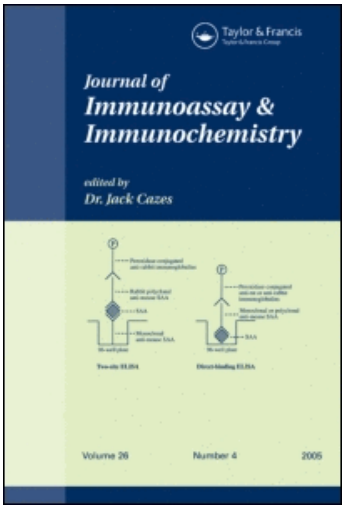

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 08 July 2002

To cite this Article Nanda, Sarita , Muralidhar, K. and Kar, S. K.(2002) 'THERMOSTABLE α-AMYLASE CONJUGATED ANTIBODIES AS PROBES FOR IMMUNODETECTION IN ELISA', Journal of Immunoassay and Immunochemistry, 23: 3, 327 — 345
To link to this Article: DOI: 10.1081/IAS-120013032
URL: http://dx.doi.org/10.1081/IAS-120013032

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JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 23, No. 3, pp. 327–345, 2002

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ABSTRACT

Thermostable α-amylase from *B. licheniformis* has been conjugated with high efficiency to goat antibodies against human, mouse, and rabbit immunoglobulins to prepare second-step reagents which can be used in Enzyme Linked Immunosorbent Assays (ELISA). Various conjugation methods, such as one- and two-step glutaraldehyde coupling and cross-linking, using heterobifunctional reagents such as sulfo-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carbonate (sulfo-SMCC) and *N*-succinimidyl-*S*-acetylthioacetate (SATA), yielded active α-amylase labeled second antibodies. Such conjugates had molecular sizes ranging between 200–300 kDa. Filter sterilized solutions of conjugates, when stored at 37°C

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DOI: 10.1081/IAS-120013032 1532-1819 (Print); 1532-4230 (Online)

for two weeks, retained 32% of their biological activity and were thermostable even after keeping for 1 h at 90°C.

INTRODUCTION

Enzyme labeled antibodies have been used widely in enzyme linked immunosorbent assays (ELISA).[1,2] Enzymes like horseradish peroxidase (HRP, EC 1.11.1.7), alkaline phosphatase (AP, EC 3.1.3.1), and β-galactosidase (β Gal, EC 3.2.1.23) are examples of such enzymes, which have been used to make antibody conjugates. The methods of conjugation of enzymes to antibodies are well established.[3–7] Of these enzymes, HRP labeled probes are most popular, as HRP of high purity is available at low cost and has several options of using substrates with different types of applications. However, HRP enzyme and its conjugates are both thermolabile and lose their activity slowly at even 4°C. Calf alkaline phosphatase (CAP) is also one of the very popular labels used for preparing antibody conjugates because of its high turnover number. However, its usefulness is also limited due to its high cost. Due to these shortcomings, there have been attempts to find reagents which have properties similar to the established reagents but are thermostable. Pencillinase (EC 3.5.2.6) has been reported to form conjugates which can retain 100% activity at 37°C for one-month.[8] But, we have observed in our laboratory that these conjugates also lose their activity, even when stored at 4°C. Besides, pencillinase is also one of the most expensive commercially available enzymes. Bacterial alkaline phosphatase (BAP) is superior because of its high thermostability and low specific activity. Attempts have been made to increase the specific activity of BAP by creating recombinant molecules by introduction of single point mutations. This recombinant BAP, with high specific activity and high thermostability, was used successfully as an enzyme label.[9] This conjugate has not been commercially exploited, probably because of high cost of the enzyme and its being available only in small quantities. Therefore, we looked into the literature for an enzyme which is rugged, easily available in large quantities, is cheap, and has the potential to be used as an enzyme label. Alpha (α) amylase (EC 3.2.1.1) of *B. licheniformis* was seen as an appropriate candidate, as it has all of these qualities. It is a heat stable enzyme which can retain 80% of its initial activity at 90°C for one hour.[10] It has a low molecular weight of 55 kDa. It is cheap and is easily available in large quantities. It has a high specific activity of 1000–1500 U/mg, which can be measured spectrophotometrically. The α-amylase molecule has several free amino groups, which can be used for conjugation with antibodies. In this communication, we highlight methods of conjuga-

tion of α-amylase with antibodies and standardization of its use in immunodetection.

