IDENTIFICATION OF DIGALACTOSYLCYSTEINE IN A GLYCOPEPTIDE ISOLATED FROM URINE BY A NEW PREPARATIVE TECHNIQUE

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1. Introduction

Normal urine contains a complex mixture of low-molecular weight carbohydrate-protein compounds, approximately half of which are in the MW range 1–5000 [1–4]. However, very few, if any, of such glycopeptides have been obtained in a pure state, perhaps due to the variable and sometimes traumatic methods used for their isolation. Some of these methods have in fact been shown to produce, from amino acids and amino sugars, both free and in peptide form, such artifacts as Amadori rearrangement products [5].

We describe here an original method of concentrating urine, which minimizes chemical modifications of the urinary constituents during the concentration process. Using this method, we have now isolated and characterized a peptide, containing a novel carbohydrate-protein crosslinkage, digalactosylcysteine.

2. Materials and methods

(a) *Urine concentration using dry Sephadex G10.* Fresh midstream urine from healthy young adult male subjects (18–22 years) was concentrated to one-third of its original volume by rotary evaporation, at 30°. Redistilled propan-2-ol was added (urine-propan-2-ol, 9:1, v/v), and the concentrate was filtered through Millipore filters type GS and VF (in series) to remove high MW material.

The filtered urine was cooled to 4° and mixed with dry Sephadex G10 until a just-mobile slurry was obtained. This procedure was found to generate considerable heat and cooling was essential (heat production is also observed when distilled water is mixed with dry Sephadex G10, in the same manner). The slurry was placed on a sintered Buchner funnel, and suction was applied. The moist gel was subsequently mixed with distilled water to produce again a just mobile slurry, and the filtration procedure was repeated. The concentrated urine and the washings were combined, and the entire procedure was repeated using fresh dry Sephadex G10, for as many times as were necessary to obtain a 100-fold concentration.

By means of the two concentration procedures described, 3 l of urine was concentrated to 10 ml, although the molarity of salt and other low molecular weight solutes remained unchanged. The Sephadex beads were regenerated by the method described below [1].

(b) *Gel filtration on Sephadex G15.* The concentrate was fractionated on a column (90 × 3.2 cm) of Sephadex G15, and the UV absorption of the eluate was monitored using an L.K.B. Uvicord (L.K.B. Producter AB, Sweden).

(c) *Peptide analysis and amino acid analysis.* Fractions eluted from the Sephadex G15 column were further fractionated on a peptide analyser (Technicon Chromobeads type P, 2% cross-linking), using the pyridine-acetic acid buffer gradient system [6].

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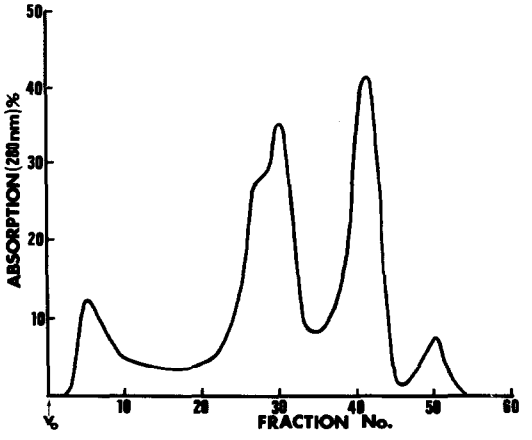

Fig. 1. Elution profile of urine concentrate on Sephadex G15, eluted with water–propan-2-ol (9:1 v/v). 7.5 ml fractions were collected. Fractions were pooled as follows: A (nos. 1–9), B (10–20), C (20–26), D (27–35), E (36–45), F (46–55).

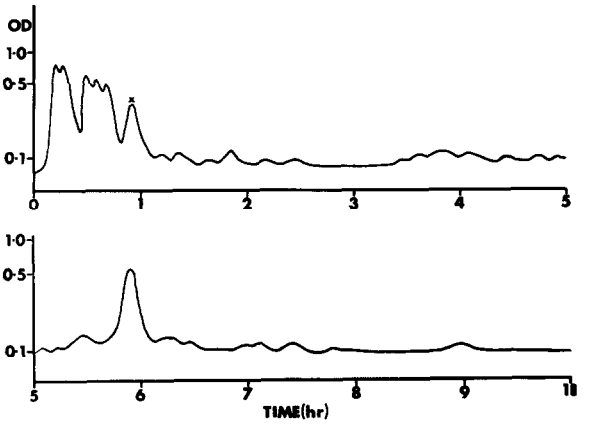

Fig. 2. Autoanalyser recording of the elution profile of fraction B from Sephadex G15, on Chromobeads type P. Eluted with a pyridine–acetic acid buffer gradient. O.D. was measured at 570 nm.

