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Short communication

Improved rapid method for the isolation, purification and identification of collagen glycosides

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Abstract

An updated method for obtaining purified glucosylgalactosyl hydroxylysine (Glc-Gal-Hyl) is presented. Marine sponge is subjected to alkaline hydrolysis followed by ion-exchange chromatography and gel filtration. Identification is confirmed by consecutive mild acid hydrolysis of the extract to produce galactosyl hydroxylysine (Gal-Hyl) and hydroxylysine (Hyl). High-performance liquid chromatography (HPLC) with a sodium acetate–acetonitrile buffer and dabsyl chloride as the derivatizing agent demonstrated the purity of the extract and its hydrolyzates. Glc-Gal-Hyl is not commercially available but this easy and efficient method can provide large amounts of it for collagen metabolism studies.

Keywords: Collagen; Glycosides; Glucosylgalactosyl hydroxylysine; Galactosyl hydroxylysine; Hydroxylysine; Saccharides; Amino acids

1. Introduction

The disaccharide glucosylgalacosyl hydroxlysine is a component of collagen. This collagen component has been isolated from the collagens of the sea anemone *Metridium dianthus*, the sea cucumber *Thyone briareus*, and bovine cornea [1–5]. Using techniques such as mass spectrometry, optical rotation and retention time on gas–liquid chromatography, it has been shown that the glucosylgalactosyl hydroxylysine from all these different sources is identical with the glucosylgalactosyl hydroxlysine from sponge *Hippospongia*. This structure has been shown to be O-α-D-glucopyranosyl-(1→2)-O-β-D-glactopyranosyl-oxy-(1→5)-L-lysine [6].

The importance of these glycosides is well documented in several areas of research. The suggestion that Gal-Hyl might be a possible glucose acceptor in the glucosylation of collagen and basement membrane by the rat kidney UDP-glucosecollagen glucosyltransferase led to one method for the isolation and purification of large quantities of the hydroxylysine–monosaccharide from commercially available marine sponge [7]. In the field of spinal cord injury, Glc-Gal-Hyl has been used to study the degradation of skin and bone collagen. This study measured the concentration of Glc-Gal-Hyl and Gal-Hyl in urine [8]. The differences in the proportional increase of either Glc-Gal-Hyl or Gal-Hyl indicate different rates of degradation of skin and bone collagen.

The method described in this paper is an improvement on the methods mentioned earlier. This method

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is easier and faster due to use of HPLC. The verification of the mild acid hydrolysis products of glucosylgalactosy hydroxylysine via HPLC as hydroxylysine monosaccharide confirms the identity of the original product.

2. Experimental

One gram of natural sea sponge is minced and washed with 100 ml of deionized water. The water is drained and the sponge air dried. The minced sponge is placed in a 50-ml centrifuge tube with a PTFE-lined cap containing 40 ml of 4 *M* KOH. The tube is sealed and placed in an oven set at 105°C for 24 h. After the tube reaches room temperature, it is centrifuged for 0.5 h at 12 000 *g* in a Sorvall RC-5B refrigerated centrifuge to remove a heavy brown residue. The supernatant is transferred to another tube and adjusted to pH 6.5 with 4 *M* $HClO_4$. The resulting precipitate is removed by centrifugation. The supernatant is saved and adjusted to pH 2 with dilute HCl and allowed to stand at 4°C overnight. The supernatant is then centrifuged at 30 000 *g* for 20 min. The supernatant collected is stored at 4°C until used.

Dowex-50 (8% cross-linked) ion-exchange resin from Sigma is placed in a side-arm flask and hydrated with deionized water. Hydrated resin is evacuated for 1 h to remove trapped air. Resin is poured into a 2×30 cm glass column and equilibrated overnight with degassed deionized water at a flow-rate of 18 ml/h.

After loading the sample, five column volumes of degassed deionized water is allowed to flow through the column overnight. The sample is eluted from the column by 50 ml of 0.5 *M* HCl followed by 1 *M* HCl (Fig. 1). Fractions of 2.5 ml are collected and kept at 4°C.

