J Endocrinol Reprod 11 (2007) 2: 69 - 75

Original Article

Preparation of horseradish peroxidase-carbamide and its use in hapten immunoassays

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Summary

Preparation of horseradish peroxidase (HRP) carbamide that is HRP linked to urea (HRP-carbamide/HRP-U) is demonstrated and its potential application in the development of enzyme immunoassays (EIAs) for haptens is described. In this new strategy, the lysine residues of HRP were acylated and then acylated HRP was activated to create highly reactive functional groups by periodate oxidation of its carbohydrate moiety and, subsequently, forming a peptide bond with one of the amino groups of urea. The resulting HRP-carbamide was then coupled to carboxylic derivatives of cortisol-21-hemisuccinate (F-21-HS), 17α-OH progesterone-3-O-carboxymethyl-oxime (17α-OHP-3-O-CMO) and nandrolone-3-O-carboxymethyl-oxime (N-3-O-CMO) to prepare enzyme conjugates adopting N-hydroxysuccinimide-carbodiimide method. The F-21-HS-U-HRP, 17α- OHP-3-O-CMO-U-HRP, and N-3-O-CMO-U-HRP enzyme conjugates thus prepared were used for the development of an enzyme linked immunosorbent assays (ELISAs) for the estimation of cortisol, 17α-OHP and nandrolone. The sensitivity of cortisol, 17α- OHP and nandrolone assays were 0.4 ng/ml, 0.05 ng/ml, and 0.12 ng/ml, respectively, and the analytical recovery ranged from 93.3% to 100%, 94.3% to 98.7%, and 93.5 % to 104%, respectively. In the present study, the strategy adopted for preparing HRP-carbamide and its subsequent use in preparing enzyme conjugate has been shown to result in an increase in thermostability of enzyme conjugate, amino group's availability in enzyme for conjugation with increase in bridge length between analyte and enzyme to reduce steric hindrance.

Key Words: HRP-carbamide, immunoassay, 17α-OHP, cortisol, nandrolone, urea.

Introduction

The chemical cross-linking method, routinely employed in the diagnostic industry, is largely derived from the field of peptide chemistry and chemical modification of proteins, due to their importance either as carrier immunogens or as enzyme labels. Therefore, the basic functional groups of proteins and concept of protein modification are relevant to enzyme immunoassay.

Of the twenty amino acids with side chains of different sizes, shapes, charges and chemical reactivity that make up the protein molecules, reactive functional groups are provided by those amino acids that have ionizable side chains. In this regard, the alkyl side chains of the hydrophobic amino acids that are located in the interior of protein molecules are chemically inert and thus not available for modification. Similarly, the aliphatic hydroxyl groups of serine and threonine are considered as water derivatives and, therefore, have low reactivity. Further, the reactivity of the available functional groups, defined in terms of their ability to undergo chemical modification, is largely determined by the sequence location and their interactions with the side chains of neighboring amino acids in the overall three-dimensional structure of protein molecule (Deshpande, 1996).

The reactive groups that are located on the protein surface and thereby exposed to the aqueous environment are available for chemical modification and conjugation purposes. In proteins, there are essentially eight hydrophilic side chains that are chemically active (Tijssen, 1985; Wong, 1993). These side chains and their functional groups are: 1) amino group of N-terminal amino acids and α-amino groups of lysine, 2) sulfhydryl groups of cysteine, 3) thioether group of methionine, 4) carboxyl group of C-terminal amino acid and β- and γ-carboxyl groups of aspartic and glutamic acids, respectively, 5) phenolic group of tyrosine, 6) imidazole group of histidine, 7) guanidinyl group of arginine, and 8) indolyl group of tryptophan.

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Of these, the first five groups are chemically most reactive. They are also normally exposed on the protein surface and form the major targets for protein cross-linking and conjugation. Several proteins also contain carbohydrates that provide useful sites for chemical modifications and cross-linking of proteins.

