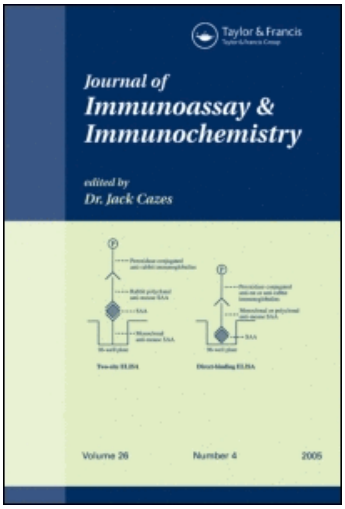

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Preparation of Horseradish Peroxidase Hydrazide and Its Use in Immunoassay

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ABSTRACT

Preparation of horseradish peroxidase (HRP) hydrazide that is HRP linked to adipic acid dihydrazide (HRP-ADH) and its use in enzyme immunoassay (EIA) is described. In this new strategy, horseradish peroxidase was conjugated to adipic acid dihydrazide using a carbodiimide coupling method. The resulting HRP-ADH was then coupled to cortisol-21-hemisuccinate (Cortisol-21-HS) to prepare enzyme conjugate. The prepared cortisol-21-HS coupled ADH-HRP (Cortisol-21-HS-ADH-HRP) enzyme conjugate was used for the development of an enzyme linked immunosorbent assay (ELISA) for direct estimation of cortisol. To the cortisol antibody coated

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microtiter wells, standard or serum samples (50 μL), along with cortisol-21-HS-ADH-HRP enzyme conjugate (100 μL) were incubated for 1 h at 37°C. Bound enzyme activity was measured by using tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) as substrate. The sensitivity of the assay was 0.05 μg/dL and the analytical recovery ranged from 92.9 to 101.7%.

Key Words: HRP-hydrazide; Direct ELISA; Immunoassay.

INTRODUCTION

The direct coupling of carboxylic derivative of a steroid to an amino group of an enzyme is a well established method in the steroid enzyme immunoassay for preparing enzyme conjugate.[1] HRP is a widely used enzyme in EIA, containing six lysine residues in sequence; however, in practice, only around one to two of these are generally available for reaction.[2] This variation in amino group content is due to changes in the reaction conditions employed for isolation of HRP from the roots of the horseradish plant.[2,3] The low yield of HRP coupled to the immunoglobulin G (IgG) using bifunctional reagents prompted Nakane et al. to investigate another method (periodate method) for coupling HRP to IgG.[4,5] The difference in amino group availability was observed in different batches of commercial preparations, which makes it difficult to establish reaction conditions applicable to more than a single batch of HRP. Therefore, adipic acid dihydrazide (ADH) has been coupled to HRP using a carbodiimide method to introduce necessary amino groups into HRP for the preparation of steroid enzyme conjugate.

ADH is a bifunctional cross linking reagent containing hydrazide groups at both ends. The reagent provides a ten-atom bridge between cross-linked molecules after conjugation. ADH has been coupled to affinity matrices,[6] 96-well microtiter plates,[7] beads,[8] avidin and streptavidin,[9] and enzymes[10] to produce hydrazide derivatives for coupling to aldehyde ligands.

The first time use of ADH is described as linker between HRP and steroid carboxylic derivatives to prepare enzyme conjugates for ELISA. The principle of the conjugation procedure exploits the activation of 28 (twenty eight) free carboxyl groups of HRP,[2] using *N*-hydroxysuccinimide-carbodiimide method and subsequently coupling to one of the hydrazide groups of ADH. In the second step, the

carboxylic derivative of the steroid was conjugated to HRP-hydrazide (HRP-ADH) using an *N*-hydroxysuccinimide-carbodiimide method. The potential application of HRP-hydrazide for preparing steroid-enzyme conjugate for the determination of an analyte is demonstrated through the development of an ELISA for direct estimation of cortisol in human serum samples.

EXPERIMENTAL

Cortisol, cortisol-3-O-carboxymethyl-oxime (Cortisol-3-O-CMO), cortisol-21-hemisuccinate (Cortisol-21-HS), horseradish peroxidase (HRP) type VI (EC 1.11.1.7), bovine serum albumin (BSA), 1-ethyl 3-(3-dimethyl-aminopropyl)-carbodiimide-HCl (EDAC), *N*-hydroxysuccinimide (NHS), adipic acid dihydrazide (ADH), glutaraldehyde (GA) gelatin, dextran T-70, gentamicin and freund's complete adjuvant (FCA), were all purchased from Sigma Chemical Company, St. Louis, MO, USA. Tetramethyl benzidine, with hydrogen peroxide solution (TMB/H_2O_2) and microtiter plate, were procured from Bangalore Genei, Bangalore, India and Greiner, Germany, respectively. All other chemicals and buffers were of analytical grade.

