*Journal of Biological Research-Thessaloniki* **12**: 173 – 186, 2009
*J. Biol. Res.-Thessalon.* is available online at http://www.jbr.gr
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# Biochemical studies on the conjugation of antibodies with turnip peroxidase using two different methods

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Received: 6 July 2009 Accepted after revision: 29 July 2009

The efficiency of conjugation between major carbohydrate rich peroxidase (TPOD) of turnip roots (*Brassica rapa*) and antibodies of rabbit anti-mouse IgGs (AM IgGs) was carried out for production of TPOD-AM IgG conjugate suitable for immunodiagnostic kits. The conjugate was prepared by two methods: one-step glutaraldehyde conjugation and periodate conjugation. The conjugates were characterized immunochemically and immuno-enzymatically. The two methods were comparable regarding the immunological activity of the conjugated products, while the ELISA titer of the conjugate prepared by the periodate method was almost double that prepared by the glutaraldehyde method. The conjugated product prepared by the one-step glutaraldehyde method was stable for at least six months at –20˚C with less than 20% loss of activity. Characterization of the TPOD and TPOD-AM IgG conjugate including Km values, optimum pH, optimum temperature, and thermal stability were carried out. The Km values of the TPOD and TPOD-AM IgG conjugate (ABTS) were 1.2 and 0.86 mM at pH 4 and 0.52 and 0.6 mM at pH 6, respectively. The Km values of these two products ($H_2O_2$) were 2 and 1.25 mM at pH 4 and 0.35 and 1.2 mM at pH 6, respectively. The optimum pH value was 3.5 for both while optimum temperature values were 55 and 50˚C, respectively. Economically, the conjugated product by the one-step glutaraldehyde method was preferable, as it requires one step for its purification with higher recovery, and immunologically comparable beside its higher stability compared with the conjugate prepared by the periodate method.

**Key words:** *Brassica rapa*, conjugation, purification, anti-mouse IgG, peroxidase.

## INTRODUCTION

Peroxidases (PODs) are heme-containing oxidoreductases having up to 20% w/w carbohydrates (Duarte-Vázquez *et al*., 2003). The only source of commercial POD is from horseradish roots which are cultivated in cool climates but not in Egypt. Economic sources of enzymes include a limited number of plants, like soybean (Sessa & Anderson, 1981), cauliflower (Lee *et al*., 1984), green peas (Halpin *et al*., 1989), peanut (Hu & van Huystee, 1989), Korean radish roots (Lee & Kim, 1994), mango (Khan & Robinson, 1994), tomato (Brownleader *et al*., 1995), strawberry (Civello *et al*., 1995), turnip roots (Hamed *et al*., 1998; Duarte-Vázquez *et al*., 2001), animals and a few species of microorganisms (Kanayama *et al*., 2002). PODs are used extensively in clinical, biochemical, biotechnological, and industrial applications as well as in the synthesis of useful compounds (Duran & Esposito, 2000; Kim & Moon, 2005).

POD has been widely used clinically in enzymatic and immunodiagnostic kits. More than 90% of the immuno-assay kits utilize POD antibody conjugates. Its commercial production has normally been by extraction from horseradish roots, as horseradish is the richest source of POD (Rashimaw, 1982). Although other sources rich in POD are locally available in Egypt, turnip was chosen as starting material in this study due to its high content of POD (Mazza *et al*., 1968; Hamed *et al*., 1998), its availability in local mar-

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kets in low price almost throughout the year, and the juicy nature of turnip roots compared with the fibrous nature of radish roots. The root of the horseradish contains a number of distinctive POD isoenzymes of which the C isoenzyme is the most abundant. The different amino-acid sequences of four isoenzymes of turnip roots from *Brassica napus* suggested the presence of at least four separate genes for POD (Mazza & Welinder, 1980).

Many methods for plant POD purification have been reported. Generally, following homogenization of the crude material, the homogenate is filtered, concentrated by precipitation, centrifuged, and the enzyme is purified by applying different chromatographic steps depending on its intended use (Gillikin & Graham, 1991; Paradkar & Dordick, 1993; Hamed *et al*., 1998). The coupling reaction should produce a conjugate that resembles the native protein. These reactions include: 1) maleimide-sulfhydryl methods using heterobifunctional coupling reagents (Hosoda *et al*., 1985; Akman *et al*., 1998), 2) sodium periodate oxidation method; still widely used in which periodate attaches a carrier molecule to carbohydrate groups (Weinryb, 1968), 3) cyanuric chloride method; used as a coupling reagent to cross-link polyethylene glycol to albumins (Jackson *et al*., 1987), 4) oligonucleotide probes method (Ruth, 1994), 5) primary amines on lysine peptide amines method (Dhawan, 2002a), 6) multivalent polystyrene microparticles and lysine peptide chains method (Dhawan, 2002b), 7) glutaraldehyde method in which glutaraldehyde links carrier molecules to the N-terminus of peptide (Saraiva *et al*., 2007).

The coupling methods rely either on the conversion of terminal sugars in the N-glycan moieties in horseradish peroxidase (HRP) to aldehyde functions and subsequent linking to amino groups in the second molecule, or on exploiting the amino groups of the enzyme molecule and appropriate reagents with succinimide ester functions. The methods are not without drawbacks due to varied reasons, including possibilities of over or under oxidation with sodium periodate (Presentini & Terrana, 1995) and inadequate coupling efficiency due to paucity or total absence of free amino groups in many commercial HRP (Nakane & Kawaoi, 1974). The lack of free amino groups has severely limited the choice of conjugation methods. The number of amino groups per molecule of the commonly used HRP preparations is reported to be six (O'Brien *et al*., 2003).

The aim of the present work is to study the efficiency of POD from the locally available turnip roots (*Brassica rapa*), for conjugation with antibodies suitable for immunodiagnostic kits using two different methods of conjugation. This will be carried out by establishing the purification procedure for the major POD and its conjugation with the antibodies.

