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Difference in Susceptibility of Tyrosine Residue to Oxidative Iodination between a Thioredoxin Box Region and a Hormonogenic Region

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Peptide fragments, isolated from proteolytic cleavage of thyroglobulin at specific sites, were examined for the iodination of tyrosine residues. The 50 kDa polypeptide, which was prepared from digestion of bovine thyroglobulin and continuous preparative SDS-PAGE, was subjected to reduction with DTT and alkylation with iodoacetic acid to generate S-carboxymethylated peptide derivative, which was further hydrolysed by endoproteinase-Asp N. Peptide products were separated by RP-HPLC, and each fraction was analyzed by LC/ESI-MS and MALDI-MS analyses. Based on the specificity of endoproteinase Asp-N and the mass spectra data, a peptide fragment turned out to correspond to a peptide, DALGCVKCPEGSYFQ (1438-1452), characterized by the presence of a thioredoxin box (CVKC) and a tyrosine residue. In addition, another peptide fragment (1453-1465) containing a thioredoxin box (CIPC) and a tyrosine residue was also observed. However, any evidence of iodination of the tyrosine residue present in these peptides was not provided. Meanwhile, tyrosine residues in the peptides, DVEEALAGKYLAGRFA (1366-1381) and DYSGLLLAFQVFLL (1290-1303) were found to be iodinated; mono- or diiodinated tyrosine residues, characteristic of a hormogenic site, existed in both peptides. In addition, the tyrosine residue in the peptide (1218-1252), corresponding to a hormonogenic site was also iodinated. Thus, there was a sharp difference of the susceptibility to oxidative iodination between the tyrosine residue in a hormonogenic site and that in a thioredoxin region. From these results, it is suggested that polypeptide region adjacent to tyrosine residues may govern the susceptibility of tyrosine to oxidative iodination.

Key words: Thyroglobulin, Diiodotyrosine, Thioredoxin, Hormonogenic, Mass spectrometry

INTRODUCTION

Thyroglobulin (Tg), a precursor protein of thyroid hormones such as 3,5,3'-triiodothyronine (T_3) and 3,5,3',5'-tetraiodothyronine (thyroxine, T_4) is the most abundant protein in the thyroid gland, where it constitutes up to 75 % of the total protein content (Chernoff and Rawitch, 1981; Gentile *et al.*, 1995; Venkatsh and Deshpande, 1999). Studies of the overall structure of thyroglobulin have been hampered by the complexity of the protein; thyroglobulin is a large molecule (M.W., 660,000) with a heterogeneity at the post-translational level such as the addition of carbohydrate, sulfate and phosphate (Gentile *et al.*, 1995). An additional factor of complexity is represented by the presence of 122 cysteinyl residues per Tg monomer, most of which are involved in intra-chain disulfide bonds and enriched in three types of cysteine-rich tandem repeats (Veneziani *et al.*, 1999). Noteworthy, some of cysteine residues are present in the region compatible with thioredoxin box (CXXC) sequence. Thyroglobulin from various mammalian species contains three highly conserved thioredoxin boxes (CXXC). Recently (Klein *et al.*, 2000) it was observed that human recombinent Tg fragment containing three thioredoxin boxes exhibited a redox activity corresponding to PDI activity at redox conditions supposed to be present in the extracellular space of thyrocytes. This redox activity was suggested to

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lead to the intermolecular cross-linking of lumenal thyroglobulin. Another peculiarity of these thioredoxin boxes region is that there exists a tyrosine residue between thioredox boxes in relatively hydrophilic region (Gentile *et al.*, 1995). On the other hand, it has been well known that the formation of T_3 and T_4 includes the primary iodination of tyrosine residue in thyroglobulin molecule and subsequent coupling of iodotyrosine residues by thyroid peroxidase. The coupling reaction takes place by the transfer of an iodophenyl group from a donor, 3-monoiodotyrosine or 3,5-diiodotyrosine residue, to an acceptor, 3,5-diiodotyrosine residue (Gregg *et al.*, 1988; Taurog, 1996). The identification of diiodotyrosine residue well manifestates that some of tyrosine residues are susceptible to oxidative iodination; according to earlier report (Gentile *et al.*, 1997), bovine thyroglobulin contains 72 tyrosine residues, of which at least 15 tyrosine residues are iodinated, and a maximum of 6-8 tyrosine residues undergo coupling to form thyroid hormones. So far, four major hormonogenic tyrosines have been identified by the isolation and sequencing of hormone-rich peptides from thyroglobulins of various animal species and comparison of their sequences with the cDNA-deduced sequences of bovine (Mercken *et al.*, 1985) and human thyroglobulin (Malthiery and Lissitzky, 1987). Tyr-5 and Tyr-130 were a couple, the most favored site, responsible for T_4 formation in many species studied. In some species, Tyr-2553 was the second most efficient T_4-forming residue, whereas Tyr-2746 was a site of the preferential synthesis of T_3 (Marriq *et al.*, 1983; Dunn *et al.*, 1987; Lamas *et al.*, 1989). Another T_4-forming couple is a pair of Tyr-1295 and Tyr-1375: in bovine thyroglobulin, these tyrosine sites, modified mostly to diiodotyrosine, were reported to be contributory in the formation of T_4 (Gentile *et al.*, 1997). Thus, the susceptibility of tyrosine residues, present in thyroglobulin molecule, to the oxidative iodination differed according to the location of tyrosine residue in thyroglobulin molecule. Noteworthy, thioredoxin boxes are close to the T_4-forming couple, Tyr 1295 and Tyr 1375. Therefore, the region containing three thioredoxin boxes was supposed to be exposed to posttranslational iodination. Nevertheless, it has received little attention wheather the tyrosine residue adjacent to a thioredox box is also iodinated. Here, we attempted to see whether tyrosine residue close to thioredoxin box may be modified by oxidative iodination, and find the difference in sensitivity of tyrosine residues to iodination between thioredoxin box region and hormonogenic region.

