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Note

Separation of iodinated compounds of L-tyrosyl-L-tyrosine from iodothyronines by Biogel P-2 column chromatography

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Previous investigations conducted in our laboratory have shown that the sequence L-tyrosyl-L-tyrosine is part of the primary structure of thyroglobulin. The dipeptide 3,5,3′,5′-tetraiodo-L-tyrosyl-L-tyrosine (I_2Tyr-I_2Tyr) extracted from trypsic digests of bovine thyroglobulin[1] has demonstrated the presence of this iodinated sequence under physiological conditions. *In vitro* experiments showed that this dipeptide is a better substrate than diiodotyrosine for the synthesis of iodothyronines[2], and we proposed a mechanism of this synthesis which appealed to a cyclic agent without breaking the peptide bond[3,4]. During these *in vitro* experiments, we developed a new technique for the separation of the iodinated compounds of tyrosyl-tyrosine from the known iodoamino acids. Various methods for the separation of iodo compounds have already been described: paper chromatography, thin-layer chromatography, ion-exchange column chromatography, Sephadex gel filtration and gas–liquid chromatography[5–10]. Up to 1975 no procedure allowing a complete separation of all the known iodoamino acids was available. Since 1975, following the work of Thomopoulos[11], we have been using a single Biogel P-2 column which separates L-monoiodohistidine, L-diiodohistidine, L-monoiodotyrosine, L-diiodotyrosine, L-triiodothyronine and L-thyroxine. But this technique does not separate the different iodinated compounds of L-tyrosyl-L-tyrosine. To obtain such a separation we performed a systematic study of the relative role of the different constituents, in particular the gel and the elution buffer.

This paper presents a new method for the separation of the iodinated tyrosines, tyrosyl tyrosines and thyronines on the basis of the pK_b value and of the iodine content.

EXPERIMENTAL

The iodination of L-tyrosyl-L-tyrosine leads to eight different iodinated compounds: ITyr-Tyr, Tyr-ITyr, I_2Tyr-Tyr, Tyr-I_2Tyr, ITyr-ITyr, I_2Tyr-ITyr, ITyr-I_2Tyr and I_2Tyr-I_2Tyr.

The synthesis and the labelling with ^{125}I or ^{131}I of these compounds were

carried out by peptidic coupling of iodotyrosines and then by isotopic exchange with radioactive iodine. A mixture of iodinated compounds of L-tyrosyl-L-tyrosine was obtained by labelling with peroxidase[12] and with a 75% iodine deficiency. I_2Tyr-I_2Tyr labelling was carried out by the chloramine T method[13]. Purification was performed on a Dowex 50-X4 column (200–400 mesh). The specific activity was 1000–1200 Ci/g. The radiochemical purity was 98%. Biogel P-2 polyacrylamide gel (200–400 mesh) (Bio-Rad Labs., Richmond, Calif., U.S.A.) was allowed to swell for 24 h at 20° in the elution buffer. The gel was then poured into plastic columns (50 × 0.9 cm I.D.) (Pharmacia, Uppsala, Sweden) and equilibrated with at least three total column volumes of the elution buffer.

We investigated the behaviour of the eight iodinated compounds of L-tyrosyl-L-tyrosine under the conditions described by Thomopoulos. Each ^{125}I- or ^{131}I-labelled product was deposited at the top of the column. Fractions of 1 ml were collected with a Gilson fraction collector. The radioactivity content for ^{125}I or ^{131}I of each fraction was measured in a dual channel Nuclear Chicago Autogamma Spectrometer with appropriate corrections for ^{131}I counts appearing in the ^{125}I channel, when needed.

Simultaneously, for each compound ^{127}I was detected on a Technicon auto-analyser by the method of Block and Mandl[7], as modified by Aquaron[14].

RESULTS AND DISCUSSION

Analysis of the results obtained with the Thomopoulos method shows that the bonding of the iodinated compounds of L-tyrosyl-L-tyrosine to a gel with an acid pH decreases with the rate of iodination. As Biogel P-2 possesses weak cationic exchange properties at alkaline pH, we can assume that the low degree of absorption of iodinated compounds is due to a reduction in their basicity compared with their less iodinated homologues. Therefore, it was logical to consider that the triiodinated compounds are less firmly bonded than the tetraiodinated ones. Hence the desorption pH of the triiodinated compounds must be lower than the desorption pH of the tetraiodinated ones. From these observations we were led to modify two constituents of the Thomopoulos method.

(1) A Biogel P-2 (minus 400) was used instead of the Biogel P-2 (200–400) in order to obtain a higher resolution of the gel filtration by the use of smaller particles.

