NEUTRON ACTIVATION ANALYSIS OF THE PEPTIDES OF THYROGLOBULIN

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A specific feature of thyroglobulin is the presence of iodine in it within wide limits (0.18-0.87%) [1]. In the protein the iodine is bound to tyrosine residues and to thyronine structures. In 1 mole of native thyroglobulin there are 118 moles of tyrosine, 5.2 moles of monoiodotyrosine, 2.9 moles of diiodotyrosine, and 1.8-2.4 moles of tetraiodotyrosine [2].

To elucidate the biosynthesis of thyronine structures and to determine the positions of the reactive tyrosine residues, thyroglobulin is usually iodinated in vitro, whereupon not only the tyrosine residues that may take part in the synthesis of the thyronine structures but also other tyrosine residues capable of adding iodine undergo iodination. On iodination in vitro, iodine is included additionally in 30% of the tyrosine residues [3, 4], which considerably complicates the elucidation of the true distribution of iodine in the protein and the original position of the iodinated tyrosine residues and thereby makes it difficult to determine the structure of thyroglobulin.

It was necessary to find the peptides richest in iodine in order to study them further by the methods of protein chemistry. Consequently, we preferred not to introduce radioactive substances from outside but used neutron activation analysis, in which the stable iodine present in the native protein becomes radioactive. The radioactivity that has appeared provides the possibility of determining the position of the iodine in the thyroglobulin molecule.

This method is very sensitive and convenient for determining micro amounts of iodine in the range from 10^{-9} to 10^{-10} g on chromatograms. The method is based on the fact that the atomic nuclei of the substance under investigation, which has been placed in a neutron flux, on being bombarded with neutrons are activated and form radioactive isotopes.

The magnitude of the induced activity is calculated from the basic formula of activation analysis [5].

$$A_t = \frac{N \cdot f \cdot \sigma \cdot \theta \cdot m}{M} \frac{(1 - e^{\lambda t})}{} e^{-\lambda \tau}.$$

The results of a determination of the amount of iodine by neutron activation in the thyroglobulin that we used showed that the protein contained 0.17 ± 0.01% of iodine (somewhat lower than the average figures). This is due to the fact that the thyroglobulin was prepared from goitrously changed human glands containing iodine in reduced amounts.

Since different amounts of hydrolyzate (1.8-2.2 mg) were deposited on the chromatograms, we evaluated the distribution of iodine between the peptides of each hydrolyzate (Table 1, vertical columns). The dashes in the table indicate amounts of iodine in the peptides of less than $3 \cdot 10^{-9}$ g. The greatest amount of iodine was concentrated in the 12th, 13th, 14th,

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TABLE 1. Distribution of Iodine in the Peptides of Thyroglobulin According to the Results of Neutron Activation Analysis

Peptide No.	Samples					
	I	II	III	IV	V	VI
1	$0,52\cdot10^{-7}$	—	—	—	$1,01\cdot10^{-7}$	—
2	$1,59\cdot10^{-7}$	$1,67\cdot10^{-7}$	$0,42\cdot10^{-7}$	$3,3\cdot10^{-7}$	—	—
3	$0,79\cdot10^{-7}$	—	—	—	$0,56\cdot10^{-7}$	—
4	—	$0,93\cdot10^{-7}$	—	$1,76\cdot10^{-7}$	—	—
5	$1,11\cdot10^{-7}$	$1,55\cdot10^{-7}$	$1,78\cdot10^{-7}$	$7,09\cdot10^{-7}$	$0,44\cdot10^{-7}$	—
6	$0,75\cdot10^{-7}$	$0,38\cdot10^{-7}$	—	—	—	—
7	$0,3\cdot10^{-7}$	$1,46\cdot10^{-7}$	—	$3,3\cdot10^{-7}$	$1,5\cdot10^{-7}$	—
8	$0,39\cdot10^{-7}$	$0,83\cdot10^{-7}$	—	$2,91\cdot10^{-7}$	$1,3\cdot10^{-7}$	—
9	$0,39\cdot10^{-7}$	—	$1,19\cdot10^{-7}$	$1,6\cdot10^{-7}$	$0,9\cdot10^{-7}$	—
10	—	—	—	—	—	—
11	$0,52\cdot10^{-7}$	—	—	$4,2\cdot10^{-7}$	$0,29\cdot10^{-7}$	—
12	$1,52\cdot10^{-7}$	$0,48\cdot10^{-7}$	$1,33\cdot10^{-7}$	$7,14\cdot10^{-7}$	$1,93\cdot10^{-7}$	—
13	$1,47\cdot10^{-7}$	$4,33\cdot10^{-7}$	$7,63\cdot10^{-7}$	$6,76\cdot10^{-7}$	$1,22\cdot10^{-7}$	—
14	$5,51\cdot10^{-7}$	$7,50\cdot10^{-7}$	$5,42\cdot10^{-7}$	$8,57\cdot10^{-7}$	$6,18\cdot10^{-7}$	$6,80\cdot10^{-7}$
15	$1,36\cdot10^{-6}$	$1,50\cdot10^{-6}$	$9,0\cdot10^{-7}$	$1,42\cdot10^{-6}$	$1,24\cdot10^{-6}$	$1,3\cdot10^{-6}$

