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

Janardarma K. Sarma
Sarah R. Hoffmann
Richard A. Houghten

Research Institute of Scripps Clinic
10666 N. Torrey Pines Road
La Jolla, California 92037

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Summary

An Enzyme Linked Immunosorbent Assay (ELISA) is described for use in the determination of β-endorphin antibody titers, as well as for the quantitation of naturally occuring levels of β-endorphin in plasma and other bodily fluids. The ability of the assay to accomodate unpurified samples containing small concentrations of β-endorphin was improved through the use of affinity purified antibodies in conjunction with a competitive inhibition ELISA. The problem of non-specific binding of β-endorphin during competitive inhibition assays was circumvented through a two-step process in which the plate was first coated with BSA, followed by a second plate coating with poly-lysine (MW4000). The second coating with poly-lysine was found necessary in order to eliminate intermolecular void spaces following initial plate treatment with BSA. Following these procedures enabled quantitation of β-EP at a level as low as 10pmoles per microtitre plate well.

Immunological reactions have been used for many years for the assay of biologically important compounds because of their high specificity and sensitivity. Antibodies used in conjunction with antigens labeled with a variety of markers have been found to be particularly useful for this type of assay. The most commonly used antigen markers have been radioisotopes and it is with these that radioimmunoassays (RIA) for β-EP were first developed (1-3). The use of radioactive isotopes, however, poses a number of problems: the reagents are costly and have a short shelf life, a gamma counter is required for reading the results, and inconvenient safety measures must be observed in the handling and disposal of the reagents. New assay procedures, such as the enzyme linked immunosorbent assay (ELISA-ref. 8), which eliminate the above mentioned limitations have recently found extensive utility because of their simplicity and ease of use.

In this paper we present procedures using ELISA methodology for the quantitation of antibody titers to the naturally occuring opioid peptide β-endorphin (β-EP) (4-7), as well as for the quantitation of both isolated and plasma β-EP levels.

Materials and Methods

Peptides were synthesized using the method of simultaneous multiple peptide synthesis (10) as recently described. $^{3}H_{2}$-(tyr-1) β-EP was synthesized as

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described earlier (9) and preserved under N_2 in water containing 5% ethanol and 5% acetic acid at 4°C. Peroxidase conjugated goat antibodies to rabbit IgG was purchased from Zymed Laboratories (Burlingame, CA); orthophenylenediamine (OPD) was obtained from Aldrich Chemicals. Bovine serum albumin (BSA), poly-L-lysine (mol. wt. 4,000-70,000), and gelatin were obtained from Sigma Chemicals (St. Louis, MO). Optical density was measured using a Titretek Multiscan type 3100 spectrometer.

Antibodies to β-endorphin. An antigen consisting of β-EP together with an N-terminal spacer of cysteine and 12-aminododecanoic acid was prepared by solid phase peptide synthesis. This cysteine containing β-EP was coupled to MBS activated keyhole lympet hemocyanine (KLH) mixed with complete Freund's adjuvant, and injected into four rabbits at multiple sites on the back and footpads. A second injection was given 14 days later omitting the mycobacterium. A final injection of the peptide KLH conjugate mixed with alum was given on the 21st day. Rabbits were routinely bled on the 7th and 14th day following the final injection to check serum anti-peptide titre levels.

Affinity purification of antibodies to β-Endorphins. Affinity purification of antibodies was achieved through standard procedures by coupling 5 mg of β-Endorphin to one gram of CNBr activated Sepharose as per manufacturer's instructions and followed by elution of the bound antibodies with 0.2 M glycine-HCl buffer pH 2.4. The pH of the solution of eluted antibodies was immediately raised to pH 7.0 with solid Tris, followed by dialysis of the resulting mixture against PBS. The affinity purified antibodies were stored in 10mM sodium phosphate buffer, pH 7.4 containing 1% BSA, 0.9% NaCl and 0.5% Tween.

Adsorption of β-Endorphin to microtitre plates.