MATERIALS AND METHODS

Materials

Thermolysin (Bacillus thermoproteolyticus rokko, EC 3.4.24.4), Sephadex G25, Sephacryl S-300 HR, EDTA, mercaptoethanol, iodoacetic acid, glycerol and cellophane film were from Sigma Chemicals Co (St. louis, MO); endoproteinase Asp-N (Pseudomonas fragi, EC 3.4.24.33) was from Roche Molecular Biochemicals (Indianapolis, IN). Various products for electrophoresis were from Bio-Rad Laboratories (St. Louis, MO). Bicinchoninic acid Protein Assay Reagent was from Pierce (Rockford, IL). HPLC grade solvents were obtained from Carlo Erba (Milan, Italy). The Vydac C-18 column (250 × 4.6 mm, 5 μm) was from The Separation Group (Hesperia, CA).

Preparation of thyroglobulin (Tg)

Bovine Tg was purchased from Pelfreeze Biologicals (Rogers, AK). The tissue was finely minced with scissors on ice. Tg was extracted briefly on ice in 0.1 M sodium phosphate, pH 7.2, and purified by fractional precipitation with 1.4-1.8 M ammonium sulfate, 50 mM Tris/HCl, pH 7.2, and gel filtration on Sephacryl S-300 HR in 50 mM Tris/HCl, pH 7.2 containing 130 mM NaCl at 4°C.

Proteolysis of Tg by thermolysin

Limited proteolysis of Tg with thermolysin was carried out as described previously (Gentile *et al.*, 1997). Thyroglobulin (1 mg/ml) in 50 mM Tris/HCl (pH 8.0) containing 130 mM NaCl was incubated with thermolysin at the enzyme/substrate ratio of 1/50 (w/w) at 30°C for 100 min. The digestion was stopped by adding EDTA (final concentration, 10 mM), and the aliquot was mixed with SDS-PAGE sample buffer (10 mM Tris/HCl, pH 6.8, 1% SDS, 5% beta-mercaptoethanol, 1.36 M glycerol, 0.0025 % bromphenol blue), and the mixture was heated in a boiling water bath for 1.5 min.

Separation and identification of products of limited proteolysis of Tg

SDS-PAGE analyses were performed according to the published procedure (Laemmli, 1970) on 10% total acrylamide gradient gels (Sigma, St.Louis, MO). The gels were stained with 0.1 % Coomassie Brilliant Blue R-250 in 20 % methanol containing 10% acetic acid (v/v), and destained in 25 % methanol (v/v) containing 10 % acetic acid

Purification of polypeptide 50 kDa

Bovine Tg was digested with thermolysin, and the digestion was stopped as described above. The fragments were precipitated in the mixture of chloroform/methanol (1:4), redissolved in SDS-PAGE sample buffer, and separated by preparative continuous-elution SDS-PAGE apparatus (Bio-Rad model 491) according to the published procedure (Gentile *et al.*, 1997). A discontinuous gel with an annular cross-section was prepared in a cylindrical assembly (diameter, 3.5 cm). The 50 ml separating gel containing 11% total acrylamide (6.3 cm high) was topped

with a 10 ml stacking gel containing 4% total acrylamide. The products from digestion of Tg (30 mg) with thermolysin were loaded onto a stacking gel, and developed in electrophoresis buffer (0.025 M Tris, 0.19 M glycine, 0.1% SDS, pH 8.2). Electrophoresis was carried at 60 mA (finally, 32 mA). Collection was started as soon as the tracking dye began to exit from the gel; 2.5 ml per tube was collected, with the pump flow rate set at 20 ml/h. The fractions were analyzed by SDS-PAGE. Fractions containing 50 kDa polypeptide were pooled and concentrated by lyophilization, freed from Tris/HCl and glycine by filtration through Sephadex G-25 column (1 × 10 cm) in distilled water, and from SDS by filtration through Extracti-gel resin (Pierce) in distilled water. The sample was lyophilized and stored at 4°C.

