Journal of Chromatography, 566 (1991) 471–480
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5815

Determination of proteolytic hydrolysis of thyroglobulin

EUGÈNE H. J. M. JANSEN*, LAMBERT DOORN and F. X. ROLAF VAN LEEUWEN

Laboratory for Toxicology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

ABSTRACT

A method for the determination of the free thyronine- and tyrosine-like amino acids in the thyroidal protein thyroglobulin is presented. The compounds of interest are monoiodotyrosine, diiodotyrosine, thyronine, diiodothyronine, triiodothyronine and tetraiodothyronine. The extent of proteolysis was followed by high-performance liquid chromatographic monitoring of both the remaining peptides and the formation of the free thyroidal amino acids. Total hydrolysis was achieved by a combination of proteolytic enzymes. A number of enzymes were tested, such as trypsin, chymotrypsin, pronase, aminopeptidase-M, carboxypeptidase-A, carboxypeptidase-P and carboxypeptidase-Y. The best combination turned out to be pronase followed by aminopeptidase-M. The relative amounts of the enzymes, with respect to the substrate thyroglobulin, and the time of incubation were optimized to achieve total proteolysis in 4 h. The method was applied successfully to samples from a toxicological experiment with sodium bromide.

INTRODUCTION

In the thyroid the synthesis of important hormones, such as triiodothyronine (T3) and tetraiodothyronine (T4), takes place in the follicular cells and the inner membrane between the follicular cells and the colloid inner space [1]. Firstly, the precursors monoiodotyrosine (MIT) and diiodotyrosine (DIT) are synthesized by binding of iodine to the amino acid tyrosine. Subsequently the iodinated tyrosines are coupled to form T3, reversed T3 (rT3) and T4. These reactions are catalysed by thyroid peroxidase [2]. Effects of toxic compounds on the thyroid will be reflected by a change in the relative amount of the iodinated hormones and precursors present in thyroglobulin [3,4]. Therefore a method is required to determine quantitatively the amount of T3, rT3, T4, MIT, DIT in thyroglobulin and also some other related analogues, such as thyronine (T0) and diiodothyronine (T2).

This paper describes the optimization of the hydrolysis of thyroglobulin by proteolytic enzymes into the free thyroid hormones and precursors. The method was developed with bovine thyroglobulin and applied to rat-thyroid homogenates from a toxicity experiment with sodium bromide.

0378-4347/91/$03.50

EXPERIMENTAL

Materials

The high-performance liquid chromatographic (HPLC) solvents were of analytical grade (Westburg, Leusden, The Netherlands). Water was purified by a Millipore-Q filtration unit. The following tyrosine and thyronine standards were used: tyrosine (TYR) obtained from Merck (Darmstadt, Germany), 3-monoiodotyrosine (MIT), 3,5-diiodotyrosine (DIT), thyronine (T0), 3,3′,5-triiodothyronine (T3), 3,3′,5′-triiodothyronine (rT3) and 3,3′,5,5′-tetraiodothyronine (T4), all obtained from Sigma, (Brunschwig, Amsterdam, The Netherlands). The enzymes aminopeptidase-M, trypsin, chymotrypsin, pronase, carboxypeptidase-A, carboxypeptidase-P and carboxypeptidase-Y were obtained from Boehringer Mannheim (Almere, The Netherlands).

Proteolytic assays

Incubation of proteolytic enzymes with thyroglobulin was performed as follows: the incubation mixtures consisted of 10 mg of thyroglobulin, 2 m*M* calcium chloride, 5 m*M* methimazole and a suitable amount of enzyme (from 0.1 to 1.0 mg) in 2 ml of Tris–HCl buffer (0.05 *M*, pH 8.0). Incubation was performed at 37°C in a water-bath. After a given time, 200 μl were taken from the mixture and 20 μl of the internal standard rT3 (350 μg/ml) were added. After centrifugation, 75 μl were injected onto the HPLC column.

