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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR β-ENDORPHIN AND ITS ANTIBODIES

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Summary

A sensitive method for quantitation of β-endorphin in biological fluids and tissues using ELISA methodology has been developed. This assay is sensitive to as low as 30 picograms of β-endorphin per measurement and can be carried out in approximately 5 hours. The determination of antibody titer levels to β-endorphin can also be quantitated using a modified double sandwich ELISA.

β-Endorphin (β-EP) is one of a number of endogenous peptides which interact with the opioid peptide receptor system. There are a number of studies which clearly indicate that β-EP exhibits pronounced opiate-like activity (1), and consequently attempts have been made to quantify β-EP under various physiological conditions in experimental animals and humans. It would be useful for those laboratories studying β-EP in biological fluids or tissues to have a simple sensitive assay method which does not require radioactive materials.

This paper deals with the development of an ELISA which can be used for quantitation of β-EP separated from biological fluids using a two-step competitive inhibition assay. Quantitation of antibody titers can also be determined in a related procedure.

Materials and Methods

Peptides were synthesized as described earlier (2). Goat α rabbit IgG-peroxidase conjugate was purchased from Zymed Laboratories and ortho-phenylenediamine (OPD) was obtained from Aldrich Chemicals. All other chemicals were obtained from Sigma Chemicals. [$^{3}H_2$-Tyr']-β-EP, 8000 cpm/ng, was used to quantitate the amounts of β-EP added to the plates. Counting efficiency was 28%. Specific activity was determined by amino acid analysis. Antibodies to β-EP were produced according to an earlier procedure by conjugating a C-terminal cysteine analogue of β-EP to KLH (3). Affinity purification of antibodies to β-endorphin was carried out according to the methods described by Engvall (4).

Modified Double Antibody Sandwich ELISA for Antibody Titer Determination

A 96-well Falcon 3070 plate was coated with approximately 40 nanograms of β-EP per well in 50 µl in PBS by incubating at 37°C overnight. Blocking with 3% BSA in PBS was achieved by adding 200 µl per well and incubating the parafilm covered plate at 37°C for 3 hours in a moist chamber. After blocking, the plate was washed with PBS/.05% Tween (X2) and H_2O (X2) followed by the addition of 50 µl of 1% BSA/PBS/Tween to all except the top row of the plate. In the top row, 100 µl of the lowest dilution of antibody serum in 1%

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BSA/PBS/Tween was added and two-fold serial dilutions were carried out to the bottom of the plate. After covering the plate with parafilm, the antibody/antigen reaction was allowed to proceed at 37°C in a moist chamber for 90 minutes. Two rabbit serum samples were tested on each plate, in duplicate, along with pre-bleed sera as a control. After completion of the antibody/antigen reaction, the plates were washed as before. One hundred microliters of goat α rabbit IgG-peroxidase conjugate diluted 1:1000 in 1% BSA/PBS/Tween was added to all wells and incubated for 1 hour at 37°C in a moist chamber. After attaching the peroxidase conjugate to antibodies of β-EP, the plates were washed as above and 70 µl of peroxidase substrate (40 mg of OPD and 40 µl of 3% H_2O_2 in 6 ml of phosphate-citrate buffer) prepared fresh was added, and the enzyme reaction was allowed to proceed for 20 minutes. The enzyme reaction was stopped by the addition of 50 µl of 4N H_2SO_4, and the resulting color was measured using a Titertek Multiskan at a wave length of 492 nm. A typical antibody titer determination is shown in Figure 1.

