Enzyme Immunoassay of Thyrotropin Releasing Hormone (TRH)

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A sensitive and specific double-antibody enzyme immunoassay (EIA) for a thyrotropin releasing hormone (TRH)-like immunoreactive substance has been developed. In order to synthesize TRH-labeled β-D-galactosidase (β-gal), a newly devised TRH derivative, pGlu–His–Pro–NH–$(CH_2)_6$–NH_2 (TRH–Hex), was employed. TRH–Hex was linked to β-gal by the *N*-(ε-maleimidocaproyloxy) succinimide coupling procedure. For competitive reactions, the TRH antibody was incubated with standard TRH and TRH–Hex–β-gal (delayed addition). Free and antibody-bound enzyme hapten were separated by using an anti-rabbit immunoglobulin G coated immunoplate. Activity of the enzyme on the plate was fluorometrically determined. The present immunoassay allows detection of 0.8 to 100 pmol/well of TRH.

Keywords TRH enzyme immunoassay; pGlu–His–Pro–NH–$(CH_2)_6$–NH–β-D-galactosidase; fluorogenic substrate; sensitive; specific; second antibody coated immunoplate

Introduction

The existence of hypothalamic thyrotropin releasing hormone (TRH) was demonstrated in 1962.[2] The isolation and characterization of TRH as the tripeptide, pGlu–His–Pro–NH_2, was carried out by Nair *et al.*[3] and Burgus *et al.*[4] in 1970. TRH releases thyrotropin (TSH)[5] and prolactin[6] from the anterior pituitary. In addition to these hormonal actions, it has become clear that TRH and its receptors are distributed in the central nervous system as well as in several peripheral organs, and TRH has been suggested to play a role as a neurotransmitter or neuromodulator.[7,8]

Radioimmunoassay (RIA) of TRH has been developed by several groups[9] using TRH-^{125}I. However, in terms of safety, sensitivity and ease of handling, RIA methods are less than satisfactory. In 1986, Fujiwara and Saita[10] reported enzyme immunoassay (EIA) for TRH using a TRH–β-D-galactosidase conjugate cross-linked through the imidazole group of His. Recently, Grouselle *et al.*[11] reported EIA for TRH using an acetylcholinesterase conjugate cross-linked through the imidazole group of His.

In the present study, we wish to report a sensitive and specific enzyme immunoassay for TRH using TRH-labeled β-D-galactosidase (TRH–β-gal) as a marker antigen, a second antibody coated immunoplate, and 4-methylumberiferyl β-D-galactopyranoside (MUG) as a fluorogenic substrate. To prepare the TRH-β-gal, a newly devised TRH derivative, pGlu–His–Pro–NH–$(CH_2)_6$–NH_2, was synthesized.

Materials and Methods

Materials Synthetic TRH was purchased from Peptide Institute Inc. (Osaka, Japan). pGlu–His–Pro–OH and β-D-galactosidase (β-gal from *Escherichia coli*) were purchased from Boehringer Mannheim Corp. (Mannheim, Germany). pGlu–His–Pro–Gly–NH_2 was purchased from Peninsula Laboratories Inc. (San Carlos, CA, U.S.A.). Bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide) and methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Goat anti-rabbit immunoglobulin G (IgG) (Cappel, 0612-0081) was purchased from Cappel Laboratories (Malvern, PA, U.S.A.).

Antiserum to TRH (i300/001) was purchased from UCB Bioproducts SA (Belgium) and the TRH-antiserum was reconstituted to 120 ml with an assay buffer (0.05 M phosphate buffer, pH 7.0, containing 0.5% BSA and 250 KIU/ml aprotinin). All other chemicals were of analytical reagent grades.