Amino acid analysis was performed on the Technicon analyser using a citrate buffer system.

(d) *Hydrolysis of samples.* Samples for amino acid analysis and paper chromatography of amino acids were hydrolysed in 6 N HCl under vacuum at 105° for 18 hr. Residual HCl was removed by rotary evaporation.

For gas–liquid chromatography or paper chromatography of sugars, samples were hydrolysed in 0.5 N HCl, under vacuum at 105° for 8 hr. Since the removal of residual HCl by evaporation destroyed monosaccharide residues, the hydrolysate was deacidified on a short column of Amberlite 1R4B (OH-form), prior to drying.

(e) *Paper chromatography.* Paper chromatography was carried out by an ascending technique on Whatman No. 1 paper, and developed by butanol–acetic acid–water (12:3:5 by vol.) and propan-2ol–water (4:1 v/v). After development, the papers were cut horizontally into strips, and material was eluted from them by downward flow using distilled water. Material was located using pyridine-ninhydrin, silver nitrate, and the Elson-Morgan reagent.

(f) *Gas–liquid chromatography.* A Pye series 104 instrument with an SE 30 column was used, having a temperature program from 140°–210° at 1.5°/min. Sugars were converted into the trimethylsilyl derivatives before injection [7]. Total volume of samples after silylation was 0.1 ml, and 1 μl quantities were injected.

(g) *Alkaline borohydride reduction.* This was performed by the procedure of Tanaka et al. [8]. The serine, cysteine and alanine contents of the samples were determined by amino acid analysis, after a 216 hr incubation in alkaline borohydride at 4°, followed by hydrolysis in 6 N HCl.

(h) *Amide nitrogen determination.* Amide nitrogen was determined by a slight modification of the procedure described by Marshall and Gottschalk [9]. Liberated ammonia was titrated using 0.001 N HCl.

(i) *Hexosamine determination.* Hexosamines were determined after paper chromatography as described above, and by gas–liquid chromatography [10].

(j) *Sequence analysis.* After application of the dansyl-Edman technique [11], dansyl-amino acids were identified by two-way chromatography on polyamide-6 layers (Camlab Ltd., Cambridge, England), in water–90% formic acid (200:3 v/v), followed by benzene–acetic acid (9:1 v/v). Spots were located under UV light.

(k) *Enzyme studies.* Carboxypeptidase A (E.C. 3.4.2.1) was obtained from Calbiochem. Leucine aminopeptidase (E.C. 3.4.1.1) was obtained from Koch-Light Laboratories Ltd. With both enzymes, digestions were performed at 37°, using an enzyme–substrate ratio of 1:20, in 0.1 M NH_4HCO_3 acetic acid buffer, pH 8.4 or 7.5.

Table 1
Composition of the urinary glycopeptide. (Equal volumes of peptide solution were analysed for amino acids and sugars respectively).

Residue	nmole/l urine	Molar ratio[a]
Aspartic acid	26	1.00
Serine	25	0.96
Glutamic acid	25	0.96
Glycine	30	1.15
Cysteine*	23	0.89
Alanine	20	0.77
Histidine	54	2.10
Galactose**	52	2.00

* Calculated as ½ cystine + cysteic acid.
** Determined by gas–liquid chromatography.
[a] Molar ratio is expressed relative to aspartic acid.

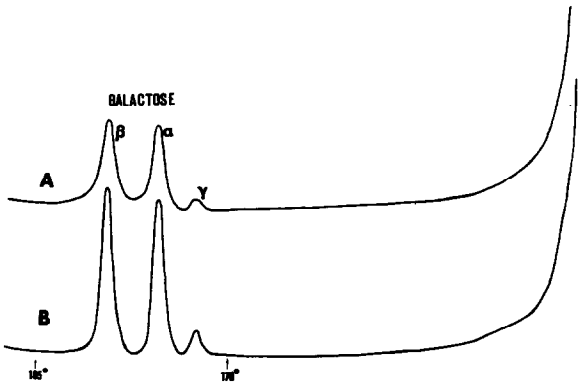

Fig. 3. Gas chromatographic analysis of purified glycopeptide. Pye series 104 analyser, SE30 column. Temp. program. 140–210° at 1.5°/min. Attenuation 2 × 10^2. A. sample. B. Standard (100 nmoles galactose). γ refers to the furanose form of galactose, α and β refer to the pyranose anomers [20].