Peptide No. 15: $M_1 \pm m_1 = 129\cdot10^{-6} \pm 0,858\cdot10^{-7}$
Peptide No. 14: $M_2 \pm m_2 = 6,66\cdot10^{-7} \pm 0,49\cdot10^{-7}$
$t = 6,32 \qquad P < 0,001$

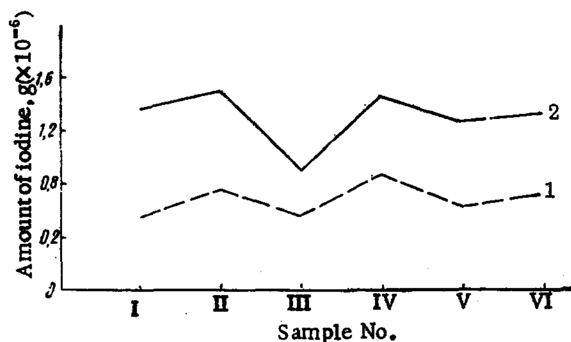

Fig. 1. Correlation between the amounts of iodine in the 14th (1) and 15th (2) peptides.

and 15th peptides (see Table 1). The results of the neutron activation analysis show that the amount of iodine in the 14th peptide varies from $5.42\cdot10^{-7}$ to $8.57\cdot10^{-7}$ g, and that in the 15th peptide from $9.0\cdot10^{-7}$ to $1.5\cdot10^{-6}$ g. On comparing these results, it can be seen that there is a definite direct correlation between the concentrations of iodine in the 15th and in the 14th peptides (Fig. 1), which shows the constancy of the distribution of iodine in the thyroglobulin peptides.

If we take the sum of the mean activities of all the peptides as 100, it can be calculated that there is 34.3% of the iodine of the protein in the 15th peptide, 17.7% in the 14th peptide, and 12.8% in the 13th peptide. Consequently, there is only 35.4% of the iodine of the protein in the remaining 12 peptides, i.e., on an average each of these peptides contains approximately 3% of the total amount of iodine in the protein.

Thus, the method of neutron activation analysis has given us the possibility of determining the amount of iodine in the native protein and also in its fractions. Furthermore, it has been established by this method that by hydrolyzing thyroglobulin with pronase it is possible to isolate three peptides containing the bulk of the iodine of the thyroglobulin.

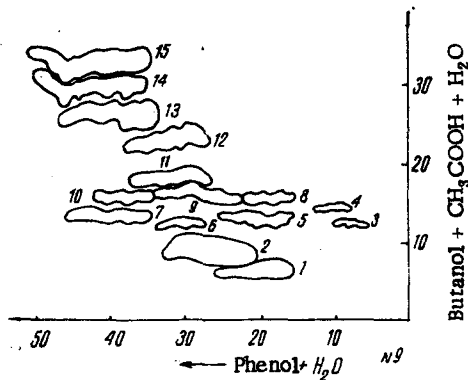

Fig. 2. Hydrolysis of thyroglobulin by pronase. Two-dimensional chromatogram in the following systems: 1) water-saturated phenol; 2) butan-1-ol—acetic acid—water (4:1:5).