HPLC with UV detection

The HPLC equipment consisted of the following components: an autoinjector (Varian, Model 9000); two solvent-delivery systems (Perkin Elmer, Series 10); two low-pressure three-way valves (Rheodyne 5301); a high-pressure valve (Rheodyne 7010); an UV–VIS detector (Linear, Model 200); and a data acquisition system (Axxiom, Analytica, Maasdijk, The Netherlands). The column system consisted of a cartridge of Chromphere C_{18} (100 mm × 3 mm I.D., 5 μm) (Chrompack, Middelburg, The Netherlands) with a concentrating column (15 mm × 3.2 mm I.D., 7 μm) (Brownlee Newgard RP-18, Inacom Instruments, Veenendaal, The Netherlands), and was thermostatted at 35°C with an electric column oven (LKB, Model 2155). The following HPLC conditions were used: after injection of 75 μl of the incubation mixture, the concentrating column was eluted for 2 min with mobile phase A. Then the analytical column was put in-line with the concentrating column and eluted for 3.5 min with mobile phase C and for 14.5 min with mobile phase D. At 11.5 min the concentrating column was switched off and eluted with mobile phase B. The total time of analysis was 20 min. Mobile phase A consisted of phosphate buffer (0.05 *M*, pH 2.2) containing 2.5 m*M* heptanesulphonic acid. Mobile phase B consisted of methanol containing 2.5 m*M* heptanesulphonic acid. Mobile phase C consisted of phosphate buffer (0.05 *M*, pH 2.5) containing 2.5 m*M* heptanesulphonic acid and methanol (65:35,

v/v). Mobile phase D consisted of phosphate buffer (0.05 *M*, pH 2.5) containing 2.5 m*M* heptanesulphonic acid and methanol (42.5:57.5, v/v).

Toxicological experiment with sodium bromide

The experimental performance and the results of the animal experiments with sodium bromide will be published in detail elsewhere [5].

RESULTS

Thyroglobulin is a glycoprotein with a high molecular mass of 660 000. In order to achieve total proteolysis for liberation of the thyroid hormones and percursors, a number of proteolytic enzymes and combinations of enzymes have been investigated. For the first incubation, in order to cleave as many peptide bonds as possible, the following enzymes were tested: trypsin, chymotrypsin and pronase. The second incubation was then performed with various amino- and carboxypeptidases. The sequences of enzymes used in this study are indicated schematically in Fig. 1.

The course of the proteolysis could be monitored with a reversed-phase HPLC system for the analysis of peptides or with a reversed-phase HPLC system with column switching, which was developed for the determination of thyroid hormones and precursors. An example of a separation of the various thyroid compounds is shown in Fig. 2. In practice, the second method turned out to be more suitable because the presence of peptide fragments could also be detected with this system.

The combination of chymotrypsin and carboxypeptidases could be expected theoretically to be a good choice. Chymotrypsin cleaves aromatic amino acids at the C-terminal side, producing peptides with thyroid-like amino acids at the C-terminus. A second incubation with carboxypeptidases should specifically release

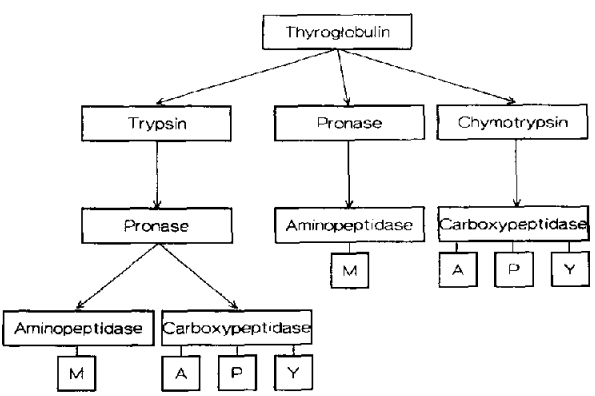

Fig. 1. Schematic presentation of the combinations of proteolytic enzymes used to hydrolyse thyroglobulin.

