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Peroxidase from *Hevea brasiliensis* (B.H.K) Mull. Arg. Leaves and its Applications

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ABSTRACT: Peroxidase from *Hevea brasiliensis* leaves (RBP) was purified by ammonium sulfate precipitation, followed by DEAE-Sephacel ion exchange chromatography and gel filtration on Sephadex G-75. RBP was conjugated to anti-rabbit IgG using three different cross-linkers : periodate, glutaraldehyde and sulfo-SMCC. The RBP-antibody conjugates were purified in a Sephadex G-200 column and the RBP-anti-rabbit IgG conjugate from the periodate oxidation was found to retain a higher peroxidase activity (68%) than when crosslinked by the glutaraldehyde (9.7%) and sulfo-SMCC (5.4%) methods. Its molecular weight (MW) is 348 kDa as determined by gel filtration chromatography in a Sephadex G-200 column. RBP-anti-human IgG conjugate prepared by the periodate oxidation method gave high yield of protein and also high peroxidase activity, as found in the case of an RBP-anti-rabbit IgG conjugate.

The RBP-anti-rabbit IgG conjugate was detected using dot blot technique. The RBP-anti-rabbit IgG conjugate can be used as a secondary antibody in the western blot technique to detect vitellogenin, a phosphoglycoprotein in mullet plasma, and HMG-CoA synthase, an enzyme in C-serum of rubber latex. Its optimal dilution is 1:200 using commercial HRP-anti-rabbit IgG conjugate as positive control. The purified RBP can also be used as a coupling enzyme for cholesterol assay.

KEYWORDS: *Hevea brasiliensis*, peroxidase, peroxidase-antibody conjugate.

INTRODUCTION

Peroxidase (E.C. 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) is a hemeprotein catalyzing the oxidation of various electron donor substrates (AH_2 : phenols, aromatic amines) at the expense of peroxide. The heme peroxidases are ubiquitous in nature with diverse physiological functions, and are classified into three groups on the basis of their amino acid sequences. Class I includes intracellular peroxidases, including cytochrome *c* peroxidase, ascorbate peroxidase, and gene-duplicated bacterial catalase-peroxidase. Class II contains secretory fungal enzymes, such as manganese peroxidase and lignin peroxidase. Class III consists of secretory plant peroxidases.[1] There is increasing interest in class III, not only to establish its physiological roles, but also for its possible industrial and analytical applications. In the plant kingdom, peroxidase is involved in lignification of cell walls,[2] and in the metabolism of the plant hormone indole-3-acetic acid (IAA) oxidation.[3] It also plays a role in the defense mechanism against attacks by pathogens,[4] and helps with salt tolerance and oxidative stress.[5] In rubber trees, peroxidase was found in newly excised bark strip, possibly in response to wounding, i.e. excision.[6] In sunflower leaves, it may also be involved in leaf senescence.[7] Peroxidase is widely used commercially as secondary antibody for research and medical diagnosis,[8-10] as indicators for reactive oxygen species formed during food processing,[11] and as catalysts for delignification of paper pulp.[12] The other areas where peroxidase could have an immediate use and economic impact are wastewater treatment,[13] soil remediation,[14] and the synthesis of polyelectrolyte complexes.[15] Recently, the combination of peroxidase and IAA has been introduced as a novel cancer therapy.[16]

Although peroxidases are found in various plant tissues, horseradish (*Amoracia rusticana*) roots are the traditional source for commercial production of peroxidases. Other cultivated plant species may soon provide peroxidases for commercial purposes because of their advantages as superior stability and increased sensitivity. An isozyme electrophoretic pattern of peroxidase from *H. brasiliensis* leaves (RBP) and C-serum of rubber latex revealed a higher peroxidase activity in leaves as compared with the latex.[17] *Hevea* leaves could thus be another natural, cheap, and convenient source of plant peroxidases. The RBP exhibits a good thermostability as it is stable up to 50 °C for 24 hr.[18]

This study describes the purification of peroxidase from rubber-tree leaves, the preparation of RBP-antibody conjugate, and the anticipated application of

RBP. RBP was used in the assay of blood cholesterol, and RBP-anti-rabbit IgG conjugate was used as a secondary antibody in detecting proteins of interest in samples analyzed by western blot technique.