Reduction and carboxymethylation of polypeptide 50 kDa

Purified polypeptide 50 kDa was dissolved in 500 µl of 0.3 M Tris/HCl, pH 8.0, containing 6 M guanidine HCl and 1 mM EDTA, and treated with dithiothreitol (10/1 molar excess with respect to cysteinyl residues) at 37°C for 2 h. The reduced peptide was carboxymethylated by iodoacetic acid (5/1 molar excess) with respect to total -SH groups at pH 8.0 at room temperature for 30 min in the dark. The sample was passed through a Sephadex G-25 column (1 × 10 cm) in 50 mM ammonium bicarbonate, pH 8.5, and lyophilized.

Digestion of carboxymthylated 50 kDa polypeptide with endoproteinase Asp-N

The reduced and carboxymethylated polypeptide was hydrolyzed with endoproteinase Asp-N at the enzyme/substrate ratio of 1/50 (w/w) in 50 mM ammonium bicarbonate containing 10% (v/v) acetonitrile, pH 8.5, at 37°C for 20 h. The reaction products were concentrated by lyophilization.

Separation of peptide fragments by HPLC

The peptides obtained by hydrolysis of peptide 50 kDa with endoproteinase Asp-N were fractionated by HPLC with a Vydac C-18 column (250 × 4.6 mm, 5 µm), which was equilibrated in 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent A), and then solvent A containing 4 % of 0.07% trifluoroacetic acid in acetonitrile (solvent B). The peptide derivative sample was injected, and the column was eluted at a flow rate of 1 ml/min with 4% of solvent B, and after 5 min, the elution was continued by a two-step linear gradient of solvent B percentage from 4 to 25% over 25 min, and from 25 to 60% over the following 60 min.

Preparation of a synthetic peptide, DVEEALAGK(DIT)LAGRFA

2 mg of DVEEALAGKYLAGRFA (M.W., 1709.42), which was obtained from Research Genetics (Huntville, AL) was iodinated in 50 mM phosphate buffer, pH 7.4 containing lactoperoxidase (0.1 unit), glucose oxidase (1 unit) and glucose (10 mM) according to the published procedure (Ma *et al.*, 1999). Diiodination of the peptide at tyrosine residue was confirmed by RP-HPLC and LC-ESI/MS analyses.

Mass spectrometry

ESI mass spectra of the peptides produced by hydrolysis of polypeptide 50K with endoproteinase Asp-N were recorded with a PE/SCIEX(API-365) electrospray triple quadruple LC/MS/MS spectrometer(University of Wisconsin Biotechnology center). LC/MS was performed on the PE/SCIEX instrument with LC interface on a Hypersil BDS C_{18} column (3 µm, 1 × 150 mm) using a gradient from 1 % acetonitrile with 0.07 % TFA for the first 0.1 min, then raised to 40% over the following 80 min, and to 65% over the next 20 min at a flow rate of 1.2 ml/h. Separately, the sample was analyzed by MALDI - TOF mass spectrometry. The sample (1 µl) was mixed on the plate with 1 µl of a saturated solution of cyano-4-hydroxycinnamic acid in acetonitrile/0.2% trifluoroacetic acid (70:30). MALDI-TOF mass spectra were acquired using a Brucker BIFLEX III equipped with a pulse nitrogen laser (337 nm). MALDI mas spectra were obtained using delayed extraction (DE) in the linear or reflector mode. Total ion accelerationvoltage was 19 kV, and signals from 300 to 500 laser shots were averaged to increase the S/N ratio of each mass spectrum. All mass spectra were externally calibrated using ubiquitin, bovine insulin, and ACTH clip (18-39).

Analytical techniques

The concentration of protein or peptide was estimated by the measurement of the absorbance at 280 nm and bicinchoninic acid Protein Assay (Pierce, Rockford, IL), respectively.

RESULTS

Proteolysis of bovine Tg by thermolysin

Crude thyroglobulin was obtained from bovine thyroids by buffer extraction and ammonium sulfate precipitation. As reported previously (Delom *et al.*, 1999), the chromatography of crude thyroglobulinon on Sephacryl 300 column yielded two major fractions of protein; an early appearing fraction, showing a suspension, and a later fraction. While the former exhibited a multimeric form of Tg on SDS-PAGE analysis, the latter showed a pattern typical of 19S thyroglobulin (unpublished data). The fractions of 19S form thyroglobulin were pooled, dialyzed against doubly distilled water, and freeze-dried. For the preparation of

polypeptide 50 kDa, 50 mg of bovine thyroglobulin was hydrolyzed with thermolysin at the enzyme/substrate ratio of 1/50 at pH 8.0 at 30°C for 80 min, and the hydrolysis products were analyzed by SDS-PAGE analyses under reducing conditions. Fig. 1 (left) indicates that the proteolytic peptides from thermolysin hydrolysis were separated nicely from each other, and characterized according to their electrophoretic mobilities.

