Thyrotropin-Releasing Hormone-Degrading Enzyme in Human Serum Is Classified as Type II of Pyroglutamyl Aminopeptidase: Influence of Thyroid Status (43107)

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Abstract. **The characteristics of thyrotropin-releasing hormone (TRH)-degrading enzyme in human serum were studied. Serum was incubated in 0.1 *M* phosphate buffer containing [*proline*-^{3}H]TRH at 37°C. A thin layer chromatography analysis of TRH degradation did not show any radioactive peak located in an acid TRH position, but apparent radioactive peaks corresponding to His-Pro and His-ProNH$_2$ occurred in the presence of *p*-hydroxymercuriphenyl sulfonic acid, an inhibitor of proline dipeptidase. With ion exchange paper chromatography, the formation of ^{3}H-labeled His-Pro and His-ProNH$_2$ was estimated as an end point in the measurement of pyroglutamyl aminopeptidase (pGlu-peptidase) activity. An assay using *p*-hydroxymercuriphenyl sulfonic acid was developed to sensitively quantitate the pGlu-peptidase. Neither bacitracin nor *p*-chloromercuribenzoic acid increased the activity of pGlu-peptidase. The addition of EDTA, dithiothreitol, and *o*-phenanthroline significantly inhibited pGlu-peptidase activity, but neither iodoacetamide nor ethylmaleimide altered its activity. The pGlu-peptidase had a stereotypic specificity for the tripeptide, pGlu-His-ProNH$_2$ of TRH, and its K_m was 44.9 μM. The pGlu-peptidase activity was not changed by either hyper- or hypothyroidism. The present data indicate that a TRH-degrading enzyme in human serum possesses a nature identical to type II of pGlu-peptidase which is not altered by thyroid status.**

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Thyrotropin-releasing hormone (TRH) is composed of a tripeptide with the sequence, pGlu-His-ProNH$_2$, and is hydrolyzed by enzymes which are implicated in its physiologic functions (1). The initial step in the breakdown of TRH in the brain involves two kinds of enzymes, pyroglutamyl aminopeptidase (pGlu-peptidase) to produce His-ProNH$_2$ and post-proline cleaving enzyme yielding pGlu-His-Pro (acid TRH) (2). Although early observations (3) on the TRH degradation in serum showed that acid TRH was the major metabolite formed, failure to form a significant amount of acid TRH has been found (4–6). In contrast, serum contains a distinct enzyme with a high specificity for the pGlu-His bond of TRH.

Recent observations have shown the possible existence of two different types of pGlu-peptidase: type 1 pGlu-peptidase closely resembles the bacterial enzyme which hydrolyzes many pGlu-containing substances, has a nature featured by a need for EDTA and dithiothreitol (DTT), and is inactivated by iodoacetamide and ethylmaleimide; type II enzyme has a narrow specificity for cleaving the pGlu-His bond of TRH and its activity is inhibited by EDTA, DTT, and *o*-phenanthroline (7, 8). Moreover, there is a different K_m for TRH in these two types of enzymes, i.e., the K_m value of type I (230–400 μM) is larger than that of type II (40–56 μM) (8). Type I pGlu-peptidase exists mainly in the brain cytosol and anterior pituitary, whereas the type II enzyme is distributed in the brain and retinal plasma membranes (7–9). The TRH-degrading enzyme in rat serum is not distinguishable from the nature of type II pGlu-peptidase (10). There are different characteristics between rat and human sera with respect to TRH degradation; no significant effect of thyroid states on TRH degradation in human serum was observed,

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in contrast to the marked effect seen in rat serum (1, 4, 6, 11). Moreover, limited information has been available concerning which type of pGlu-peptidase is responsible for TRH degradation in human serum. The present study was designed to increase the sensitivity for measurement of pGlu-peptidase activity by adding *p*-hydroxymercuriphenyl sulfonic acid (*p*HMSA), an inhibitor of proline dipeptidase (12), and evaluate whether human serum possesses a type II pGlu-peptidase activity which can be altered by thyroid status.

