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QUANTITATIVE DETERMINATION OF HEXOSAMINES IN GLYCOPROTEIN BY ION-EXCHANGE CHROMATOGRAPHY

ROBERT M. ZACHARIUS

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pa. 19118 (U.S.A.)

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SUMMARY

An ion-exchange chromatographic method for the quantitative determination of glucosamine and galactosamine in glycoproteins is described. The hexosamines are completely separated from interfering peptides of the acid hydrolysate using a small column of cation exchanger in a manner described earlier by Boas. Chromatographic separation of the hexosamine fraction and the determination of glucosamine and galactosamine are accomplished on an amino acid analyzer with ninhydrin.

INTRODUCTION

During a study of the hexosamine content of κ-casein and potato glycoprotein in this laboratory, a method was required for quantitatively determining the hexosamines. While glucosamine and galactosamine are readily determined in the presence of each other by cation-exchange chromatography, this is limited to their presence unbound or in small peptides. The partial hydrolysis of glycoproteins, a condition for minimum loss of amino sugars, yields peptides with elution volumes in the chromatographic separation which overlap those of the hexosamines. This does not permit hexosamine determination by amino group reagents. Lee *et al.*[1] employed a neocuproine reducing sugar reagent with the column effluent to avoid interference by amino compounds. On the other hand, Fanger and Smyth[2] prefractionated the partial hydrolysate on an anion exchanger, eluting the amino sugars and lysine with a volatile buffer of N-ethylmorpholine. The hexosamines were finally determined with ninhydrin on a short amino acid analyzer column.

In this laboratory, limited success was experienced with the neocuproine method[1] because of some continuing interference by peptides, presumably by those containing hydroxy amino acids. An alternative method is reported here which utilizes a prefractionation of the hydrolysate as described by Boas[3], followed by chromatographic separation of the hexosamine fraction and determination with ninhydrin.

EXPERIMENTAL*

Columns and resins

Prefractionating columns consisted of 5–5.5 ml of Amberlite IR-120 (ref. 2) (200–400 mesh) resin packed in 140 × 9 mm I.D. nonjacketed glass columns. The resin was poured in segments. The columns were readied and regenerated after each sample according to the procedure of Boas[3]. Analytical chromatography was carried out in a water-jacketed 290 × 9 mm I.D. glass column packed in segments with Amberlite IR-120 resin to a height of 265 mm. Crushed resin, 25–30 μm particles, was sized by the Hamilton procedure[4].

Hexosamines and amino acids

D-Glucosamine hydrochloride and glycyl-L-leucyl-L-tyrosine were purchased from Mann Labs. (New York, N.Y., U.S.A.) and D-galactosamine hydrochloride from Pfanstiehl Labs. (Waukegan, Ill., U.S.A.). D-Mannosamine hydrochloride, L-serine, L-threonine, and L-tyrosine were obtained from Calbiochem (Los Angeles, Calif., U.S.A.).

Proteins

β-Lactoglobulin A and κ-casein were prepared by the Dairy Laboratory of the Eastern Regional Research Center. Sperm whale myoglobin was purchased from Schwarz/Mann (Orangeburg, N.Y., U.S.A.) and the iron removed by conventional procedure. A potato protein preparation was made in this laboratory from the expressed tuber juice by ammonium sulfate precipitation and dialysis in the usual manner.

Analyzer system

The modules and arrangement comprising an amino acid analyzer were used. A Milton Roy piston pump delivered the eluting buffer and another the ninhydrin reagent. When neocuproine was the developing reagent, the modules included a Technicon proportioning pump in lieu of the second piston pump and a 95° reaction bath assembled and employed in the manner described by Lee *et al.*[1]. The colored ninhydrin reaction products were measured with two Technicon colorimeters, using a 570-nm and a 450-nm filter with 15-mm flow cells. The 450-nm colorimeter was employed in the neocuproine assembly. The results were plotted on a 12-in. multiple-point recorder.