Time course study of the adsorption of $^3H_2$-β-Endorphin to microtitre plates. Several commercial firms market microtitre plates. Three types of polystyrene plates (Falcon, CoStar, and Linbro) and one type of polyvinylchloride plate (CoStar) were examined. The adsorption of β-EP to these microtitre plates was studied using $^3H_2$(tyr-1) β-EP. Approximately 10,000 cpm of $^3H_2$-β-EP (equivalent to approximately one ng) was made up in either sodium phosphate buffer pH 7.5, containing 0.9% NaCl (PBS), or carbonate/bicarbonate buffer pH 9.6. and immediately added to each well. After incubation for various lengths of time, at 37°C, the well contents were directly transferred to a vial containing scintillation fluid. Three additional 100 µl washes of the well were carried out with the given buffers and these added to the scintillant. The decrease in the number of counts with time was indicative of the amount of β-EP bound to the plate.

Variation of β-Endorphin binding to different microtitre plates. The above mentioned plates were compared for their ability to adsorb β-EP in a manner which best presented its antigenic sites to β-EP antibodies. To test this, general principles of ELISA as outlined by Engval (8) were followed. Plates were coated with three different concentrations of β-EP in PBS using 50 µl/well, covered with parafilm, and kept at 37°C overnight. The next day the plates were washed with PBS twice and 100 µl of a fixed dilution (1:3125 in° this case) of serum antibodies were added and these incubated for 90 minutes at 37°C after covering the plate with parafilm. After completion of the antigen/antibody reaction, the plates were washed with PBS Tween three times and water twice. One hundred microliters of peroxidase conjugated goat antibodies to rabbit IgG at a dilution of 1:1000 in PBS/Tween was added and incubated for one hour at 37°C in a moist chamber. The plates were then washed as in the previous step and 70 µl of peroxidase substrate containing hydrogen peroxide and OPD (40 microliters of 3% H_2O_2 with 40 mg of prepared OPD in 6 ml H_2O) were added and the reaction allowed to proceed for 20 minutes. The reaction

was stopped by the addition of 50 µl of 4N H_2SO_4 to each well. The resulting color was measured at a wave length of 492 nm using a Titretek spectrophotometer.

Optimal conditions for the blocking of uncoated areas on the plate. Falcon 3070 polystyrene plates were coated with 100 picograms of human β-EP in 50 µl of PBS/well by drying overnight at 37°C. After this treatment the plates were washed twice with PBS. Two hundred microliters of various concentrations of BSA, gelatin and poly-L-lysine (mol. wt. 4,000) dissolved in PBS were added, the plates were covered with parafilm and kept at 37°C for 3 hr in a moist chamber. The plates were then washed three times with PBS/Tween and twice with distilled water. Subsequent steps were the same as in the ELISA procedure described above.

Modified double antibody sandwich ELISA for β-EP antibody titer determination.

A 96-well Falcon 3070 polystyrene plate was coated with approximately 40 nanograms of β-EP per well in 50 µl of PBS as in the previous experiment. Blocking with 3% BSA in PBS was done by adding 200 µl per well and maintaining the parafilm covered plate at 37°C for 3 hr in a moist chamber. The plate was then washed as described above with PBS/Tween and H_2O, and 50 µl of 1% BSA PBS/Tween were added to all wells, except the first row. In the first row wells 100 µl of the lowest dilution of antibody serum in 1% BSA PBS/Tween were added and two-fold dilutions were done serially by taking 50 µl from the first row, mixing it ten times in the next row, and repeating this procedure to the end of the plate and discarding the final 50 µl. After covering the plate with parafilm, the antibody/antigen reaction was allowed to proceed at 37°C in a moist chamber for 90 minutes. Addition of conjugate and substrate were done as in the ELISA procedure outlined previously. Two different rabbit serum samples were tested in duplicate on each plate along with preimmunization sera from each rabbit which were used as controls.

Quantitation of β-EP.

Two-site sandwich ELISA for β-EP. Different concentrations of β-EP standards and/or unknown concentrations in biological fluids were coated on the plate in PBS buffer as in the antibody titer determination. Fixed dilutions of antibody were added, followed by peroxidase tagged goat antibodies to rabbit IgG, and the color was developed and measured as described above.