Subsequently, the proteolytic fragments were separated by preparative continuous-elution SDS-PAGE, and the molecular weight of fraction of interest was determined by SDS-PAGE as shown in Fig. 1 (right). The fractions (No 51-55), corresponding to 50 kDa polypeptide, were pooled and freeze-dried. Approximately 1 mg of relatively pure polypeptide of 50 kDa was obtained, indicating that the yield of purification of polypeptide 50 kDa is approximately 2%.

Endoproteinase-Asp N-catalyzed proteolysis of carboxymethylated polypeptide 50 kDa and separation of hydrolysis products by RP-HPLC

The polypeptide 50 kDa was reduced with DTT and subsequently carboxymethylated with iodoacetic acid. The carboxymethylated peptide, which was separated from excess reagents, was digested with endoproteinase Asp-N, and the digested products were fractionated by reverse-phase HPLC on a Vydac C-18 column (250 × 4.6 mm, 5 μm). The typical chromatogram is shown in Fig. 2. Although the profile of HPLC appeared to be reproducible between repeated experiments, the separation of peptides was not satisfactory under the condition used. This led to the necessity of LC/ESI-MS analyses, combined with MALDI-TOF, and rather than ES/MS analyses for the identification of components of each peak. Each peak or each fraction of more than two peaks was freeze-dried, and stored at below -15°C.

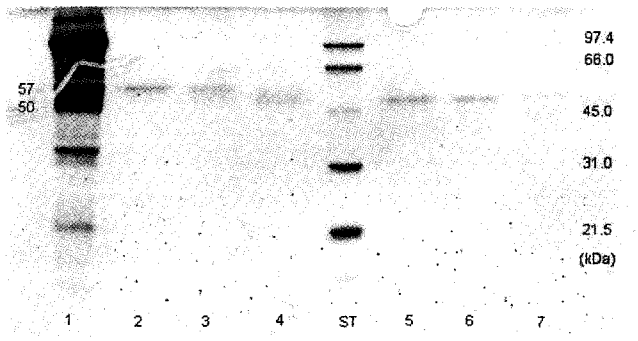

Fig. 1. SDS-PAGE of the fractions from preparative SDS-PAGE of the digested thyroglobulin. The products from the digestion of thyroglobulin with thermolysin were subjected to continuous elution preparative SDS-PAGE, and 2 ml fractions were collected at a flow rate of 12 ml/h. The aliquot of each fraction was subjected to 10 % acrylamide SDS-PAGE under reducing condition. **1**, proteolytic peptides; **2**, fraction 67; **3**, fraction 63; **5**, fraction 55; **6**, fraction 51; St, molecular standards.

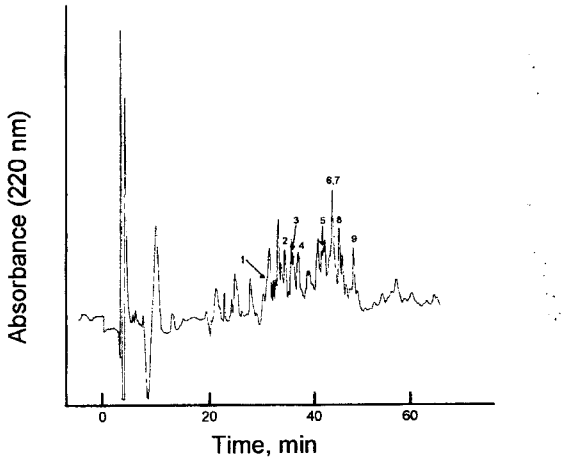

Fig. 2. RP-HPLC of peptides from digestion of carboxmethylated 50 kDa polypeptide. Peptide sample, produced from proteolysis of 50 kDa polypeptide by endoproteinase Asp-N, was injected into Nova-Pak C_{18} cartridge (0.8 × 10 cm), which was eluted with the gradient concentration of 0.1% TFA and acetonitrile (0.07% TFA) at a flow rate of 1 ml/min.

Analyses of carboxymethylated peptides by LC/ESI-MS or MALDI-TOF mass spectrometry

The respective sample from each fraction, after freeze-drying, was subjected to the analysis by MALDI-TOF or LC/ESI-MS, and the mass spectrum of each sample was analyzed in comparison with the data of mass spectra already published (Gentile *et al.*, 1997). First, the mass, corresponding to the theoretical mass (1732.9) of the peptide fragment **1** (DALGCVKCPEGSYFQ) containing a thioredoxin box (CVKC) and a tyrosine residue, was examined among peptide fragments containing specific cleavage sites at aspartic acid (Fig. 3), which is generated from the selective proteolysis of thyroglobulin by endoproteinase Asp N. When each fraction was subjected to MALDI TOF mass spectrum analysis (Fig. 4A), the sample from fraction 1, composed of several compounds, was observed to exhibit the protonated molecular ion (MH^+, 1733.6) expected for the peptide fragment **1** (DALGCVK CPEGSYFQ). Next, when the same sample was analyzed by LC-ESI/MS, it was observed that double charge component ion (867.8) and MH^+ (1734.0) were present in the spectrum of the corresponding component peak, as shown in Fig. 5A. By computational calculation, the mass value was estimated to be 1732.8. This value was in good agreement with that expected for peptide 1438-1452, produced by endoproteinase Asp-N by a specific cleavage at Asp-1438 and Asp-1453, based on the cDNA-derived sequence (Gentile *et al.*, 1997). In an attempt to investigate the possible iodination of tyrosine residue in peptide