## MATERIALS AND METHODS

### *Purification of peroxidase*

#### Enzyme extraction

Unless otherwise stated, all purification procedures were carried out at 4-7°C. Turnip roots were cut into cubes and juiced. The juice was filtered through four layers of cheesecloth; the filtrate was designated crude homogenate. The filtrate was precipitated by solid ammonium sulfate up to 75% saturation. After staying overnight, the precipitate was collected on Beckman C-21 cooling centrifuge at 11000 × g for 15 min and dissolved twice in a minimal volume of 0.05 M sodium acetate buffer (pH 5.6). The clear supernatants were pooled and designated as ammonium sulfate precipitate while the remaining precipitate was discarded.

#### Chromatography on phenyl Sepharose CL-4B column

The enzyme sample (ammonium sulfate precipitate) was dissolved in 0.05 M sodium phosphate buffer containing 1 M ammonium sulfate, pH 6.8 (equilibration buffer, EB), and applied onto degassed preswollen phenyl Sepharose CL-4B column (20 × 1.6 cm i.d.), previously equilibrated with the same EB. The proteins bound to the hydrophobic resin were eluted using a stepwise decrease in ammonium sulfate concentration (1 M, 0.8 M of ammonium sulphate and phosphate buffer, pH 6.8). The fractions were collected in 2 ml volume at a flow rate of 30 ml $h^{-1}$. Absorbance of each fraction was determined at 280 nm for protein detection using a Shimadzu spectrophotometer UV-100-2. The POD activity was assayed in each tube. Fractions containing enzyme activity were pooled and stored at –20°C until use.

#### Chromatography on Con-A Sepharose column

Glycoproteins containing PODs were isolated according to the method of Parkhouse *et al*. (1981) using Con-A Sepharose column. The bound proteins were eluted using 0.5 M a-methyl mannoside in run-

ning buffer. Fractions were collected in 2 ml volume at a flow rate of 30 ml $h^{-1}$. Absorbance was recorded at 280 nm. The POD activity was assayed and fractions containing activity were pooled and stored at –20°C until use.

Peroxidase assay

POD activity was determined according to the method of Childs & Bardsley (1975), using ABTS as a reducing substrate, in a reaction mixture (1 ml) containing 6 mM $H_2O_2$, 0.36 mM ABTS, 100 mM sodium acetate buffer (pH 6) and POD concentration which gave linear response over a period of 3 min. The change in absorbance at 405 nm was followed at 1 min intervals. One unit of POD activity was defined as the amount of enzyme that oxidized 1 mmol ABTS per min at 25°C under the assay conditions.

*Purification of rabbit anti-mouse immunoglobulins (IgGs)*

Anti-mouse IgG antibodies (AM IgGs) were prepared by intramuscular injection of mouse IgG into rabbits using the protocol of Hudson & Hay (1989). The rabbit AM IgGs were purified using the protein G-Sepharose CL-4B column according to Hudson & Hay (1989).

*Coupling of the AM IgGs with TPOD*

Glutaraldehyde method (method I)

The AM IgGs were conjugated to the turnip peroxidase (TPOD) (666 units and 19.2 mg), according to the one-step purification method of Boorsma & Kalsbeek (1975). Briefly, 1% of glutaraldehyde (0.05 ml) was added under gentle stirring to 3 ml of 0.1 M phosphate buffer, pH 6.8, containing 2.5 mg protein of AM IgGs and the TPOD enzyme. After staying for two hours at room temperature, the reaction mixture was dissolved in 0.1 M PBS, pH 7.2 for 2 hrs. Next, the solution was centrifuged at 15000 × g for 30 min at 4°C and the precipitate was discarded. The conjugated AM IgG antibodies were purified from the unconjugated antibodies using a Sephadex G-200 column.

Sodium periodate method (method II)

The AM IgGs were conjugated to the TPOD according to the method of Hudson & Hay (1989). Briefly, 200 µl of freshly prepared 0.1 M sodium periodate solution was added while stirring for 20 min to TPOD (427 units) until a greenish brown color was obtained. Next, the mixture was dissolved in 0.001 M sodium acetate buffer, pH 4.4 for 4 hrs. Then, the pH was raised to 9 using 20 µl of 0.1 M sodium carbonate buffer, pH 9.5. Eight mg $ml^{-1}$ of AM IgGs were added to the mixture while stirring for 2 hrs at room temperature. Subsequently, 100 µl of freshly prepared sodium borohydride (4 mg $ml^{-1}$) were added to the mixture and incubated for 2 hrs at 4°C. Finally, the mixture was dissolved in a buffer containing 0.1 M Tris-HCl and 1 M NaCl (pH 8) for 2 hrs. The conjugated antibodies were purified from the unconjugated antibodies using a Sephadex G-200 column.

*Purification of the conjugated antibodies*

The TPOD-AM IgG conjugate was purified using gel filtration on Sephadex G-200. The conjugate product was applied on top of a Sephadex G-200 column (90 × 1.6 cm i.d.). The column was equilibrated and eluted with 0.1 M Tris-HCl (pH 8) containing 1 M NaCl and 0.1 mM thiomersal.

*Enzyme linked immunosorbent assay (ELISA)*

The reactivity of the conjugates obtained as crude or purified fractions was examined in 96-well microtiter plates using the ELISA test described by Ricoux *et al.* (2000). Briefly, each well was coated overnight with 100 µl of monoclonal mouse anti-albumin IgG (0.5 µg protein/well) dissolved in coating buffer (50 mM sodium carbonate, pH 9.6). Non-specific sites were blocked for 1 hr by the addition of 100 µl of the blocking solution (3% gelatin dissolved in coating buffer) at 37°C. Each well was incubated with 100 ml of human serum albumin (1 mg/well) dissolved in washing buffer at 37°C for 60 min. Serial dilutions (100 ml) from the prepared rabbit TPOD-AM IgG conjugate dissolved in washing buffer were added to each well and incubated at 37°C for 60 min. A quantity of 100 µl of commercial anti-mouse IgG (whole molecule) POD conjugate diluted (1:5000) in washing buffer was added to each well as reference and incubated for 60 min at 37°C. After each step, the wells were thoroughly washed three times with washing buffer (0.01 M PBS, pH 7.4 containing 0.05% Tween-20). Finally, 100 µl of substrate buffer (0.33 mg OPD/ml dissolved in citrate buffer, pH 4.5 containing 0.04% hydrogen peroxide) were added to each well. The reaction was stopped after 20 min by the addition of 20 µl of a 1:20 dilution of sulfuric acid and the absorbance values were determined at 490 nm with ELISA

reader. All the values were recorded in duplicate and the mean results were obtained. A standard curve between log conjugate dilution and log optical density (OD) was plotted. The dilution that gives 0.5 OD at 490 nm was taken as the ELISA titer.