Two-step competitive ELISA for β-EP. In accordance with the principles of two step competitive inhibition ELISA, the optimal concentration of β-EP to be coated was determined by checkerboard titration against various dilutions of antibody. This procedure revealed that one nanogram of β-EP gave sufficient optical density with a 1:1600 dilution of antibody. Using this information, a study of the competition between plate bound β-EP and externally added free β-EP for antibodies against β-EP was carried out. As a rule, 1 nanogram of β-EP was coated onto the plate in 200 µl PBS at 37°C overnight, followed by blocking with 3% BSA/PBS for 3 hrs. All wells except the first row wells were then filled with 50 µl of 0.1% poly-L-lysine (mol. wt. 4,000) in 1% BSA PBS/Tween. In the first row wells 100 µl of either standards or unknowns of β-EP diluted with 0.1% poly-L-lysine (mol. wt. 4,000) in 1% BSA PBS/Tween were added and serial two-fold dilutions were done as described above. One hundred microliters of serum diluted 1:1600 with 0.1% poly-L-lysine (mol. wt. 4,000) in 1% BSA PBS/Tween was then added to each well, mixed, and incubated 90 minutes at 37°C in a moist chamber after covering with parafilm. The development and reading were carried out as described above.

This procedure could be simplified in the following manner. The samples and standards in 1% BSA/PBST are preincubated for two hours with antibodies of an appropriate fixed dilution in plastic tubes. After this incubation period, 20 microliters of concentrated poly-L-lysine (Ave. Mol. Wt. 4,000) was added to each tube to make a final concentration of 0.1%. The poly-L-lysine was added just prior to the addition of aliquots to the wells of the plate.

In our later experiments we found that of the plates tested, Linbro plastic plates from Flow laboratories gave no nonspecific binding to the plate surface of competitively added β-Endorphin. The use of polylysine with these plates was thus not needed. Competitive inhibition on these plates is done in essentially the same manner as for other plates with the following conditions changed: i) the plates are coated with 1 ng β-Endorphin in 50 microliters of buffer per well overnight at 37°C and completely dried; ii) as in the modification previously described the standards and samples are preincubated for two hours with antibodies of appropriate dilution at 37°C, then added to the wells without addition of poly-L-lysine.

Results

Binding of β-EP to microtitre plates. Figure 1 shows the results for a typical binding of β-EP in different buffers to the two plastic types used for microtitre plates, polystyrene and polyvinylchloride. It is evident from this data that maximum binding occurs in 18-24 hrs and that plates coated with β-EP in PBS exhibit better binding than those coated with alkaline buffer. In addition, Figure 1 shows that the polystyrene plates are slightly more effective at binding β-EP than polyvinylchloride plates. The comparison of three brands of polystyrene plates and one brand of polyvinylchloride plate for best presentation of β-EP antigenic sites are shown in Table 1. Both Linbro polystyrene plates and Costar polyvinylchloride plates showed low blank valves with no β-EP coated on the plate but relatively poor binding of β-EP, while Falcon 3070 and Costar plates showed higher blank values, but also a higher net antigenic site exposure for a fixed amount of antigen. Falcon 3070 plates were therefore used for the remainder of the study.

Table 1

Binding of β-Endorphin to Various Microtiter Plates

Picograms of β-Endorphin Absorbed	Polystyrene Plates			Polyvinylchloride Plates
	Falcon	CoStar	Linbro	CoStar
None(blank)	0.365 Net O.D.	0.385 Net O.D.	0.101 Net O.D.	0.189 Net O.D.
100	0.184	0.194	0.102	0.105
300	0.425	0.430	0.236	0.200
1000	0.600	0.599	0.382	0.470

Values are O.D. of the peroxidase activity measured at 492 nm after deducting the corresponding blanks. Comparison of different types of plates for the proper antigenic site presentation after adsorption of a β-Endorphin dissolved in 50 microliters overnight at 37°C in phosphate buffer with 0.9% NaCl at fixed antibody dilution (1:3125) without blocking the uncoated area. Values are average of duplicates.