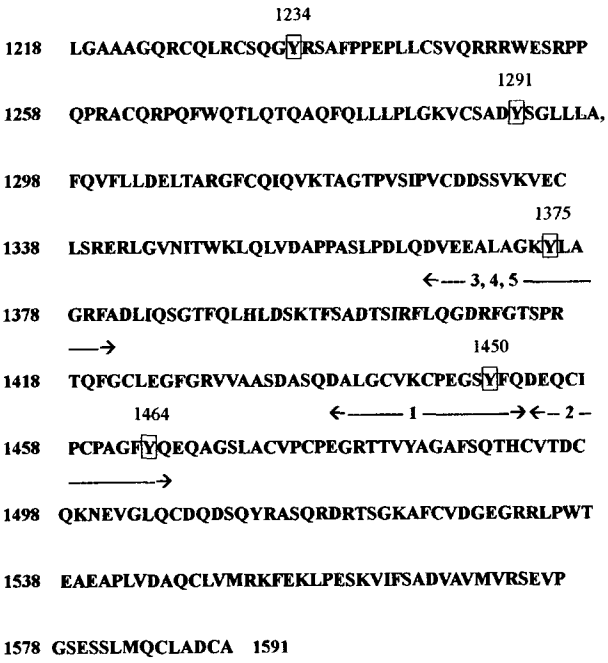

Fig. 3. Map of amino acid sequence of 50 kDa polypeptide (1298-1417). Peptide **1** and **2**, fragment with thioredoxin box; peptide **3**, **4** and **5**, fragment with tyrosine, monoiodotyrosine and diiodotyrosine residue, respectively.

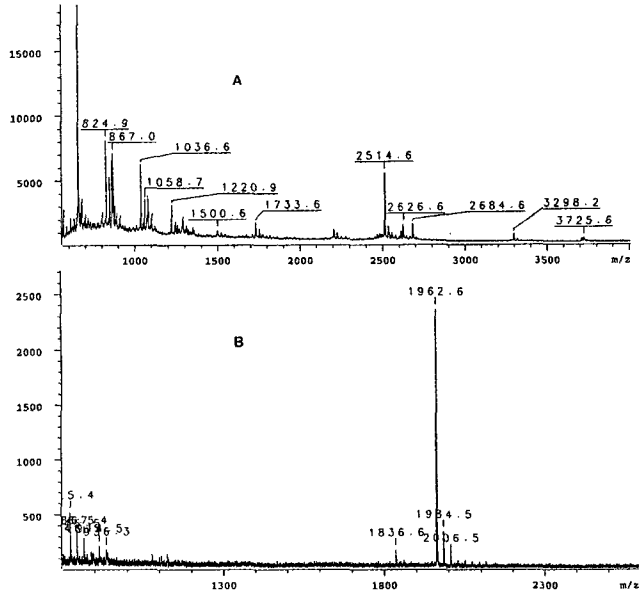

Fig. 4. MALDI- mass spectra of peptides. Fraction 1 (A) and fraction 9 (B), shown in Fig 2, were subjected to MALDI-TOF mass analysis as described in Materials and Methods.

1438-1452, the molecular mass compatible with that expected for the same peptide, in which one atom (+126) or two atoms (+252) of iodine had been added to Tyr-1450, was examined among all fractions of peptide fragments. However, any mass value, indicating the existence of a monoiodotyrosine (mass value, 1859) or a diiodotyrosine (mass value, 1985) residue at position 1450 was not detected in the whole fractions, suggesting that the tyrosine residue close to the thioredoxin box (peptide 1442-1445) was not modified by oxidative iodination. Next, the mass value corresponding to the theoretical mass (1586.8) of the peptide peptide (1453-1465) fragment **2** (EQCIPCPAGFYQ) containing a thioredoxin box (CIPC) and a tyrosine residue, which was produced from hydrolysis of thyroglobulin by endoproteinase Asp-N through a cleavage at Asp-1453 and Glu-1466, was examined among all fractions (Fig. 3). As shown in Table I, the mass value (1586.0), expected for the peptide fragment **2**, was observed with fraction 2. Meanwhile, the mass value, compatible with that expected for the same peptide, in which one or two iodine atoms had been added to Tyr-1464, was not found among other fractions despite repeated experiments. Thus, it is suggested that the tyrosine residue in the thioredoxin box (peptide 1466-