EXPERIMENTAL

α-Amylase of *B. licheniformis*, glutaraldehyde, chromogenic substrates, ELISA plates, centricon tubes with 100 kDa and 300 kDa cut off, dialysis wells, and membrane filters were purchased from Sigma Chemical Co., St. Louis, MO, USA, Antibodies against human, rabbit, and mouse immunoglobulins were raised in goat and then purified in the laboratory, or purchased from M/S Genei, Bangalore, India. HRP conjugates and their substrates were purchased from M/S Genei, Bangalore, India. Plasticware, disposable microfuge tubes, and Eppendorf pipettes were purchased from M/S Tarsons, Calcutta, India. All other reagents were of analytical grade. A Dynatech 250 model, ELISA reader was used for this study.

Conjugation of α-Amylase to Different Antibodies—One Step Glutaraldehyde Method

α-Amylase was coupled to goat second antibodies to human, rabbit, and mice immunoglobulin (IgG) by slight modification of the published procedure.[11] One mg α-amylase and 1 mg goat IgG in 100 μL of 0.1 M phosphate buffered saline (PBS), pH 7.4, and 6 μL of 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, were incubated with constant shaking at 4°C overnight. 20 μL of 1 M glycine was added next and incubated for 1 h at room temperature (RT) with constant shaking. The resulting conjugate was passed through the Centricon tubes with 100 kDa cut off membrane to remove unconjugated enzyme and stored in 200 μL 0.1 M PBS, pH 7.4, at 4°C.

Two-Step Glutaraldehyde Method

We followed a procedure which was a slight modification of the procedure given earlier.[3,4] One milligram α-amylase in 200 μL of 0.1 M PBS, pH 7.4, and 6 μL of 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, were incubated overnight at 4°C with constant shaking. Excess glutaraldehyde was removed by dialysis in 0.02 M carbonate buffer, pH 9.5, using dialysis wells (Millipore, USA). 0.25 mg goat antibody in 100 μL 0.02 M carbonate buffer, pH 9.5, was then added, pH adjusted to >9.0 with 0.2 M carbonate buffer, pH 9.5, and incubated with constant shaking overnight at 4°C. Then, 20 μL

of 1 M glycine was added and incubated for 1 h at RT. The conjugate was passed through Centricon tubes with 100 kDa cut off membrane to remove unconjugated enzyme and then was stored in 200 μL 0.1 M PBS, pH 7.4. This method was standardized by optimizing with different (a) concentrations of glutaraldehyde (b) pH for conjugation (c) ratios of enzyme and antibody (d) time and temperatures for conjugation, and (e) concentrations of second antibodies.

Periodate Method

This was done by modification of the method given earlier.[5] One milligram of α-amylase in 200 μL distilled water was incubated with 1.5 mg of sodium periodate overnight at 4°C with constant shaking. Excess periodate was removed by dialysis using dialysis wells (Millipore, USA). Then, 0.50 mg of goat antibody in 100 μL carbonate buffer was added and pH adjusted to 9.5 with 0.2 M carbonate buffer, pH 9.5. Then, incubation continued with constant shaking overnight at 4°C. After that, 100 μL of sodium borohydride was added and incubated for 1 h at RT. The conjugate was passed through Centricon tubes with 100 kDa cut off membrane to remove the unconjugated enzyme and then was stored in 200 μL of 0.1 M PBS, pH 7.4 at 4°C.

Maleimidobenzoyl-*N*-hydroxysuccinimide Ester (MBS) Coupling

We followed the published method[6] with slight modification. Ten microlitres of MBS solution in dioxane (10 mg/mL) was added to 0.50 mg goat antibody in 100 μL of 0.1 M PBS, pH 7.4, and incubated for 1 h at RT. Excess MBS was removed by dialysis using a dialysis well (Millipore, USA). One milligram α-amylase was added next and kept shaking overnight at 4°C. Then, 2 μL of 10 mM mercaptoethanol was added and incubated for 1 h at RT. The conjugate was passed through Centricon tubes with 100 kDa cut off membrane to remove the unconjugated enzyme and stored in 200 μL of 0.1 M PBS, pH 7.4, at 4°C.