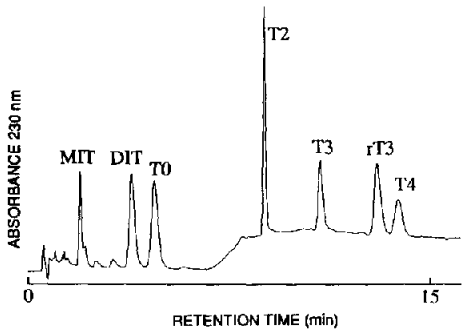

Fig. 2. HPLC separation of thyroid hormones and precursors. The abbreviations are indicated in the text.

these aromatic amino acids. In practice, however, this sequence was not successful, since the liberation of the thyroid hormones and precursors was far from complete. Also a great number of peptides were still present, as shown in Fig. 3.

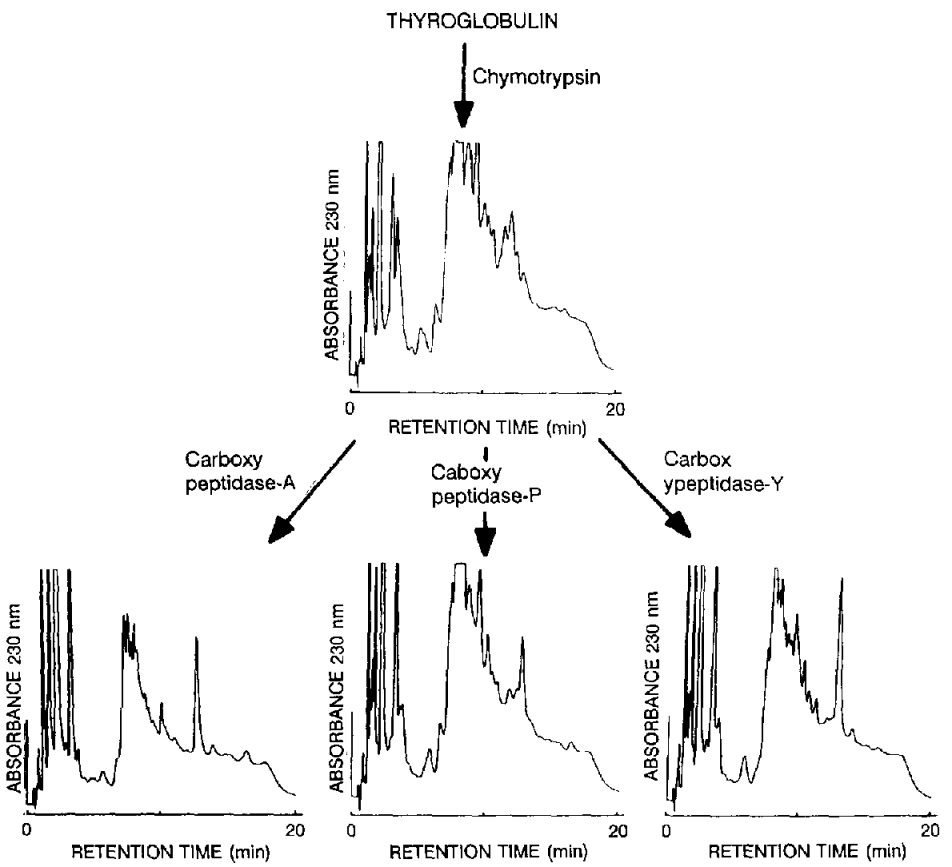

Fig. 3. Chromatograms of the incubation mixtures of thyroglobulin with chymotrypsin followed by various carboxypeptidases.

The same kind of chromatogram was also observed after treatment with trypsin.

Proteolysis with pronase, however, showed a rather clean chromatogram with respect to peptide peaks as shown in Fig. 4. An additional pretreatment with trypsin, followed by pronase, did not show any improvements. As a final incubation, the enzyme aminopeptidase-M gave the best results with respect to the intensity of the thyroid hormones and precursors. The quantitative results are listed in Table I, expressed as percentages of the peak heights in the chromatogram after treatment with the combination of pronase and aminopeptidase-M.