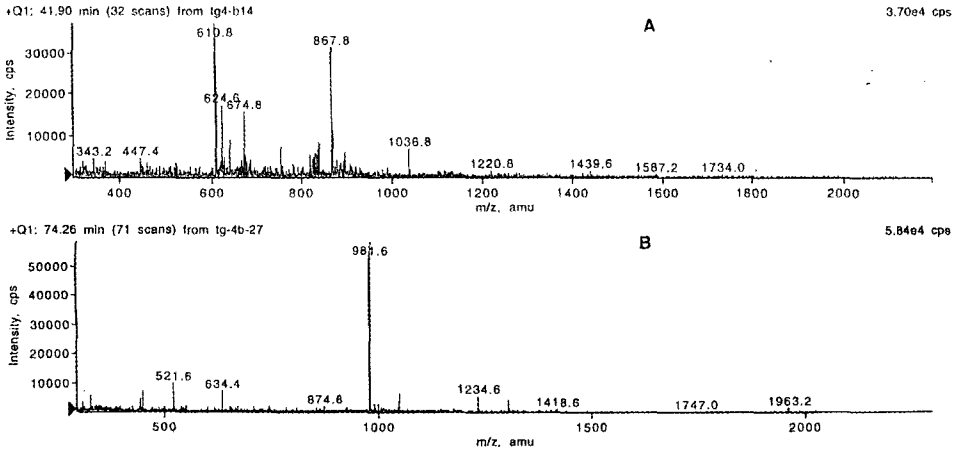

Fig. 5. ESI/MS spectra of peptides. Fraction 1 (A) and fraction 9 (B), obtained in Fig 2, were subjected to LC-ESI/MS mass analysis as described in Materials and Methods.

Table I. Analyses by ESI/MS of the products from digestion of reduced and alkylated peptide 50 kDa with endoproteinase Asp-N

Fraction No	Measured mass	peptide	Theoretical mass	Type of residue
1	1732.8	1438-1452	1732.9	Tyr-1450
	1599.0	1290-1303	1599.0	Tyr-1291
2	1586.0	1453-1465	1586.8	Tyr-1464
3	1850.4	1290-1303	1850.7	DIT-1291
4	4293.6	1355-1393	4294.4	MIT-1375
	4422.0	1355-1393	4420.3	DIT-1375
5	1709.4	1366-1381	1709.8	TYR-1375
6	4262.9	1218-1252	4262.6	MIT-1234
7	4390.1	1218-1252	4388.7	DIT-1234
8	1835.3	1366-1381	1835.8	MIT-1375
9	1961.7	1366-1381	1961.7	DIT-1375

*MIT, monoiodotyrosine; DIT, diiodotyrosine

1469) region may also be resistant to oxidative iodination *in vivo* system.

In the subsequent experiment, we turned to the modification of tyrosine residues in hormonogenic region. In this study, we examined the possible iodination of tyrosine residue in the hormonogenic donor-containing peptide (1366-1381), DVEEALAGKYLAGRFA, since this tyrosine residue had been reported to be highly susceptible to oxidative iodination. First, the mass values corresponding to the same peptide (1366-1381) with diiodotyrosine (+252) residue, produced by endoproteinase Asp-N through a specific cleavage at Asp-1366 and Asp-1382, was investigated among all fractions. As exhibited in Fig. 4B, fraction 9 showed the predominant existence of m/z 1962.6, compatible with a protonated molecular ion expected for the peptide **5** in which two iodine atoms (+252) had been added to Tyr-1375. ESI/MS data (Fig. 5B) of the same sample showed two ions; double charge component ion (981.6) and MH^+ (1963.2). From computational calculation, the mass value was estimated to be 1961.7, which corresponds to the theoretical value (Table I) for a peptide, [DVEEALAGK(DIT)LAGRFA, DIT=diiodotyrosine]. In comparative study, where the peptide from fraction 9 was compared with the corresponding synthetic peptide [DVEEALAGK(DIT)LAGRFA], it was found that the peptide (fraction 9) was the same as the synthetic specimen in the chromatographic behavior and mass spectra (unpublished data). In the similar way, the mass value corresponding to the same peptide **3** or **4** (1366-1381) with tyrosine residue or monoiodotyrosine (+126), was examined among all fractions. In the MALDI-TOF analyses of each group of several peaks, it was found (Fig. 6A) that the pooled sample of fractions (42-46 min) contained the protonated molecular ion (MH^+) of around 1710 and that of 1836.8, implying the presence of two peptides, DVEEALAGKYLAGRFA (1366-1381), and [DVEEALAGK(MIT)LAGRFA, MIT = monoiodotyrosine]. The MALDI-TOF analysis of each peak indicates that the mass of 1710 is from fraction 5, and the mass of 1836.8 from fraction 8. Further study employing LC-ESI/MS (Fig. 6B) provides the positive evidence that fraction 5 contains a peptide, DVEEALAGKYLAGRFA, based on the calculated mass value (1709.4); double charge component ion (856.2) and MH^+ (1710). In comparative study, it was observed that the chromatographic behavior of the peptide (fraction 5) was the same as that of