Buffers and reagents

Sodium citrate buffer, 0.35 *N*, was prepared from Baker's reagent grade salt adjusted to pH 5.28 $\pm$ 0.02 (ref. 5). The preservative was 0.1 ml octanoic acid per liter of buffer. Ninhydrin reagent, procured from Pierce (Rockford, Ill., U.S.A.), was prepared according to the procedure of Spackman *et al.*[5]. Neocuproine hydrochloride was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and the reagent was prepared as described for procedure II of the publication of Lee *et al.*[1].

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* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Hydrolysis of protein

Proteins and glycoproteins (2.6–10 mg) were hydrolyzed with 3–6 ml of 4 *N* HCl for 4 h in a 100° water-bath in evacuated sealed tubes. Hydrolysates were taken to dryness at 40° with a rotary evaporator until free of mineral acid.

Prefractionation

The residues from hydrolysis were dissolved in approximately 2 ml distilled water and passed through the small Amberlite IR-120 (H^+) prefractionating column. The column was washed with 15 ml distilled water (removing unabsorbed sugars, acidic amino acids, and peptides), and eluted with 9.5–18 ml 2 *N* HCl. The column was further eluted with 9–10 ml 4 *N* HCl. When studying the ion-exchange retention of hydrolytic fragments of β-lactoglobulin A, the column was water washed and further eluted with 4 *N* NH_4OH. All eluates were concentrated separately on the rotary evaporator at 40° to dryness until free of the eluting agent.

Analysis

Each of the residues was dissolved in distilled water and applied to the analyzer column either in its entirety or as a volumetric aliquot. With the ninhydrin method, samples in volumes as high as 0.350 ml were examined on the analytical column. The samples were washed into the column with three 0.020-ml aliquots of the eluting buffer. Elution of the column was carried out at 55° and at a flow-rate of 60.0 ml/h with 0.35 *N*, pH 5.28 ± 0.02 citrate buffer. Ninhydrin reagent was pumped at the rate of 20.9 ml/h with the second piston pump. Ninhydrin flow was initiated 15 min after the start of column elution. When the reagent was neocuproine, the module arrangement and flow-rates described under procedure II of Lee *et al.*[1] were employed. Peak areas were determined as with automatic amino acid analyzers, whereby the area is determined from half the peak height multiplied by the peak width.

RESULTS AND DISCUSSION

With the column length employed in this study, glucosamine and galactosamine were satisfactorily separated on the analyzer with maximum peak heights at 62.5 and 70 min, respectively. Separations carried out with stepwise lengthened analyzer columns indicated that a resin column of 300 mm would permit a complete return to the baseline between the two hexosamine peaks. This, however, was not essential to the quantitation.

Recovery determinations were made on standard binary solutions of the hexosamines at two levels differing by a factor of ten (Table I). No hexosamine was found in the water wash from the prefractionating column. After elution of the column with 9.5 ml of 2 *N* HCl, a small amount of residual hexosamines could still be recovered with further elution using 4 *N* HCl. However, 16 ml of 2 *N* HCl fully eluted the tenfold hexosamine sample. Since the sample not pretreated showed a ± deviation from 100% recovery, the 90–96% recovery totals of those that had undergone column pretreatment indicate that some losses had probably occurred in the manipulations. Greater care was taken in subsequent operations.