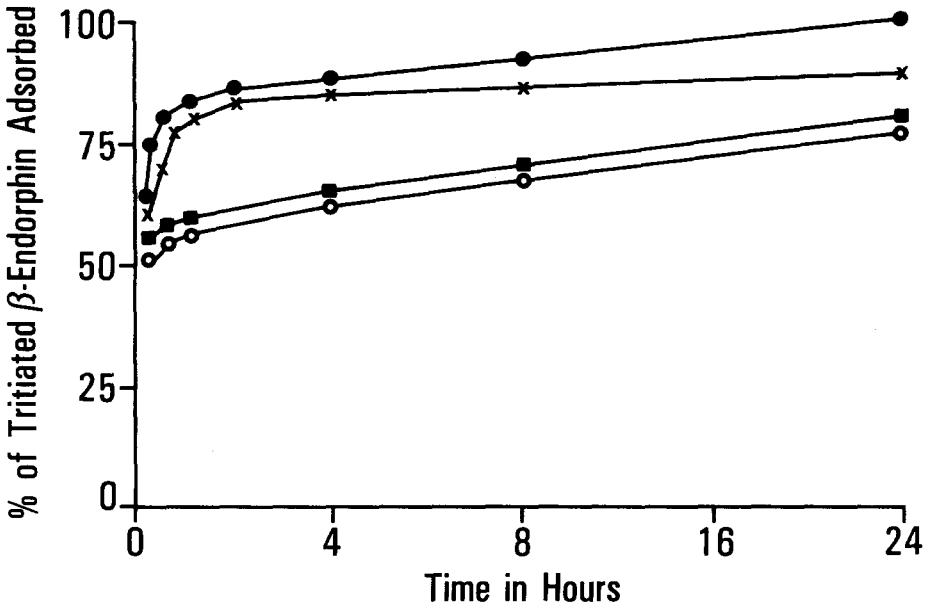

FIG. 1

Time course of adsorption of β-Endorphin to microtitre plates. Falcon polystyrene pH 7.4 (PBS) (●); Costar polyvinychloride pH 7.4 (carbonate-bicarbonate) (x); Falcon polystyrene pH 9.6 (carbonate- bicarbonate) (■); Costar polyvinylchloride pH 9.6 (carbonate- bicarbonate) (o).

Optimal blocking conditions for non-competitive, two site sandwich ELISA. For the Falcon 3070 polystyrene plates the non-specific binding of unpurified antibodies was observed to be high, resulting in a low signal to noise ratio with decreasing concentrations of β-EP bound to the plate. This necessitated blocking the rest of the surface area of the well with some other agent without sacrificing the antibody/antigen reaction. Bovine Serum Albumin (BSA), fetal calf serum, normal rabbit serum, gelatin, polylysine, and polyethyleneimine were tried at various concentrations. Optimal concentrations were determined for BSA, gelatin, and polylysine. Polylysine and BSA were found to be effective at low concentrations (10^{-3} mg/ml). In the case of gelatin, however, slight changes in the blocking concentration caused drastic reductions in the antibody/antigen reaction over the controls. Polylysine, although it worked well, is relatively expensive and the antibody/antigen reaction was significantly decreased in comparison to that using the BSA blocked β-EP. Hence, blocking with 3% BSA for 3 hrs was preferred.

β-EP antibody titer determination.

Typical results for the determination of β-EP antibody titers in serum are shown in Figure 2. A low titer serum was chosen for this example in order to emphasize the ease with which one can determine titers, even in cases in which the differences between the pre-bleed and inoculated serum are relatively small. The reproducability of this method was better than ± 10%.

Quantitation of β-EP.

Noncompetitive, two-site sandwich ELISA for β-EP. It is well-documented that β-EP sticks to many surfaces, including glass and polystyrene (11). We took advantage of this property to quantify β-EP levels using a two site sandwich ELISA. Using this method, as little as 300 picograms of β-EP can

easily be detected as shown in Figure 3. Since, however, human plasma has a β-EP concentration of approximately 150 picograms per ml (2-4) a more sensitive assay was needed. Efforts to do this by either diluting the antibody or incubating for longer periods of time were not successful. The use, however, of affinity purified antibodies for this two site type sandwich ELISA improved the sensitivity to as low as 10 picograms per well with excellent reproducibility as can be seen in Figure 4.