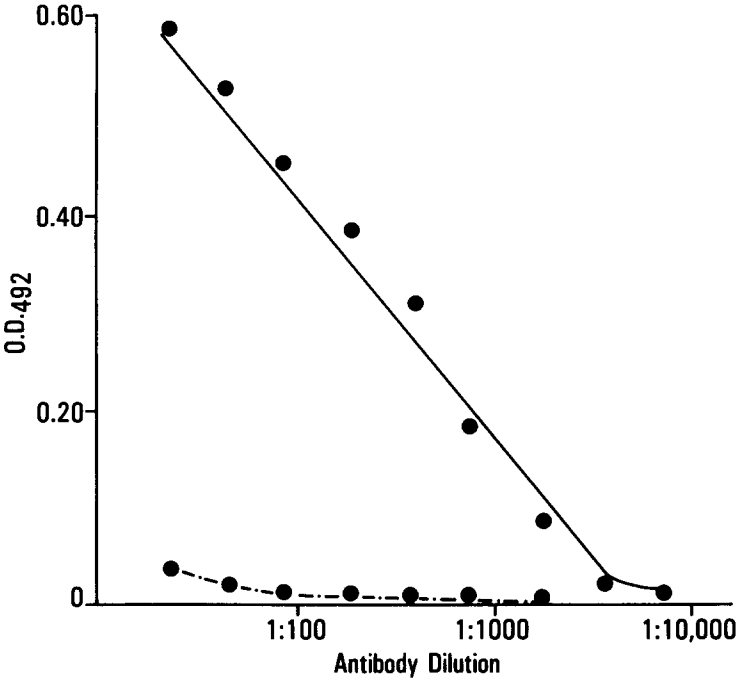

Fig. 1. Quantitation of β-endorphin antibody titers.

Modified Double Antibody Sandwich ELISA for β-EP Quantitation

Different concentrations of β-EP were coated on the plate as described above. Fixed dilutions of rabbit antibodies were added, followed by goat α rabbit IgG-peroxidase conjugate and substrate. In the same manner, affinity purified antibodies were utilized for quantitation. Figure 2 represents standard curves obtained with sera antibodies and affinity purified antibodies, respectively.

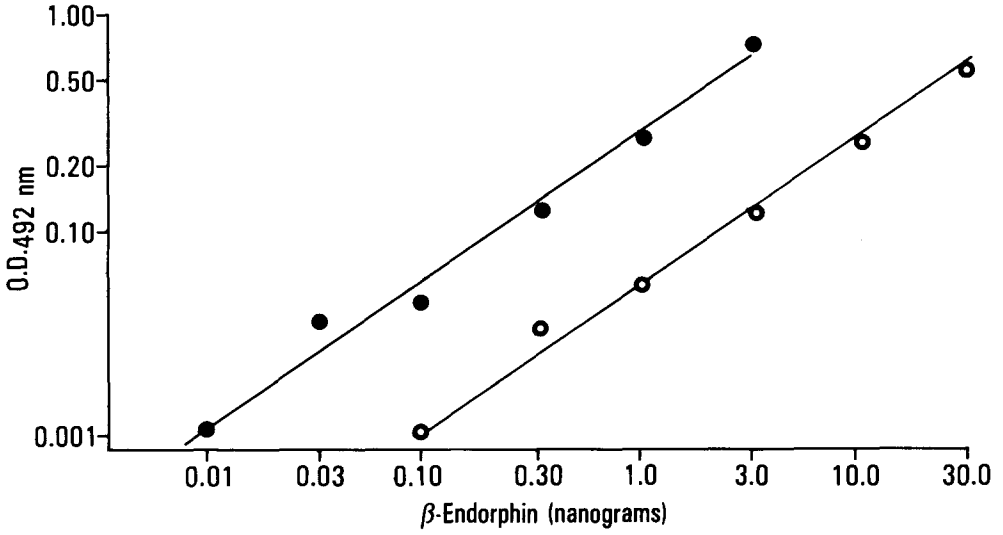

Fig. 2. Typical standard curve for β-endorphin in a modified double antibody sandwich method using unpurified antibody (•) and affinity purified antibodies (o).