Z–Pro–NH–$(CH_2)_6$–NH–Boc $(Boc)_2O$ (1 eq) in dioxane was added to an ice-chilled solution of NH_2–$(CH_2)_6$–NH_2 (5 eq) in H_2O and the mixture was stirred overnight. After the evaporation of dioxane, the product was dissolved AcOEt. The organic phase was washed with H_2O, dried over Na_2SO_4 and concentrated. The obtained NH_2–$(CH_2)_6$–NH–Boc was used in the next step without further purification. A mixture of Z–Pro–OH [prepared from 12.9 g (37.0 mmol) of the CHA salt], NH_2–$(CH_2)_6$–NH–Boc (8.0 g, 37.0 mmol), WSCD (6.4 ml, 37.0 mmol) and HOBt[12] (5.7 g, 37.0 mmol) in DMF (100 ml) was stirred overnight. The solution was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was crystallized from *n*-hexane, followed by recrystallization from AcOEt with *n*-hexane; yield 12.5 g (76%), *Rf* 0.6 ($CHCl_3$: MeOH = 10 : 0.5), *Anal.* Calcd for $C_{24}H_{37}N_3O_5$: C, 64.40; H, 8.33; N, 9.40. Found: C, 64.15; H, 8.33; N, 9.40.

Z–pGlu–His–Pro–NH–$(CH_2)_6$–NH–Boc Z–Pro–NH–$(CH_2)_6$–NH–Boc (1.1 g, 2.5 mmol) was dissolved in MeOH (20 ml) and hydrogenated over a Pd catalyst for 2 h. The solution was filtered, the filtrate was

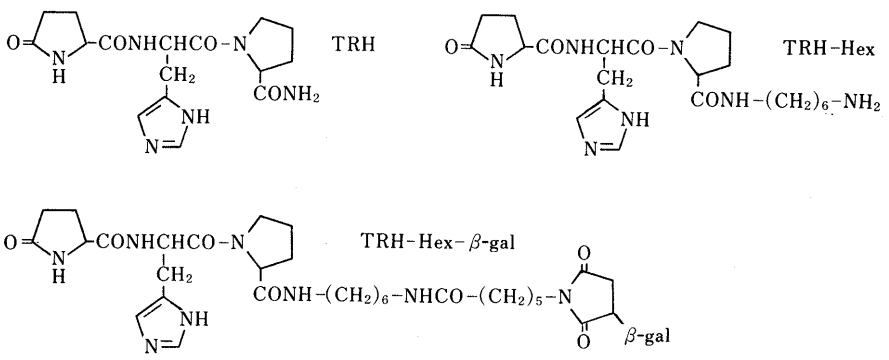

Fig. 1. Structure of TRH Derivatives

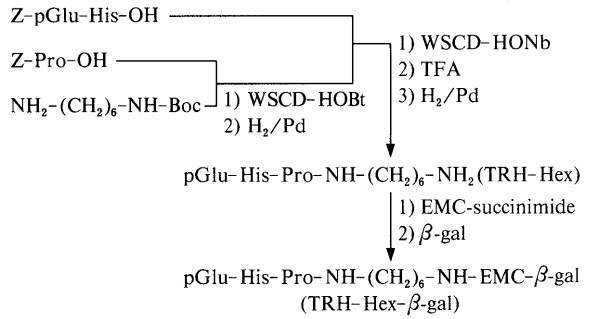

Fig. 2. Synthetic Procedure for TRH–Hex–β-Gal

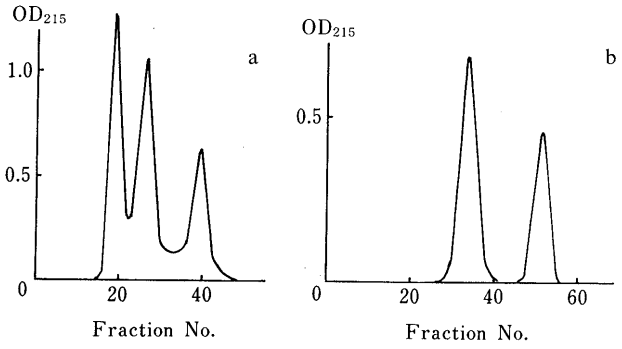

Fig. 3. Purification of TRH-Labeled β-D-Galactosidase

a. Gel-filtration of EMC–TRH–Hex on Sephadex G-15: column: 1.0×64 cm, fraction: 1.8 ml, elution: 0.05 M phosphate buffer, pH 7.0.

b. Gel-filtration of TRH-linked β-D-galactosidase on Sephacryl S-300: column: 1.5×59 cm, fraction: 1.8 ml, elution: 0.05 M phosphate buffer, pH 7.0, containing 0.1 mM $MgCl_2$.