Coupling with Heterobifunctional Reagents

Coupling was performed with little modification of the published procedure.[12] Six milligrams of α-amylase in 200 μL of 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2, was incubated with 2 mg of sulfo-

SMCC for 30 min at RT with constant shaking. Excess crosslinker was removed by passing through Centricon tubes with 30 kDa cut off membrane and the cross-linked enzyme was used immediately. For thiolation 1 mg goat antibody in 1 mL of 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2, was incubated with 40 μL of 8 mg SATA in dimethyl sulfoxide (DMSO; 8 mg/mL) for 30 min at RT with constant shaking. Excess SATA was removed by passing through Centricon tubes with 100 kDa cut off membrane. SATA modified antibody was activated by incubation for 2 h at RT with 100 μL of 0.5 M hydroxylamine solution in 0.1 M sodium phosphate buffer, pH 7.2, containing 10 mM ethylenediaminetetraacetate (EDTA) for 2 h at RT. Excess hydroxylamine was removed by passing through Centricon tubes with 100 kDa cut off membrane. The thiolated antibody was incubated with maleimide activated enzyme in 4 : 1 ratio overnight at 4°C with constant shaking. Conjugate was passed through Centricon tubes with 100 kDa cut off membrane and stored in 0.1 M PBS, pH 7.4.

Characterization of the Formed Conjugate

The α-amylase second antibody conjugate was characterized for its molecular weight and its thermostability. The molecular weight of the formed conjugate was determined by it's mobility in 5% native PAGE, as well as its ability to pass through, or to be retained in, centricons tubes with 100 kDa and 300 kDa cut off membranes, respectively. The thermostability of the formed conjugate was established by observing activity of the conjugates stored at 4°C, 25°C, RT, 37°C, 60°C, and 90°C for various periods of time.

Selection of Substrate to Achieve Maximum Sensitivity

Substrates that were used for colorimetric estimation of enzyme activity were starch and iodine,[13] 4-nitrophenylmaltoheptaoside, α-glucosidase (EC 3.2.1.20; 14), and blue starch.[15]

Checking the Ability of the Conjugates to be Used as Immunodetectors

The ability of the conjugates to immunodetect was observed in immunoassay tubes and ELISA using different (a) dilutions of conjugate,

(b) temperatures and times of incubation with conjugate, (c) concentrations of coated antigen, (d) checkerboard analysis with different dilutions of antisera and conjugate, and (e) termination of ELISA with or without alkali.

Application of Conjugate in Detection of Different Analytes in ELISA

Analyte detection in direct, indirect, and competitive ELISA modes was used. The validity of its use was established by observing the inter-assay and intra-assay coefficient of variation (CV).

Direct Detection of Coated Human, Rabbit, and Mouse IgG

Direct ELISA was set up using the conventional method.[16] Human, rabbit and mouse IgG were used for coating at 250–2 ng IgG/well using 0.1 M PBS, pH 7.4, or 0.01 M carbonate buffer, pH 9.5. Blocking was done with 1.0% BSA in 0.01 M PBS, pH 7.4, for 1 h at 37°C. Conjugates were used at a dilution of 1 : 500 and incubated for 1 h at 37°C. The plates were then washed with 0.1% Tween 20 in 0.01 M PBS, pH 7.4. To observe the activity, 50 μL of substrate (ETG_7PNP and α-glucosidase) was added to wells and plates and incubated at 37°C for 30 min. The reaction was terminated with 100 μL of 0.5 N KOH and the color was read at 405 nm in the ELISA reader.

Indirect Detection of Antigen Specific Antibodies Present in the Sera of Filarisis Patient

Fifty microlitres of a 20 μg/mL solution of a soluble antigen from *Setaria digitata* adult was used to coat wells in 0.01 M carbonate buffer, pH 9.5, at 4°C. Blocking was done with 1.0% bovine serum albumin (BSA) in 0.01 M PBS, pH 7.4, for 1 h at 37°C. Sera from different filaria patients were serially diluted and added to antigen coated wells and incubated overnight at 4°C. The wells were then washed with 0.1% Tween 20 in 0.01 M PBS, pH 7.4, and incubated with 1 : 500 diluted goat antihuman α-amylase conjugated antibodies for 1 h at 37°C. After washing, 50 μL of substrate (ETG_7PNP and α-glucosidase) was added and incubated for 30 min at 37°C. The reaction was terminated with 100 μL of 0.5 N KOH. The color was read at 405 nm in an ELISA reader.

Competitive Detection of Buffalo Luteinizing Hormone (buLH)

The assay was set up, essentially, as described for measuring buffalo prolactin.[17] Fifty microlitre of 1 μg/mL solution of buLH was used to coat A 96 well plate in 0.1 M PBS, pH 7.4. Blocking was done with 1.0% BSA in 0.01 M PBS, pH 7.4. Anti buLH serum at dilution of 1 : 500 and serially diluted buLH (250–2 ng) were added together to appropriate wells and incubated together at 4°C overnight. After this, the plate was washed with 0.1% Tween 20 in 0.01 M PBS, pH 7.4. Incubation with 1 : 500 diluted conjugate was then done for 1 h at 37°C. After washing, 50 μL of substrate (ETG_7PNP and α-glucosidase) solution was added to all wells and incubated for 30 min at 37°C. The reaction was terminated with 100 μL of 0.5 N KOH and the color was read at 405 nm using the ELISA reader.

