Enzyme Linked Immunosorbent Assay for β-Endorphin in Human Plasma

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We established a highly sensitive and specific double-antibody enzyme linked immunosorbent assay for β-endorphin (β-EP). For competitive reactions, the β-EP-antibody was incubated with β-EP standard (or sample) and β-D-galactosidase-labeled β-EP (delayed addition). Free and antibody-bound labeled antigen were separated by using an anti-rabbit immunoglobulin G coated immunoplate. The enzyme activity on the plate was fluorometrically determined. The minimal detection limit was approximately 0.4 fmol/well (10 pmol/l). Using this assay system, β-EP-like immunoreactivity (-LI) in human plasma was determined. The level of β-EP-LI in extracted human plasma from 6 normal subjects was 2.44±0.68 pmol/l. High performance liquid chromatography analysis of the plasma of a normal subject revealed a single immunoreactive form which eluted with the same retention time as that of synthetic β-EP.

Keywords β-endorphin; human plasma; highly sensitive ELISA; β-D-galactosidase-labeled antigen; fluorogenic substrate

β-Endorphin (β-EP) is an endogenous opioid peptide which has received considerable attention because of its potent opiate-like activity and others.[1] β-EP is composed of 31 amino acids and its sequence is contained in the structure of β-lipotropin, which, as yet, has no defined biologic role.

Radioimmunoassay (RIA) has been widely used to establish the identity of β-EP in biological samples ever since Guillemin *et al.*[2] developed the assay system, but in terms of safety, sensitivity and ease of manipulation, the RIA method is still less than satisfactory.

Recently, some investigators have made an effort to develop enzyme linked immunosorbent assay (ELISA) systems for β-EP: In 1986, Sarma *et al.*[3] reported a two-step competitive inhibition type ELISA for β-EP, and in 1988, Hochhaus *et al.*[4] reported a biotin-avidin-based ELISA for β-EP. In the present study, we report a highly sensitive and specific ELISA for β-EP using β-D-galactosidase-labeled β-EP as a marker antigen, a second antibody-coated immunoplate and 4-methylumbelliferyl β-D-galactopyranoside as a fluorogenic substrate. This assay system is the most sensitive and simple of all β-EP ELISA systems described so far. Using this assay system, plasma β-EP levels in normal subjects were measured.

Materials and Methods

Materials β-EP, γ-EP, Met-enkephalin, Leu-enkephalin, bradykinin and angiotensin III were purchased from Peptide Institute Inc. (Osaka, Japan). Bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide) and 4-methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β-D-Galactosidase (β-Gal from *Escherichia coli*) and goat anti-rabbit immunoglobulin G (IgG) were purchased from Boehringer Mannheim Corp. (Mannheim, Germany) and Organon Teknika Corp. (Westchester, PA, U.S.A.), respectively.

Antiserum to β-EP (i456/001) was purchased from UCB-Bioproducts S. A. (Belgium). The lyophilized β-EP-antiserum was reconstituted to 200 ml with an assay buffer (0.05 M phosphate buffer, pH 7.4, containing 0.5% BSA, 1 mM $MgCl_2$ and 250 KIU/ml aprotinin). All other chemicals were of an analytical reagent grade.

Preparation of Plasma Extract Human plasma samples were obtained from 6 normal subjects. Blood was collected at 10:00 a.m. into a chilled tube containing 500 KIU/ml aprotinin and 1.2 mg/ml ethylenediaminetetraacetic acid (EDTA). After centrifugation (3000 rpm, 4 °C, 15 min), plasma was collected and stored at −40 °C until use. Human plasma (2 ml) was diluted five-fold by 4% acetic acid (AcOH), pH 4.0, and loaded on reversed phase C18 cartridges (Sep-Pak C18, Waters Co. Inc., Milford, MA, U.S.A.). After washing with 4% AcOH (10 ml), the immunoreactive β-EP was eluted with 70% acetonitrile (MeCN) in 0.5% AcOH, pH 4.0 (3 ml). The eluates were concentrated by spin-vacuum evaporation, lyophilized, reconstituted to 100 μl with the assay buffer and subjected to ELISA. Recovery of this extraction procedure was 72±10% ($n=6$).

Preparation of Enzyme-Labeled Antigen β-EP was conjugated with β-Gal by EMC-succinimide according to the method of Kitagawa *et al.*[5] β-EP (0.33 mg) in 0.05 M phosphate buffer, pH 7.0 (0.6 ml), was stirred with EMC-succinimide (0.50 mg) in tetrahydrofuran (60 μl) at 20 °C for 60 min. Then, the EMC-β-EP thus obtained was purified with a Sephadex G-15 column (1.0×65 cm) using a 0.05 M phosphate buffer, pH 7.0, as an eluate. Purified EMC-β-EP fractions were combined with β-Gal (2.60 mg) by stirring at 25 °C for 50 min. The β-Gal conjugate was then applied to a Sephacryl S-300 column (1.5×55 cm) and eluted with 0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$. β-Gal-labeled β-EP fractions were collected and stored at 4 °C after the addition of 0.2% BSA and 0.1% NaN_3.