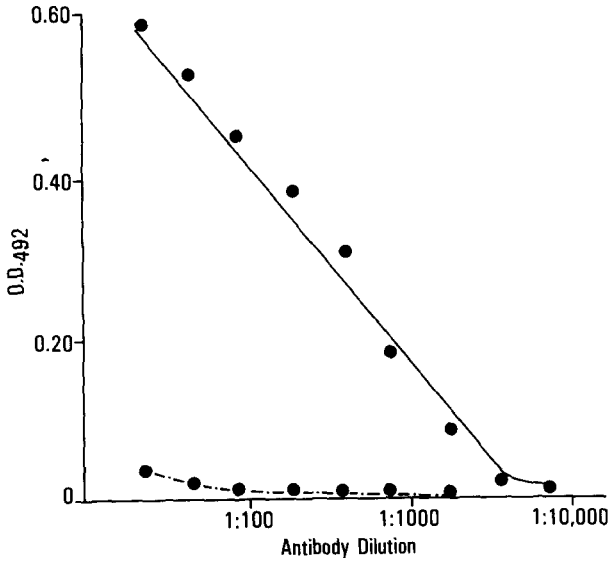

FIG. 2

Quantitation of β-endorphin antibody titers by ELISA. Antisera from rabbit immunized with β-endorphin conjugated to KLH (———); prebleed titers from the same rabbit (·–·–·).

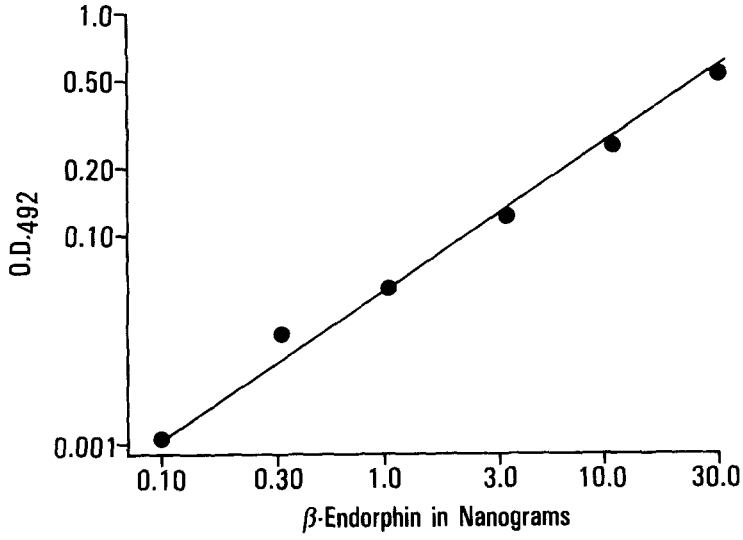

FIG. 3

Standard curve for β-EP in a modified double antibody sandwich method using unpurified polyclonal antisera.

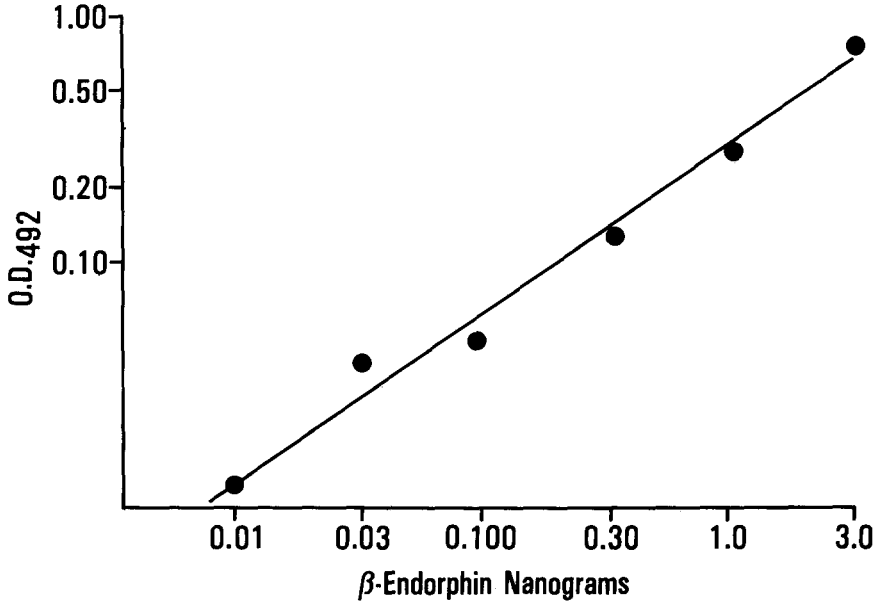

FIG. 4

Standard curve using affinity purified polyclonal antibodies in a modified double antibody sandwich ELISA.