Buffer

1. The most frequently used buffer was 10 mM phosphate containing 0.9% NaC1(10 mM PBS), pH 7.0, (Na_2HPO_4 $2H_2O$: 0.895 gm/L and NaH_2PO_4 $2H_2O$: 0.39 gm/L) and 0.1% NaN_3 or thimerosal.
2. HRP conjugate dilution buffer was 10 mM acetate buffer (10 mM AB), pH 5.6 (CH_3COONa: 0.84 gm/L and 1 N CH_3COOH 1.5 mL/L), containing 0.1% thimerosal, sodium salicylate, dextran T-70, and 0.3% BSA.
3. Microtiter well blocking and stabilizing buffer was 10 mM PBS containing 0.2% BSA, 01% gelatin, thimerosal, dextran T-70, ethylene diamine tetraacetic acid dipotassium salt (EDTA K-Salt) and 0.01% gentamicin sulfate.

Biological and Immunological Materials

Normal rabbit serum (NRS) was collected from non-immunized New Zealand white rabbits.

Primary antibody: Cortisol antiserum was generated against cortisol-3-O-CMO-BSA as an immunogen in New Zealand white rabbit.[11]

Second antibody: Antispecies antiserum as second antibody was generated in goats using purified normal -γ globulin of rabbit as an immunogen.[11]

Preparation and Dilution of Cortisol-21-HS-ADH-HRP Conjugate

To 10 mg of HRP, 20 mg of NHS, and 40 mg of EDAC, one milliliter of water was added. The reaction mixture was vortexed and kept overnight at 4°C. After overnight incubation at 4°C, one milliliter of ammonium carbonate containing 100 mg of ADH was added and the reaction mixture was further vortexed and kept overnight at 4°C. After incubation, the reaction mixture was passed through a G-25 column previously equilibrated with 10 mM PBS containing 0.1% thimerosal; the brown fraction of HRP-hydrazide was directly collected and kept at −30°C for future use.

To 5 mg of cortisol-21-HS, 10 mg of NHS, and 20 mg of EDAC, 400 μL of 1:1 mixture of dioxan and dimethyl formamide (DMF) and 100 μL of water was added and the reaction mixture was vortexed and kept overnight at 4°C. To the activated cortisol-21-HS, one milliliter of HRP-hydrazide (approximately containing 1 mg of HRP) was added and the reaction mixture was further vortexed and incubated overnight at 4°C. After incubation, the reaction mixture was passed through a G-25 column, previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown coloured portion was directly collected and, to it, a pinch of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added and kept at −30°C in aliquots for future use.

The optimal dilution of cortisol-21-HS-ADH-HRP conjugate (1/8000) was determined by checkerboard assay. The diluted conjugate was stored in the conjugate dilution buffer at 4°C for future use. This was stable for more than one year at 2–8°C.

Preparation of Cortisol Standards in Charcoal Stripped Serum

Six cortisol working standards, se.g., 0 μg/dL, 1 μg/dL, 3 μg/dL, 10 μg/dL, 20 μg/dL, and 40 μg/dL were prepared in charcoal stripped serum, as described earlier.[12]

Coating of Microtiter Plates

Wells of microtiter plates were coated with 225 μL of diluted cortisol-3-0-CMO antibody (1/8000) by an immunobridge technique described elsewere.[11]

Preparation of Substrate Solution

Substrate solution was prepared from TMB/H_2O_2 solution (Bangalore Genei, Bangalore, India). According to the manufacturer protocol, 100 μL of TMB/H_2O_2 solution was diluted to 2 mL (1:20 dilution), in water. This solution was prepared just before its use.

One Step ELISA Procedure

To the cortisol antibody coated wells, 50 μL of cortisol standards or serum samples were added in duplicate. The 100 μL of cortisol-21-HS-ADH-HRP conjugate was added to all the wells and incubated for 1 h at 37°C. After incubation, the contents of the wells were decanted and washed under running tap water five to six times by filling, decanting, and tapping. Finally, for measuring the bound enzyme activity, 100 μL of substrate solution was added to all the wells and incubated for 15 min at 37°C. The reaction was stopped by adding 100 μL of 0.5 M H_2SO_4 and the colour intensity was measured at 450 nm in a Tecan-Spectra ELISA plate reader.

