ISOLATION AND CHARACTERIZATION OF A GLYCOPEPTIDE FROM SHEEP THYROGLOBULIN*

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Thyroglobulin is a glycoprotein containing galactose, mannose, fucose, glucosamine and sialic acid (Ujejski et al., 1955; Wollman and Warren, 1961; Robbins, 1963).

Recently we described a procedure for the efficient release of I^{131}-labeled amino acids from I^{131}-labeled thyroproteins that entailed the use of the proteolytic enzyme, Pronase, obtained from Streptomyces griseus (Tong et al., 1963). Proteolysis of thyroglobulin by this enzyme resulted in release of a nondialyzable glycopeptide. Spiro and Spiro (1963) also reported the release of glycopeptides from calf thyroglobulin by the action of Pronase. The present report deals with the isolation, purification and partial characterization of a glycopeptide from sheep thyroglobulin.

EXPERIMENTAL

Proteolysis of Thyroglobulin and Isolation of Glycopeptide

Thyroglobulin was prepared from a saline extract of sheep thyroid glands by fractionation with ammonium sulfate

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as described by Roche et al., (1947). Further purification of the protein was achieved by gel filtration on Sephadex G-200 (unpublished work). Five hundred mg of the purified thyroglobulin were dissolved in 10 ml of a buffer composed of 0.07 M Tris-HCl, 0.14 M sodium chloride and 0.014 M calcium chloride, at pH 8.0, and hydrolyzed for 20 hours at 37°, with 10 mg of Pronase (obtained from the California Corporation for Biochemical Research, Los Angeles). The small amount of insoluble material formed was removed by centrifugation, and the supernatant fraction was concentrated, in vacuo at 40°, to one third its original volume. The concentrated supernatant fraction* was either used directly for gel filtration on Sephadex G-25 or subjected to alcohol fractionation as follows: It was cooled to 4° and treated with 4 volumes of cold 95 % ethanol. The mixture was allowed to stand at 4° for 20 hours. The precipitate formed was separated by centrifugation and dissolved in 2-5 ml of water; the resulting solution+ was used for gel filtration.

Gel filtration was carried out on Sephadex G-25 columns as described by Porath (1960). Columns were equilibrated with 0.1 N acetic acid, which was also used for elution. The specific conditions employed for the gel filtration are described in the legends of figures 1 and 2. All column separations were done at room temperature, and each eluate was collected in fractions of 2.5-5.0 ml. The protein content of each fraction was determined from its absorbancy at 280 mμ. Aliquots of the fractions were also analyzed for bound hexose,

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* Henceforth referred to as the Pronase digest.

+ Henceforth referred to as the alcohol-insoluble fraction.

hexosamine and sialic acid. For isolation purposes the fractions corresponding to each peak were pooled and lyophilized.

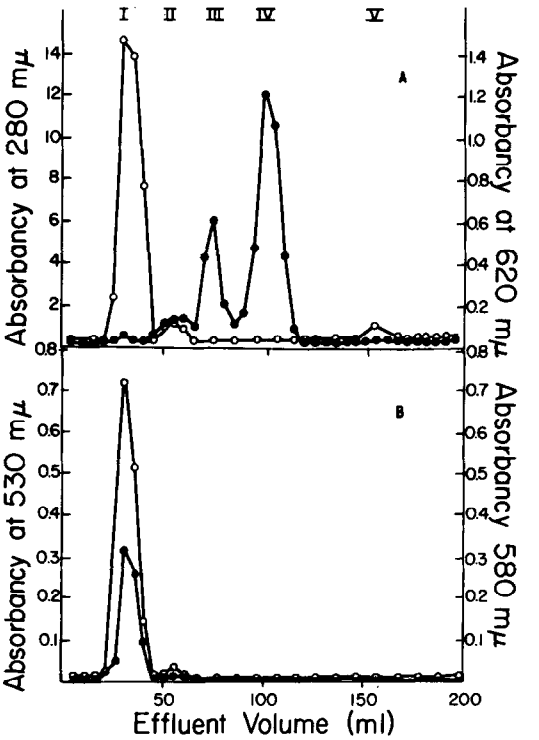

Fig. 1 - Gel filtration of Pronase digest of sheep thyroglobulin. Digest equivalent to 350 mg of protein was loaded on a 0.9 x 75 cm column of Sephadex G-25. Eluant, 0.1 M acetic acid; flow rate, 20 ml per hour; fraction collected, 5.0 ml per tube. For hexosamine, hexose and sialic acid determinations, 0.1-0.5-ml aliquots were used.