Comparison of α-Amylase Conjugates with Other Conjugates and Labels

α-Amylase conjugates were compared with HRP conjugates as regards to their thermostability and limit of detection (LOD) by using the formula: Any value > Blank value = 3SD. Different characteristics of established enzyme labels used for conjugation were reviewed and compared with α-amylase.

RESULTS

The conjugation methods that worked well were those using homobifunctional agents like glutaraldehyde[18] and the heterobifunctional agent like the sulfo-SMCC-SATA (Table 1, Fig. 1). Comparing the activity of conjugates formed by one- and two-step glutaraldehyde methods, it was found that the latter method had better activity at increasing dilutions (Fig. 2). The conjugation was best achieved at alkaline pH of 9.5 (Fig. 3). The enzyme α-amylase could be conjugated to goat antibodies raised against rabbit, human and mouse IgG by using 50–100 μL of 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4/mL of enzyme solution. Lower concentrations of glutaraldehyde formed better conjugates than the higher ones. Conjugation was found to be achieved in 2 h at 37°C or in 24 h at 4°C (Table 2). Different molar ratios of enzyme: antibody were used to prepare the conjugates. Increasing the enzyme concentration, while keeping that of antibody constant, or vice versa, was found to affect the activity of the formed conjugate (Fig. 4).

Table 1. Methods of Conjugation Tried for Conjugation of α-Amylase with Antibody

Methods of Conjugation	Enzyme Activity Retained After Passing Through Centricon Tube with 100 kDa Cut Off Membrane	Enzyme Activity Which Passed Through Centricon Tube with 100 kDa Cut Off Membrane
Glutaraldehyde method	+	−
MBS method	−	+
Periodate method	−	+
Crosslinking with SMCC-SATA	+	−

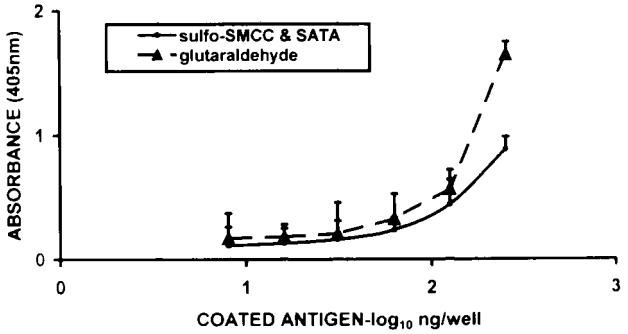

Figure 1. Immunodetection of coated antigen by α-amylase conjugates formed by various methods.

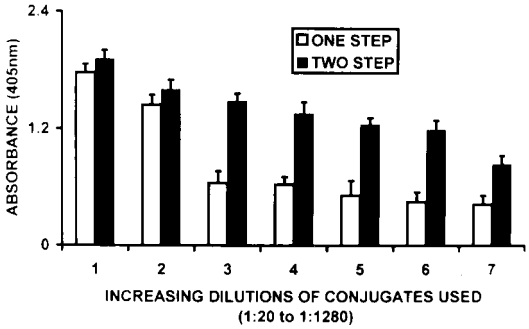

Figure 2. Comparison of conjugates formed by glutaraldehyde methods.

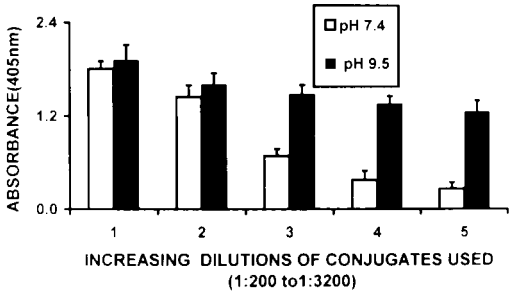

Figure 3. Activity of conjugates formed in buffers of different pH's.

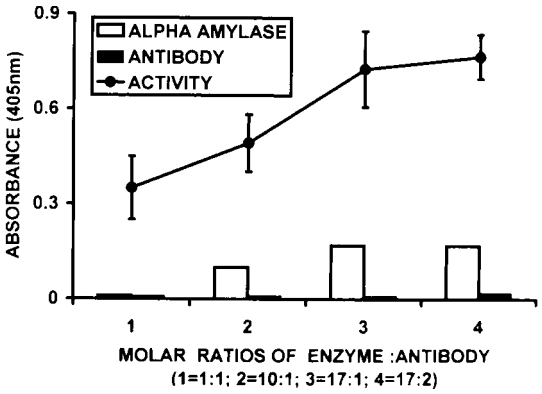

Figure 4. Activities of conjugates formed with different ratios of enzyme : antibody molecules.