Release of Cortisol Bound to Cortisol Binding Globulin (CBG)

The Cortisol-21-HS-ADH-HRP conjugate was diluted in HRP conjugate buffer which contains 0.1% sodium salicylate. The 100 μL of diluted conjugate was added to all the wells and incubated for 1 h at 37°C in the assay procedure. The sodium salicylate present in the conjugate buffer binds to CBG and releases the cortisol from it.

RESULTS

Sensitivity

The lower detection limit of the assay that is the concentration equivalent to B0 -2SD is 0.05 μg/dL of serum after a thirty-fold determination of B_0 binding.

Table 1. Recoveries of cortisol from exogenously spiked pooled serum.

Pooled Serum	Cortisol added (μg/dL)	Expected (μg/dL)	Obtained (μg/dL)	Recovery (%)
Basal	0	–	2.7	–
Low	3	5.7	5.8	101.7
Medium	15	17.7	17.4	98.3
High	30	32.7	30.4	92.9

Specificity of Antibody

Cortisol-3-0-CMO-BSA antibody had less than 0.1% cross reaction with naturally occuring C_{27}, C_{21}, C_{19} and C_{18} steroids except for cortisone (3.4%) and 17-α−OH-progesterone (5.6%). However, the cross reaction with synthetic glucocorticoids, such as dexamethasone and prednisolone, were 0.8 and 7%, respectively.

Analytical Recoveries

The ability of the assay to accurately quantify cortisol in serum samples was tested. Low, medium, and high concentrations (3–30 μg/dL) of cortisol were added exogenously to the three fractions of pooled serum (blood collected around midnight).

After addition, the concentration of cortisol was determined and recovery was calculated in each fraction of serum. The recoveries ranged between 92.9% and 101.7%. (Table 1)

DISCUSSION

The developed direct ELISA for estimation of cortisol in serum samples using cortisol-21-HS-ADH-HRP enzyme conjugate is a single step, rapid, and simple. The use of enzyme conjugate with a longer bridge in ELISA increases the sensitivity and specificity of assay, as compared with enzyme conjugate with shorter bridge length.[13]

In EIA, HRP is a widely used enzyme, containing six lysine (amino) groups. Availability of amino groups in HRP for derivatization depends

on the extraction conditions employed for its isolation from the roots of the horseradish plant. During purification, a component of horseradish plant, sinigrin, is prone to releasing allylisothiocyanates,[3,14] which are likely to react with enzymes' amino groups. Any resulting thioureas will be quite stable, so these lysines are effectively lost as useful handles for derivatization. Such differences in amino group availability have been observed, which make it difficult to establish reaction conditions which can be used for more than one batch of HRP. Therefore, in the present procedure, the enzyme HRP has been made functional by introducing the required amino (hydrazide) group by preparing its hydrazide. The HRP-hydrazide preparation exploits activation of twenty eight free carboxyl groups of HRP by an activated ester method and subsequently coupling to one of the hydrazide groups of ADH. The HRP-hydrazide was utilized to couple to steroid carboxylic derivatives by an activated ester method.

Rennke and Venkatachalam[15] used this route for preparing poly-aminated HRP (pKa 8.4–9.2) by reaction with hexanediamine and a carbodiimide, and noted no change in pH optimum or enzyme activity with a colorimetric substrate. Interestingly, polymerization of the HRP appears to have been avoided in this preparation. Gershoni et al.[10] prepared the enzyme hydrazide and its use was confined for the general staining of glycoproteins and other glycoconjugates on protein blots. The principle of the staining involves generation of aldedydes from the sugars of glycoconjugates by periodate oxidation, which then reacts with the hydrazide group of the enzyme-hydrazide and the bound enzyme-hydrazide was demonstrated histochemically.

For the first time, the use of ADH as linker between HRP and steroid carboxylic derivative to prepare enzyme conjugate for use in enzyme immunoassay has been demonstrated. The developed procedure is a simple method for attaching a hapten carboxylic group to HRP with an increased bridge length, which is one of the desirable features of hapten immunoassay[16] for achieving sensitive and specific assay.

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