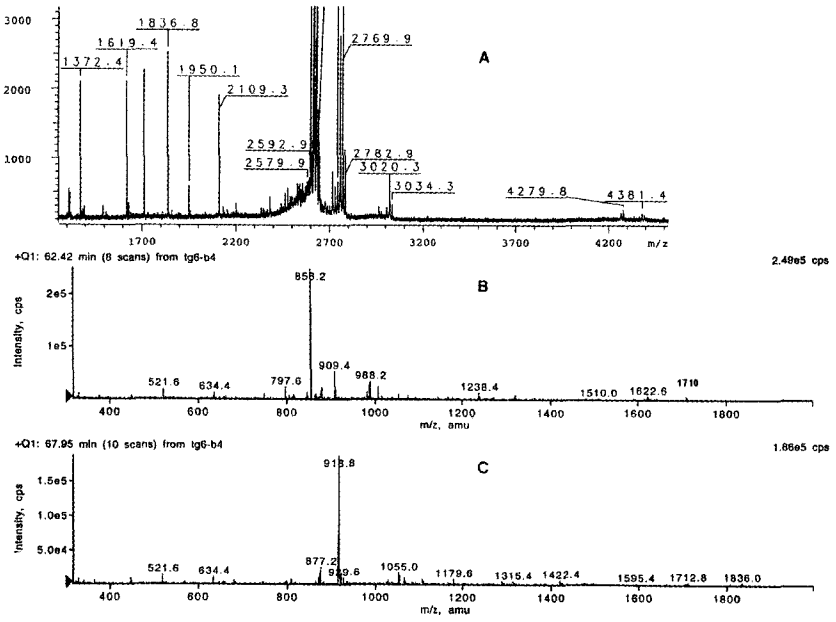

Fig. 6. Mass spectra of peptides. MALDI-TOF mass spectra of peptides from total fractions (A) between 42 and 46 min in RP-HPLC (Fig. 2), and ESI/MS spectra of fraction 5 (B) and fraction 8 (C).

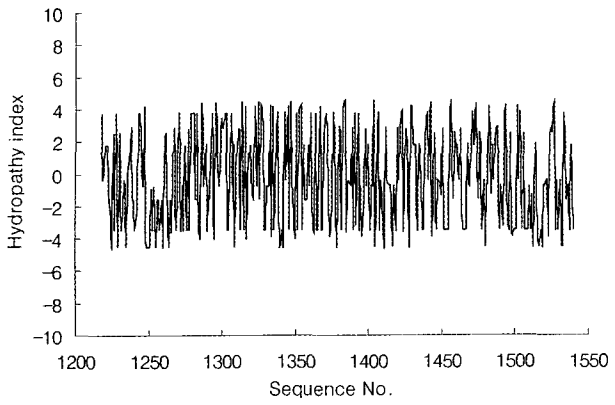

Fig. 7. Hydropathy profile of 50 kDa polypeptide (1298-1417)

synthetic specimen (DVEEALAGKYLAGRFA). The separate LC-ESI/MS analysis (Fig. 6C) of fraction 8 showed the presence of double charge component ion (918.8), and single charge component ion (MH^+, 1836.0). The calculated mass value was estimated to be 1835.3, corresponding to the mass value for [DVEEALAGK(MIT)LAGRFA, MIT = monoiodotyrosine], which is compatible with the addition of one iodine atom (+126) to Tyr-1375. Noteworthy, the above two iodinated peptides, peptide **4** and **5** from fraction 8 and 9, respectively, were reproducibly observed in repeated experiments. In comparison, the order for the relative amount of three derivatives at Tyr-1375 was diiodotyrosine derivative>monoiodotyrosine derivative> tyrosine derivative. Thus, it seems that Tyr-1375 is favorably exposed to oxidative iodination in vivo system. Subsequently, the iodination of Tyr-1234, a hormonogenic acceptor site, was investigated. It was confirmed from LC-ESI/MS analyses (Table I) that fraction 6 contained the peptide with a mass value of 4262.9, corresponding to the value expected for a peptide (1218-1252) containing monoiodotyrosine at Tyr-1234. Similarly, fraction 7 was found to contain a peptide with a mass value of 4390.1, compatible with the value expected for a peptide (1218-1252) containing diiodotyrosine at Tyr-1234. In an attempt to examine the chemical property of the polypeptide region around tyrosine residues, the hydropathy profile of 50 kDa polypeptide was prepared. As demonstrated in Fig. 7, it seems that the polypeptide region around thioredoxin boxes is somewhat more hydrophilic than that around the hormonogenic sites. Thus, the hydrophilicity in polypeptide region may not promote the susceptibility of tyrosine residues to peroxidase-catalyzed oxidative iodination.