Chemical modification of existing functional groups of protein is sometimes essential for several practical purposes. These include (a) activation of the inactive carbohydrates to functional groups for further chemical reactions, (b) inter-conversion into one another either to change the specificity or to increase their reactivity, and (c) incorporation of spacer arms to reduce steric hindrances as well as to decrease the influence of local environment (Kabakoff, 1980; Tijssen, 1985; Wong, 1993; Basu et al., 2006).

We describe for the first time the use of urea as a homo-bifunctional coupling reagent for the preparation of HRP-carbamide and its use in the development of immunoassays for haptens. In this new strategy, amino groups (-NH_2) of HRP were blocked by acetic acid N-hydroxysuccinimide followed by oxidation of carbohydrate moieties of HRP by sodium meta-periodate to form reactive aldehyde group (-CHO) and, subsequently, forming a Schiff's base with one of the amino groups of urea. The resultant HRP-carbamide was conjugated to carboxylic derivatives of steroids using the N-hydroxysuccinimide-carbodiimide method. The potential application of HRP-carbamide for the preparation of enzyme conjugate is demonstrated through the development of ELISAs for direct estimation of haptens like cortisol, 17α- OHP, and nandrolone.

Materials and Methods

Materials

Cortisol, cortisol-3-O-carboxymethyl-oxime (F-3-O-CMO), cortisol-21-hemisuccinate (F-21-HS), 17α-OHP, 17α-OHP-3-O-CMO, nandrolone, nandrolone-3-O-carboxymethyl-oxime (N-3-O-CMO) and other steroids were purchased from Steraloids Inc. Ltd., 94 Tachbrook Street, London SW 1V 2NB, England. Horseradish peroxidase (HRP) type VI (EC 1.11.1.7), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide-HCl (EDAC), N-hydroxysuccinimide (NHS) and Freund's complete adjuvant (FCA) were all purchased from Sigma Chemical Company, St. Louis, MO, USA. TMB/H_2O_2 substrate solution was purchased from Arista Biologicals Inc., USA. Microtiter plates were procured from Greiner-Bio-One, Germany. All other chemicals and buffer salts were of analytical grade.

Normal Rabbit Serum (NRS) was collected from New Zealand white rabbits. Anti-species antiserum (as a second antibody) was generated in goat using purified rabbit γ-globulin as an immunogen (Shrivastav, 2004). Polyclonal antiserum for cortisol, 17α-OHP, and nandrolone were generated against F-3-O-CMO-BSA, 17α-OHP-3-CMO-BSA and N-3-O-CMO-BSA, respectively, as immunogens, in New Zealand white rabbits (Shrivastav, 2004). NRS and second antibody were used for coating microtiter plate. The buffer was 10 mM phosphate (10 mmol/L PB), pH 7.0 ($Na_2HPO_4.2H_2O$: 0.895 g/l and $NaH_2PO_4.2H_2O$: 0.39 g/L) containing 0.9% NaCl and 0.1% NaN_3. The HRP conjugate dilution buffer was 10mmol/L acetate buffer (10 mmol/L AB), pH 5.6 (CH_3COONa: 0.84 g/L and 1mol/L CH_3COOH 1.5 ml/L), containing 0.1% thimerosal, dextran T-70 and 0.3%, BSA. The microtiter well blocking and stabilizing buffer was 10 mmol/L PB containing 0.9% NaC1, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid: di-potassium salt (EDTA: K salt), and 0.01% gentamicin sulfate.

Preparation of HRP–Carbamide (HRP-U/ HRP-Urea)

To incorporate urea to HRP enzyme, 1 ml of water per 1 mg of HRP and 1.5 mg of acetic acid N-hydroxysuccinimide were added. The reaction mixture was vortex-mixed and kept overnight at 4º C to block –NH_2 group of HRP. Thereafter, the reaction mixture was extensively dialyzed against water, and carbohydrate moieties of HRP were oxidized by addition of 10 µL of 0.01mol/L sodium meta-periodate to form aldehyde group (-CHO). Activated HRP (HRP-aldehyde) was passed through Sephadex G-25 column. A brownish fraction of activated HRP from G-25 column was directly collected in a vial containing 10 mg of urea. The reaction mixture was kept overnight at 4ºC to form Schiff-base between –CHO group of activated HRP and –NH_2 group of urea. The Schiff-bases formed were stabilized by addition of 1 µL of 5 mol/L sodium cyanoborohydride in 1 mol/L NaOH and kept for 3 hr at 4ºC. After stabilization, the conjugate was passed through Sephadex G-25 column, and the brownish fractions of HRP coupled with urea were pooled.