A. ●----●----● Protein (280 mμ) o----o----o Hexose (620 mμ).

B. o----o----o Hexosamine (530 mμ) ●----●----● Sialic acid (580 mμ).

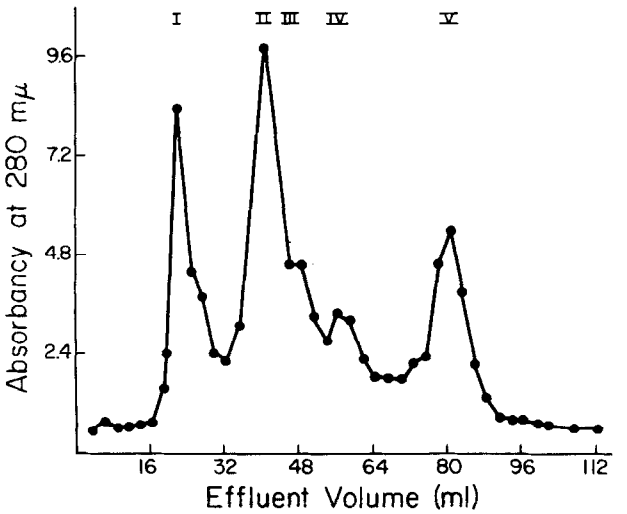

Fig. 2 - Gel filtration of the ethanol-insoluble fraction from Pronase digest of sheep thyroglobulin. Material obtained from 425 mg of protein was loaded on a 0.9 x 65 cm column of Sephadex G-25. Eluant, 0.1 M acetic acid; flow rate, 13.5 ml per hour; fraction collected, 2.7 ml per tube.

Analytical Procedure

Hexosamine was estimated by the method of Rondle and Morgan (1955). After preliminary experiments, hydrolysis with 1 N HCl for 6-8 hours at 100° was adopted for release of bound hexosamine from both thyroglobulin and glycopeptides. Hexose was estimated by the anthrone procedure of Südhof *et al.* (1955). The samples were hydrolyzed with 0.05 N H_2SO_4 for one hour at 90°, and the sialic acid liberated was determined by the method of Svennerholm (1958).

Hexose and hexosamine were separated by the method of Anastassiadis and Common (1958), and subjected to paper chro-

matographic analysis with the solvent system consisting of ethyl acetate, pyridine, acetic acid and water in the proportions 5:5:2:3. Sugars were identified by spraying with a benzidine:acetic acid reagent (Block et al, 1958).

Zone electrophoresis was carried out on cellulose acetate strips with a barbiturate buffer (pH 8.6 and ionic strength 0.05) at a potential of 20 v/cm. Peptide bands were located by means of ninhydrin spray and by staining with amidoblack.

Nitrogen was determined by the Nessler reaction as described by Koch and McMeekin (1924). Protein was estimated either by the method of Lowry et al. (1951), with bovine serum albumin as standard, or by measuring the absorbancy at 280 mμ. Iodine analyses were kindly performed by Dr. G. La Roche of the Donner Laboratory.

RESULTS AND DISCUSSION

Studies on the hydrolysis of thyroglobulin by Pronase revealed that, in 8 hours, up to 90 % of the hexose was released into the trichloroacetic acid (TCA)-soluble fraction (Unpublished observations). A 20-hour period of hydrolysis was, however, employed to ensure maximum proteolysis. When the absorbancy (at 280 mμ) of the eluate obtained by gel filtration of the Pronase digest on Sephadex G-25 was determined, 5 peptide peaks were detected. A typical chromatographic pattern is shown in figure 1. Most of the hexosamine, hexose and sialic acid was found in peak I, thus showing that this peak contained the major glycopeptide released by Pronase digestion of thyroglobulin. It should be noted, however, that the materials in peaks II and V also contained small amounts of carbohydrate. The glycopeptide in peak I had a relatively

high molecular weight, as judged by the fact that it emerged immediately after the holdup volume of the column (25 ml).

When the alcohol-insoluble fraction obtained from the Pronase digest was subjected to gel filtration on Sephadex G-25, its chromatographic pattern was similar to that of the Pronase digest (Fig. 2). The fractions represented by each peak were pooled and lyophilized. The values for the molar ratios of nitrogen-to-hexosamine in the materials isolated from the different peaks are shown in Table I. They indicate that a major portion of the carbohydrate of thyroglobulin is firmly bound to a single alcohol-insoluble peptide. This peptide is, however, soluble in TCA.