concentrated, and the residue was redissolved in DMF (10 ml). To the solution, Z–pGlu–His–OH (1.0 g, 2.5 mmol),[13] WSCD (0.48 ml, 2.75 mmol) and HONB[14] (0.45 g, 2.75 mmol) were added, and the mixture was stirred overnight. The solution was removed by evaporation, and the residue was dissolved in AcOEt. The organic phase was washed with 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystalized from AcOEt with ether; yield 1.1 g (64%), *Rf* 0.75 ($CHCl_3$: MeOH : H_2O = 8 : 3 : 1), *Anal.* Calcd for $C_{35}H_{49}H_7O_8 \cdot 0.5H_2O$: C, 59.64; H, 7.15; N, 13.91. Found: C, 59.62; H, 7.33; N, 13.91.

H–pGlu–His–Pro–NH–$(CH_2)_6$–NH_2 (TRH–Hex) A TFA treated sample of the above tripeptide (330 mg, 0.47 mmol) was dissolved in DMF (10 ml) and hydrogenated over a Pd catalyst for 3 h. The solution was filtered, the filtrate was concentrated, and the residue was re-dissolved in H_2O. The H_2O phase was washed with ether and concentrated. The product was purified by gel-filtration of Sephadex G-10 using 1 N AcOH as an eluent, followed by lyophilization; yield 180 mg (83%), FAB-MS Calcd for $C_{22}H_{35}N_7O_4 + H^+$: 462.3. Found: 462.4, Amino acid ratios in 6 N HCl hydrolysate: Glu 1.00, His 0.96, Pro 0.98 (recovery of Glu, 81%).

Preparation of Enzyme-Labeled Antigen, TRH–Hex–β-Gal TRH–Hex was conjugated with β-gal by EMC-succinimide according to the method of Kitagawa *et al.*[15] TRH–Hex (0.5 mg) was dissolved in 0.05 M phosphate buffer, pH 7.0 (0.5 ml), and an aliquot of tetrahydrofuran (50 μl) containing EMC-succinimide (0.5 mg) was added. The mixture was stirred at 20 °C for 60 min, then applied to a Sephadex G-15 column (1.0×64 cm) pre-equilibrated with 0.05 M phsophate buffer, pH 7.0. The column was eluted with the same buffer. Individual fractions (1.8 ml each) that showed absorbance at 215 nm were collected. The pooled TRH–Hex–EMC fractions (No. 25—28 in Fig. 3a) were combined with β-gal (1.6 mg) by stirring at 20 °C for 60 min. The β-gal conjugate was applied to a Sephacryl S-300 column (1.5×59 cm) pre-equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$, and was eluted with the same buffer. Individual fractions (1.8 ml each) that showed absorbance at 215 nm were collected. The pooled TRH–Hex–β-gal fractions (No. 30—39 in Fig. 3b) were stored at 4 °C after the addition of 0.2% BSA and 0.1% NaN_3.

Assay Procedure for TRH For assay, the above-mentioned assay buffer

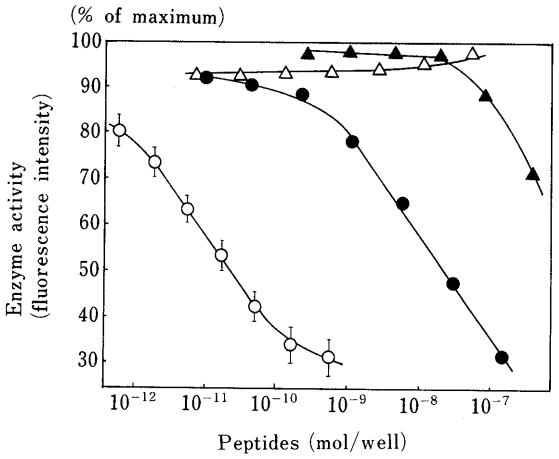

Fig. 4. Dose Response Curves

Inhibition Curves of Various TRH Related Peptides in EIA by Competition between TRH–Hex–β-Gal and Various Peptides toward Anti-serum i300/001: TRH (○), TRH–Hex (●), pGlu–His–Pro–OH (△), pGlu–His–Pro–Gly–NH_2 (▲).