Materials and Methods

*p*HMSA, bacitracin, EDTA, DTT, and iodoacetamide were purchased from Sigma Chemical Co., St. Louis, MO. *p*-Chloromercuribenzoic acid (*p*CMBA), hydroxymercuri-benzoic acid sodium salt (HMBS), *o*-phenanthroline, 8-hydroxyquinoline, ethylmaleimide, and pGlu were purchased from Wako Chemical Co., Osaka, Japan. Benzamidine hydrochloride was obtained from Tokyo Kasei Industrial Co., Tokyo, Japan. TRH, acid TRH, pGlu-His-Pro-Gly, Glu-His-ProNH$_2$, His-Pro, His-ProNH$_2$, and cyclo(His-Pro) were supplied by Tanabe Pharmaceutical Co., Osaka, Japan. Pro and ProNH$_2$ were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. pGlu-His-Pro-GlyNH$_2$, pGlu-His-Gly, and luteinizing hormone-releasing hormone (LHRH) were purchased from Peninsula Laboratories, Inc., Belmont, CA. [*Pro*-2,3,4,5-^{3}H]TRH was purchased from New England Nuclear, Boston, MA (specific activity = 106.3 Ci/mmol). Human blood samples were centrifuged at 1500*g* for 20 min, and the sera were used for experiments.

Thin Layer Chromatography (TLC) Analysis of TRH Degradation. TLC was used to estimate TRH, acid TRH (pGlu-His-Pro), His-Pro, Pro, ProNH$_2$, and cyclo(His-Pro). Ten microliters of human serum were incubated in 20 μl of phosphate buffer (0.1 *M* KH_2PO_4, pH 7.4) containing 200 μM *p*HMSA at 37°C for 10 min, followed by the addition of 10 μl of 20 pmol of [^{3}H]TRH, which was previously purified by TLC in the same solvent system as described below. After incubation at 37°C for 50 min, 10 μl of chilled 99% methanol were added to terminate the reaction. Twenty microliters of each mixture were applied on a silica gel-60 TLC plate (#5553; Merck, Darmstadt, Federal Republic of Germany), and developed in an ascending system ($CHCl_3$:CH_3OH:29% NH_4OH = 140:60:20, v/v/v). Plates (the type of plastic) were cut out with scissors and extracted in 99% methanol followed by centrifugation at 1500*g* for 20 min. The supernatants were transferred into scintillation vials containing Aquasol 2 (New England Nuclear). Radioactivity was measured with an Aloka scintillation spectrometer with automatic quenching controller.

Ion Exchange Paper Chromatography Analysis of TRH Degradation. Ion exchange paper chromatography was used to separate His-Pro and His-ProNH$_2$ from other degrading products of TRH. [^{3}H]TRH was incubated with human serum in the presence of *p*HMSA as described above, and the reaction was terminated with methanol. According to the method described by Bauer and Nowak (13, 14), 50 μl of mixture were applied to ion exchange paper chromatography (P81; Whatman Ltd., Maidstone, England) and developed in 1 *M* acetic acid. Neither TRH, acid TRH, cyclo(His-Pro), Pro, nor ProNH$_2$ is located in a start segment (the first three peptides visualized by Pauly's reagent and the last two peptides by ninhydrin reaction). The data are compatible with the previous observations (13, 14). The start segments containing His-Pro and His-ProNH$_2$ were separated and extracted twice in 90% methanol. After centrifugation at 1500*g* for 10 min, the supernatant was evaporated to dryness and dissolved in 20 μl of distilled water. Then it was analyzed by TLC as described above.

Formation of cyclo(His-Pro). Because pGlu-peptidase hydrolyzes TRH to yield His-ProNH$_2$ which is easily, nonenzymatically cyclized to produce cyclo(His-Pro) (15), formation of cyclo(His-Pro) from TRH in human serum was estimated as described previously (15). As described above, 20 pmol of [^{3}H]TRH were incubated with human serum at 37°C for 30 min. Acetic acid (5 *N*) was added to terminate the reaction. After adding 1.4 μmol of unlabeled cyclo(His-Pro) to each tube, the reaction mixture was applied on a TLC, heated at 95°C for 30 min, and developed in $CHCl_3$:CH_3OH (5:2, v/v). Cyclo(His-Pro) was visualized by spraying with saturated with I_2 in $CHCl_3$, and a spot was scraped out and placed into a scintillation vial containing Aquasol 2.

Detection of pGlu-Peptidase Activity in Human Serum. The formation of ^{3}H-labeled His-Pro and His-ProNH$_2$ from [^{3}H]TRH was estimated as an end point in measuring pGlu-peptidase activity. Routinely, 10 μl of human serum were preincubated in 20 μl of 0.1 *M* phosphate buffer containing *p*HMSA (200 μM as the final concentration) and/or several inhibitors at 37°C for 10 min and further incubated in 10 μl of 2 pmol of [^{3}H]TRH at 37°C for 30 min. Ten microliters of chilled 99% methanol were added, and the reaction tube was placed in ice. Ten microliters of each reaction mixture were applied to ion exchange paper chromatography as described above. The start segments containing His-Pro and His-ProNH$_2$ were cut out, and the radioactivity of TRH degradation products was determined.