*Protein determinations*

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

*SDS-PAGE*

Electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA). Preparation of gels, samples, and electrophoresis was performed according to the conditions described by Laemmli (1970).

*Effect of pH*

Activity of purified TPOD and TPOD-AM IgG conjugate fractions was assayed at different pH values using 0.01 M citrate phosphate buffer for pH 3 to pH 5.5, and 0.01 M sodium phosphate buffer for pH 6 to pH 7.

*Thermal stability*

The thermal stability of the TPOD and TPOD-AM IgG conjugates was studied. Both TPOD and TPOD-AM IgG conjugates were incubated for 15 min in 10 mM citrate phosphate buffer, pH 4 at different temperatures ranging from 10 to 90˚C prior to substrate addition and the residual TPOD activity was assayed using ABTS as substrate under standard assay conditions.

## RESULTS AND DISCUSSION

*Purification of TPOD*

TPOD was prepared from 4.5 kg of turnip roots. The initial specific activity was 9.8 units/mg of protein (Table 1). The ammonium sulfate precipitation of TPOD increased the specific activity to 15.2 units/mg of protein with 1.5 fold purification and 38.8% recovery. The ammonium sulfate precipitate of TPOD was applied on phenyl Sepharose CL-4B. Most of the TPOD activity was bound to the matrix and eluted by buffer lacking ammonium sulfate. The typical elution profile is shown in Figure 1. Most of the TPOD activity was recovered in the third peak with 8667 units and 318.6 mg of protein with specific activity of 27.2 units/mg of protein and fold purification of 2.7 from

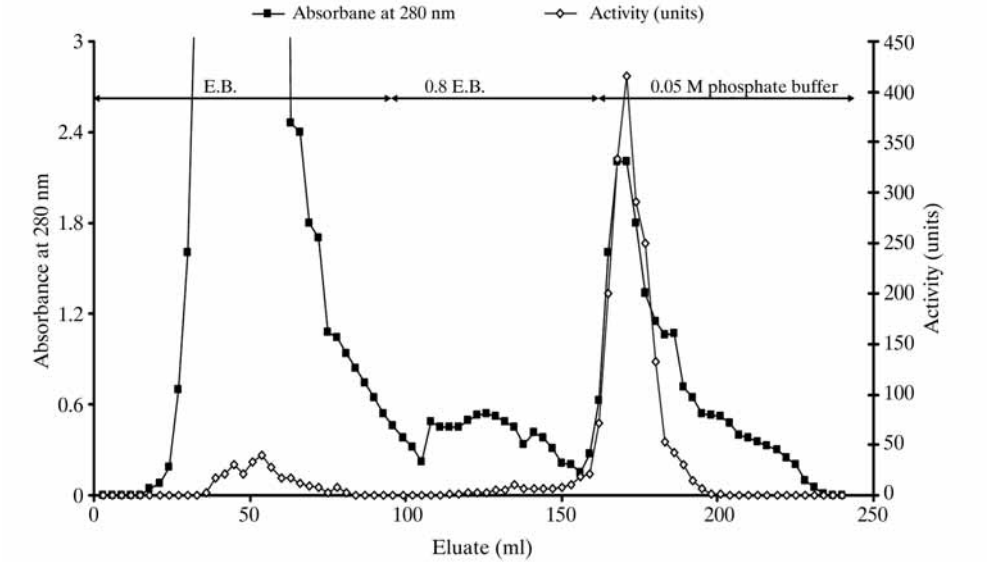


FIG. 1. Typical elution profile for the hydrophobic interaction chromatography of the TPOD (ammonium sulfate precipitate) on phenyl Sepharose CL-4B column. The fractions were collected in 2 ml volume at a flow rate of 30 ml $hr^{-1}$. Absorbance of each fraction was measured at 280 nm and TPOD activity was detected using ABTS. EB is the equilibration buffer (0.05 M sodium phosphate containing 1 M ammonium sulfate, pH 6.8).

Table 1. Summary of the purification of turnip (*B. rapa*) POD

| Purification step | Protein (mg) | Activity (units)* | Specific Activity (unit/mg protein) | Fold Purification | Recovery (%) |
|---|---|---|---|---|---|
| Crude homogenate | 3400 | 33300 | 9.8 | 1.0 | 100.0 |
| Ammonium sulfate precipitate | 1450 | 21249 | 15.2 | 1.5 | 38.8 |
| Phenyl Sepharose | 318.6 | 8667 | 27.2 | 2.7 | 26.0 |
| Con-A Sepharose | 115.8 | 7551 | 65.2 | 6.6 | 22.6 |

* one unit of POD activity is the amount of enzyme that oxidized 1 mmol ABTS/min at 25°C under assay conditions

the crude extract (Table 1). Many PODs from different higher plants have been characterized as glycoproteins having up to 20% w/w carbohydrates (Duarte-Vázquez *et al*., 2001). The carbohydrate content of turnip POD is similar to that of Korean radish (9-14%) (Lee & Kim, 1994) and higher than that of wheat germ (4.1-7.9%) (Converso & Fernández, 1995). However, PODs having carbohydrate contents higher than that of turnip are those from soybean (15%) (Schmitz *et al*., 1997), peanut (22%) (Hu & van Huystee, 1989), and isozyme C of HRP (18%) (Ohlsson *et al*., 1977).