TABLE I

ANALYSES OF FRACTIONS OBTAINED BY GEL FILTRATION OF THE ALCOHOL-INSOLUBLE FRACTION OF A PRONASE DIGEST OF SHEEP THYROGLOBULIN

Conditions of gel filtration are described in the legend of figure 2. Eluates representing each peak were pooled and lyophilized before analysis.

Peak	Total nitrogen	Total hexosamine	Nitrogen:hexosamine, molar ratios
	μg	μg	
I	1800	4500	5.1
II	5100	490	132.9
III	1580	20	1009
IV	320	10.9	375.2
V	340	3.6	1206

A comparison of the absorbancies, at 280 mμ, of the various peaks in figures 1 and 2 shows that fractionation with

alcohol resulted in a substantial concentration of the glycopeptide in peak I. When the glycopeptide was subjected a second time to gel filtration on Sephadex G-25, it emerged as a single component within the same effluent volume as it had in the first fractionation. Electrophoresis of the glycopeptide on cellulose acetate showed the presence of a single component.

Amino acid analyses of the glycopeptide revealed the presence of aspartic acid, glutamic acid, serine, threonine, alanine, proline, valine, tyrosine, lysine, arginine, glycine and leucine. Histidine, methionine, isoleucine and cysteine were found in thyroglobulin but not in the glycopeptide. Aspartic acid was identified as the N-terminal residue of the glycopeptide. Digestion of the glycopeptide with carboxypeptidase resulted in the release of several amino acids. In this respect, this peptide differs from the "protease fragment" isolated from ovomucoid, which is resistant to the action of carboxypeptidase (Hartley and Jevons, 1962).

Analyses of constituent sugars in the glycopeptide showed the presence of glucosamine, galactose, mannose and fucose. These sugars were also found in the parent protein. A quantitative analysis of the carbohydrates in thyroglobulin and in the glycopeptide is presented in Table II. The peptide is quite rich in sialic acid, hexose and glucosamine. The nitrogen content was reduced from 14.3 % for the native thyroglobulin to 6.0 % for the glycopeptide. Since the yield of the peptide from thyroglobulin was approximately 5 to 6 %, we have estimated that at least 60 % of the carbohydrate of thyroglobulin is in this glycopeptide.

It is of interest to note that the glycopeptide contained little or no iodine, a finding which suggests that the iodine-

TABLE II

NITROGEN AND CARBOHYDRATE CONTENT OF SHEEP THYROGLOBULIN AND GLYCOPEPTIDE (Peak I)

Values are expressed as g per 100 g of material. No corrections have been made for moisture content.

Component	Thyroglobulin	Glycopeptide
Nitrogen	14.3	6.0
Hexosamine	1.8	14.7
Hexose	4.5	34.5
Sialic acid	1.4	10.7
Protein*	92.0	36.0
Iodine	0.7	0.03

* Estimated by the procedure of Lowry et al. (1951).

containing portion of the thyroglobulin is not located in the immediate proximity of the major carbohydrate-containing unit.

REFERENCES

Anastassiadis, P. A., and Common, R. H., Can. J. Biochem. Physiol. 36, 413 (1958).

Block, R. J., Durrum, E. L., and Zweig, G., A manual of paper chromatography and paper electrophoresis, Academic Press, Inc., New York, 1958, p. 182.

Hartley, F. K., and Jevons, F. R., Biochem. J. 84, 134 (1962).

Koch, F. C., and McMeekin, T. L., J. Am. Chem. Soc. 42, 2066 (1924).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 293, 265 (1951).

Porath, J., Biochim. Biophys. Acta 39, 193 (1960).

Robbins, J., J. Biol. Chem. 238, 182 (1963).

Roche, J., Derrien, Y., and Michel, R., Compt. Rend. Soc. Biol. 141, 2229 (1947).

Rondle, C. J. M., and Morgan, W. T. J., Biochem. J. 61, 586 (1955).

Spiro, R. G., and Spiro, M. J., Fed. Proc. 22, 538 (1963).

Südhof, H., Kellner, H., and Schulte, N., Z. Physiol. Chem. 300, 68 (1955).

Svennerholm, L., Acta Chem. Scand. 12, 547 (1958).

Tong, W., Raghupathy, E., and Chaikoff, I. L., Endocrinol. 72, 931 (1963).

Ujejski, L., and Glegg, R. S., Can. J. Biochem. Physiol. 33, 199 (1955).

Wollman, S. H., and Warren, L., Biochim. Biophys. Acta 47, 251 (1961).