Table 2. Activity of Conjugates Formed at Different Time and Temperature

Time of Incubation	Temperature of Incubation	Activity (A_{405}) at the Dilution of 1 : 500
24 h	4°C	0.780 ± 0.059
02 h	37°C	1.000 ± 0.038

The substrate starch and iodine and the blue starch were found to be unsuitable for the purpose of immunodetection because of their poor sensitivities. The substrate which could be used for immunodetection consisted of 1 mmol/L 4,6-ethylidene-1-*p*-nitrophenyl-α-D-maltoheptaoside(ETG_7PN),

10 mM $MgCl_2$, 50 mM NaCl, 25000 U/L α-glucosidase in phosphate buffer, pH 7.4. The reaction that is catalyzed by α-amylase using this substrate is given below:

$$5\text{ET-G}_7\text{PN} + \alpha\text{-Amylase} \rightarrow 2\text{ET-G}_5 + 2\text{G}_2\text{PN} + 2\text{ET-G}_4 + 2\text{G}_3\text{PN} + \text{ET-G}_3 + \text{G}_4\text{PN}$$

$$2\text{G}_2\text{PN} + 2\text{G}_3\text{PN} + \alpha\text{-Glucosidase-} \rightarrow 4\text{p-PN} + 10\ \text{Glucose}$$

α-Amylase (1,4-α-D-glucanohydrolase; EC 3.2.1.1) hydrolyses ETG_7PN to maltodioside, maltotrioside, and maltotetraoside nitophenyl fragments.

α-Glucosidase (α-D-glucoside glucohydrolase; EC 3.2.1.20) hydrolyses G_2PN and G_3PN to yield *p*-nitrophenol and glucose. Five moles of substrate (ETG_7PN) hydrolyze to yield 4 moles of *p*-nitrophenol. 100–200 μL of 0.5 N potassium hydroxide (KOH) terminates the reaction and deepens the yellow color that is formed. The substrate remained active for a year when kept at 4°C in the lyophilized form. Each vial was reconstituted with 3.5 mL of distilled water and 50 μL/well of substrate was sufficient to observe the activity of the conjugate. A reconstituted vial, when stored for 2 weeks at 4°C, did not deteriorate in its activity.

To optimize the concentrations of different components, and to obtain better sensitivity, various concentrations of *p*-nitrophenylmaltoheptaoside were used with a single concentration of α-glucosidase (25 U/mL). Higher concentrations of *p*-nitrophenylmaltoheptaoside gave higher blank values (Fig. 5). Therefore, 1 mg/mL was the selected as the working concentration for this substrate. Increasing units of α-glucosidase above 25 U/mL in the assay mixture improved detectable activity but, unfortunately, the cost of the assay also increased. Thus, this substrate medium was then prepared as a 1 mL cocktail consisting of α-glucosidase 25 Units, 1 mg/mL *p*-nitrophenyl-maltohepataoside containing 0.01 M $MgCl_2$, and 0.01 M PBS, pH 7.4.

The incubation temperature affected the amount of product formed. A bell shaped curve was obtained when the substrate was incubated at different temperatures. The optimum temperature was observed to be 37°C, as the blank at that temperature was colorless and the activity was high. The absorbance at 405 nm increased linearly with increasing time of incubation. The color developed was stable. The plates were read directly after color development, or the reaction was terminated by addition of alkali (0.5 N KOH) and then absorbance was read at 405 nm. There was enhancement of absorbance with alkali. Whenever the plates could not be read immediately, the reaction was not terminated with alkali, but was kept at 4°C overnight and read the next day without loss of sensitivity in immunodetection.

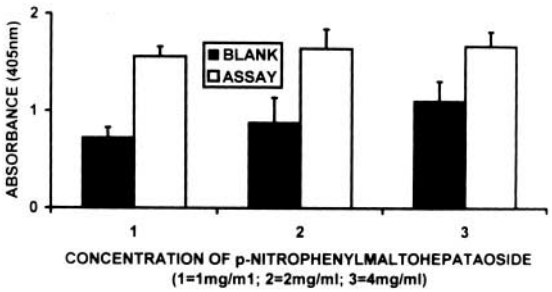

Figure 5. Activity of α-amylase conjugate with different concentrations of substrate.