(l) *Regeneration of Sephadex G10.* The used Sephadex was washed with water, soaked in 6 M urea, and washed free of urea with copious water. The clean beads were dried by successive soaking in 20–100% ethanol (in steps of 10%), and were finally air dried in a warm atmosphere.

3. Results

Fig. 1 shows the elution profile obtained after fractionation of the urine concentrate on Sephadex G15. Fraction B, when applied to the peptide analyser, gave the elution profile shown in fig. 2. On examination of these fractions from the peptide analyser, only one contained bound neutral sugar. Paper chromatography of this fraction (X, fig. 2), gave two spots, one always remaining at the origin, and the other moving as a compact spot with an Rf of 21 in butanol–acetic acid–water and Rf 68 in propan-2ol–water. In both solvents, a positive ninhydrin and weak silver nitrate reaction were obtained. The hexosamine (Elson-Morgan) reaction was negative.

After elution from the paper, equal volumes of the peptide solution were taken to dryness, and analysed for amino acids and carbohydrates after appropriate hydrolyses. The results are shown in table 1. No hexosamine was present, and galactose was the only sugar detected (fig. 3).

The total yield of the glycopeptide per litre of urine was 20–30 nmoles (approx. 30 μg/l). The dansyl-Edman degradation indicated the following sequence: Cys–Glu–His–Ser–His–Asp–Gly–Ala. Since the peptide contained serine, an alkaline borohydride reduction was performed to determine whether the galactose was linked to serine. Amino acid analyses before and after the alkaline reduction indicated that no loss of serine or any other amino acid occurred, and there was no increase in the alanine content of the peptide.

Digestion of the peptide with carboxypeptidase for 1 hr released alanine: the remainder of the peptide was resistant to this enzyme. In order to determine the position of the sugar residues, sequential degradation of the peptide with leucine aminopeptidase was attempted. After a 1 hr incubation (pH 8.4), a new component (Rf 30 in butanol–acetic acid–water) could be detected by paper chromatography. This component was eluted and hydrolysed in 0.5 N HCl for 8 hr. Galactose could then be detected by gas–liquid chromatography, and cysteine by paper chromatography. No galactose or cysteine was present in the residual peptide, indicating that both residues of galactose were linked to cysteine.

Digestion of the peptide with leucine aminopeptidase at pH 7.5 for a period of 6 hr, followed by two-way paper chromatography gave the following results: after 1 hr, only the cysteinylgalactose com-

ponent was released; after 3 hr, glutamic acid and histidine could also be seen, together with a trace of serine; after 6 hr, in addition to the aforementioned amino acids, aspartic acid, glycine and alanine could also be detected.

Amide nitrogen determinations indicated that no amide nitrogen was present.

4. Discussion

Gel filtration on Sephadex G15, of urine concentrated by the method described in this paper, yielded 6 fractions (fig. 1) all of which gave positive protein- and carbohydrate-reactions. When fraction B was further fractionated on the peptide analyser, 36 separate peptides were obtained. Each of the other 5 fractions contained 20–30 peptides. Thus the simple Sephadex elution profile has been shown to contain well over 100 peptides. This large number of peptides, having similar molecular weights, may account for the difficulty other workers have experienced in obtaining reproducible results for the composition of urinary constituents, since these peptides may aggregate [12]. In the work described in this paper, reproducibility was good, and in repeated experiments the galactosylpeptide was eluted in the same position both from Sephadex G15 columns and from the peptide analyser.

A possible disadvantage of the dry Sephadex G10 concentration procedure is the loss of low MW peptides into the beads, and of other peptides by adsorption to the bead surface. However, the presence of urea in urine minimizes adsorption, and the addition to the urine of propan-2-ol further prevents adsoption. Concentration of urine by an ultrafiltration technique (using ultrafilters having a MW cut-off of 1000) did in fact give slightly higher yields of material, but also caused the production of a number of artifactual Amadori rearrangement compounds (Lote and Weiss, unpublished observation).