Assay Procedure for β-EP For assay, the above-mentioned assay buffer and the washing buffer, 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20, were used. Second antibody-coated immunoplates were prepared as previously reported[6] using Microwell Maxisorp F8 plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG. A test tube containing 100 μl of the diluted solution of the β-EP-antiserum, i456/001, and 100 μl of each sample solution or standard solution was incubated at 4 °C for 15 h, then 50 μl of the diluted solution of the enzyme-labeled antigen 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 20 °C for 5 h, then washed 4 times with the washing buffer. After washing, 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 2 h. The resulting fluorescence intensity (λ_{ex} 360 nm, λ_{em} 450 nm) of each well was measured with a MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

High Performance Liquid Chromatography (HPLC) Analysis of Plasma HPLC was performed using a reversed phase C18 column (Cosmosil 5C18-AR, 4.6×150 mm, Nacarai Tesque, Inc., Kyoto, Japan). The HPLC consisted of a model 610 dual pump system (Waters Co. Inc., Milford, MA, U.S.A.). The extract from human plasma (5 ml) by the Sep-Pak C18 cartridge mentioned above was reconstituted to 100 μl with 15% MeCN in 0.1% trifluoroacetic acid (TFA), and applied to the column. The immunoreactive β-EP was eluted with a linear gradient of MeCN (from 15% to 60% in 50 min) in 0.1% TFA. The flow rate was 1 ml/min and the fraction size was 1 ml. The fractions were concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 μl with the assay buffer and analyzed to ELISA.

Results

Standard Curve for β-EP The typical standard curve for this ELISA of β-EP is shown in Fig. 1. The minimal

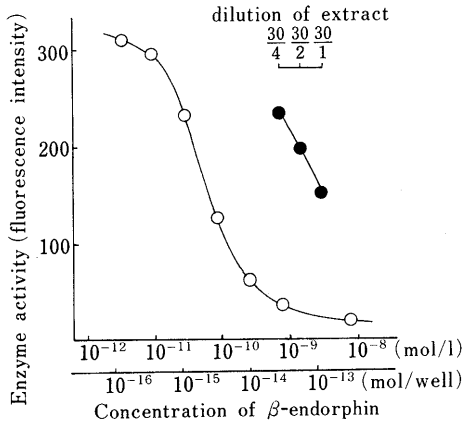

Fig. 1. Standard Curve of Synthetic β-EP and Dilution Curve of Plasma Extract Obtained with Antiserum i456/001 by ELISA

Synthetic β-EP (○), plasma extract (●).

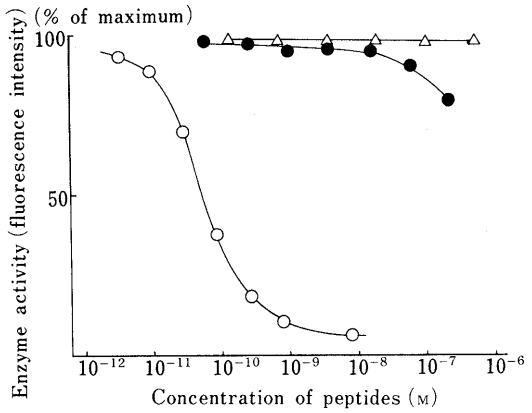

Fig. 2. Inhibition Curves of Various Peptides in ELISA by Competition between β-D-Galactosidase Labeled β-EP and Various Peptides toward Antiserum i456/001

β-EP (○), γ-EP (●), other peptides (Met-enkepharin, Leu-enkepharin, bradykinin, angiotensin III). (△).

TABLE I. Levels of β-EP-LI in Human Plasma

Subject	Age (years)	Sex	β-EP-LI (pmol/l)
1	31	M	3.76
2	33	M	2.45
3	35	M	2.11
4	35	M	2.31
5	38	M	1.88
6	45	M	2.10
Mean ± S.D.			2.44 ± 0.68

M; male.

detectable quantity in the ELISA was as little as 0.4 fmol/well of synthetic β-EP, and the 50% binding intercept was 2.26 fmol/well (57 pmol/l). An intraassay variation of 5% and interassay variation of 10% were obtained at 2.26 fmol/well. Serial dilution of the plasma extract showed a parallel displacement of enzyme-labeled β-EP to synthetic β-EP in this ELISA.

Specificity of the Antiserum (i456/001) Immunospecificity of the antiserum (i456/001) was examined by ELISA using β-Gal-labeled β-EP. As shown in Fig. 2, the cross-reactivity of the N-terminal fragment of β-EP, γ-EP, was less than 0.01%. Structurally related peptides, Met-enkephalin and Leu-enkephalin, as well as structurally unrelated peptides, bradykinin and angiotensin III, hardly inhibited the binding of β-Gal-labeled β-EP with the β-EP-antibody.