Preparation of F-21-HS-U-HRP, 17α-OHP-3-CMO-U-HRP and N-3-O-CMO-U-HRP conjugates

F-21-HS, 17a-OHP-3-CMO and N-3-O-CMO were conjugated to HRP-carbamide (HRP-urea). For this purpose, 5 mg of F-21-HS or 17α-OHP-3-CMO or

N-3-O-CMO were separately added to a tube containing 200 µL of dimethyl formamide and 200 µL of dioxan. 100 µL of distilled water containing 10 mg of N-hydroxysuccinimide and 20 mg of 1-ethyl-3- (3-dimethyl-amino-propyl) carbodiimide-HCl were added to each steroid solution. The reaction mixtures were vortex-mixed and kept overnight in refrigerator to activate the steroid. Thereafter, each activated steroid was added separately to 1 ml of HRP-cabamide solution and incubated for 24 hr in a refrigerator. The conjugates were passed through Sephadex G-25 columns. The conjugates were kept at –30º C in aliquots mixing with ethylene glycol (50% v/v) for future use.

Preparation of F-21-HS-HRP, 17α-OHP-3-CMO-HRP and N-3-O-CMO-HRP conjugates

F-21-HS, 17α-OHP-3-CMO and N-3-O-CMO were conjugated to HRP. For this purpose, 5 mg of F-21-HS or 17α-OHP-3-CMO or N-3-O-CMO were separately added to a tube containing 200 µL of dimethyl formamide, and 200 µL of dioxan. 100 µL of distilled water containing 10 mg of N-hydroxysuccinimide and 20 mg of 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide-HCl were added to each steroid solution. The reaction mixtures were vortex-mixed and kept in refrigerator to activate the steroid. After overnight incubation, each activated steroid were added separately to 1mg/ ml of HRP solution and incubated for 24 hr in a refrigerator. The conjugates were passed through Sephadex G-25 columns. The conjugates were kept at –30° C in aliquots mixing with ethylene glycol (50% v/v) for future use.

Coating of polystyrene wells with F-3-O-CMO or 17α -OHP-3-CMO or N-3-O-CMO antibodies

Polystyrene wells of microtiter plate were coated immunochemically either with F-3-O-CMO, 17α-OHP-3-CMO or N-3-O-CMO antibody following the method of Shrivastav et al (2003). Briefly, 250µL of (1:1000) diluted NRS in water was added in each well of microtiter plate and incubated overnight at 37ºC. The plates were washed in running tap water and 250µL of ARGG (1:4000) diluted in 10mmol/L PBS was added to all the wells of microtiter plate and incubated for 2 hr at 37ºC. After washing, 200µL of (1:4000) diluted F-3-O-CMO or (1:32000) diluted 17α-OHP-3-CMO or (1:4000) diluted N-3-O-CMO antibody was added to the wells of microtiter plates and incubated for 2 hr at 37ºC. The blocking and stabilizing buffer was added (250 µL) to all wells and incubated for 1 hr at 37ºC. The plates were dried and kept under desiccation at 4ºC for further use. The optimal dilutions of antibody to cortisol, 17α-OHP, nandrolone and their respective enzyme conjugates were worked out by checkerboard assay.

Standard preparation

Six cortisol standards (0, 1, 3, 10, 30 and 60 µg/ 100µL), six 17α-OHP standards (0, 0.2, 0.8, 2, 10 and 50ng/µL), and six nandrolone standards (0, 0.2, 0.8, 2, 10 and 50ng/µL), were prepared in pooled steroid-stripped serum, following the method described elsewhere (Shrivastav, 2004). Stripping of steroids from the serum was carried out by adding charcoal at a concentration of 50mg/mL to pooled serum followed by filtration through 0.45 µm membrane filter.