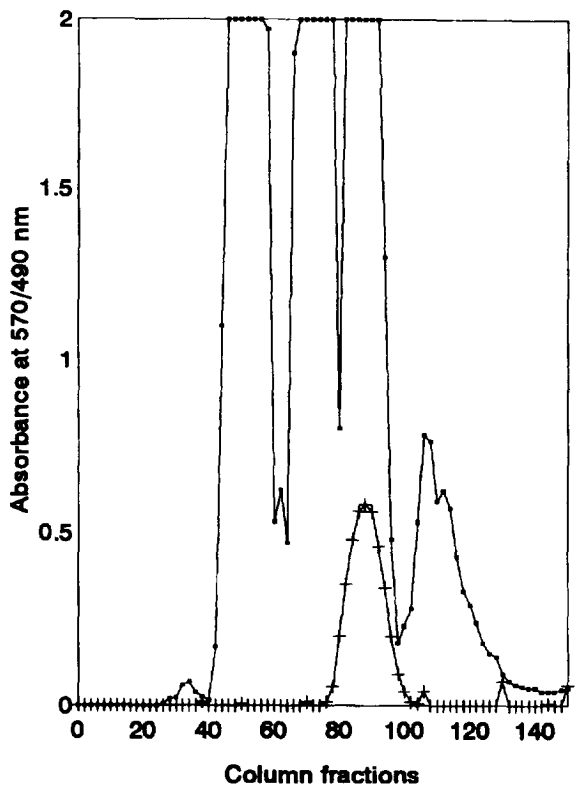

Fig. 1. Ion-exchange chromatography of sponge hydrolysate. Step elution by water, 50 ml of 0.5 *M* HCl, and 1 *M* HCl: (□) positive for amino acids; (+) positive for carbohydrates.

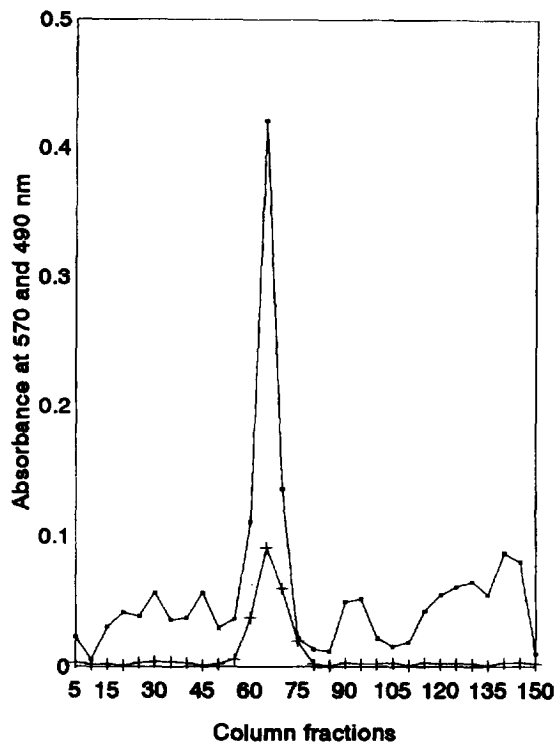

Fig. 2. Chromatogram of second elution of fractions positive for both amino acids and carbohydrates: (□) amino acids; (+) carbohydrates.

All fractions are tested for carbohydrates using the phenol-sulfuric acid method [9] and for amino acids via the ninhydrin method [10]. Fractions that tested positive for both are pooled and neutralized with dilute NAOH (Fig. 1). The crude Glc-Gal-Hyl is lyophilized and reconstituted in 5 ml of degassed deionized water and rechromatographed on a 1×50 cm column packed with the same ion-exchange resin used earlier. After applying the sample, the column is washed with deionized water for 4 h at 15 ml/h followed by 0.5 *M* HCl, overnight. The sample is eluted from the column with 1 *M* HCl and collected in 1.5-ml factions. Fractions are assayed for carbohydrate and amino acid content as before. Fractions positive for both carbohydrates and amino acids are pooled, neutralized with dilute NAOH (Fig. 2), and lyophilized.

The lyophilized sample is rehydrated with 6 ml of deionized water and filtered using a 0.2-μm syringe filter. A 2-ml aliquot is applied to a 1×60 cm column packed with Bio-Gel P-2 (Bio-Rad Labs.) that had been equilibrated with degassed deionized water. Water is used as the eluant. Fractions collected are about 1 ml (10 drops). Multiple runs are made to chromatograph the entire sample. Fractions are stored at 4°C.

Fractions from the P-2 column are tested for the presence of salts using a saturated silver nitrate solution as the precipitant. Ninhydrin and phenol-sulfuric acid methods are utilized to confirm the presence of amino acids and carbohydrates, respectively. Salt, carbohydrate and amino acid content data is graphed and fractions positive for amino acids and carbohydrate, but negative for salts are pooled (Fig. 3). This pool of fractions represented our final product, Glc-Gal-Hyl.