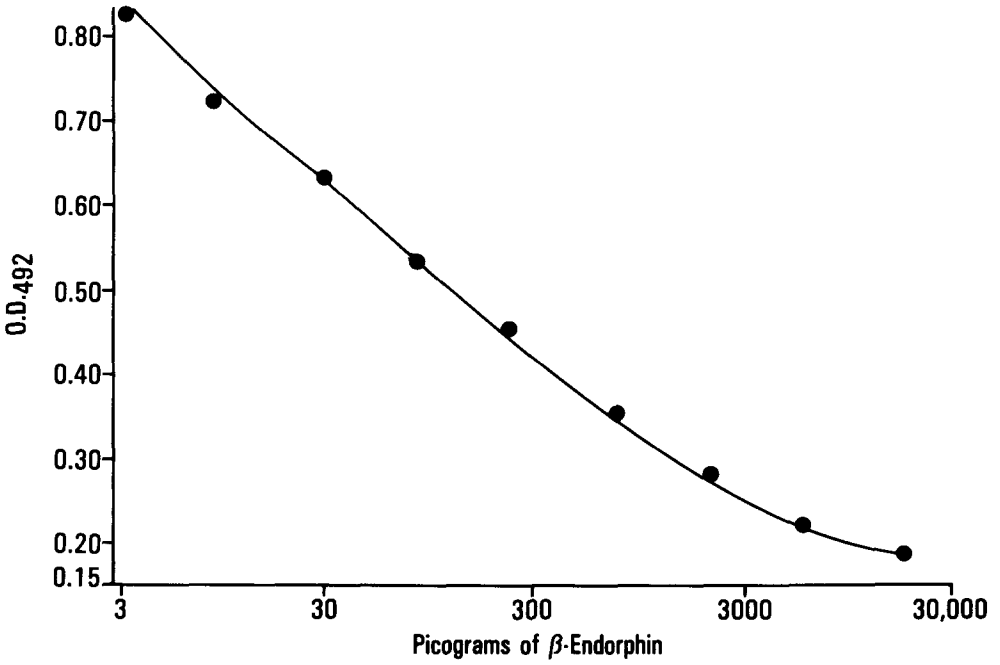

Fig. 3. Inhibition of the binding of β-EP antibodies to β-EP bound to polystyrene wells by β-EP in solution.

Two-Step Competitive Inhibition Assay

In accordance with the principle of the two-step competitive inhibition ELISA technique (5), the optimal concentration of β-EP to be used in coating the plates was determined by checkerboard titration against various dilutions of antibodies. This procedure revealed that one nanogram of β-EP seemed to be sufficient for obtaining a usable optical density of a 1:1600 dilution of antibody. Generally, 1 nanogram of β-EP in 200 µl PBS was incubated on the plates at 37°C overnight followed by blocking with 3% BSA/PBS for 3 hours. All wells except those of the top row were filled with 100 µl of 0.1% poly-L-lysine (mol. wt. 4000) in 1% BSA/PBS/Tween. To the top row, standards or samples diluted in 0.1% poly-L-lysine (mol. wt. 4000) plus 1% BSA/PBS/Tween were added. Serial three-fold dilutions were carried out. One hundred microliters antibody serum, diluted 1:1600 in 0.1% poly-L-lysine (mol. wt. 4000) plus 1% BSA/PBS/Tween, was added and incubated 90 minutes at 37°C in a moist chamber after covering with parafilm. The rest of the procedure was repeated as in the determination of antibody titers. A typical standard curve is given in Figure 3.

Results and Discussion

A simple ELISA method has been developed for β-EP and its antibodies. This type of ELISA can be used for antibody titer determinations during the course of antibody production as well as for checking cross-reactivity of antibody with other peptides.

Quantitation of β-EP can be done in two ways depending upon the cross-reactivity of the antibody and the titers obtained. If β-EP can be partially or completely purified, the "sticky" property of β-EP can be utilized to estimate its concentration using a modified double antibody sandwich ELISA method. Using β-EP antibodies in sera, the sensitivity of this assay is as low as 300 picograms per well. Using affinity purified antibody, the sensitivity was found to be as low as 30 picograms per well.

A second method of quantitation of β-EP is by a two-step competitive inhibition assay. This procedure showed that the externally added β-EP was being bound to the plastic surface, as seen in the control wells, when there was no precoating of β-EP on the plates. However, inclusion of poly-L-lysine (mol. wt. 4000) inhibited this binding. The sensitivity of such an assay is as low as 10 picograms per well. A typical standard curve is given in Figure 3. The assay as described above can be done in less than 5 hours.

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