Estimation of K_m of pGlu-Peptidase in Human Serum. After serum was incubated in phosphate buffer containing *p*HMSA at 37°C for 10 min, it was incubated with varying doses of [^{3}H]TRH at 37°C for 30 min. Methanol was added and a reaction mixture was developed with ion exchange paper chromatography as described above.

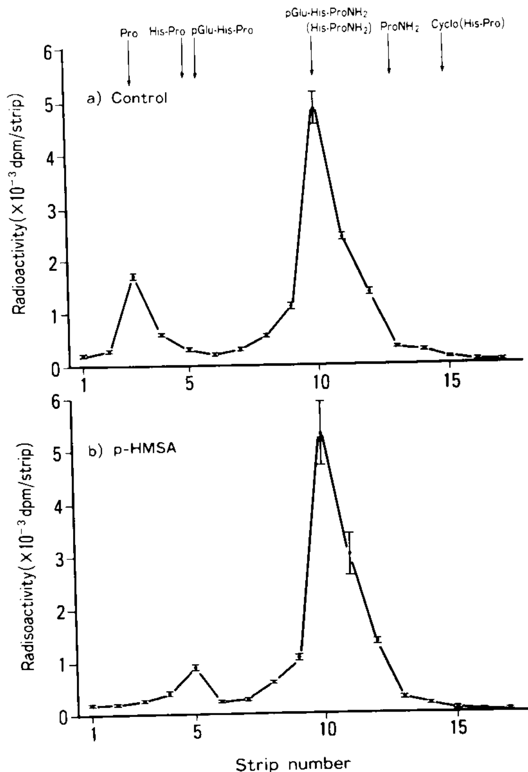

Figure 1. A TLC analysis of TRH degradation. Human serum was incubated with [^{3}H]TRH in the presence or absence of 200 μM *p*HMSA at 37°C for 50 min and subjected to TLC in a solvent of $CHCl_3$:CH_3OH:NH_4OH (140:60:20, v/v/v) as described in Materials and Methods. Plates were cut out and radioactivity was measured. Peptides were visualized by Pauly's and ninhydrin reagents. The data are expressed as the mean ± SE. Three samples were used in each group. (a) Control group. (b) *p*HMSA-added group.

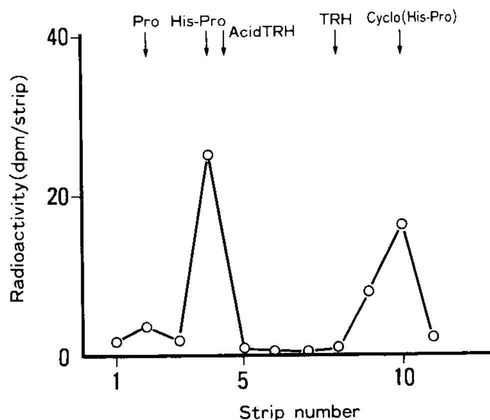

Figure 2. A TLC analysis of methanol extracts from the start segments of ion exchange paper chromatography. After serum was incubated with [^{3}H]TRH in the presence of 200 μM *p*HMSA, the incubation mixture was subjected to ion exchange paper chromatography in a solvent system of 1 *M* acetic acid. The start segment was extracted in 90% methanol. The extract was evaporated to dryness, dissolved in water, and analyzed by TLC as described in Figure 1. The plates were cut out and radioactivity was determined. Peptides were visualized with Pauly's and ninhydrin reagents. Each point was expressed as a result of duplicate determinations.

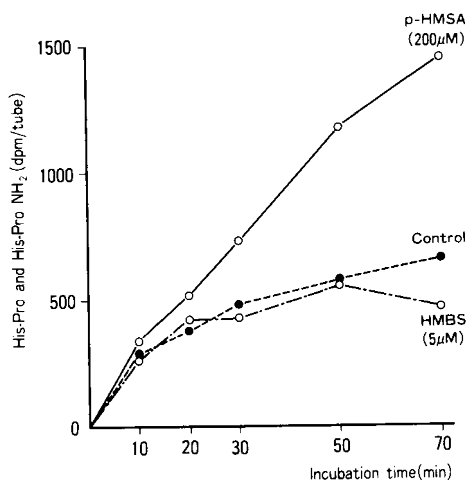

Figure 3. Time course of pGlu-peptidase activity in human serum. Human serum was incubated with [^{3}H]TRH in the presence or absence of 200 μM *p*HMSA or 5 μM HMBS at 37°C as described in Materials and Methods. After the end of each incubation, chilled 99% methanol was added, and ^{3}H-labeled His-Pro and His-ProNH_2 were determined by ion exchange paper chromatography. The data are expressed as a representative result of three replications. Two samples were used in each group.