Both β-lactoglobulin A and sperm whale apomyoglobin are proteins devoid of carbohydrates and amino sugars. These two proteins were utilized to study the

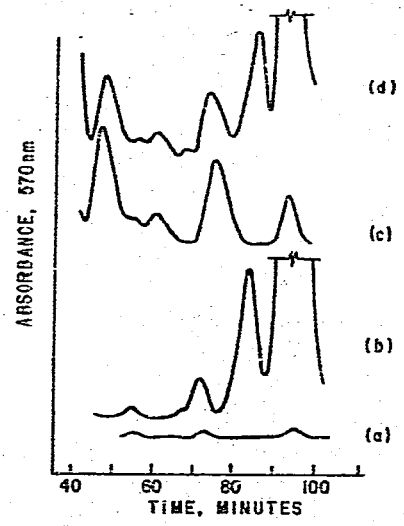
TABLE I

DETERMINATION OF HEXOSAMINES IN STANDARD BINARY SOLUTIONS

The concentrations of Experiments 1 and 2 are tenfold dilutions of Experiment 3. Experiment 3 represents a mixture containing 0.1172 μM galactosamine and 0.0580 μM glucosamine in 100 μl of 0.01 M HCl.

Experiment	*Pretreatment*	*% Recovered*	
		Glucosamine	*Galactosamine*
1	None	94.2	103.7
2	IR-120; 9.5 ml 2 N HCl	92.8	87.5
2 wash	9 ml 4 N HCl	3.1	3.8
	Σ 2 + 2 wash	95.9	91.3
3	IR-120; 16 ml 2 N HCl	92	89.6
3 wash	10 ml 4 N HCl	0	0
	Σ 3 + 3 wash	92	89.6

effects of the prefractionating column on the removal of peptides with elution volumes overlapping those of glucosamine and galactosamine. The results of elution of a mild acid hydrolysate of β-lactoglobulin A using 2 N HCl followed by 4 N HCl and ultimately stripping the column with 4 N NH_4OH are shown in Fig. 1. Without pre-



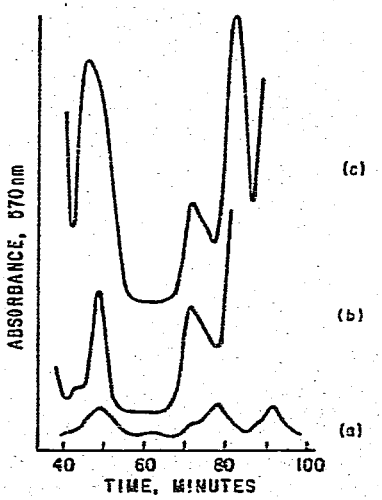


Fig. 1. Chromatography of a partial hydrolysate (4 N HCl, 4 h, 100°) of β-lactoglobulin A on a 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 N sodium citrate buffer. Ninhydrin reagent. (a) Partial hydrolysate eluted from the prefractionating IR-120 column with 2 N HCl. (b) Fraction from the continued elution of the prefractionating column (a) with 4 N HCl. (c) Fraction from the continued elution of (b) with 4 N NH_4OH. (d) Partial hydrolysate chromatographed directly on the analytical column.

Fig. 2. Chromatography of a partial hydrolysate (4 N HCl, 4 h, 100°) of sperm whale apomyoglobin on a 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 N sodium citrate buffer. Ninhydrin reagent. (a) Partial hydrolysate eluted from the prefractionating IR-120 column with 2 N HCl. (b) Fraction from the continued elution of the prefractionating column (a) with 4 N HCl. (c) Partial hydrolysate chromatographed directly on the analytical column.

treatment of the hydrolysate before analytical chromatography, ninhydrin-positive material is especially evident partially overlapping the galactosamine region (70 min). Only a very small portion of this peak is eluted with 2 *N* HCl, while 4 *N* acid removes an additional amount plus some interfering material in the glucosamine region of the chromatogram. Further amounts of interfering peptides are obtained from the pre-fractionating column with NH_4OH. Sperm whale apomyoglobin yielded a similar elution pattern (Fig. 2).