Carbohydrates may modulate physicochemical properties of the glycoproteins, mediate their biological activity (Rademacher *et al*., 1988), and affect their storage stability (Tigier *et al*., 1991; Nie *et al*., 1999). The removal of the carbohydrate moiety normally affects POD kinetics (Tigier *et al*., 1991), antigenicity and resistance to protease attack (Hu & van Huystee, 1989), and thermal stability (Sánchez-Romero *et al*., 1994). A simple reproducible procedure for the purification of TPOD having carbohydrate and including hydrophobic interaction chromatography and Con-A affinity chromatography was established. The specific activity increased to 65.2 units/mg of protein with a 22.6% recovery (Fig. 2, Table 1). The Con-A purified TPOD was found to be almost pure with a molecular weight of 35 kDa (Fig. 3A). This result is analogous to that reported by Duarte-Vázquez *et al*. (2001) for POD purified from *B. napus* (L.) roots, but differs from that reported by Rudrap-

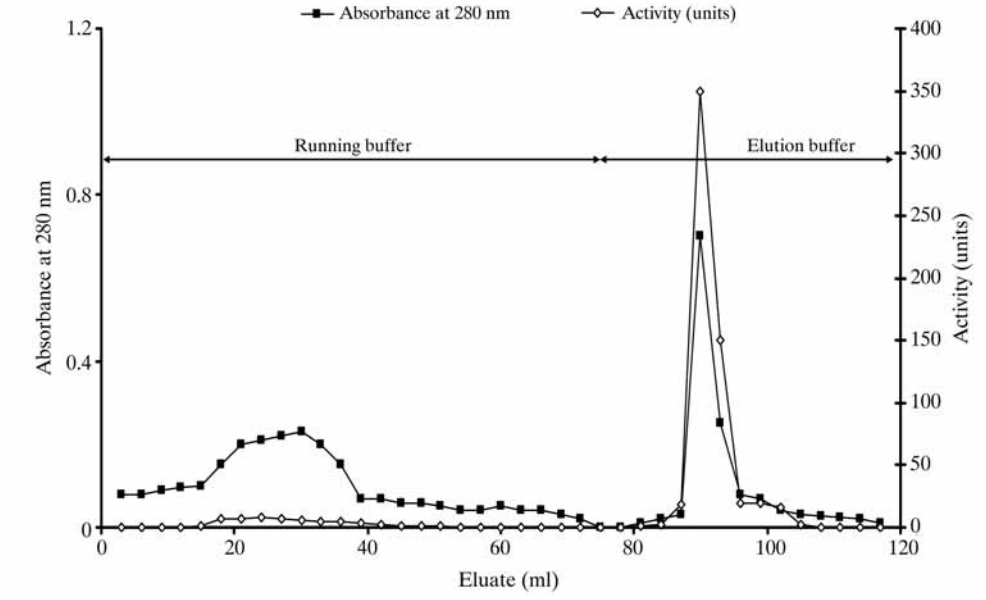


FIG. 2. Typical elution profile for the affinity chromatography of phenyl Sepharose purified TPOD on Con-A column (20 × 1.2 cm). The fractions were collected in 2 ml volume at a flow rate of 30 ml $hr^{-1}$. Absorbance at 280 nm was recorded. The TPOD activity was detected using ABTS.

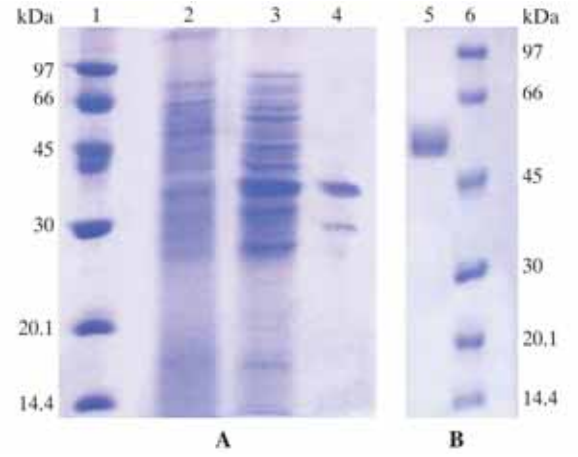


FIG. 3. 12% SDS-PAGE of crude turnip juice (lane 2), phenyl Sepharose purified TPOD (lane 3), Con-A purified TPOD (lane 4), and purified AM IgG (lane 5). Lanes 1, 6: molecular weight marker.

pa *et al*. (2007) for POD purified from red beet hairy roots (*Beta vulgaris* L.) which had a molecular weight of 45 kDa.

*Purification of rabbit IgGs*

Anti-immunoglobulins are needed for conjugation with TPOD. In this investigation, anti-mouse immunoglobulins (AM IgGs) were prepared in rabbit using the traditional protocol, and purified using protein G-Sepharose column. The purified IgGs were concentrated by precipitation with 75% ammonium sulfate. A quantity of 22 mg of AM IgGs was recovered from 4 ml of rabbit antiserum. The purity of AM IgGs was examined using SDS-PAGE (Fig. 3B).

The extensive use of POD as a label in immunochemical analysis justifies the improvement of the critical conjugation steps required in every case. Several methods of coupling have been developed to meet the needs in different fields, including immunocytochemistry, immunohistochemistry, and immunoassays.

*Conjugation of purified TPOD to AM IgG*

The two most widely used methods of POD coupling, coupling with periodate (Tijssen & Kurstak, 1984) and coupling with glutaraldehyde in one step (Avrameas & Ternynck, 1969) were compared. TPOD (666 units and 19.2 mg of protein) was conjugated to 8 mg AM IgGs using 1% glutaraldehyde (method I). The conjugated TPOD-AM IgG was dissolved (in 10 kDa dialysis bag) in 0.1 M Tris-HCl buffer containing 1 M NaCl, 0.1 mM thiomersal, pH 8 (EB) for 2 hrs, with several changes of the dissolving buffer. The conjugate sample containing 390 units and 27.8 mg of protein with specific activity of 14 units/mg of protein was applied on Sephadex G-200 column (Fig. 4, Table 2). Three peaks were observed by chromatography on Sephadex G-200. The first peak eluted with the void

