ON THE STRUCTURE OF GALACTOSYLHYDROXYLYSINE AND GLYCOPEPTIDES DERIVED FROM BOVINE TRACHEAL CARTILAGE

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SUMMARY : Galactosylhydroxylysine isolated from the alkaline hydrolysate of bovine tracheal cartilage was shown to be O-β-D-galactopyranosylhydroxylysine. The structures of several hydroxylysine-containing glycopeptides suggested the existence of a unique sequence around the glycosylated site with one exception in that an amino acid other than arginine was present in the third position from the glycosylated residue. Isolation of glycopeptides with an identical hexapeptide sequence yet with a different carbohydrate side chain suggested that the attachment of glucose to a polypeptide-bound galactose is not regulated by a short amino acid sequence in the glycopeptide region.

Collagens and related proteins have been shown to have the carbohydrate prosthetic groups linked O-glycosidically to hydroxylysine (1,2). Our primary interest is the mechanism of glycosylation of the limited hydroxylysine residues in these proteins. Morgan et al. (3) suggested that the sequence Gly-X-Hyl-Gly-Y-Arg of vertebrate collagens is a minimal structure required for glycosylation of the hydroxylysine. This idea was expanded by our findings on invertebrate collagens (4-6). Some of their suggestions, however, did not seem to be consistent with our previous work (4-6). Because of the limited amount of data on vertebrate glycopeptides, it was not possible to evaluate the suggestion that different observations may reflect the difference between vertebrates and invertebrates. In the present work, the structures of hydroxylysine-containing glycopeptides derived from bovine tracheal cartilage have been determined and compared with those of vertebrate and invertebrate collagens. The structure of

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Gal-Hyl* derived from the cartilage has also been examined in order to see if the specificity preventing further glycosylation after the formation of monosaccharide side chains may reside in this structure.

MATERIALS AND METHODS

Bovine tracheal cartilage was defatted with acetone and chopped into small pieces which were extracted with 3M $MgCl_2$ to remove the proteoglycans. The residues were washed thoroughly with water and incubated with Pronase P (Kaken Kagaku Co., Tokyo) at pH 8.0. After 40 hr., cetylpyridinium chloride was added to the digest and the precipitates were removed by centrifugation. The supernatant was concentrated and subjected to gel filtration through Sephadex G-25 as described previously (4-6). The main hexose-containing fraction was further fractionated by conventional chromatographic techniques (4-6). Six homogeneous glycopeptides were obtained in sufficient quantity for the structure determinations. The amino acid sequence and the carbohydrate compositions were determined as described previously (5). Glc-Gal-Hyl and Gal-Hyl were prepared from the alkaline hydrolysate of bovine tracheal cartilage as reported previously (5,7). Repeated gel filtration through Bio-Gel P-2 with 0.1N acetic acid permitted the isolation of pure compounds. The carbohydrate side chain of each glycopeptide was analyzed for its carbohydrate composition and by detecting either Glc-Gal-Hyl or Gal-Hyl in alkaline hydrolysates on a high voltage paper electrophoretogram. N-Acetylated Gal-Hyl was prepared by treating Gal-Hyl with acetic anhydride in aqueous $NaHCO_3$, followed by gel filtration through Bio-Gel P-2. Aspergillus oryzae β-galactosidase was purified from Taka-diastase Sankyo (8). Galactostat was obtained

* Abbreviations used are: Gal-Hyl for galactosylhydroxylysine and Glc-Gal-Hyl for glucosylgalactosylhydroxylysine.

Table I. The degree of hydrolysis of various galactosides by Aspergillus oryzae β-galactosidase. Reaction was performed in 0.1M pyridine-acetate for 18hr. with 0.05 unit of the enzyme at pH 5.0 and 40°C. One unit of the enzyme will release 1 μmole of p-nitrophenol per min. from p-nitrophenyl β-galactopyranoside at pH 5.5 and 37°C.

Substrate	Hydrolysis, %[1)]
Gal-Hyl	8[2)]
N-Acetylated Gal-Hyl	98
Phenyl α-D-galactoside	0
Phenyl β-D-galactoside	99
Lactose	93
4-O-β-D-Galactosyl-D-glucitol	69

1) As determined by quantitating galactose gas-chromatographically.
2) 0.125 unit of the enzyme was used and the degree of hydrolysis was estimated by densitometric tracing at 500 nm of the paper electrophoretogram of the reaction mixture after stained with cadmium-ninhydrin.

from Worthington Biochemical Corp. Dinitrophenylation of Gal-Hyl was carried out in a manner similar to that reported previously (1,2).

RESULTS AND DISCUSSION

A. oryzae β-galactosidase, free of α-galactosidase activity, released galactose completely from N-acetylated Gal-Hyl as shown in Table I. The result was consistent with the finding that Gal-Hyl was absent on a paper electrophoretogram of the alkaline hydrolysate of the reaction mixture. This enzyme also cleaved the galactosidic bond of Gal-Hyl slowly (Table I). These observations were in contrast to the reported findings that E. coli β-galactosidase hydrolyzed N-acetylated Gal-Hyl less effectively and did not attack Gal-Hyl itself (10). The almost complete destruction by galactose oxidase (Fig. 1) indicated that galactose residue possessed the D-pyranosyl configuration (9). Dinitrophenylation showed that Gal-Hyl had two free amino groups. These results are consistent with the structure

Table II. The structures of collagen glycopeptides derived from bovine tracheal cartilage.