When the two hexosamines were added to the β-lactoglobulin A hydrolysate and pretreated on the IR-120 column, the 2 *N* HCl eluate yielded a good separation pattern and constant baseline. A 100% recovery of both amino sugars was obtained with 15 ml of 2 *N* acid (Table II). Similar results were obtained with the hexosamines added to sperm whale apomyoglobin with a 98% recovery of both compounds using 18 ml of 2 *N* acid (Fig. 3; Table III). The material eluted between 64 and 80 min with 4 *N* HCl, partially overlapping the galactosamine position, represents peptide material indigenous to the myoglobin hydrolysate.

The glycoprotein, κ-casein, was examined for its hexosamine content using the neocuproine procedure as described by Lee *et al.*[1]. With no pretreatment of the hydrolysate, a steeply changing baseline was observed in the hexosamine region of the chromatogram (Fig. 4), interfering with an accurate determination of their peak areas. It also raised doubts as to the complete composition of the peaks regarded as

TABLE II

DETERMINATION OF HEXOSAMINES ADDED TO β-LACTOGLOBULIN A

Experiments 1 and 2 represent 3 mg partially hydrolyzed β-lactoglobulin A to which was added 0.1172 μ*M* galactosamine and 0.0580 μ*M* glucosamine to give a total volume of 1.03 ml.

Experiment	*Pretreatment*	*% Recovered*	
		Glucosamine	*Galactosamine*
1	IR-120; 12 ml 2 *N* HCl	89.2	86.0
1 wash	10 ml 4 *N* HCl	11.5	14.0
	Σ 1 + 1 wash	100.7	100
2	IR-120; 15 ml 2 *N* HCl	100	101.7
2 wash	10 ml 4 *N* HCl	0	trace
	Σ 2 + 2 wash	100	101.7

TABLE III

DETERMINATION OF HEXOSAMINES ADDED TO MYOGLOBIN

Experiments 1 and 2 represent 4 mg partially hydrolyzed sperm whale apomyoglobin to which was added 0.1172 μ*M* galactosamine and 0.0580 μ*M* glucosamine to give a total volume of 0.430 ml.

Experiment	*Pretreatment*	*% Recovered*	
		Glucosamine	*Galactosamine*
1	IR-120; 16 ml 2 *N* HCl	86.5	90.7
1 wash	11 ml 4 *N* HCl	6.1	<1
	Σ 1 + 1 wash	92.6	91
2	IR-120; 18 ml 2 *N* HCl	98.1	97.6
2 wash	10 ml 4 *N* HCl	0	0
	Σ 2 + 2 wash	98.1	97.6

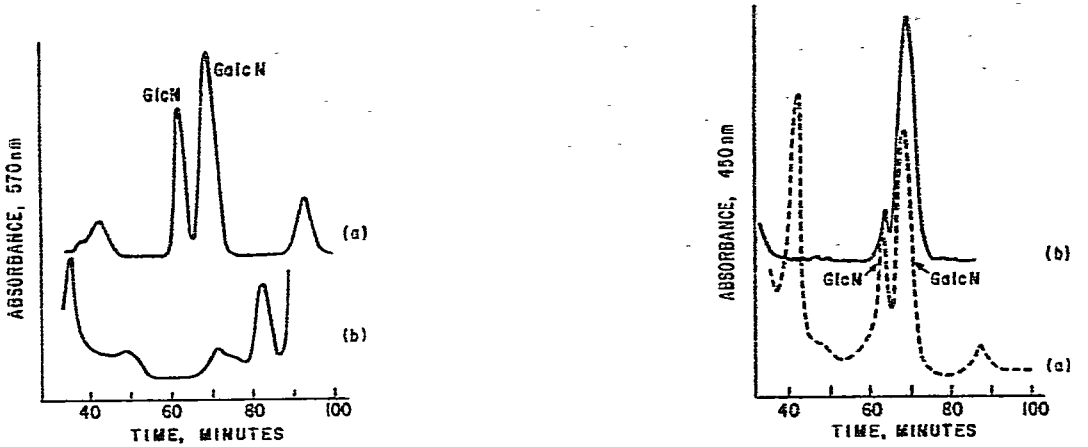


Fig. 3. Chromatography of glucosamine and galactosamine added to a partial hydrolysate (4 *N* HCl, 4 h, 100°) of sperm whale apomyoglobin on a 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 *N* sodium citrate buffer. Ninhydrin reagent. (a) Fraction eluted from the prefractionating IR-120 column with 2 *N* HCl. (b) Fraction from the continued elution of the prefractionating column with 4 *N* HCl.