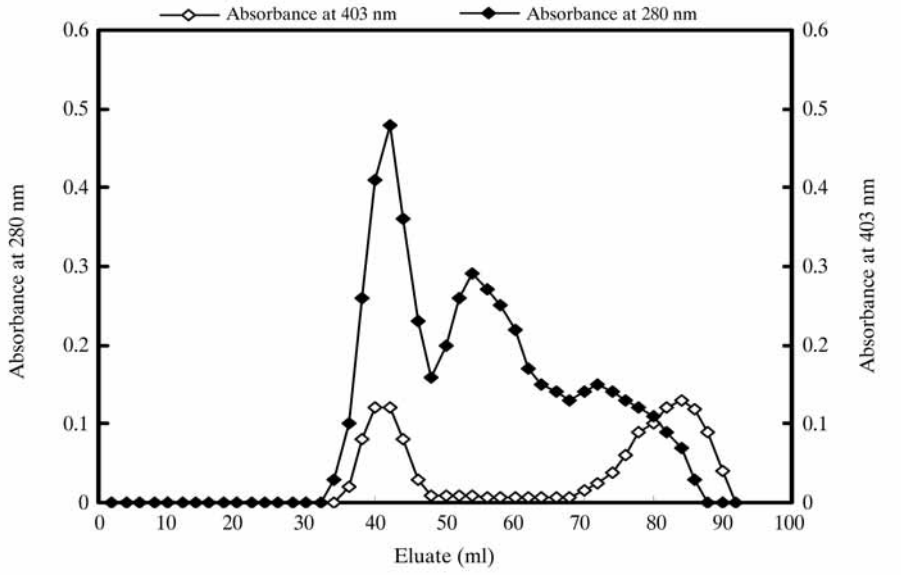


FIG. 4. Typical elution profile for gel filtration chromatography of the TPOD-AM IgG conjugate prepared by method I on Sephadex G-200 column. Fractions were collected in 2 ml volume at a flow rate of 12 ml $hr^{-1}$. Absorbance of each fraction was measured at 280 and 403 nm.

Table 2. Conjugation of the phenyl Sepharose purified TPOD to AM IgG by method I

| Sample number | Protein (mg) | Activity (units) | Specific Activity (unit/mg protein) | Recovery (%) | ELISA titer |
|---|---|---|---|---|---|
| 1 | 19.2 | 666 | 34.7 | 100.0 | – |
| 2 | 27.8 | 390 | 14.0 | 58.5 | 1:10 |
| 3 | 3.0 | 26 | 8.6 | 3.9 | 1:2 |
| 4 | 3.4 | 233 | 68.5 | 35.0 | 0 |

Sample number: 1 = Phenyl Sepharose purified TPOD, 2 = TPOD-AM IgG conjugate, 3 = Purified TPOD-AM IgG conjugate, 4 = Unconjugated TPOD

volume consists of immunoglobulin (around 160 kDa) conjugated to the TPOD (around 35 kDa) containing 26 TPOD units and 3 mg of protein with specific activity of 8.6 units/mg of protein. The second peak with molecular weight around 160 kDa represents the free immunoglobulins while the third peak represents the free POD. Also, using method II for conjugation, TPOD (427 units and 8 mg of protein) was conjugated to 8 mg AM IgGs using 0.1 M sodium periodate. The conjugated TPOD was dissolved in 0.1 M Tris-HCl buffer containing 1 M NaCl, 0.1 mM thiomersal, pH 8 for 2 hrs. The conjugate sample containing 217 units and 6.5 mg of protein with specific activity of 33.4 units/mg of protein was applied on Sephadex G-200 column (Fig. 5). A similar chromatographic pattern was observed by chromatography on Sephadex G-200. The first peak eluted with the void volume consists of immunoglobulin conjugated to the TPOD containing 5.58 units and 0.46 mg of protein with specific activity of 12.1 units/mg of protein (Table 3). The second peak represents the free immunoglobulins while the third peak represents the free POD.

Tresca *et al*. (1995) compared the efficiency of the coupling of HRP to anti-C-reactive protein (CRP) with periodate and glutaraldehyde in one or two steps. Periodate appeared to be more consistently effective as a coupling agent than glutaraldehyde, and the one-step coupling method was more efficient than the two-steps one. This was shown in the chromatographic data, by differences in immuno-enzymatic activities and by titration curves. The low coupling yields consistently observed with glutaraldehyde in the dif-

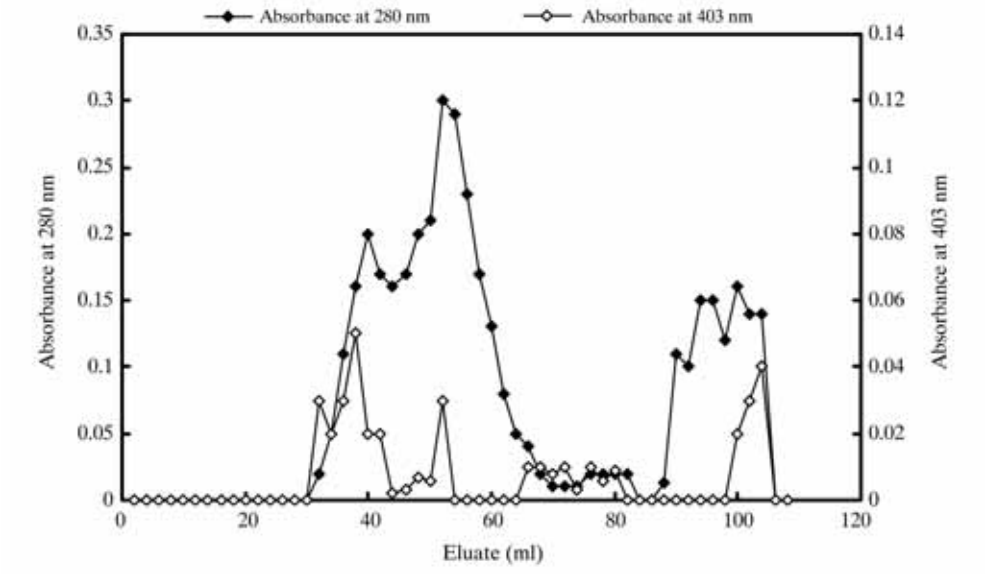


FIG. 5. Typical elution profile for gel filtration chromatography of the TPOD-AM IgG conjugate prepared by method II on Sephadex G-200 column. Fractions were collected in 2 ml volume at a flow rate of 12 ml $hr^{-1}$. Absorbance of each fraction was measured at 280 and 403 nm.