Glyco-peptide	Structure	Sugar unit
BC-1	Gly-Ile-Hyp-Gly-Ala-Hyl*-Gly-Ser	Glc-Gal
BC-2	Thr-Gly-Pro-Hyl*-Gly-Ala-Arg	Glc-Gal
BC-3	Gly-Pro-Hyl*-Gly-Ala-Hyp-Gly-Glu-Arg	Gal
BC-4	Gly-Pro-Hyl*-Gly-Asp-Arg	Glc-Gal
BC-5	Gly-Pro-Hyl*-Gly-Ala-Arg	Glc-Gal
BC-6	Gly-Pro-Hyl*-Gly-Asp-Arg	Gal

Hyl* represents the glycosylated hydroxylysine residue.

of O-β-D-galactopyranosylhydroxylysine for Gal-Hyl. The same compound had been obtained by a partial acid hydrolysis of Glc-Gal-Hyl derived from the renal glomerular basement membrane (10) and from the sponge (9). Since Glc-Gal-Hyl had been shown to be 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine (9,10), our study confirmed that Gal-Hyl represented the incomplete form or the intermediate structure in the biosynthesis of the Glc-Gal-Hyl moiety.

The structures of six glycopeptides derived from the cartilage are summarized in Table II. The unique sequence Gly-X-Hyl-Gly-Y-Arg generally found in collagen glycopeptides (3-6) can be seen with one exception in which hydroxyproline replaces arginine. Since many collagen glycopeptides of diverse origins conformed this general sequence (3-6), it is likely that the sequence is required for glycosylation of hydroxylysine in a collagen polypeptide, although in some cases exception to the rule occurs (5,6,11). This apparent discrepancy may be explained by the possible existence of multiple forms of galactosyltransferase. In fact, two galactosyl

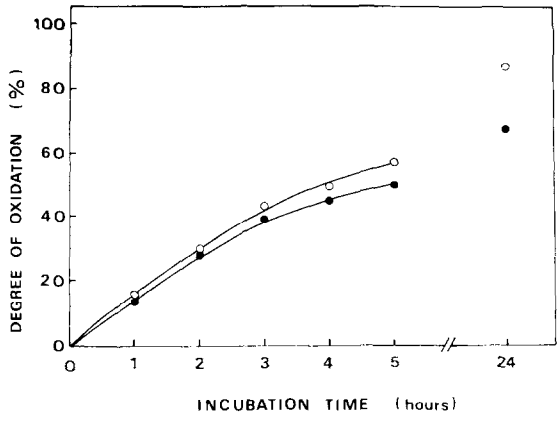

Fig. 1. Oxidation of Gal-Hyl isolated from the alkaline hydrolysate of bovine tracheal cartilage and its N-acetylated form by galactose oxidase. Gal-Hyl(0.32 μmole, ●) or N-acetylated Gal-Hyl(0.1 μmole, ○) was incubated with 2 ml of the galactostat reagent at 40°C. The degree of oxidation was determined by the increase in absorbance at 425nm.

transferases with different specificiy have been reported (12,13). Another explanation may be that arginine in this sequence is not essential for glycosylation as suggested in a recent work by Aguilar et al. (11).

As seen in the Table II, the amino acids found around glycosylated hydroxylysine are those encountered most frequently in collagen, such as glycine, proline, alanine and asparatic acid. The result is in accordance with our previous study on invertebrate collagens (4-7), whereas it is in contrast to the reported observation that the amino acids occurring rarely in collagens, such as methionine and histidine, are found in the linkage region in vertebrate glycopeptides (3). Thus, the present study makes it clear that the presence of such amino acids is not necessarily required for the glycosylation process of vertebrate and invertebrate collagen polypeptides.

Another interesting point arises from the results in Table II. The amino acid sequences of glycopeptides BC-4 and BC-6 are

identical while the carbohydrate chains are different. They may represent the microheterogeneity at a distinct site in collagen polypeptide chain (11), but the details remain to be elucidated. The determination of the structure of Gal-Hyl in the present work excludes the possibility that these glycopeptides have the structural difference in Gal-Hyl moiety.

The present findings provide the contrary view to the suggestion (3,11) that the sequence Gly-Pro-Hyl-Gly prevents the glucose-transferring activities, since glycopeptides BC-2, BC-4 and BC-5 have normal disaccharide chains. In invertebrates, some glycopeptides with this sequence, yet with a disaccharide side chain have been reported (4-7). Thus the present study suggests that a short amino acid sequence around the glycosylated site does not dictate whether a carbohydrate side chain is to be completed or not. The factor(s) restricting the side chain to the monosaccharide in these instances must be sought elsewhere.

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