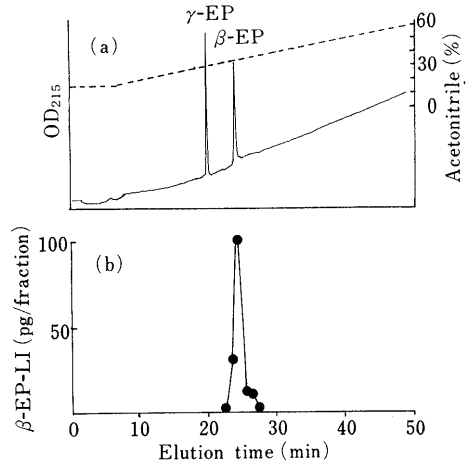

Fig. 3. Reverse Phase HPLC Profile of Plasma Extract on a C18 Column

(a) Chromatograph of synthetic β-EP and γ-EP standards monitored at 215 nm. (b) Immunoreactive profile of human plasma (5 ml). β-EP-LI (●), extracted plasma sample was reconstituted in 15% MeCN in 0.1% TFA and injected onto HPLC. The MeCN gradient is indicated by the dotted line. The HPLC conditions are described in the Methods section.

Measurement of β-EP-LI in Human Plasma by ELISA The proposed ELISA was applied to the determination of β-EP-LI in human plasma samples from 6 volunteers (31 to 45 years old). Table I shows the concentrations of β-EP-LI in human plasma; The mean was 2.44 ± 0.68 pmol/l.

HPLC Analysis of Plasma Human plasma extract was subjected to reversed phase HPLC in order to study the molecular variants of β-EP-LI present in human plasma. Extract from human plasma by a Sep-Pak C18 cartridge was applied to reversed phase HPLC on a C18 column. The elution profile of β-EP-LI in human plasma is shown in Fig. 3. β-EP-LI was eluted as one major peak at the elution position of standard synthetic β-EP.

Discussion

Using β-Gal-labeled β-EP as a marker antigen, an anti-rabbit IgG coated immunoplate, and MUG as a fluorogenic enzyme substrate, we developed a highly sensitive and specific ELISA for the quantification of β-EP. Since the discovery of β-EP by Li *et al.*[7] from the pituitary of camel in 1976, RIA methods for β-EP were first developed.[2,8]

Although RIA for β-EP has been widely used, it has some disadvantages: 1) A γ-counter is expensive and has to be used in a radioisotope room. 2) The radioligand has a short half life. 3) Radioisotopes may be health hazards. Some investigators recently reported an ELISA of β-EP, which would retain the advantages of RIA systems and minimize the disadvantages. Sarma *et al.*[3] first reported a two-step competitive ELISA with a detection limit of 3 fmol/well, and Hochhaus *et al.*[4] subsequently reported a biotin–avidin-based ELISA with 50% binding at 5 fmol/assay and a detection limit of 0.5 fmol/assay. The ELISA reported here, using β-Gal-labeled antigen, second antibody-coated immunoplate and MUG as a fluorogenic

substrate, was highly sensitive and specific for the quantification of β-EP, and a sharp standard inhibition curve was obtained. This ELISA standard curve was concentration-dependent, with 50% binding at 2.26 fmol/well and minimal detectability at 0.4 fmol/well. The sensitivity was higher than other ELISA methods for β-EP. Furthermore, the use of a second antibody-coated immunoplate make the assay procedure quite simple. In addition, β-Gal-labeled antigen is stable in an aqueous form at 4 °C for at least one year, unlike the iodinated form which must be made and purified once a month.

We applied the present assay system to the determination of β-EP in human plasma. By an extraction procedure with a Sep-Pak C18 cartridge from human plasma (2 ml) combined with this ELISA, the present method made it possible to determine β-EP levels in human plasma; that is, the minimal detectable quantity in this method was as little as 0.5 pmol/l (20 amol/well). The levels of β-EP-LI in plasma from 6 normal subjects at 10:00 a.m. were 2.44 ± 0.68 pmol/l, similar to those reported by Nakao *et al.*[9]

Immunoreactive β-EP present in human plasma extract was identified. After treatment with a Sep-Pak C18 cartridge, the extract was submitted to reverse-phase HPLC and analyzed by ELISA. As seen in Fig. 3, the major peak of immunoreactive β-EP was eluted at the position identical with synthetic β-EP. No other peak of immunoreactive β-EP was detected in the elution fractions by the present ELISA. Thus it was proven that the β-EP-LI in human plasma is identical to authentic β-EP.

This simple and sensitive ELISA will be extremely useful for the measurement of human β-EP in biologic fluids and tissues.

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