Table 3. Conjugation of the Con-A Sepharose purified TPOD to AM IgG by method II

| Sample number | Protein (mg) | Activity (units) | Specific Activity (unit/mg protein) | Recovery (%) | ELISA titer |
|---|---|---|---|---|---|
| 1 | 8.0 | 427 | 53.4 | 100.0 | – |
| 2 | 6.5 | 217 | 33.4 | 50.8 | 1:1.5 |
| 3 | 0.46 | 5.58 | 12.1 | 1.31 | 1:3 |
| 4 | 2.8 | 90 | 32.0 | 21.0 | 0 |

Sample number: 1 = Con-A Sepharose purified TPOD, 2 = TPOD-AM IgG conjugate, 3 = Purified TPOD-AM IgG conjugate, 4 = Unconjugated TPOD

ferent tests do not seem to be due to the inactivation of the glutaraldehyde, which can spontaneously and reversibly polymerize in concentrated solution (Kawahara *et al*., 1992).

The percent recovery of the POD coupled with antibodies by method I was 3.9% of the total initial activity (Fig. 4, Table 2) while that of the periodate method (method II) accounted for 1.31% (Fig. 5, Table 3). Also, regarding the immuno-enzymatic activity of the conjugated products prepared by the different methods, the binding of the TPOD-AM IgG conjugate using method II to the antigen-antibody complex was almost two times more efficient than using method I. This may be due to the blocking of the amino terminal of the TPODs with allyl isothiocyanate during extraction steps (Nakane & Kawaoi, 1974), where the allyl isothiocyanate reacts with free amino groups at neutral and alkaline pH to produce N-allylthiocarbamoyl amino acids (Cejpek *et al*., 2000), and the carbohydrate content in the TPOD which forms an unlimited number of coupling sites (Duarte-Vázquez *et al*., 2001).

The periodate coupling method for horseradish POD in the study of Tresca *et al*. (1995) was much more efficient than that of the glutaraldehyde method, as the activity of coupling products was about 100 times greater than that of the product obtained after the conjugation of HRP with glutaraldehyde. The differences in efficiency can be ascribed to the fact that periodate induced more coupling sites than glutaraldehyde. Periodate is therefore a better coupling agent for preparing conjugates to be used in ELISA, in which conjugate size does not hinder accessibility to the antigen. On the other hand, if the size of the conjugate is a major disadvantage, which may be the case in immunohistochemistry and immunocytochemistry techniques (Boorsma & Streefkerk, 1976), glutaraldehyde coupling can be used with the reservation that the yield will be low.

### *Km value*

The Km values of TPOD and TPOD-AM IgG conjugate exhibited typical Michaelis-Menten behavior with respect to ABTS as substrate. Km values (ABTS) were 1.2 and 0.86 mM at pH 4, and 0.52 and 0.6 mM at pH 6, respectively (Table 4), which are comparable to that reported for TPOD at pH 6 (0.7 mM and 0.47 mM, Duarte-Vázquez *et al*., 2000, 2001). Recently, the Km value for ABTS at pH 6 of 0.56 mM has been

Table 4. $K_m$ and $V_{max}$ for purified TPOD and TPOD-AM IgG conjugate with ABTS or $H_2O_2$ as a substrate at different pH values

| pH value | Substrate | Purified TPOD | | TPOD-AM IgG conjugate | |
|---|---|---|---|---|---|
| | | $K_m$ (mM) | $V_{max}$ (μmmol/min/mg protein) | $K_m$ (mM) | $V_{max}$ (mmol/min/mg protein) |
| 4 | ABTS | 1.2 | 260 | 0.86 | 10.0 |
| 6 | ABTS | 0.52 | 9 | 0.6 | 0.71 |
| 4 | $H_2O_2$ | 2.0 | $10^4$ | 1.25 | 1.4 |
| 6 | $H_2O_2$ | 0.35 | 1300 | 1.2 | 0.66 |

reported for TPOD by Quintanilla-Guerrero *et al*. (2008). However, these values are lower than that of acidic HRP but higher than that of basic HRP (Hiner *et al*., 1996). Purified TPOD and TPOD-AM IgG conjugate exhibited distinct Michaelis-Menten constants with respect to $H_2O_2$ as substrate. Km values ($H_2O_2$) were 2 and 1.25 mM at pH 4 and 0.3 and 1.2 mM at pH 6, respectively (Table 4). These values differed from that reported for red beet (0.113 mM) at pH 6 (Rudrappa *et al*., 2007) and Siam weed (0.123 mM) (Rani & Abraham, 2006). However, recently a Km value as low as 0.018 mM was reported by Quintanilla-Guerrero *et al*. (2008).

*pH optimum*

The optimum pH was found to be 3.5 for both purified TPOD and TPOD-AM IgG conjugate prepared by method I (Fig. 6). Optimum pH value for TPOD and TPOD-AM IgG conjugate differs from that reported by Rudrappa *et al*. (2007) for red beet (*B. vulgaris* L.) POD which has an optimum pH of 5.5, as well as from that reported by Duarte-Vázquez *et al*. (2001) for TPOD. PODs are specific for $H_2O_2$ as substrate but can use a number of H donors such as ABTS and guaiacol. Optimum pH values for HRPC activity using ABTS and guaiacol as H donors were 4.5 and 5.5, respectively (Vojinovic *et al*., 2004). The pH of optimum activity for TPOD was 5.5, very similar to that of tomato (5.3-5.5) (Heidrich *et al*., 1983) and soybean (5.5) (Sessa & Anderson, 1981) PODs.

*Effect of temperature*

The effect of temperature on ABTS conjugation reaction with $H_2O_2$ catalyzed by TPOD-AM IgGs conjugate and TPOD purified from turnip (*B. rapa*) roots was examined under the standard assay conditions. The reaction was carried out at a temperature range of 10 to 70°C and the residual activity of the enzyme was measured. The enzymatic activity of purified TPOD increased with increasing temperature up to 45°C. After that, the activity remained almost at the same level, up to 70°C (Fig. 7). On the contrary, the activity of TPOD-AM IgG conjugate increased with increasing temperature up to 40°C and after that the activity decreased with increasing temperature up to 65°C (Fig. 7).