The combination of pronase and aminopeptidase-M was further optimized with respect to the proportion of enzyme to thyroglobulin and the time of incubation. In Fig. 5 the peak heights of DIT and T4 are shown, both as a function of the relative amount of pronase (1:10, 25, 50 and 100) and as a function of the time of incubation. The behaviour of MIT and T3 was similar to that of DIT and T4, respectively. From Fig. 5 it can be concluded that a proportion of pronase of 1:10 (w/w) gives the best results, especially for T4 (and T3). With this concentration the proteolysis is almost complete after 1 h and complete after 2–4 h. The influence of the second proteolytic enzyme in sequence, aminopeptidase-M, is shown in Fig. 6. After treatment with pronase for 1 h, the incubation with aminopeptidase-M gives an additional increase in peak heights for MIT, DIT, T3 and T4 of 5, 0, 10 and 25%, respectively. The proportion of aminopepti-

TABLE I

RELATIVE CONCENTRATIONS OF MIT, DIT, T3, T4, TYROSINE (TYR) AND TRYPTOPHAN (TRP) IN BOVINE THYROGLOBULIN AFTER PROTEOLYTIC HYDROLYSIS BY DIFFERENT COMBINATIONS OF PROTEOLYTIC ENZYMES

The HPLC peak height after treatment with pronase and aminopeptidase-M is set to 100%.

Proteolytic enzyme	Relative concentration (%)					
	MIT	DIT	T3	T4	TYR	TRP
Pronase	91	98	85	69	87	91
Pronase + AP-M	100	100	100	100	100	100
Trypsin	41	35	58	15	36	30
Trypsin + pronase	87	92	85	69	95	89
Trypsin + pronase + AP-M	94	92	91	85	101	92
Trypsin + pronase + CP-A	94	88	82	71	104	89
Trypsin + pronase + CP-P	93	87	76	70	106	87
Trypsin + pronase + CP-Y	94	87	76	68	100	83
Chymotrypsin	46	33	82	6	41	39
Chymotrypsin + CP-A	105	55	76	53	91	91
Chymotrypsin + CP-P	51	46	91	38	58	52
Chymotrypsin + CP-Y	62	51	70	69	66	61