(2) The replacement of the second eluent with pH 9.0 by a linear pH gradient was expected to produce a better separation of the iodinated compounds of I_2Tyr-I_2Tyr because of the difference in pK values for each compound. To obtain a good separation of all the iodinated compounds we used a linear pH gradient starting with a 0.05 M Tris–maleate buffer (pH 5.3) and ending with a 0.05 M Tris–HCl buffer (pH 9.0), at a 6 ml/h flow-rate for 24 h.

As can be seen from the elution curve (Fig. 1) and from Table I, a complete separation of five iodinated compounds of L-tyrosyl-L-tyrosine is achieved with this technique. Using the classical ion-exchange column chromatography these five compounds were eluted as a single component[1]. The method described by Sorimachi[9,10] allows a separation of most but not of all the compounds and metabolites related to LT_3. As mentioned earlier, the Thomopoulos method separates only two iodohistidines and two iodotyrosines from the thyronines[11]. None of these methods provided the separation needed in our specific case, *i.e.* the separation of iodinated compounds

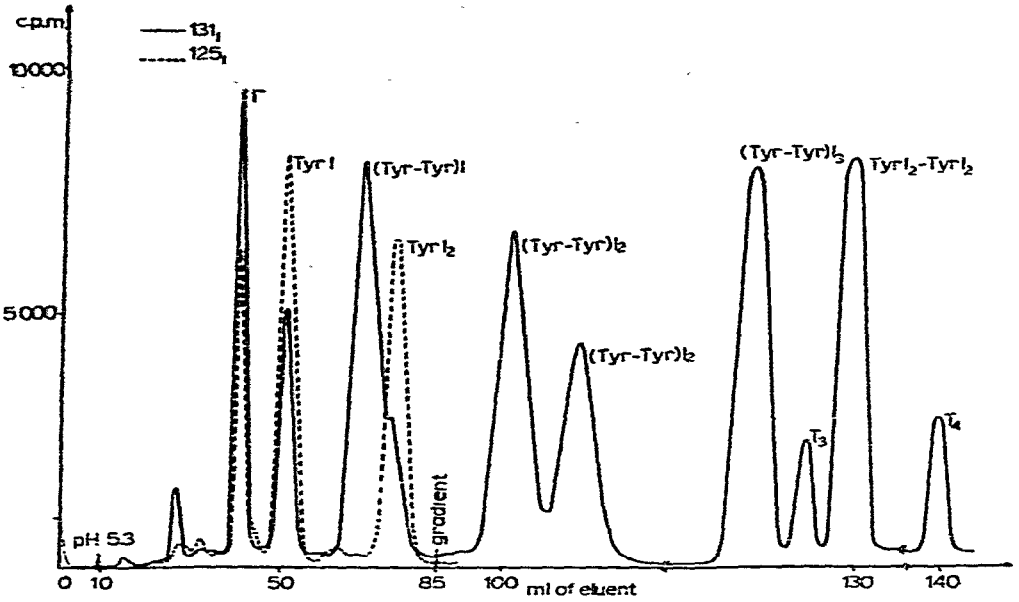

Fig. 1. Elution pattern of radioactive iodoamino acids on a Biogel P-2 (minus 400) column equilibrated and eluted at a flow-rate of 6 ml/h for 24 h with 0.05 *M* Tris–maleic acid–NaOH at pH 5.3 followed (arrow) by a linear pH gradient starting with a 0.05 *M* Tris–maleate buffer (pH 5.3) and ending with a 0.05 *M* Tris–HCl buffer (pH 9).

of L-tyrosyl-L-tyrosine present in the digests obtained from *in vitro* iodination both chemically and enzymatically.

Our new procedure should be helpful to predict tentatively the structure of unknown metabolites of I_2Tyr-I_2Tyr by comparing their R_F values to those of authentic iodotyrosines, iodotyrosyl-tyrosines and iodothyronines. It can also provide information on the structure–mobility relationship in gel filtration column chromatography. This technique has also been adapted to the problem of identification and purification of minor iodinated components in thyroglobulin digests, in the study of iodothyronine biosynthesis from I_2Tyr-I_2Tyr, and in *in vitro* iodination experiments performed in our laboratory.

TABLE I

ELUTION VOLUME/VOID VOLUME RATIOS AND pH DATA FOR EIGHT IODINATED COMPOUNDS

Iodinated compounds	V_e/V_0	*pH*
I_2Tyr-Tyr	1.8	5.9
Tyr-I_2Tyr	2.3	6.1
ITyr-ITyr	3.5	6.6
I_2Tyr-ITyr + ITyr-I_2Tyr	8.9	8.5
T_3	10	8.9
I_2Tyr-I_2Tyr	10.7	9
T_4	11.4	9

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