ELISA procedure

To the cortisol, 17α-OHP or nandrolone antibody-coated wells 25 µL of cortisol or 50 µL of 17α-OHP, 50 µL of nandrolone standards, controls or samples were added. The 100 µL of appropriate dilution of respective hapten-HRP conjugate (with or without urea bridge) was added to all wells and incubated for 1 hr at 37º C. Thereafter, the wells were decanted and washed in running tap water for 5-6 times by filling, decanting and flicking. The 100µL of substrate solution (tetramethyl benzidine/ hydrogen peroxide; TMB/H_2O_2) was added to all the wells and incubated for 20 min at 37º C. The reaction was stopped by adding 100 µL of 0.5 (M) H_2SO_4 and the color intensity was measured at 450 nm in Tecan-Spectra microplate reader.

Checking of thermo-stability of enzyme conjugates

Enzyme conjugates prepared with or without acetylated amino groups were kept at 37^{0}C in working dilutions and their enzymatic activity was determined using ELISA procedure as described above.

Checking of immuno-reactivity of enzyme conjugates

Enzyme conjugates, prepared with or without acetylated amino groups, were kept at 37º C in working dilutions. The enzyme conjugate that has lost its enzymatic activity was used as unknown and their immuno-reactivity was determined using ELISA procedure described above.

Results

Sensitivity

The lowest detection limit of the cortisol, 17α-OHP and nandrolone assays (concentration equivalent to B_0 – 2SD) using enzyme conjugate with urea bridge were 0.4 ng/ml, 0.05 ng/ml, and 0.12 ng/ml of serum, respectively,

after thirty-fold determination of B_0 binding, whereas using enzyme conjugate without urea bridge were 2.8 ng/ml, 0.09 ng/ml, and 7.31ng/ml, respectively.

Specificity of antibody

Cortisol, 17α- OHP and nandrolone antibody had less than 0.1% cross-reaction with naturally occurring C_{27}, C_{21}, C_{19} and C_{18} steroids. However, the cross-reaction of prednisolone (a synthetic glucocorticoid) was 25% in cortisol assay, 2.1% of progesterone in 17α-OHP assays and 100 % cross-reaction of testosterone in nandrolone assay using enzyme conjugate without urea bridge. When enzyme conjugates with urea bridge were used in respective assays, the percent cross- reaction of cross reacting steroids was further reduced to 0.01% of prednisolone (a synthetic glucocorticoid) in cortisol assay, 1.8% of progesterone in 17α-OHP assay and, 10 % of testosterone in nandrolone assay.

Analytical recoveries

The ability of the respective assays to accurately quantify cortisol, 17α-OHP and nandrolone in serum samples was tested. Low, medium and high concentrations (1.0-20ng/ml) of cortisol, 17α-OHP and nandrolone were added exogenously to three fractions of pooled serum. After addition, the concentration of cortisol, 17α-OHP and nandrolone were determined and the recovery was calculated for each fraction of serum. The recovery ranged from 93.3% to 100.0%, 94.3% to 98.7%, and 93.5 % to 104 % using enzyme conjugate with urea bridge, whereas recovery ranged from 85.8% to 98.7% and 83.4% to 110.6% in cortisol and 17α-OHP assays, respectively using enzyme conjugate without urea bridge. Tables 1-3 represent the recovery profile for cortisol, 17α-OHP and nandrolone, respectively.

Thermostability study

Directly prepared enzyme conjugates were less stable then the enzyme conjugates prepared using acylated enzyme. There is complete loss of enzyme activity after 96 hr of incubation with directly prepared enzyme conjugates at 37° C. On the other hand, enzyme conjugates prepared using acylated enzyme retained more than 85% of enzyme activity after 96 hr of incubation at 37° C. The thermostability study of enzyme conjugates revealed that the enzyme conjugates prepared using enzyme where amino groups are acylated with an organic acid has more thermostability than directly prepared enzyme conjugates.