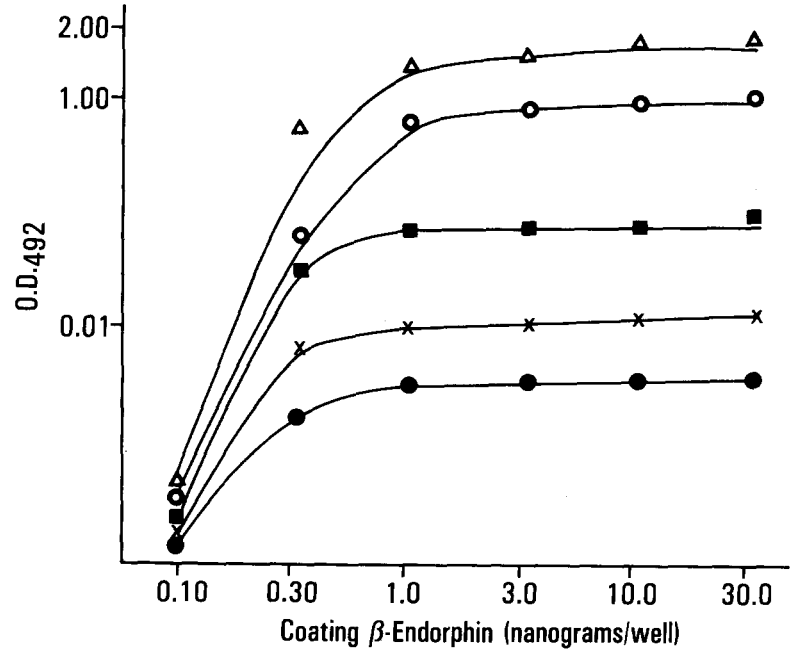

FIG. 5

Optical density as a function of the concentration of antigen used for plate coating with antiserum diluted: 1 to 15,600 (●); 1 to 3125 (x); 1 to 625 (■); 1 to 125 (o); and 1 to 25 (Δ).

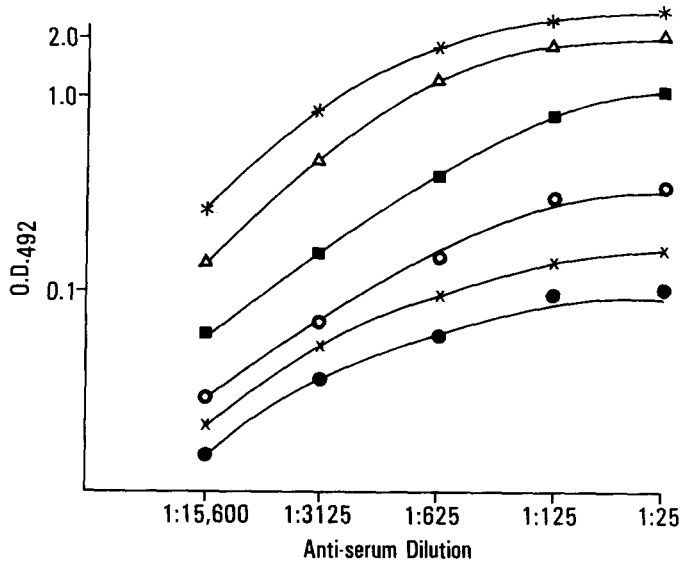

FIG. 6

Optical density as a function of antiserum dilution with wells coated with β-Endorphin (nanograms per well): 0.1 (●); 0.3 (x); 1.0 (o); 3.0 (■); 10.0 (Δ); 30 (*).

