents. In the present paper the purification and properties of a ß-N-acetylhexosaminidase and its action on glycopeptides containing O-seryl and O-threonyl linked N-acetylgalactosamine are described.

EXPERIMENTAL AND RESULTS

Purification and Properties of ß-N-Acetylhexosaminidase

The purification of the enzyme present in bovine spleen homogenates was carried out by fractional precipitation with ammonium sulfate (crude enzyme) followed by chromatography on TEAE and CM Cellulose columns (highly purified enzyme). The anomeric specificity of the enzyme was shown to be of the ß-type. Several natural and synthetic glycosides were found to be substrates for the enzyme (see also Weissmann et al., 1964). The K_m values x 10^3 for ß-phenyl-N-acetyl-D-glucosaminide, ß-phenyl-N-acetyl-D-galactosaminide and ß-p-nitrophenyl-N-acetyl-D-glucosaminide were 2.0, 1.1 and 1.4 respectively. The enzyme requires for its activity a terminal non-reducing

*On leave from the Oklahoma Medical Research Institute, Oklahoma City, Oklahoma, U. S. A.

N-acetylhexosamine residue. The increase in specific activity, referred to the homogenate as unity, was 42 fold for the crude enzyme and 20 000 fold for the highly purified enzyme.

Preparation and Properties of Glycopeptides from OSM

Ovine submaxillary gland glycoprotein (OSM) was prepared as previously described (Graham and Gottschalk, 1960) with the modification that the precipitation of glycoproteins and proteins to be discarded was carried out at pH 4.5 instead of pH 4.0. Yield 5% of the dry weight of the glands. The compound displayed a single and almost symmetrical peak in the analytical centrifuge. It contained about 40% carbohydrate (as residues) consisting of equimolar amounts of N-acetylneuraminic acid and N-acetylgalactosamine. 250 mg OSM was exhaustively treated with Pronase and the digest fractionated on a Sephadex G25 column. The pooled fractions of the sialoglycopeptide peak reacting with both ninhydrin and resorcinol were submitted to neuraminidase action and the enzyme was removed by a second passage through a Sephadex G25 column.

The sialic acid-free glycopeptides were separated from sialic acid on a Dowex I-X2 (formate form) column and the effluent was concentrated to dryness. Yield about 50 mg containing 50% of the galactosamine of OSM. On the Dowex I column about 35% of the applied glycopeptides, expressed in terms of galactosamine, were retained. The quantitative analysis of the glycopeptide fraction is shown in Table I. The near lack of dicarboxylic acids in the glycopeptide fraction (OSM contains 0.84 moles dicarboxylic acids/mole galactosamine) would indicate that a considerable amount of the glycopeptides, applied to the Dowex I column, contained aspartyl and glutamyl residues with unsubstituted ß- and γ-carboxyl groups respectively and were therefore retained on the column. In fact, when the column was eluted with 2 N acetic acid, analysis of the eluate showed the presence of 20% of the total dicarboxylic acids. Another 58% of dicarboxylic acids was found in the pool of free amino acids and small peptides emerging from the first Sephadex column

Table I. Amino acid composition of glycopeptide fraction

(expressed in moles per 1 mole of galactosamine)

Aspartic acid	0.03	Proline	0.36
Glutamic acid	0.08	Glycine	0.56
Serine	0.62	Alanine	0.30
Threonine	0.67	Valine	0.17
Lysine	0.01	Leucine	0.07
Arginine	0.09		

after the sialoglycopeptide peak. The average molecular weight of the glycopeptides was about 660 (potentiometric titration) and the molar ratio amino acids/hexosamine (after hydrolysis of the glycopeptides) was found to be 3.3. These data would indicate that on an average the glycopeptides were tetrapeptides with 1.3 residues of N-acetylgalactosamine attached.