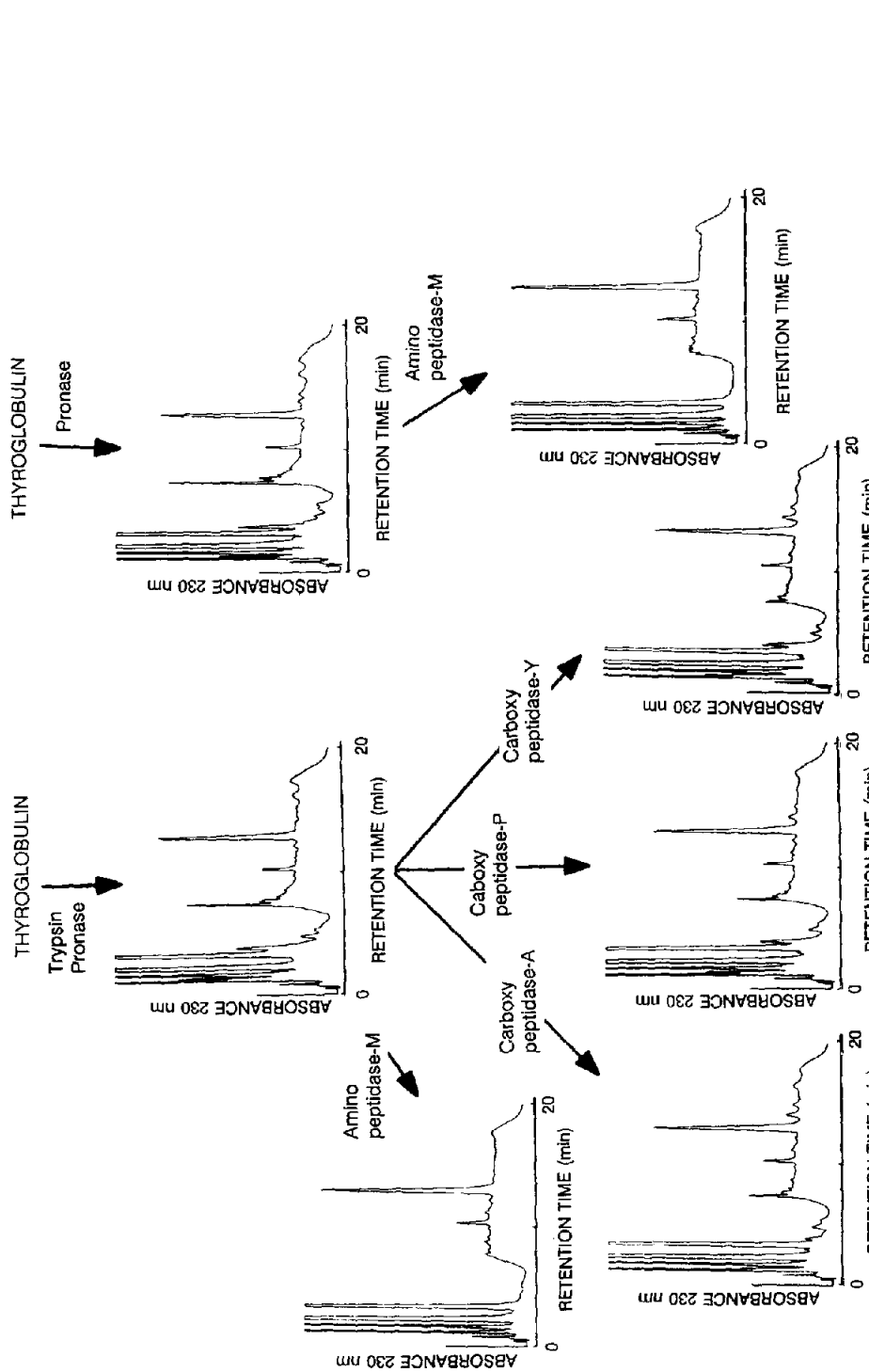

Fig. 4. Chromatograms of the incubation mixtures of thyroglobulin with combinations of trypsin, pronase, aminopeptidase-M and various carboxypeptidases.

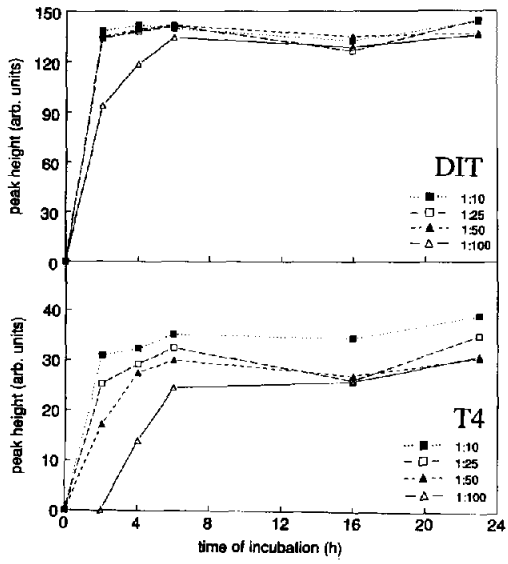

Fig. 5. HPLC peak heights of DIT and T4 in thyroglobulin as a function of time and the proportion of pronase in the mixture.