Table 3. Activity of Conjugate Observed After Passing Through Centricons with Different Mol. Wt. Cut off Membrane

Centricon Used	Activity Observed in Retentate	Activity Observed in Elute
100 kDa	+	−
300 kDa	±	±

Table 4. Thermostability of α-Amylase Conjugated Antibody Conjugate at Different Temperatures

Temp. of Incubation for 1 h	Activity (A_{405}) at 1 : 500 Dilution
25°C	0.147 ± 0.056
37°C	1.668 ± 0.054
60°C	1.340 ± 0.035
90°C	0.434 ± 0.047

Electrophoretic mobility analysis, using 5% native PAGE, indicated that the conjugates formed were of approx. 200–300 kDa in molecular size. This was confirmed when activity was observed in conjugates passed through the Centricon tubes with 100 kDa and 300 kDa cut off membranes (Table 3). Activity was observed in the retentate of the former and the filtrate of the latter Centricons only.

The thermostability studies showed that the conjugate remained active after remaining for an hour at 90°C, for 2 h at 60°C and for several days at 37°C (Table 4, Fig. 6).

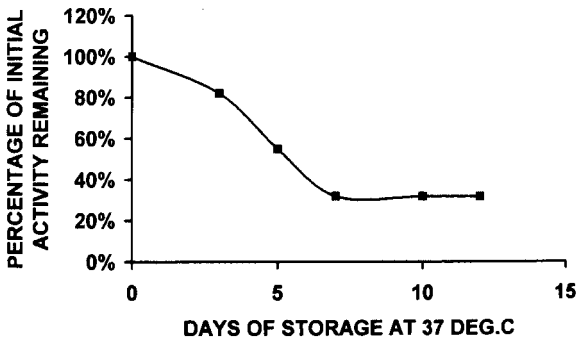

Figure 6. Shelf life of α-amylase conjugate at 37°C.

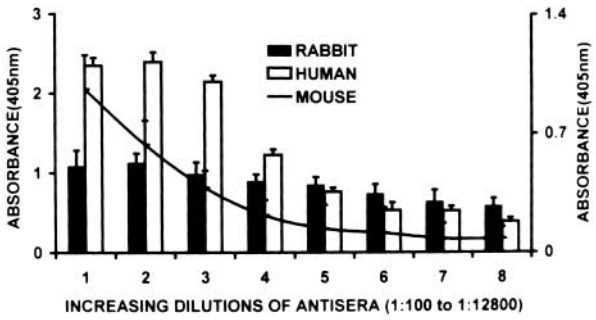

Figure 7. Immunodetection of antisera titre by α-amylase conjugate.

The formed conjugates were passed through Centricon tubes with 100 kDa cut off membranes and were stored in sterile 0.1 M PBS, pH 7.4, at 4°C. The stored conjugates retained good activity for 1 year. For longer storage, conjugates were frozen at −20°C in 50% glycerol. Under these conditions, they retained activity for 2 years.

The α-amylase conjugate's ability to act as a reagent for immunodetection was checked in various ways. Its ability to act as a probe was checked at various dilutions (Fig. 3) with different analytes, such as coated rabbit IgG as antigen (Fig. 1) and to detect antibody titres in rabbit, human, and mouse sera (Fig. 7). The conjugate was observed to be sensitive when used at different dilutions to antisera used also at different dilutions in the checkerboard assay (Fig. 8). The conjugate showed equal ability for immunodetection after its incubation at 37°C for 1 h or after incubation at 4°C for 24 h (Fig. 9). The α-amylase conjugated antibodies

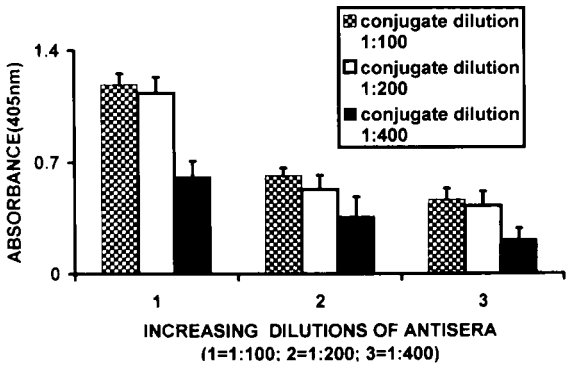

Figure 8. The checkerboard assay of α-amylase conjugate with different dilutions of antisera and conjugate.