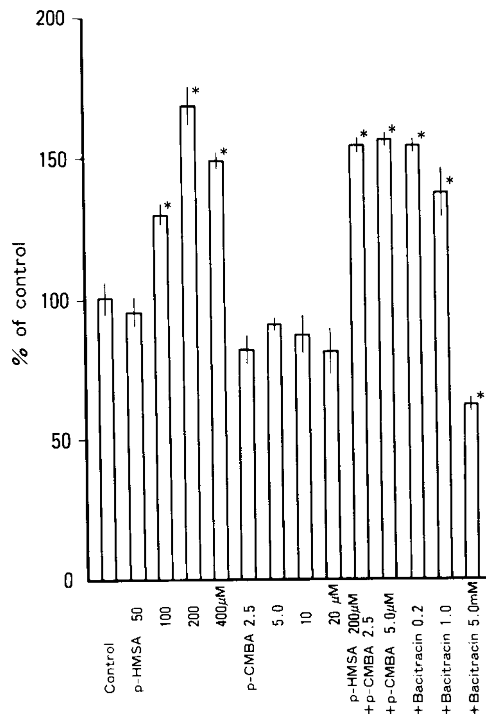

Figure 4. Effects of *p*HMSA on pGlu-peptidase. Serum was incubated with [^{3}H]TRH in the presence of varying doses of *p*HMSA, *p*CMBA, and bacitracin at 37°C for 30 min. The pGlu-peptidase activity was determined as described in Materials and Methods. The data are expressed as percentage of the control enzyme activity with the mean ± SE. Three samples were used in each group. *$P < 0.05$ differs from the control group.

Table I. Effects of Enzyme Inhibitors and TRH Analogues on pGlu-Peptidase Activity in Human Serum[a]

Enzyme inhibitors	n	% of control activity
Control	6	100.0 ± 6.9
EDTA	6	65.3 ± 9.6[b]
o-Phenanthroline	5	57.5 ± 7.6[b]
8-Hydroxyquinoline	6	98.1 ± 26.5
Iodoacetamide	6	104.8 ± 16.3
Ethylmaleimide	5	91.1 ± 12.6
Benzamidine	5	94.6 ± 16.8
*p*CMBA	4	103.2 ± 21.4
DTT	5	60.0 ± 11.4[b]
DTT + EDTA	3	57.9 ± 8.4[b]
DTT + EDTA + *o*-phenanthroline	6	54.4 ± 4.4[b]

TRH analogues	n	% of control activity
Control	4	100.1 ± 6.9
pGlu-His-ProNH$_2$ (TRH)	4	25.5 ± 9.6[b]
pGlu-His-Pro (acid TRH)	4	37.5 ± 3.1[b]
pGlu-His-Pro-Gly	4	28.9 ± 16.0[b]
pGlu-His-Pro-GlyNH$_2$	4	54.3 ± 10.9[b,c]
pGlu-His-Gly	4	103.2 ± 2.9[c]
pGlu-His-Trp-Ser-Tyr--- (LHRH)	4	26.0 ± 6.5[b]
Glu-His-ProNH2	4	95.2 ± 6.4[c]
γ-Butyrolactone-γ-carbonyl-His-ProNH$_2$ (DN1417)	4	97.9 ± 6.2[c]
pGlu	4	102.0 ± 1.0[c]

[a] Human serum was incubated with [^{3}H]TRH in the presence of each inhibitor (1 m*M*) or analogue (100 μM) at 37°C for 30 min. The pGlu-peptidase activity was determined as described in Materials and Methods. The data are expressed as percentage of the control enzyme activity with the mean ± S.E.
[b] $P < 0.05$ differs from the control group.
[c] $P < 0.05$ differs from the TRH-added group.

Evaluation of pGlu-Peptidase in Patients with Thyroid Dysfunction. The blood samples were obtained from normal subjects, patients with hyper- and hypothyroidism. They were centrifuged at 1500*g* for 20 min at 4°C and stored at −20°C until assay. Thyroxine (T_4), triiodothyronine (T_3), and thyroid-stimulating hormone (TSH) in sera were measured with commercially available kits (Dainabot's radioimmunoassay kits for T_4 and T_3 and Daiichi's radioimmunometric radioimmunoassay kit for TSH).