Fig. 4. Chromatography of a partial hydrolysate (4 *N* HCl, 4 h, 100°) of κ-casein on 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 *N* sodium citrate buffer. Neocuproine reagent. (a) Direct chromatography of the partial hydrolysate on the analytical column. (b) Partial hydrolysate eluted from the prefractionating IR-120 column with 2 *N* HCl.

hexosamines. An improved separation was obtained when the hydrolysate was pretreated with the Boas-type column and the 2 *N* HCl eluate examined on the analytical column (Fig. 4). Hexosamine values for both κ-casein and a potato protein preparation obtained with the neocuproine reagent were in excellent agreement with those obtained with the ninhydrin reagent following the same column pretreatment and chromatography (Table IV).

TABLE IV

COMPARISON OF NEOCUPROINE AND NINHYDRIN REAGENTS IN DETERMINING GLYCOPROTEIN HEXOSAMINE

	*Neocuproine**		*Ninhydrin**	
	% *Glucosamine*	% *Galactosamine*	% *Glucosamine*	% *Galactosamine*
Potato protein	0.228	—	0.249	—
κ-Casein	0.044	0.579	0.053	0.533

* Mean value of two determinations.

The use of 18 ml of 2 *N* HCl has proven to be most conducive to complete removal of the two hexosamines from prefractionating columns reported in this study. As much as 15 mg of caseins (1% hexosamine)[6] and as little as 1 mg of purified milk fat globule membrane protein (6% hexosamine)[7] have been partially hydrolyzed and successfully pretreated for hexosamine recovery from these columns.

Column effluents of serine, threonine, tyrosine, and glycylleucyltyrosine were found to produce peaks on the analyzer with the neocuproine reagent, although none had elution volumes interfering with the determination of glucosamine and galactosamine. However, it would not be incongruous to expect other peptides containing hydroxyamino acids to respond similarly with neocuproine and cause some interference in the determination of the hexosamines.

In this analytical system, mannosamine and galactosamine were found to have identical elution volumes. However, mannosamine has rarely been encountered as a constituent of glycoproteins.

During the course of this study, four commercially purchased samples of D-galactosamine regarded as chromatographically pure by their suppliers were found to be contaminated with glucosamine. These samples had been prepared from natural sources and the impurity varied from 5.0–16.5%. The synthetic preparation of galactosamine utilized in this study was found to be devoid of glucosamine.

Treatment of acid hydrolysates of glycoproteins was recommended by Boas[3] for improvement of the Elson–Morgan method for determining hexosamine. Although Boas had designed the modification in order to eliminate sugar–amine interactions responsible for abnormally high color values, this study finds it valuable in the ion-exchange chromatographic method for hexosamines. It improves the results obtained with neocuproine and permits the use of amino group reagents such as ninhydrin. Thereby, the final analytical step can be carried out directly on an amino acid analyzer.

REFERENCES

1 Y. C. Lee, J. R. Scocca and L. Muir, *Anal. Biochem.*, 27 (1969) 559.
2 M. W. Fanger and O. G. Smyth, *Anal. Biochem.*, 34 (1970) 494.
3 N. F. Boas, *J. Biol. Chem.*, 204 (1953) 553.
4 P. B. Hamilton, *Anal. Chem.*, 30 (1958) 914.
5 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
6 R. M. Zacharius and R. Greenberg, to be published.
7 J. J. Basch, H. M. Farrell and R. Greenberg, *Biochim. Biophys. Acta*, in press.