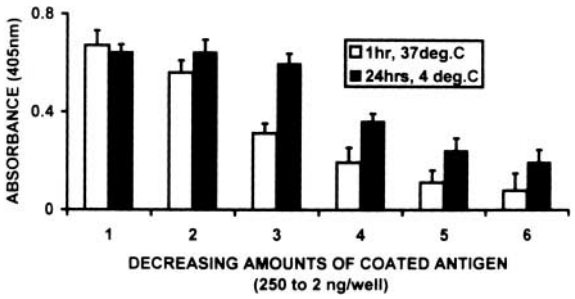

Figure 9. Immunodetection by α-amylase conjugate after incubation at different times and temperatures.

have been found to be useful in detecting different concentrations of rabbit IgG coated to wells in direct ELISA (Figs. 1 and 10). The goat antihuman IgG α-amylase conjugate was used to detect titres of parasite antigen specific antibodies in Filariasis patient sera by indirect ELISA (Table 5). Similarly, the goat antirabbit IgG α-amylase conjugate was used to detect the competing buLH in competitive ELISA (Fig. 11). Intra-assay and inter-assay coefficients of variations (CV) were observed to be 2–7% and 5–13%, respectively. The α-amylase conjugates were compared with HRP conjugate in several important aspects, viz., thermostability, ability to immundetect, cost of the reagent, etc. (Table 6). The immunodetection of coated rabbit IgG by α-amylase, as well as HRP conjugated goat antirabbit antibody in direct ELISA, showed a correlation coefficient of 0.97. The characteristics

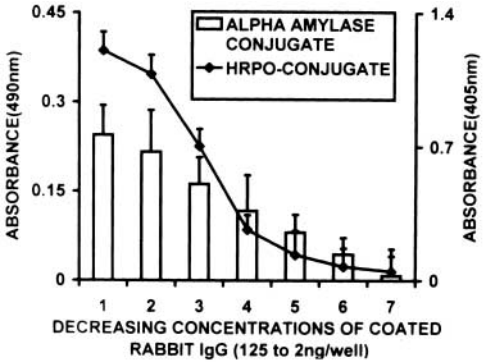

Figure 10. Comparison of immunodetection of coated rabbit IgG by α-amylase and HRPO conjugate.

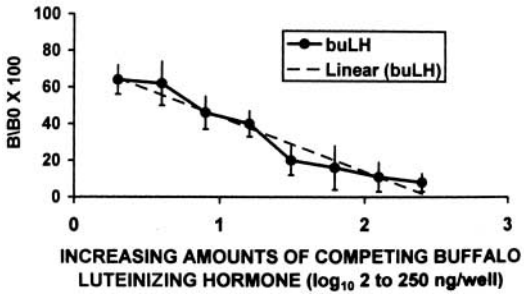

Figure 11. Detection of competing buffalo luteinising hormone by α-amylase second antibody conjugate in competitive ELISA.

Table 5. Immunodetection of Antibody Titre of Parasite Antigen Specific of Filariasis Patient Sera Samples by α-Amylase Conjugated Antibodies

Sample No. of Filariasis Patient Sera	Antisera Dilution 1 : 100	Antisera Dilution 1 : 200	Antisera Dilution 1 : 400	Antisera Dilution 1 : 800
1	0.334 ± .016	0.237 ± .030	0.166 ± .030	0.132 ± 0.026
2	0.385 ± .040	0.213 ± .023	0.132 ± .042	0.108 ± 0.012
3	0.284 ± .019	0.274 ± 0.036	0.239 ± .017	0.209 ± 0.015
4	0.274 ± .036	0.153 ± .050	0.103 ± .035	0.086 ± 0.025
5	0.222 ± .050	0.163 ± .020	0.124 ± .020	0.114 ± 0.014
Blank	0.074 ± .004	0.060 ± .010	0.055 ± .009	0.050 ± 0.010

Table 6. Characteristics of HRPO Conjugated Antibody Compared with α-Amylase Conjugated Antibody

Characterisitics	HRPO-Conjugate	α-Amylase-Conjugate
Cost of conjugate	Costly	Much cheaper
Cost of substrate	Cheaper	Costly
Thermostability of conjugate	Poor	Good
Limit of detection of antigen	10–1000 ng/well	10–1000 ng/well
Intraassay CV, interassay CV	7–13%, 2–7%	5–13%, 2–7%
Background in immunodetection	High	Low
Reading of plate after addition of substrate	Should be read within 30 min–1 h	Can be read within 30 min–12 h

Table 7. Comparison of α Amylase with Other Important Commercially Available Enzyme Labels

Enzyme Labels	Mol. Wt. (kDa)	Storage Temp.	Sp. Activity	Cost/1000 U ($)
α-Amylase	55	2–8°C	500–1500 U/mg	00.27
Horseradish peroxidase, RZ approx 3.0	45	−0°C	1000 U/mg	05.06
β.Galactosidase (*E. coli*)	116	−0°C	600–1200 U/mg	40.90
Alkaline phosphatase (bovine)	85	2–8°C	2000–4000 U/mg	22.30
β.Lactamase	27	2–8°C	1500–3000 U/mg	35.06

of α-amylase is theoretically compared with the known data of the other commercially available enzyme labels (Table 7).