Statistical significance was analyzed with Duncan's multiple range test.

Results

Estimation of TRH Degradation Products by TLC. As shown in Figure 1, two radioactive peaks were found on TLC. The first radioactive peak corresponded to Pro and the second peak to TRH and His-ProNH$_2$. No radioactive peak was observed in acid TRH ($R_f = 0.29$). The addition of *p*HMSA led to the appearance of a radioactive peak corresponding to His-Pro ($R_f = 0.24$) instead of Pro. The data indicate that *p*HMSA acted as an inhibitor of proline dipeptidase in human serum (12).

Detection of His-Pro and His-ProNH$_2$. Figure 2 shows the TLC analysis of methanol extracts from the start segments of ion exchange paper chromatography. The first radioactive peak was recognized as His-Pro and the second as cyclo(His-Pro). Because cyclo(His-Pro) was not found in the start segment in the present system using ion exchange paper chromatography and His-ProNH$_2$ is easily cyclized to produce cyclo(His-Pro) (15), the radioactivity corresponding to cyclo(His-Pro) appeared to result from the conversion of ^{3}H-labeled His-ProNH$_2$ to [^{3}H]cyclo(His-Pro).

Effect of *p*HMSA on Cyclo(His-Pro) Formation in Human Serum. Because His-ProNH$_2$ which results from TRH degradation is nonenzymatically cyclized to produce cyclo(His-Pro), we measured cyclo(His-Pro) formation as described in the Materials and Methods. The addition of *p*HMSA (200 μM) slightly, but significantly, increased cyclo(His-Pro) formation (the control group, 6.52 ± 0.13, $n = 12$ versus the *p*HMSA-added group, 8.11 ± 0.64 cyclo(His-Pro) formed fmol/30 min/tube, $n = 12$, $p < 0.05$), but the amount was only 0.04% of the original concentration of [^{3}H]TRH. Bacitracin (1 m*M*) was not observed to stimulate cyclo(His-Pro) formation in human serum (6.76 ± 0.38 cyclo(His-Pro) formed fmol/30 min/tube, $n = 12$).

Effect of *p*HMSA on pGlu-Peptidase Activity. Figure 3 depicts the time-dependent formation of His-Pro and His-ProNH$_2$ which indicates the activity of pGlu-peptidase. The assay sensitivity to detect pGlu-peptidase in the presence of *p*HMSA progressively increased with the period of incubation. After 70-min incubation, the sensitivity to detect pGlu-peptidase activity was 3.5 times as great in the *p*HMSA-added group as in the control group. The conversion ratio of [^{3}H] TRH into ^{3}H-labeled His-Pro and His-ProNH$_2$ was 1.38% 30 min after incubation with 200 μM *p*HMSA. The addition of HMBS, a drug inhibiting post-proline cleaving enzyme, did not stimulate the pGlu-peptidase activity. The serum that was previously heated at 63°C for 30 min did not significantly degrade [^{3}H]TRH (data not shown here). As shown in Figure 4, *p*HMSA caused a dose-dependent increase in the pGlu-peptidase activity (the 200 μM *p*HMSA-added group, 168.1 ± 7.1% of the control activity), but *p*CMBA or bacitracin, drugs inhibiting post-proline cleaving enzyme, did not significantly increase the pGlu-peptidase activity. The addition of 5 m*M* bacitracin caused a significant decrease in the *p*HMSA-stimulated activity of pGlu-peptidase (62.1 ± 2.3% of the control activity). The data are in agreement with the previous observations (13) that this

Table II. Effects of Thyroid Status on pGlu-Peptidase Activity[a]

Group	T_4	T_3	TSH	pGlu-peptidase
Normal persons (n = 5)	8.8 ± 1.3	1.43 ± 0.21	1.51 ± 0.13	8.8 ± 0.4
Hyperthyroidism (n = 8)	23.7 ± 1.3[b]	4.75 ± 0.39[b]	<0.01	7.6 ± 1.7
Hypothyroidism (n = 5)	3.2 ± 0.5[b]	0.45 ± 0.21[b]	60.9 ± 11.3[b]	9.7 ± 1.3

[a] Sera from normal persons and patients with hyper- and hypothyroidism were incubated with [^{3}H]TRH in the presence of 200 μM *p*HMSA 37°C for 50 min. The pGlu-peptidase activity was determined as described in Materials and Methods. The data are expressed as the mean ± S.E. T_4, T_3, TSH, and pGlu-peptidase activity are shown as μg/dl, ng/ml, microunits/ml, and fmol/ml/min, respectively.
[b] $P < 0.05$ differs from the normal group.

large dose of bacitracin resulted in the inhibition of pGlu-peptidase.