was used. Second antibody-coated immunoplates were prepared as previously reported[16] using Microwell Maxisorp F8 plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG. A test tube containing 100 μl of anti-TRH-serum, i300/001, and each sample (or standard, 100 μl) was incubated at 4 °C for 24 h, then the diluted enzyme-labeled antigen (50 μl) was added. The test tube was further incubated at 4 °C for 24 h. The antibody-antigen solution (100 μl) from each test tube was added to the second-antibody-coated immunoplate. The plate was incubated at 4 °C overnight, then washed 4 times with the washing buffer (0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20). Next 0.1 mM MUG (200 μl) in a substrate buffer (0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$) was added to each well. The plate was again incubated at 37 °C for 3h. The resulting fluorescence intensities (λ_{ex} 360 nm, λ_{em} 450 nm) of each well were measured with a MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

Results and Discussion

Standard Curve The typical calibration curve for EIA for TRH is shown in Fig. 4. A linear displacement of enzyme-labeled TRH by synthetic TRH was obtained, when plotted as a semilogarithmic function from 0.7 to 100 pmol/well of TRH. The minimum amount of TRH detectable by the present EIA system was 0.7 pmol/well (0.4 pg/well).

Specificity of the Antiserum, i300/001 Immunospecificity of the antiserum (i300/001) was examined by EIA using TRH–Hex–β-gal. The displacement curves of various TRH-related peptides are shown in Fig. 4. TRH–Hex exhibited approximately 0.1% cross-reactivity as compared with synthetic TRH, and the displacement curve was parallel to that of TRH. The TRH analogue, pGlu–His–Pro–Gly–NH_2, also inhibited the binding of TRH–Hex–β-gal with TRH-antibody, but reduced cross-reactivity (0.001%). However, the carboxy free TRH analogue, pGlu–His–Pro–OH, hardly inhibited the binding of TRH–Hex–β-gal with the TRH-antibody. Thus, it was shown that the TRH-anti-serum, i300/001, can recognize precisely the carboxy-terminal amide structure of TRH.

Since the TRH, pGlu–His–Pro–NH_2, has no free amino and carboxyl group, it is difficult to prepare a TRH-linked enzyme under mild conditions. So we introduced haxamethylenediamine in carboxy-terminal of TRH to produce pGlu–His–Pro–NH–$(CH_2)_6$–NH_2 (TRH–Hex). TRH–Hex has a free amino group which makes it possible to introduce

TRH into an enzyme by a maleimide type bifunctional cross-linking reagent under mild conditions. We introduced β-D-galactosidase into TRH–Hex using EMC-succinimide according to the method of Kitagawa[15] to produce TRH–Hex–β-gal safely under mild conditions. Furthermore, TRH–Hex–β-gal has a free imidazole group of His which is considered to be important for the biological activity of TRH.[17] Thus, our method is superior to the diazo-coupling method through the imidazole group of His reported by Fujiwara[10] and Grousselle.[11] Using the TRH–Hex–β-gal as a marker antigen, an anti-rabbit IgG coated immuno-plate, and MUG as a fluorogenic enzyme substrate, we have developed a sensitive and specific EIA for the quantitation of TRH. The calibration curve was linear in the range of 0.7 to 100 pmol/well and maximum sensitivity was 0.7 pmol/well (0.4 pg/well) which was almost the same as that of RIA method[8] and EIA methods reported by Fujiwara[10] and Grousselle.[11] The cross-reactivity of TRH-Hex was found to be 0.1%. A TRH analogue, pGlu–His–Pro–Gly–NH_2, also inhibited the binding of TRH–Hex–β-gal but its reduced cross-reactivity (0.001%) was negligible. A carboxy free TRH analogue, pGlu–His–Pro–OH, had no cross-reactivity. Thus it was shown that the TRH-antiserum, i300/001, can recognize precisely the carboxy-terminal amide structure of TRH. Thus, TRH was distinguished from other TRH related peptides by the present EIA system. This simple and specific EIA for TRH using TRH–Hex–β-gal as a marker antigen may be effective method in research on TRH.

References and Notes

1) Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; CHA, cyclohexylamine; AcOH, acetic acid; TFA, trifluoroacetic acid; AcOEt, ethyl acetate; HOBt, 1-hydroxybenzotriazole; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboximide; WSCD, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; DMF, dimethylformamide.
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