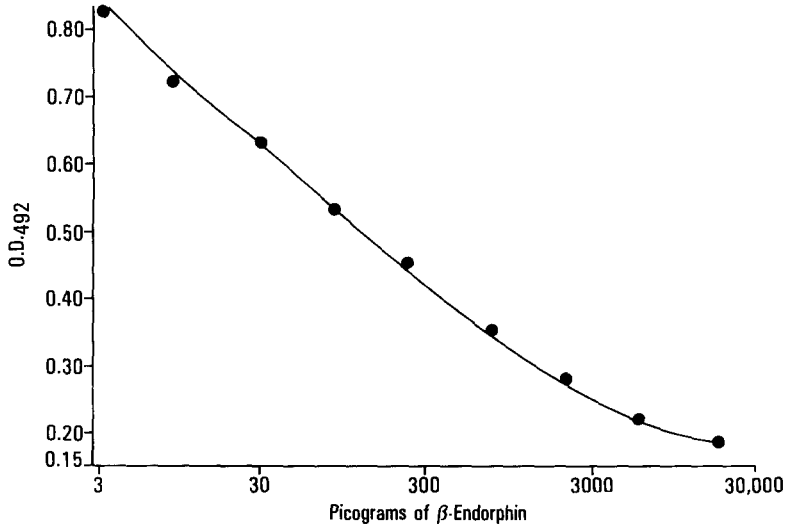

FIG. 7

Competitive inhibition of the binding of antibodies against β-endorphin bound to polystyrene wells by β-endorphin in solution.

Two step competitive inhibition assay. Two site sandwich ELISA requires the use of relatively pure β-EP. The assay works well in cases where the peptide has been separated from other materials by talc extraction, HPLC purification, or gel filtration. The necessary purification of β-EP from

samples of plasma, brain, CSF, etc., however, is time consuming and losses of desired material occur. To circumvent these difficulties a two step competitive inhibition type ELISA was developed which uses plasma directly. The optimal serum dilution and antigen concentration were determined as shown in Figures 5 and 6. As a final procedure for the competitive inhibition ELISA of β-EP, addition of 0.1% poly-L-lysine (mol. wt. 4,000) to the 1% BSA PBS/Tween solution used for diluting antibodies and antigen proved to be successful. A typical standard curve is shown in Figure 7.

Discussion

Through the systematic evaluation of the assay parameters a simple ELISA 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 β-EP antibodies with other peptides.

Quantitation of β-EP can be done in two ways depending upon the cross-reactivity and titers of the antibodies available. If the β-EP to be used is partially or completely purified, the "sticky" properties of the peptide can be effectively utilized to estimate its concentration by a two-site sandwich ELISA method. Depending on the given β-EP antibody titers levels, and whether affinity purified antibodies are used the sensitivity of this assay can be enhanced to measure as low as 10 picograms per well.

A second method of quantitation of β-EP is through the use of a two-step competitive inhibition assay. Initial attempts to quantitatively determine β-EP levels by this method failed. It was determined that the externally added β-EP was being bound to the plastic surface of the microtitre plate and that this binding increased linearly with increasing concentrations of standard β-EP. Attempts were made to prevent this nonspecific binding of externally added β-EP by coating the plates with various blocking agents including BSA, poly- lysine, gelatin, fetal calf serum, normal rabbit serum, spermidine and Triton- X-100. None of these were found to be completely satisfactory. Poly-L-lysine (MW 4,000), however, worked quite well to prevent non-specific binding of the added β-EP. This suggests that small void spaces on the microtiter plate had been left unblocked by the relatively large BSA molecule (MW 60,000), thus allowing the added B-EP to adsorb to the plate. Attempts to block with various higher molecular weight poly-L-lysines were unsuccessful.

Using this principle of void blocking, a sensitive ELISA has been developed for β-EP. The sensitivity of such an assay is as low as 10 picograms (3 fmoles) per well. Using this method directly with plasma samples, the level of β-EP in rat plasma was found to be 0.180 ± 0.02 nanograms/ml. This value compares well with that found in the literature of 0.260 ng/ml (13).

The possible general applicability of this procedure to other "sticky" peptides is currently being investigated.

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