PODs of many plants have an optimum temperature around 25°C (Castillo Leon *et al*., 2002). The optimum temperature of free purified phenyl Sepharose TPOD showed its maximum activity at 55°C (Fig. 7) which is different from that recorded for vanilla bean POD. The latter, showed its maximum activity at 16°C and retained 78% of its activity at 5°C (Márquez *et al*., 2008) which makes it suitable to be considered as a cold-active POD (Iyo & Forsberg, 1999). The optimum temperature for TPOD-AM IgG conjugate was 50°C (Fig. 7).

Arrhenius plot of the data from 10 to 70°C gave a straight line with both purified TPOD and TPOD-AM IgG conjugates (Fig. 8). The relation between the rate of enzymatic reaction and activation energy

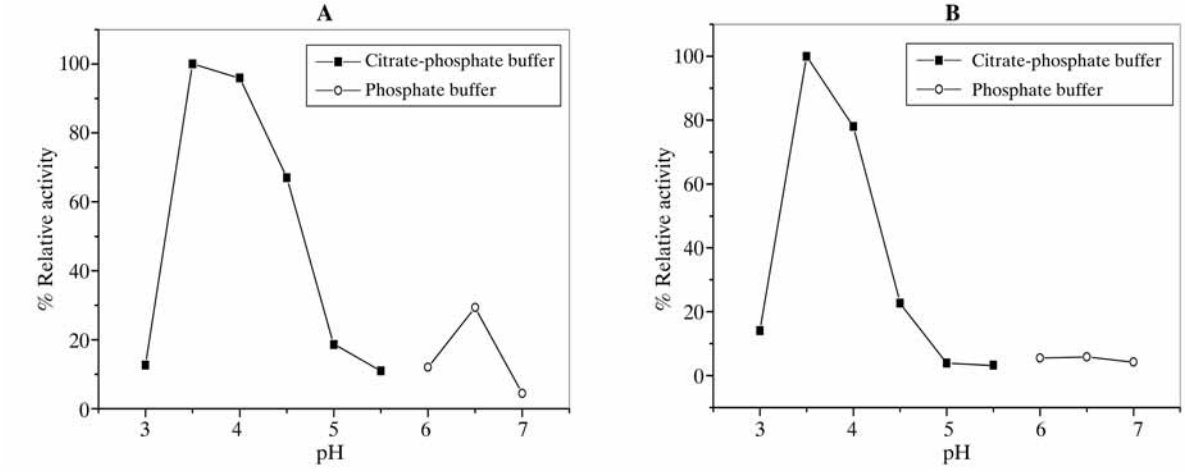


FIG. 6. pH optima of phenyl Sepharose purified TPOD (A) and TPOD-AM IgG conjugate (prepared by method I) after Sephadex G-200 column (B). The buffers used were 0.01 M citrate phosphate buffer (pH 3-5.5) and 0.01 M sodium phosphate buffer (pH 6-7).

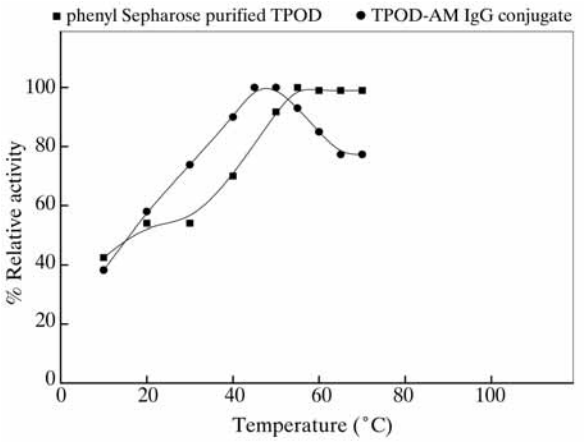


FIG. 7. Temperature optima on phenyl Sepharose purified TPOD and TPOD-AM IgG conjugate (prepared by method I) after Sephadex G-200 column.

($E_a$) is given by the empirical equation of Arrhenius:

$$E_a = \frac{2.3026\, R \log V_2/V_1\, T_1\, T_2}{T_2 - T_1}$$

where: $V_1$ = first enzyme activity
$V_2$ = second enzyme activity
$T_1$ = first absolute temperature
$T_2$ = second absolute temperature
$R$ = the gas constant (8.314472 J $K^{-1}$ $mol^{-1}$)

The $E_a$ (the difference in the free energy of the substrates in the ground state and the transition state for the reaction) can be determined from the slope of the Arrhenius plot (the dependence of log activity on 1/T) multiplied by 2.3026 *R*. The activation energy ($E_a$) was calculated to be 16.3 and 17.5 kJ $mol^{-1}$ for phenyl Sepharose purified POD and TPOD-AM IgG conjugate, respectively.

The activation energy ranges from 11.4 kJ $mol^{-1}$ for green chilli purée POD (Ahmed *et al*., 2000) to 557 kJ $mol^{-1}$ for tomato POD (Anthon *et al*., 2002). Recently, the activation energy for TPOD was reported to be 113.9 kJ $mol^{-1}$ by Quintanilla-Guerrero *et al*. (2008). Usually, the loss of activity of cold-active enzymes is severe at temperatures near 50°C, which could be due to the unfolding of the enzyme or to an alteration in the interaction between the enzyme and the substrate, without a significant or irreversible modification of the three-dimensional structure of the enzyme (Gerday *et al*., 1997).