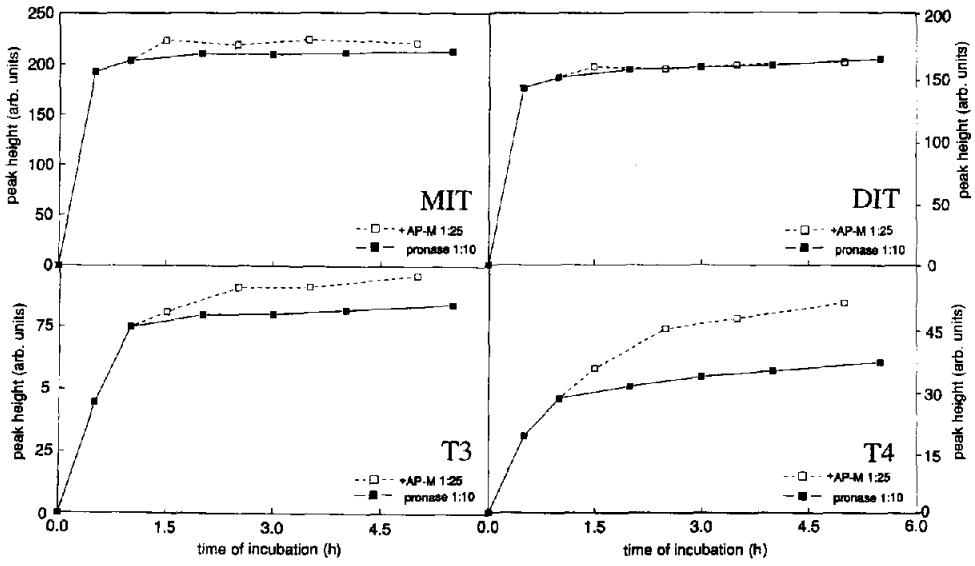

Fig. 6. HPLC peak heights of MIT, DIT, T3 and T4 in thyroglobulin after proteolysis with pronase and subsequent treatment with aminopeptidase-M as a function of time of incubation.

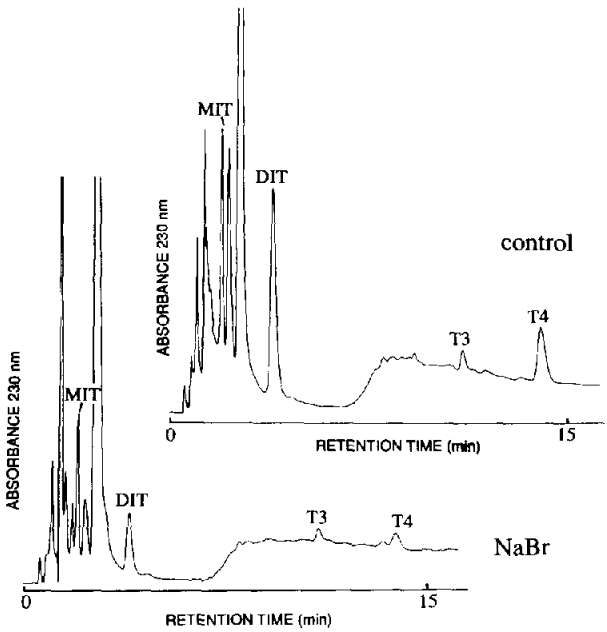

Fig. 7. Chromatograms of rat thyroid extracts after complete proteolytic hydrolysis. (Top) A control male rat; (bottom) a male rat treated with sodium bromide.

dase-M in this experiment was 1:25 (w/w). A proportion of 1:10 did not show an additional improvement (data not shown).

In conclusion, the best results were obtained with the following procedure: treatment with pronase (1:10, w/w) for 1 h, followed by treatment with aminopeptidase-M (1:25, w/w) for 2 h.

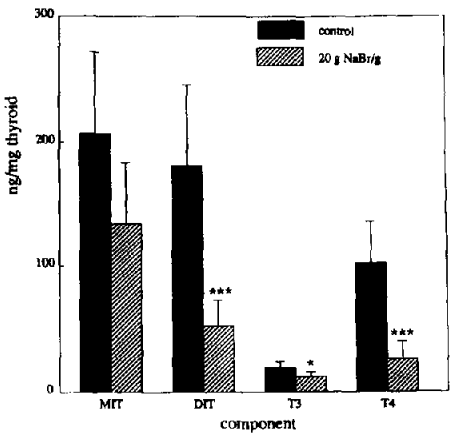

Fig. 8. Summarized results of a toxicological experiment with sodium bromide. Data were obtained from HPLC peak heights after total proteolytic hydrolysis with pronase followed by aminopeptidase-M.