Immunoreactivity

The enzyme conjugates which had lost enzyme activity after 96 hr of incubation at 37° C were tested for the retention of immunoreactivity by utilizing them as unknown. The immunoreactivity study revealed that though the enzyme conjugates have lost their enzyme activity, they retained the immunoreactivity.

Discussion

We demonstrate for the first time the use of urea as a homo-bifunctional coupling reagent for the preparation of HRP-carbamide and its use in the development of ELISA

Table 1: Recovery of cortisol from exogenously spiked serum pools using F-21-HS-HRP and F-21-HS-Urea-HRP.

Serum pool no.	Spiked cortisol (µg / dL)	F-21-HS-HRP			F-21-HS-Urea - HRP		
		Observed value (µg/dL)	Expected value (µg / dL)	% Recovery	Observed value (µg/dL)	Expected value (µg / dL)	% Recovery
A	-	8.9	-	-	10.4	-	-
B	5	13.6	13.9	97.8	15.4	15.4	100.0
C	10	17.7	18.9	93.6	20.3	20.4	99.5
D	15	21.0	23.9	87.8	23.7	25.4	93.5
E	20	24.8	28.9	85.8	28.0	30.4	93.3

Table 2: Recovery of 17 QH - P from exogenously spiked serum pools using 170 OH-P- HRP and 170 H - P - Urea - HRP.

Serum pool no.	Spiked cortisol (µg / dL)	F-21-HS-HRP			F-21-HS-Urea - HRP		
		Observed value (µg/dL)	Expected value (µg / dL)	% Recovery	Observed value (µg/dL)	Expected value (µg / dL)	% Recovery
A	-	4.43	-	-	2.40	-	-
B	2.5	6.61	6.64	99.5	4.71	4.97	94.7
C	5.0	8.30	7.50	110.6	7.38	7.47	98.7
D	15.0	15.98	19.14	83.4	16.49	17.47	94.3

Table 3: Recovery of nandrolone from exogenously spiked serum pools using nandrolone-3-CMO-Urea-HRP.

Serum pool no.	Nandrolone added (µg/mL)	Observed value (µg/mL)	Expected value (µg / mL)	% Recovery
Pool A	-	0.93	-	-
Pool B	2.0	3.05	2.93	104.0
Pool C	5.0	5.82	5.93	98.1
Pool D	10.0	10.22	10.93	93.5

for direct estimation of haptens like cortisol, 17α-OHP and nandrolone in serum. The assays thus developed are sensitive, accurate and specific. In the present study, the strategy adopted for preparing HRP-carbamide and its subsequent use in preparing enzyme conjugate may result in improvement in EIA like i.e., a) increase in thermostability of enzyme conjugate, b) increased availability of amino groups in enzyme for conjugation, and c) increase in bridge length between analyte and enzyme to reduce steric hindrance.

In the present study, acylation of the lysine residues of HRP with N-hydroxysuccinimide resulted in an increased thermostability as compared to native enzyme. Rennke and Venkatachalam (1979) showed that the derivaization of HRP with succinic anhydride yielded a polycarboxylated molecule (pKa < 4) which had no detectable effect on the pH optimum or enzyme activity in a colorimetric assay. Guedon et al. (1979) observed similar retention of activity during extensive biotinylation. Ugarova et al. (1978a, b, c, 1979) investigated the effect of derivatizing the amine groups of HRP-C in more detail. Anhydrides of mono- and dicarboxylic acids were capable of acylating four lysine residues at 0° C and pH 8.0, while more bulky trinitrobenzenesulphonic acid (TNBS) modified three lysine residues. Increasing temperature facilitated further derivatization. For example, TNBS modified four groups at 22° C and pH 9.5, and all six-lysine residues at 40° C and pH 8.5.