By altering the time of hydrolysis, we can produce different proportions of Glc-Gal-Hyl, Gal-Hyl, and Hyl (Fig. 4). Optimal conditions for obtaining Gal-Hyl are: a 20-μl aliquot of the final pooled P-2 fractions is hydrolyzed with 20 μl of 0.8 *M* HCl at 110°C for 31 min. After reaching room temperature,

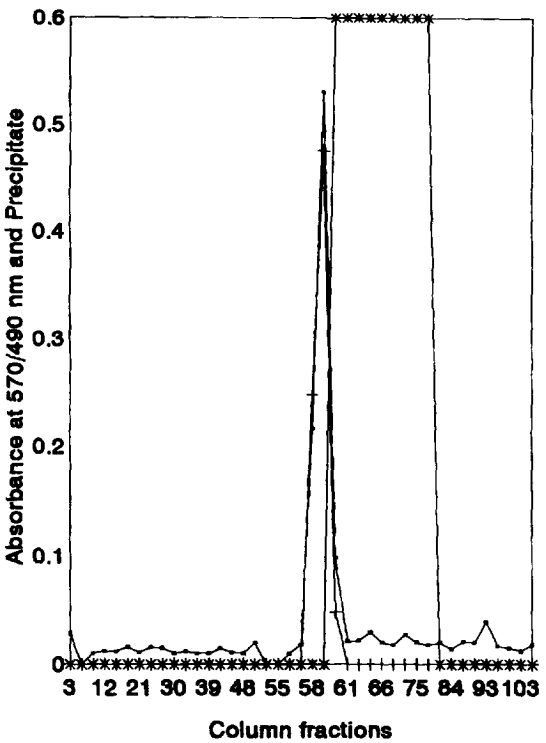

Fig. 3. Chromatogram of pooled fractions desalted on a P-2 column: (□) amino acids; (+) carbohydrates; (×) precipitate appeared.

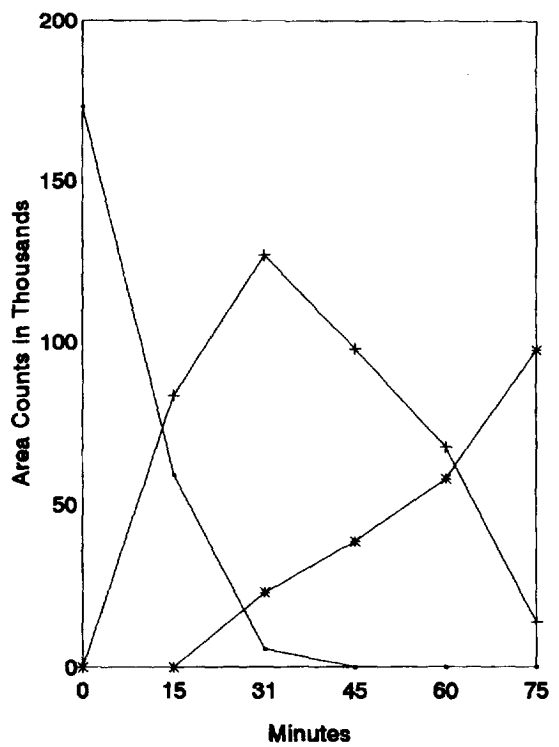

Fig. 4. Hydrolysis products: (□) Glc-Gal-Hyl; (+) Gal-Hyl; (×) Hyl.

17.5 μl of 0.8 *M* NAOH is added to partially neutralize the acid. This will convert 73% of the Glc-Gal-Hyl to Gal-Hyl. Ultimately, it is possible to remove both sugar moieties and have only Hyl as the end product. The first two unresolved peaks at 17.11 and 17.33 min in Fig. 5A represent Glc-Gal-Hyl. With our procedure Glc-Gal-Hyl appears as a double peak relatively free of contaminants. Fig. 5B illustrates almost complete conversion of Glc-Gal-Hyl to Gal-Hyl, which appears as a single peak at 18.10 min. The prominent peak at 20.10 min in Fig. 5C represents Hyl.

Aliquots of hydrolyzed and unhydrolyzed Glc-Gal-Hyl are chromatographed on a Spectra-Physics HPLC unit using dabsyl chloride (4-dimethylaminoazobenzen-4′-sulfonyl chloride from Sigma) as a derivatization agent. The column used is a 100×4.6 mm, 5 μm, C_{18} reversed-phase column (Keystone Scientific). The standard amino acid program provided by the manufacturer is altered to permit complete separation of Glc-Gal-Hyl, Gal-Hyl, and hydroxylysine. All chemicals/reagents are HPLC grade or better. The eluents are acetonitrile (Burdick and Jackson) and 0.025 *M* sodium acetate (EM Science) with 4% MeOH (Malinckrodt) pH 5.1. Separation is accomplished by a gradient profile that starts at 20% acetonitrile, increases to 30% by 6 min, 42.5% by 16 min, 47.5% by 20 min, 62.5% by 27 min, 100% by 29 min, and back to 20% at the end of 35 min. The flow-rate is maintained at 1 ml/min. and the detector is set at 436 nm. The system's integrator then converts the detectors signal to area counts. Area counts are later converted to concentration units. A commercially prepared amino acid standard