This method was applied in a toxicological experiment to assess the possible toxic effects of sodium bromide on the thyroid. Fig. 7 shows chromatograms of a sample from a control animal and a treated animal. From this figure it appears that the proteolysis of rat thyroglobulin is complete in real samples. In this experiment significant differences where observed between treated and control animals, as shown schematically in Fig. 8.

DISCUSSION

Reversed-phase HPLC with a step gradient has proved to be an excellent method for the quantitation of thyroid hormones and their precursors [6–8]. Column switching has been used to allow only the compounds of interest into the analytical column [9]. The same HPLC system has also proved to be suitable for monitoring the extent of proteolysis of thyroglobulin. Both the existence of peptides and the increase in peak heights of the compounds of interest can be demonstrated. When the peak heights of the thyroid hormones have reached their maximum values and all the peptides have disappeared, it can be concluded that the proteolysis is complete. The optimal combination of proteolytic enzymes turned out to be pronase followed by aminopeptidase-M which was described also earlier [10]. Theoretically, the combination of chymotrypsin and a carboxypeptidase should also liberate the free aromatic (thyroidal) amino acids. In practice, however, it appeared that these particular amino acids do not behave as normal aromatic amino acids.

In contrast to earlier reported hydrolysis procedures [10–13], the optimized condition of proteolysis can be performed in 4 h: 1 h of incubation with pronase and a subsequent 3-h incubation with aminopeptidase-M. For a few samples the whole procedure of proteolysis and HPLC analysis can be performed within one working day. Larger number of samples (up to 50) can be processed within one working day and prepared for overnight automatic HPLC analysis.

The general applicability of the method described here was shown in a toxicological experiment of the effects of sodium bromide in rats. The thyroid homogenates were subjected to the proteolysis described in this report. It appeared that the method worked well on these real samples from an animal experiment, and total proteolysis was achieved.

REFERENCES

1 T. J. Visser, in B. A. Cooke, R. B. J. King and H. J. van der Molen (Editors), *Hormones and their Action, Part I*, Elsevier, Amsterdam, 1988, p. 81.
2 S. C. Werner and S. H. Ingbar, *The Thyroid*, Harper & Row, New York, 1978.
3 F. X. R. van Leeuwen, J. G. Loeber and M. A. M. Franken, *Arch. Toxicol., Suppl.*, 12 (1988) 93.
4 F. R. Fullerton, R. J. Kushmaul, R. L. Suber and N. A. Littlefield, *J. Toxicol. Environ. Health*, 22 (1987) 175.
5 F. X. R. van Leeuwen, L. Doorn and E. H. J. M. Jansen, in preparation.

6 N. M. Alexander and M. Nishimoto, *Clin. Chem.*, 25 (1979) 1757.
7 P. R. Kootstra, H. H. van den Broek, E. A. Hogendoorn, C. E. Goewie and J. J. M. de Vijlder, *J. Chromatogr.*, 458 (1988) 175.
8 L. Doorn, R. Both-Miedema, E. H. J. M. Jansen and F. X. R. van Leeuwen, *Pharm. Weekblad Sci. Ed.*, 11 (1989) N4.
9 L. Doorn, E. H. J. M. Jansen and F. X. R. van Leeuwen, *J. Chromatogr.*, in press.
10 A. Taurog, G. Riesco and P. R. Larsen, *Endocrinology*, 99 (1976) 281.
11 K. Inoue and A. Taurog, *Endocrinology*, 81 (1967) 319.
12 K. Sorimachi and N. Ui, *J. Biochem.*, 76 (1974) 39.
13 M. Rolland, R. Aquaron and S. Lissitzky, *Anal. Biochem.*, 33 (1970) 307.