Enzyme activity was remarkably stable to lysine modification; no loss in activity was detected under any one of conditions described above. An introduction of extra negative charges by succinylation caused a broadening of the pH-dependence around the same optimum (5.5-6.0), and additionally showed greater resistance to thermal inactivation than native HRP (Ugarova et al., 1978a). The succinylated derivative of HRP showed a 90-fold increase

in stability at 56° C over the native enzyme, which was attributed to a reduction in mobility around the heme group. Similarly Ryan et al. (1994) observed substantial stabilization at 75° C when HRP was derivatized with bis-succinimide esters; these were able to derivatize five lysine residues at room temperature, and no cross linking were observed under the condition used.

The direct coupling of carboxylic derivative of a steroid to an amino group of enzyme is well-established method in the steroid enzyme immunoassay for preparing enzyme conjugate (Dent and Aslam, 1998a). In the present study, HRP acylation is followed by oxidation of carbohydrate moieties of HRP by sodium meta-periodate to form reactive aldehyde group (-CHO) and subsequently forming a peptide bond with one of the amino groups of urea.

HRP is a widely used enzyme in EIA, containing six lysine residues in sequence; however, in practice, only one or two of these are generally available for reaction (Dent and Aslam, 1998b). This variation in amino group content is caused by changes in extraction conditions used for the isolation of HRP from roots of horseradish plant (Ornstein, 1966; Dent and Aslam, 1998c). The low yield of HRP coupled to IgG by the use of bifunctional reagents, namely glutaraldehyde, carbodiimide, cyanuric chloride, bis-diazotized O-dianisidine, and p-p'-difluoro-m, m-dinitrophenyl sulfone (FNPS), and so forth, prompted Nakane and Kawaoi (1974) to investigate another method (periodate method) for the conjugation of HRP to IgG. Comparative coupling efficiency studies were carried out with the use of glutaraldehyde, periodate, and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as cross-linking reagents (Boorsma and Streefekerk, 1979; Jeanson et al., 1988) for the preparation of HRP-IgG conjugate. These studies revealed that the most efficient HRP-IgG conjugate was obtained by periodate method. In practice, the difference in amino group availability in different batches of commercial preparation of HRP were observed (Basu et al., 2003; Shrivastav, 2004), which makes it difficult to establish standard reaction conditions that could be applicable for more than one batch. Introduction of bifunctional reagent like urea in HRP enzyme where amino groups are not available makes HRP enzyme functional in term of availability of amino group.

Enzyme immunoassay is popular in the field of clinical applications for its simplicity in measurement. In steroid immunoassay, it is always required to conjugate carrier protein with steroid derivative for immunogen preparation to generate antibody. Similarly, enzyme is required to conjugate with steroid derivative for the preparation of enzyme conjugate to monitor antigen-antibody interaction. Hence, there is an increase or decrease of labeled steroid recognition by antibody that affects sensitivity of the assay. The incorporation of urea between steroid derivatives and enzyme might have increased the distance between steroids and enzyme. As a result, there may be an easy access for native steroid to antibody binding sites, resulting in better displacement of enzyme conjugate leading to increased sensitivity and specificity. The reason of getting sensitive and specific assays of cortisol, 17α-OHP and nandrolone using cortisol, 17α-OHP and nandrolone coupled through urea bridge than directly coupled with enzyme may be due to differential kinetic interaction with respective antibody and this can be further studied.

In conclusion, for the first time HRP-carbamide was prepared, and while doing so we acylated amino group that resulted in the thermostability of enzyme carbamide complex. The HRP-carbamide reagent may also be used for coupling to nucleic acid for nucleic acid hybridization assay and to protein for preparing enzyme conjugates for immunoassay and immunochemistry.

Acknowledgments

The National Institute of Health and Family Welfare, New Delhi, India supported this study. We are grateful to Profs. D. Nandan, M. C. Kapilashrami, N. K. Sethi and K. Kalaivani, for their keen interest and encouragement.

References

Basu A, Shrivastav TG, Kariya KP (2003) Preparation of enzyme conjugate through adipic acid dihydrazide as linker and its use in immunoassays. *Clin Chem* 49: 1410-1412.

Basu A, Nara S, Chaube SK, Rangari, K, Kariya KP, Shrivastav TG (2006) The influence of spacer-containing enzyme conjugate on the sensitivity and specificity of enzyme immunoassays for hapten. *Clin Chim Acta* 366: 287-292.