Estimation of K_m of pGlu-Peptidase in Human Serum. According to a Lineweaver-Burk's plot (data not shown here), the K_m of pGlu-peptidase for TRH was calculated to be 44.9 μM.

Effect of Enzyme Inhibitors and TRH Analogues on pGlu-Peptidase Activity. As shown in Table I, EDTA, DTT, and *o*-phenanthroline significantly inhibited pGlu-peptidase activity, whereas iodoacetamide, ethylmaleimide, or benzamidine had no effect on its activity. The addition of 100 μM TRH caused a significant inhibition of the enzyme activity. A potent inhibitory effect was observed following the addition of acid TRH and pGlu-His-Pro-Gly, but the inhibitory effect of pGlu-His-Pro-GlyNH_2 was feeble and pGlu-His-Gly did not inhibit the enzyme activity. LHRH was observed to be a significant competitor for pGlu-peptidase, the data being compatible with the previous observations (13). Glu-His-ProNH_2 or DN1417 (Takeda Pharmaceutical Co., Osaka, Japan) did not inhibit the pGlu-peptidase activity.

Effect of Thyroid Status on pGlu-Peptidase Activity. As shown in Table II, neither hyper- nor hypothyroidism changed the enzyme activity.

Discussion

It is clear from the present study that acid TRH did not form during incubation of TRH with human serum and that *p*CMBA, bacitracin, or HMBS did not change pGlu-peptidase activity. These data are in agreement with the previous reports (4–6) which indicated that human serum did not contain a post-proline cleaving enzyme which was previously designated as a TRH-deamidating enzyme.

The present data demonstrate that human serum possesses pGlu-peptidase activity. Previous observations have determined Pro as an end point in determining pGlu-peptidase activity (5). Employing ion exchange paper chromatography, His-Pro and His-ProNH_2 have been also estimated to express the pGlu-peptidase activity (13, 14). However, these methodologies did not precisely focus on the subsequent changes in TRH degradation. Indeed, the linear formation of His-Pro and His-ProNH_2 from TRH was not observed in the absence of *p*HMSA as shown in the present study. In contrast, the addition of *p*HMSA, a drug inhibiting proline dipeptidase, caused a progressive increase in His-Pro and His-ProNH_2 which were determined as an end point in the measurement of the enzyme activity, resulting in the increase in sensitivity to detect pGlu-peptidase. The increase was due apparently to accumulation of His-Pro, because proline was not detected in the presence of *p*HMSA. A conventional method to determine the pGlu-peptidase activity has employed a system in estimating cyclo(His-Pro) formation (15). However, the conversion ratio of cyclo(His-Pro) from TRH added was 0.04% in human serum, while the pGlu-peptidase assay system as described here could detect 1.38% of TRH added as its degradation products. Eventually, the highly sensitive, convenient, and reliable assay to estimate pGlu-peptidase activity was developed by using *p*HMSA.

The pGlu-peptidase activity was significantly inhibited by metal chelators, EDTA, and *o*-phenanthroline, but not by sulfhydryl-enzyme-blocking reagents, iodoacetamide, or ethylmaleimide. These data are consistent with the action of the previously described "thyroliberinase" thought to be a metalloenzyme (10, 13). The present data on the inhibitory effects of TRH analogues on pGlu-peptidase indicate a restricted specificity of the enzyme which requires the entire tripeptide molecule of TRH as a substrate. In view of these observations, the present results showing that the K_m of pGlu-peptidase in human serum was smaller than the reported value of type I pGlu-peptidase (8) have led to the conclusion that a TRH-degrading enzyme in human serum is classified as type II of pGlu-peptidase.

Because the activity of TRH-degrading enzyme in rat serum has been reported to be changed by thyroid status (1, 4, 16), we measured the pGlu-peptidase activity in sera from patients with hyper- and hypothyroidism. However, neither hyper- nor hypothyroidism caused a significant change in the pGlu-peptidase activity. These data are compatible with the previous observations (4, 6, 11) and extended to the delineation of effects of thyroid status on type II pGlu-peptidase activity in human serum. The different effects of thyroid status on TRH degradation in human and rat sera remain to be elucidated.

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