Although aldoses are known to react with cysteine under certain conditions to give thiazolidines [15], such a structure is not possible in the glycopeptide described here, since the amino group of the cysteine residue is free, as evidenced by its availability for dansylation. The lability of the cysteinylgalactose bond to mild acid hydrolysis, supports the contention that the linkage is glycosidic and therefore *S*-glycosidic. The release of two moles of galactose per mole peptide suggests that the carbohydrate is present as digalactose. The nature of the linkage between the galactose residues is still under investigation.

The octapeptide which has been isolated contains an *S*-glycosidic linkage between galactose and cysteine. No linkage of this type has been previously described. A carbohydrate linkage to serine was excluded since alkaline borohydride treatment did not decrease the serine content or increase the alanine content of the peptide. It is likely that the cysteinylgalactose linkage would undergo β-elimination in a manner analogous to serine linkages, but in the present case this would not be expected, since the cystein is N-terminal, and adjacent to the carboxyl group of glutamic acid [13, 14]. An N-glycosidic linkage of the galactose moiety to asparagine (or glutamine) can also be excluded, since enzymic degradation of the peptide followed by paper chromatography indicated that both amino acids were present in the dicarboxylic form, not as amides. This finding was confirmed by amide nitrogen determinations.

Enzymic degradation with exopeptidases provides confirmation of the peptide structure. At pH 8.4, over a period of 1 hr, leucine aminopeptidase does not degrade the peptide beyond the cysteine residue, since the highly charged glutamic acid residue is a barrier [16]. At pH 7.5 over a period of 6 hr, the peptide was progressively degraded. At both pH 8.4 and 7.5 the rate of removal of the cysteinylgalactose was slow compared to non-substituted cysteine. Carboxypeptidase digestion of the peptide, at pH 8.4, removes the alanine residue, but the sequence Asp–Gly is relatively resistant [17, 18].

The source of the urinary galactosyl peptide is as yet unknown, but it is of interest that thioglycosidases, cleaving *S*-glycosidic bonds, are widely distributed in many organisms, including man [19]. It seems likely that an *S*-glycosidic linkage of the type described here could be the natural substrate for such an enzyme.

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References

[1] A. Lundblad, Biochim. Biophys. Acta 101 (1965) 46.
[2] R. Bourrillon and J. Vernay, Biochim. Biophys. Acta 117 (1966) 319.
[3] M.G. Cherian and A.N. Radhakrishnan, Indian J. Biochem. 3 (1966) 101.
[4] E.H.F. McGale and F.R. Jevons, Clin. Chim. Acta 14 (1966) 528.
[5] C.J. Lote and J.B. Weiss, in press.
[6] Technicon Peptide Analyser Instruction Manual, T-67-101.
[7] K.M. Brobst and C.E. Lott, Cereal Chem. 43 (1966) 35.
[8] K. Tanaka, M. Bertolini and W. Pigman, Biochem. Biophys. Res. Commun. 16 (1964) 404.
[9] R.D. Marshall and A. Gottschalk, Glycoproteins (Elsevier Publishing Co., Amsterdam, London, New York, 1966) p. 151.
[10] M.D.G. Oates and J. Schrager, J. Chromatog. 28 (1967) 232.
[11] D. Beale, Chromatographic and Electrophoretic Techniques, 3rd ed., Vol. 1 (W. Heineman Medical Books, London, 1969) p. 204.
[12] E.H.F. McGale, Advan. Carbohydrate Chem. 24 (1969) 435.
[13] G. Riley, J.H. Turnbull and W. Wilson, J. Chem. Soc. (1957) 1373.
[14] R. Carubelli, V.P. Bhavanandan and A. Gottschalk, Biochim. Biophys. Acta 101 (1965) 67.
[15] M.P. Schubert, J. Biol. Chem. 130 (1939) 601.
[16] E.L. Smith and D.H. Spackman, J. Biol. Chem. 212 (1955) 271.
[17] K. Hofmann and M. Bergman, J. Biol. Chem. 134 (1940) 225.
[18] R.P. Ambler, in: Methods in Enzymology, Vol. 9, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York and London, 1967) p. 155.
[19] I. Goodman, J.R. Fouts, E. Bresnick, R. Menegas and G.H. Hitchings, Science 130 (1959) 450.
[20] J.R. Clamp, G. Dawson and L. Hough, Biochim. Biophys. Acta 148 (1967) 342.