DISCUSSION

α-Amylase can be conjugated to antibodies using glutaraldehyde and heterobifunctional agents, i.e., SMCC and SATA. Glutaraldehyde provides the bridge between the amine group of antibody and the enzyme. α-Amylase is rich in lysine and arginine residues which would be providing the free amino groups required for conjugation. The SMCC linked to the enzyme provides the amine group, which can react with free SH groups available on SATA

treated antibody.[12] As α-amylase is not a glycoprotein and has no carbohydrate groups attached to it, the periodate method fails to provide conjugation. The inability of the MBS method to form the conjugate is due to the absence of free SH groups on the antibody, needed for conjugation.

Of the two glutaraldehyde methods, the two-step method has been optimized for conjugation. Small amounts of glutaraldehyde, and shorter exposure to it, provides better conjugation. Larger amounts of glutaraldehyde and longer exposures may encourage aggregate formation, which may decrease the activity and efficiency of the conjugate.

The substrate containing ETG_7PNP and α-glucosidase is most sensitive to be used by the α-amylase conjugate for immunodetection, because of the coupled reaction. However, as this is an expensive substrate, there is a need to find other substrates, e.g., fluorescent substrates which can be used with this conjugate for immunodetection.

We have shown that the α-amylase conjugate can be effectively used in immunodetection in ELISA. It can detect analytes with high sensitivity. Its sensitivity is on par with the HRP conjugate. Its intra-assay and inter-assay coefficients of variation are well within the permissible limits. Its very low blank values show that its nonspecific reactions are quite low, compared to HRP conjugates.

The native enzyme is known to be rugged and heat stable. It has been reported that α-amylase retains 80% of its activity when kept at 90°C for 1 h.[10,19] The conjugate is seen to retain the thermostable characteristic of the enzyme label. This provides an advantage over other available enzyme labels.

Of the commercially available enzyme labels, α-amylase is the cheapest (Table 7). It has high specific activity. It has a low molecular weight. It is readily available in quantity, and it can remain active for more than two years in refrigerated condition. Moreover, it is of bacterial origin and, therefore, this enzyme can be made available in abundance in the laboratory.

In the near future, it may be possible to improve upon this conjugate. A thermostable α-amylase with higher specific activity, e.g., α-amylase from archaebacterium, which is thermostable even at 100°C, has been isolated. It has a molecular size of 100 kDa and has specific activity of 3000 U/mg.[20] This α-amylase may be a good candidate to prepare α-amylase conjugates with better sensitivity and thermostability.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Science & Technology, Govt. of India, for providing financial assistance for this research. The

moral and administrative support given by the Principal, Daulat Ram College and Head of the Biochemistry Department, Delhi University, in this research project was invaluable.

ABBREVIATIONS

α, Alpha; AP, Alkaline phosphatase; β Gal, β-Galactosidase; BSA, Bovine serum albumin; BAP, Bacterial alkaline phosphatase; BuLH, Buffalo luteinising hormone; CV, Coefficient of variation; CAP, Calf alkaline phosphatase, DMSO, Dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetate; ELISA, Enzyme linked immunosorbent assay; ETG_7PN, Ethylidene 1-*p*-nitrophenyl-α-D-maltoheptaoside; G_2PN, Nitrophenyl maltodioside; G_3PN, Nitrophenyl maltotrioside; G_4PN, Nitrophenyl maltotetraoside; HRP, Horseradish peroxidase; IgG, Immunoglobulin G; KOH, Potassium hydroxide; LOD, Limit of detection; MBS, Maleimidobenzoyl-*N*-hydroxysuccinimide ester; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffered saline; PN, Nitrophenol; RT, Room temperature; SATA, *N*-Succinimidyl-*S*-acetylthioacetate; SD, Standard deviation; SulfoSMCC, Sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane1-carbonate.

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Received March 6, 2001
Accepted June 20, 2001
Manuscript 3032

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