Type of Linkage between N-Acetylgalactosamine and Peptide

It is evident from Table I that in the glycopeptides only serine and threonine are conceivable as sugar acceptors. On treatment of the glycopeptide fraction with 0.5 N NaOH at 0° for 20 hr (see Anderson et al., 1963) followed by acid hydrolysis, only 504 μmoles of serine and 576 μmoles of threonine were recovered from 900 and 960 μmoles of serine and threonine respectively present in 1 gram of untreated glycopeptide, whereas the other amino acids were recovered quantitatively. When the experiment was extended to 68 hr, 456 and 475 μmoles of serine and threonine respectively were recovered. The combined loss of 929 μmoles of serine and threonine coincided with the appearance of 900 μmoles of free N-acetylhexosamine in the assay prior to acid hydrolysis. When DL-seryl-glycyl-glycinamide and L-seryl-leucyl-leucine were treated similarly, the losses of serine did not exceed 10%. These data can only be interpreted as resulting from ß-elimination of N-acetylhexosamine residues linked O-glycosidically to the hydroxyl groups of peptide-bonded serine and threonine residues.

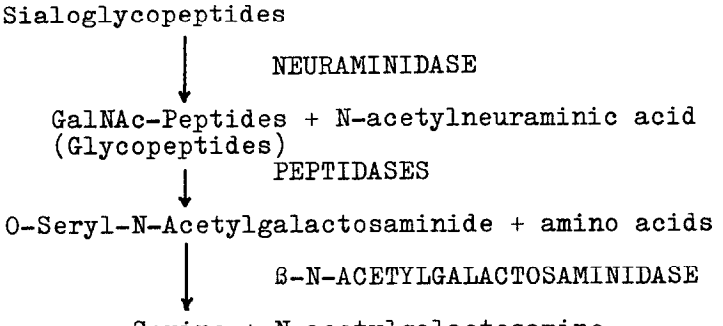
Enzymic Degradation of Glycopeptides

When the glycopeptides were treated with crude enzyme added in portions (0.025 M citrate buffer, pH 4.3, 37°), 40.0, 75.9 and 94.7% of the total hexosamine were released after 48, 96 and 144 hr respectively; paper chromatography of the digest revealed in addition to free N-acetylgalactosamine all the amino acids listed in Table I. The presence of peptides in the digest freed from proteins was seen from a 40% increase of the ninhydrin value (corrected for hexosamine) after acid hydrolysis of the digest. The highly purified enzyme released under same conditions 6.7, 9.8 and 13.2% respectively of the total hexosamine. From sialoglycopeptides only 4.5 and 6.5% of hexosamine were split off after 54 and 97 hr incubation respectively with the crude enzyme.

DISCUSSION

These results indicate that in OSM, as prepared above, at least 50% of the total hexosamine is linked ß-O-glycosidically to the OH-groups of seryl and threonyl residues of the peptide and that this linkage is susceptible to N-acetylhexosaminidase. The enzymic cleavage takes place only after removal of sialic acid and after shortening of the glycotetrapeptide by at least one or two amino acid residues. The shortening is effected by peptidases the presence of which in the crude enzyme was also evidenced by its action at pH 4.3 on L-seryl-L-leucyl-L-leucine. This type of peptidases is quite distinct from the known exopeptidases. The occurrence of these peptidases and ß-N-acetylhexosaminidase in the same tissue and their activity at the same pH favours the view that the sequence of reactions shown in the diagram may be the physiological degradation mechanism.

DIAGRAM



A similar scheme would hold for O-threonyl-N-acetyl-galactosaminide.

The relative proportion of glycosidic-ester linkages (Graham et al., 1963) and O-seryl (threonyl)-glycosidic linkages in various OSM preparations is at present under investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Peter Anders, M-P-I für Virusforschung, for the quantitative amino acid analyses. This research has been supported in Part by Grants GM 10917-02 and AI 04954-02 of the U. S. Public Health Service and by the Deutsche Forschungsgemeinschaft.

REFERENCES

Anderson, B., Hoffman, P., and Meyer, K. (1963). Biochim. Biophys. Acta, 74, 309.
Graham, E. R. B., and Gottschalk, A. (1960). Biochim. Biophys. Acta, 38, 513.
Graham, E. R. B., Murphy, W. H., and Gottschalk, A. (1963). Biochim. Biophys. Acta, 74, 222.
Weissmann, B., Hadjiioannou, S., and Tornheim, J. (1964). J. Biol. Chem., 239, 59.