*Thermal stability*

The effect of temperature on the stability of TPOD and TPOD-AM IgG conjugate was examined (Fig. 9). The thermal stability studies showed that both purified phenyl Sepharose TPOD and TPOD-AM IgG conjugates were thermolabile above 50°C (Fig. 9) where 95% of the enzyme activity was lost upon incubation for 15 min at 60°C at pH 6. The enzymatic activity was completely lost at 90°C, which is different from vanilla POD which retained the highest activity at 70°C at pH 7 (47% residual activity) while at pH 9, it retained only 12% activity and the activity was completely lost at pH 11 (Márquez *et al*., 2008). PODs have been reported to be the most heat-stable en-

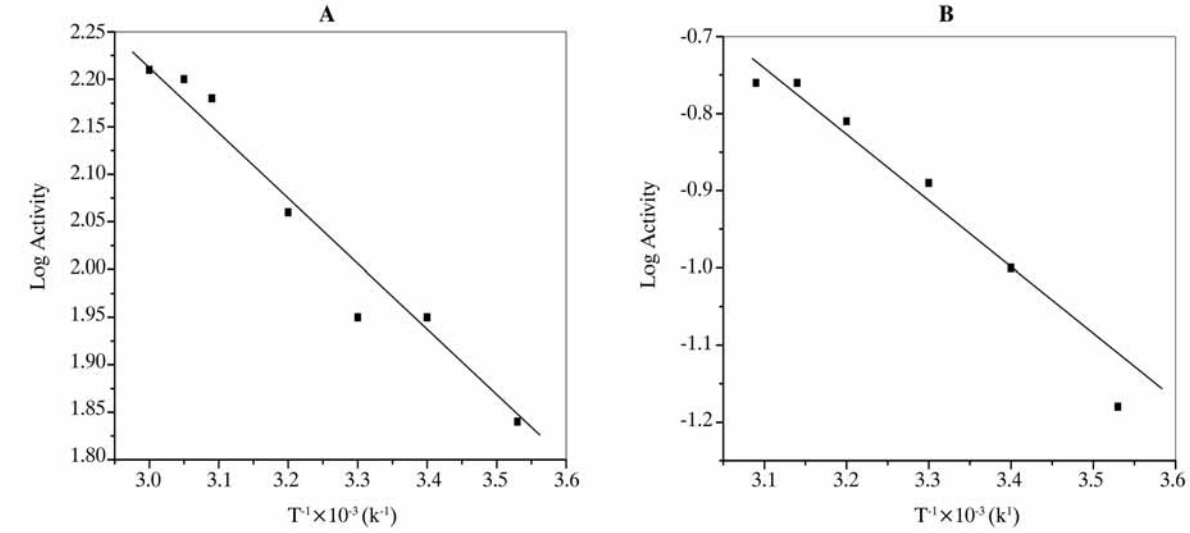


FIG. 8. Arrhenius plot of log activity versus 1/temperature for phenyl Sepharose purified TPOD (A) and TPOD-AM IgG conjugate (prepared by method I) after Sephadex G-200 column (B).

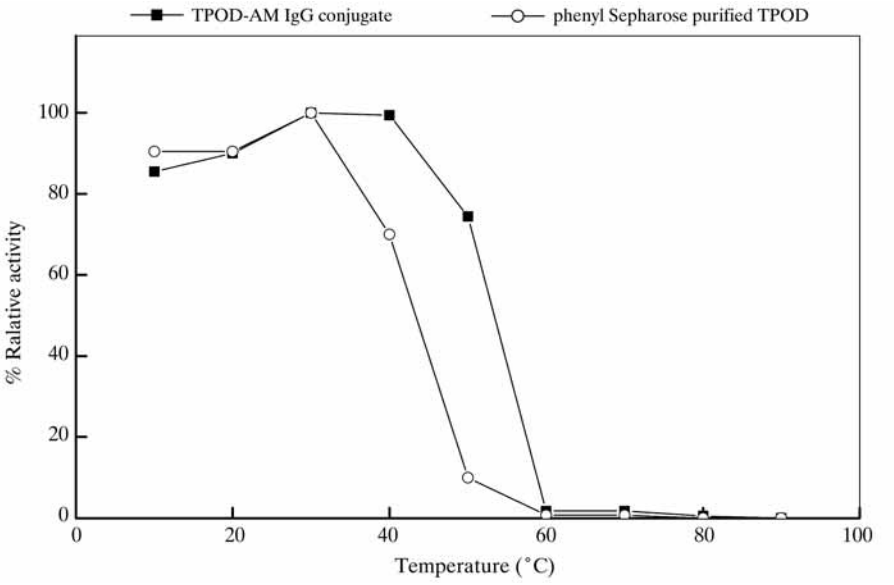


FIG. 9. Thermal stability of phenyl Sepharose purified TPOD and TPOD-AM IgG conjugate (prepared by method I).

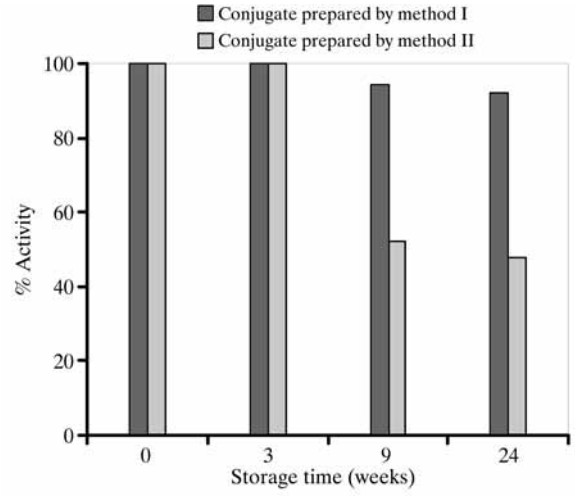


FIG. 10. Activity (%) of the TPOD-AM IgG conjugate prepared by method I and method II after different times of storage.

zymes in plants; for example, a treatment at 121°C for 6 min is needed to inactivate green peas POD (Vamos-Vigyazo, 1981). However, temperature enzyme activity resistance depends on the source of the enzyme, purity of the enzyme as well as on the assay conditions, especially pH and the nature of the substrate employed.

The stability studies of the two conjugated products showed that the conjugated product prepared by method I is stable for at least six months at –20°C with less than 20% loss of activity, while the stability of the same product prepared by method II decreased after one month under the same conditions (Fig. 10).

*Conclusions*

In conclusion, the results of the two methods were comparable regarding the immunological activity of the conjugated products. The ELISA titer of the conjugate prepared by periodate (method II) was almost double that of glutaraldehyde (method I). The opposite is true with respect to the amount of product. Stability studies of the two conjugated products clearly indicated that the conjugated product prepared by method I is more stable than that prepared by method II. Economically, the conjugated product of method I was preferable, as it requires one purification step, has higher recovery, and is immunologically comparable, beside its higher stability, compared with the conjugate prepared by method II.

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