Boorsma DM, Streefekerk JG (1979) Periodate or glutaraldehyde for preparing peroxidase conjugates. *J Immunol Methods* 30: 245-255.

Dent AH, Aslam M (1998a) The preparation of protein-small molecucle conjugate. In Aslam M. and Dent A., (Eds), *Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences*, Ch. 6, pp 364-482, Macmillan Reference Ltd: London.

Dent AH, Aslam M (1998b) The preparation of protein-small molecucle conjugate. In Aslam M and Dent A (eds) *Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences*, Ch. 3, p 120, Macmillan Reference Ltd, London.

Dent AH, Aslam M (1998c) Enzymes. In Aslam M and Dent A (Eds), *Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences*, Ch. 3, p 121. Macmillan Reference Ltd, London.

Deshpande SS (1996) Conjugation techniques. In: Deshpande SS (Ed), *Enzyme Immunoassays from Concept to Product Development*. Ch. 4, pp 72-110, Chapman & Hall, New York.

Guedon JL, Ternynck T, Averameas S (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. *J Histochem Cytochem* 27: 1131-1139.

Jeanson A, Cloes JM, Bouchet M, Rentier B (1988) Comparison of conjugation procedure for the preparation of monoclonal antibody-enzyme conjugates. *J Immunol Methods* 111: 261-270.

Kabakoff DS (1980) Chemical aspects of enzyme immunoassay. In: Maggio ET (Ed), *Enzyme Immunoassay*, pp 71-104, Boca Raton, Florida.

Nakane PK, Kawaoi A (1974) Peroxidase-labeled antibody: a new method of conjugation. *J Histochem Cytochem* 22: 1084-1091.

Ornstein, L. Discussion on "enzyme-labeled antibodies for light and electron microscopic localization of antigens" by PK Nakane et al. J Histochem Cytochem 1966, 14, 790.

Rennke HG, Venkatchalam MA (1979) Chemical modification of horseradish peroxidase. Preparation and characterization of tracer enzymes with different isoelectric points. *J Histochem Cytochem* 27:1352-1353.

Ryan O, Smith MR, Fagain CO (1994) Thermostabilized chemical derivatives of horseradish peroxidase. *Enzym Microb Technol* 16: 501-505.

Shrivastav TG (2004) Incorporation of different bridge length linkers in enzyme and its use in the preparation of enzyme conjugates for immunoassay. *J Immunoassay Immunochem* 25: 215-225.

Shrivastav TG, Basu A, Kariya KP (2003) Substitution of carbonate buffer by water for IgG immobilization in enzyme linked immunosorbant assay. *J Immunoassay Immunochem* 24: 191-203.

Tijssen P (1985) *Practice and Theory of Enzyme Immunoassay*, Elsevier, Amsterdam.

Ugarova NN, Rozhkova GD, Vasileva TE, Berezin IV (1978a) Chemical modification of epsilon-NH2 groups of lysine residues in horseradish peroxidase. Accessibility of these groups to different modifying agents. *Biokhimiia* 43: 793-797.

Ugarova NN, Rozhkova GD, Berezin IV (1978b) Chemical modification of epsilon-amino lysine groups in horseradish peroxidase. Its effect on catalytic properties and spatial structure of the enzyme. *Biokhimiia* 43: 1242-1250.

Ugarova NN, Rozhkova GD, Berezin IV (1978c) Chemical modification of lysine epsilon-NH2-groups in horseradish peroxidase. Its effect on enzyme stability. Temperature dependence of thermo-inactivation constants for native and modified peroxidase. *Biokhimiia* 43:1382-1389.

Ugarova NN, Rozhkova GD, Berezin IV (1979) Chemical modification of the epsilon-amino groups of lysine residues in horseradish peroxidase and its effect on the catalytic properties and thermostability of the enzyme. *Biochim Biophys Acta* 570: 31-42.

Wong SS (1993). *Chemistry of Protein Cnjugation and Cross-Linking*. Boca Raton: Florida.