DISCUSSION

Earlier, it had been reported that thyroglobulin was subjected to post-translational modifications such as sulfation, phosphorylation, iodination and addition of carbohydrates (Gentile *et al.*, 1995). In particular, bovine thyroglobulin contains 18 tyrosine residues, some of which are known to be modified by oxidative iodination (Taurog, 1996). Thus far, it has been of much interest to see whether there is a different susceptibility to oxidative iodination between a hormonogenic tyrosine and a non-hormonogenic tyrosine residue (Chernoff and Rawitch, 1981; Gentile, 1995). In the present study, we examined the iodination of tyrosyl residues in 50 kDa polypeptide fragment (1218-1591) of bovine thyroglobulin, which is characterized by the existence of seven tyrosine residues and triply-conserved thioredoxin (CXXC) boxes. Preparative continuous SDS-PAGE under reducing condition was necessary for the separation of proteolytic fragments of thyroglobulin bearing many disulfide bonds. The preparation of 50K polypeptide was reproducible in repeated experiments, and the purification yield amounts to approximately 2%. Thus, it is assumed that the proteolysis of thyroglobulin by thermolysin is relatively selective and complete. In the meantime, the hydrolysis of carboxymethylated 50 kDa polypeptide by endoproteinase Asp-N was incomplete even either after longer (>18 h) incubation or with excess amount of enzyme; sometimes, the proteolysis results in a partial aggregation of peptide, which is supposed to be mediated by nonpolar binding, since the aggregated material could be dissolved by the addition of SDS. Moreover, the aggregation seemed to be enhanced by guanidium salt, which was included for the S-alkylation of protein. Previously (Gentile *et al.*, 1997), the identification of hormonogenic sites by the sequencing of hormone-rich peptides of bovine Tg was performed by a ES/MS and FAB/MS spectrometry. In our studies, LC/ESI-MS, in combination with MALDI mass spectrometry, was employed for the detection of oxidative iodinatin of tyrosine residues present in 50 kDa polypeptide. Actually, since each peak in HPLC was composed of a few compounds, MALDI-MS technique was very useful for the prognosis of expected mass values. In this study, the oxidative iodination of Tyr-1234, Tyr-1291 and Tyr-1375 was confirmed as reported previously (Gentile *et al.*, 1997). Especially, the peptide with diiodotyrosine residue at 1375, appearing in the fractions 8 and 9, was found to be the most prominent among the iodotyrosine-containing peptides, suggesting that the tyrosine residue at Tyr-1375 was the most susceptible to the oxidative iodination by thyroid peroxidase in vivo system. In addition, Tyr-1234 and Tyr-1291 were also modified by oxidative iodination, although to a lower extent than Tyr-1375. In the meantime, despite the extensive study on the possibe iodination of tyrosine residues, Tyr-1450 and Tyr-1464, adjacent to thioredoxin boxes such as CVKC or CIPC were not modified by oxidative iodination, indicating that the tyrosine residue in thioredoxin region may not be exposed to posttranslational oxidative iodination. There-

fore, it is supposed that the amino acid sequence around the tyrosine residue might contribute to govern the susceptibility of tyrosine residue to the oxidatve iodination. Resistance of the tyrosine residue in thioredoxin region to oxidative iodination might be explained by the assumption that thyroid peroxidase may not associate with the polypeptide region containing thioredoxin boxes, which may be different from the region containing the hormonogenic site. In support of this, the hydropathy plot of 50 kDa polypeptide shows that the region containing three thioredoxin boxes, far away from the T4-forming couple (Tyr-1234 and Tyr-1375), is relatively more hydrophilic than the hormonogenic region in 50 kDa polypeptide. Alternatively, the tyrosine residues in thioredoxin region may be protected from the thyroid peroxidase-generated HOI, which was observed to be a diffusible modifier of tyrosine residue in other protein (Seo *et al.*, 1999). This might be supported by the recent report (Klein *et al.*, 2000) that the association of thyroglobulin monomer through thioredoxin boxes may result in the formation of thyroglobulin dimer or multimers, following PDI activity - mediated formation of disulfide bonds in thioredoxin boxes. In addition, the thioredoxin box region may exert an antioxidant action against the oxidative iodination. Presumably in support of this supposition, it had been proposed that the tyrosine radicals in thyroglobulin molecule was continuously utilized for the oxidation of thiol, glutathione, catalyzed by thyroid peroxidase (Nakamura *et al.*, 1989). In further support, thyroglobulin molecule expressed some antioxidant action in Cu^{2+}- catalyzed oxidation of thyroglubulin (Lee and Sok, 2000). These observations may be consistent with the notion that the susceptiblity of tyrosine residues to oxidative modification may differ according to the physico-chemical property of the region where the tyrosine residue exists.

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