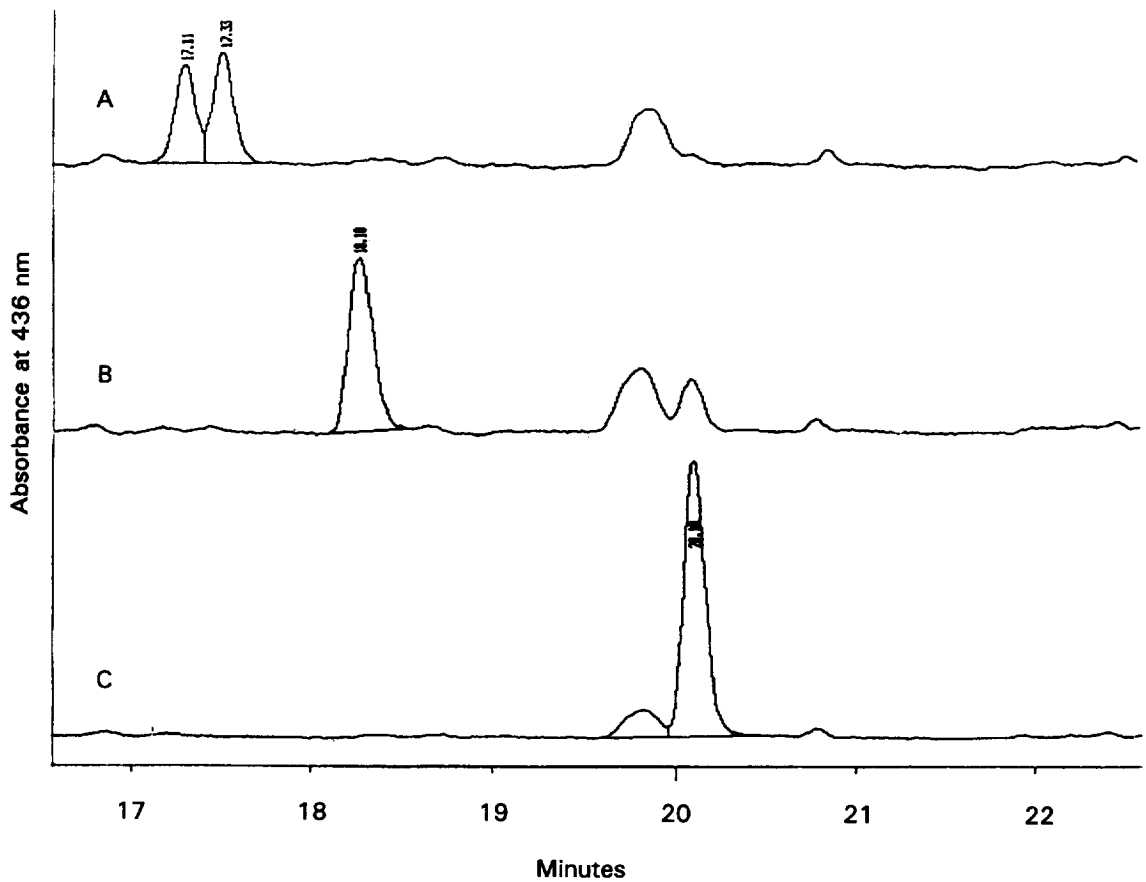

Fig. 5. HPLC chromatograms: A, Glc-Gal-Hyl; B, Gal-Hyl produced by mild hydrolysis of Glc-Gal-Hyl; C, Hyl produced by complete hydrolysis of Glc-Gal-Hyl.

mix from Sigma is used to identify the Hyl peak. Concentrations are calculated based on the response factor of hydroxylysine.

3. Results

The procedure described above produced one gram of purified Glu-Gal from 100 g of marine sponge in a short period of time with equipment available in most biochemical laboratories.

4. Conclusions

This method makes commercially unavailable hydroxylysine glycosides simple and cost-effective to obtain and facilitates their use in collagen research. The ratio of Glc-Gal-Hyl to Ga-Hyl is distinctly different in skin collagen as compared to bone collagen. Therefore, hydroxylysine glycosides can be used as indicators for the tissue source of urinary collagen metabolites.

Acknowledgments

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