EXPERIMENTAL

We obtained the iodine-containing protein (thyroglobulin) by Karlsson's method [6] in our modification [7], and the peptides of the thyroglobulin by proteolytic enzymatic hydrolysis without the preliminary reduction of the disulfide bonds of this protein. For hydrolysis we used pronase of Japanese manufacture (2% of the amount of protein). The thyroglobulin was hydrolyzed in a volatile buffer [0.1 M $(CH_3CH_2)_3N$ and 0.1 M CH_3COOH] at pH 8.0, and the samples of protein amounted to 1.8-2.2 mg. Hydrolysis was performed at 38-39°C for 5 h. After the end of hydrolysis, the enzyme was inactivated by heating the reaction mixture in the boiling water bath for 5 min, and then the hydrolyzate was centrifuged, the small amount of precipitate was rejected, and the supernatant liquid was dried in vacuum over P_2O_5. The dry residue was dissolved in water and the solution was deposited on paper for two-dimensional chromatography in the following systems: 1) water-saturated phenol, and 2) butan-1-ol—acetic acid—water (4:1:5). Thanks to the standard conditions for the hydrolysis of thyroglobulin and for the chromatography of the hydrolyzate that had been developed, we observed an identical reproducible distribution of the peptides on the chromatograms (Fig. 2).

Since in the induction of activity the activation not only of iodine but also of such elements as sodium and chlorine, which give peaks similar to those of iodine is possible, before investigating thyroglobulin, the chemical composition of which does not include sodium and chlorine ions, we excluded contamination the source of which could be the chromatographic paper. For this purpose, before chromatography the paper was washed chromatographically with 3% acetic acid for three days, which completely eliminated chlorine and sodium ions.

The chromatograms of the enzymatic hydrolyzate of thyroglobulin obtained were revealed with a 0.015% solution of ninhydrin in aqueous butanol. The sections of the paper with the peptides were cut out and numbered. Then these strips of paper were packed into 3 × 3 cm polyethylene envelopes. As standard, a solution of ammonium iodine ($1 \cdot 10^{-6}$ g) was deposited on a piece of chromatographic paper of the same size cut from the same chromatograms. The envelopes were sealed in a spirit flame. Corresponding sections of the chromatogram without peptides were packaged likewise and were used as controls. The samples under investigation, the standard, and the control were placed in the channel of a reactor with a neutron flux of 10^{13} neutrons/cm^2·sec and were irradiated for 5 min.

The activities of the sample and of the standard at the photo peak of 0.44 MeV were measured directly after irradiation in an apparatus consisting of a UCD-1 scintillation detector and a AI-100 100-channel amplitude analyzer. The results were calculated from the formula given above. The results for the 14th and 15th peptides, as those richest in iodine, were treated statistically.

The amount of iodine in the native thyroglobulin was determined by the same method as for the protein peptides. On the chromatographic paper prepared for activation analysis was deposited 5-mg portions of thyroglobulin and it was then subjected to neutron activation under the conditions described.

In view of the fact that on two-dimensional chromatography the superposition of two peptides in the same spot is possible, we checked the peptides for homogeneity by paper electrophoresis. The sections of paper with the 14th and 15th peptides were cut out from the chromatogram and eluted by water from paper strips arranged in parallel [8]. Hydrogen sulfide was passed through the solution of the peptides eluted in order to decolorize the ninhydrin, and then to decrease the volume and increase the concentration the solution was evaporated in vacuum over P_2O_5. Then it was filtered through a Pasteur pipette with glass

wool (glass wool as uniform as possible) and in this way the excess of sulfur and the products of the decomposition of the ninhydrin were removed. The solution was dried in previously weighed crucibles with a capacity of about 1.5 ml. The peptides were dried, dissolved, and deposited on paper for electrophoresis (1-1.5 mg in 5 μl of water). After this procedure, electrophoresis was performed in various buffers (CH_3COOH + CH_3COONa; KH_2PO_4 + Na_2HPO_4; veronal and medinal) at various pH values (4.7, 6.8, and 8.6, respectively). In order to reveal the spots on the electrophoretograms dried in the air at room temperature, the latter were sprayed with a solution of ninhydrin, thanks to which it was possible to reveal all the finest fractions, down to amino acids. In each case, electrophoresis of the peptides on paper gave a single ninhydrin-positive spot which, in our opinion, confirms the homogeneity of the peptides isolated chromatographically.

SUMMARY

The peptides of thyroglobulin obtained by enzymatic hydrolysis with pronase and by two-dimensional paper chromatography have been investigated by neutron activation analysis in order to determine the micro amounts of iodine in each peptide. The greatest amount of iodine is present in the 2nd, 7th, 12th, 13th, 14th, and 15th peptides. The bulk of the iodine is present in the 14th and 15th peptides, which contain 17.7 and 34.3%, respectively, of the total iodine. There is a definite direct correlation between the concentrations of iodine in them.

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