Materials and Methods

Fresh mature rubber-tree leaves of the RRIM 600 variety were collected from a rubber plantation at the Songkhla Rubber Research Center. Vitellogenin and anti-vitellogenin were gifts from Mr. Peerapong Puengyam, Department of Biochemistry, Faculty of Science, Prince of Songkla University. Human serum was provided by Ms. Yoawamarn Sutivichit, Regional Medical Sciences Center Songkhla. HMG-CoA synthase and anti-HMG-CoA synthase were prepared in our laboratory.[19] Sephadex G-25, G-75 and G-200 were purchased from Amersham Pharmacia Biotechnologies, Uppsala, Sweden. DEAE-Sephacel, anti-rabbit IgG, anti-human IgG, horseradish peroxidase (HRP), HRP-anti-rabbit IgG conjugate, HRP-anti-human IgG conjugate, sodium periodate ($NaIO_4$), glutaraldehyde, cholesterol oxidase, 4-aminoantipyrine, and *o*-dianisidine were purchased from Sigma Chemical Company, St. Louis, MO, USA. Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce, Rockford, IL, USA. All other chemicals used were of analytical grade and purchased from local suppliers.

RBP Purification

All purification steps were carried out at 4 °C, unless otherwise specified. Fresh, mature *Hevea* leaves (100 g) were washed and homogenized in 500 ml of 100 mM Tris-HCl, pH 7.5, for 10 min. The homogenate was filtered through cheesecloth to remove the leaf debris, and the crude extract separated as a greenish-brown supernatant by centrifugation for 20 min at 11,000 × *g*. Solid $(NH_4)_2SO_4$ was added to the supernatant up to 40 % saturation. The suspension was stirred for 5 hr and then centrifuged for 20 min at 11,000 × *g*, after which precipitate was discarded. Protein in the supernatant was precipitated by 80 % sat. $(NH_4)_2SO_4$ overnight and then centrifuged for 30 min at 15,000 × *g*. The enzyme pellet was dissolved in a minimum volume of 50 mM Tris-HCl, pH 7.5. Undissolved material was removed by centrifugation for 10 min at 1,000 × *g*. The dark brown enzyme was subsequently applied to a DEAE-Sephacel column (1.5 × 24 cm) equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, followed by stepwise elution with 0.3 M NaCl in the same buffer. The first peak of peroxidase active fractions was pooled and concentrated by centrifugation at 1,000 × *g* through a CF-25 centriflo membrane (25,000 MW Cut-Off). The concentrated peroxidase was further purified in a Sephadex G-75 column (0.9 × 30 cm) and eluted with 10 mM Tris-HCl, pH 7.5. The enzyme was eluted out in the first peak. High peroxidase activity fractions were pooled and concentrated as described above. After having been purified in the Sephadex G-75 column, the partially purified enzyme was kept at -20 °C for further study. The same purification procedure was carried out on other batches of rubber leaves and yielded similar results.

Peroxidase Activity Assay

Peroxidase activity during purification was determined with *o*-dianisidine as a reducing substrate[20] in a mixture consisting of 2.79 ml of 50 mM sodium acetate, pH 5.4, 0.1 ml of 25 % *o*-dianisidine in methanol, 0.1 ml of 0.1 M H_2O_2 and 10 µl of purified enzyme. The activity was monitored by following the increase in absorbance at 460 nm of the reaction product. One unit of the enzyme was defined as the amount of the enzyme forming 1 µmol of the product in 1 min, based on the extinction coefficient of *o*-dianisidine of 1.13 $\times 10^4$ M^{-1} cm^{-1}.

Protein Determination

The protein in all samples at various steps was determined by the Lowry method, [21] using bovine serum albumin (BSA) as standard.

Preparation of RBP-Antibody Conjugate

The purified RBP was conjugated with either anti-rabbit IgG or anti-human IgG using one of the following three cross linkers : periodate, glutaraldehyde [22] and sulfo-SMCC. [23]

With the periodate oxidation method, the purified RBP (5 mg protein) in 1.0 ml of 0.3 M sodium bicarbonate, pH 8.1 (freshly prepared) and 0.1 ml of 1 % fluorodinitrobenzene in absolute ethanol was mixed gently for 1 hr at 30 °C. Sodium periodate (0.06 M, 1 ml) in distilled water was then added and mixed for 30 min, followed by 1 ml of 0.06 M ethylene glycol. The solution was mixed well for another hour at 30 °C. The mixture was dialyzed three times against 1 liter of 0.01 M sodium carbonate, pH 9.5, at 4°C overnight and centrifuged at 1,000 × *g* and at 4°C for 10 min to remove the insoluble material. Anti-rabbit IgG or anti-human IgG (5 mg) was added to the supernatant, mixed gently and incubated for 3 hr at 30 °C, followed by the addition of $NaBH_4$ (5 mg), gently mixed and left at 4°C for at least 3 hr up to overnight. The RBP-antibody conjugate formed was dialyzed three times against 1 liter of 50 mM Tris-HCl, pH 7.5, at 4°C overnight. The dialyzed RBP-antibody conjugate was centrifuged at 1,000 × *g* for 10 min to remove the precipitate and the supernatant containing RBP-antibody conjugate was further

purified.

Using glutaraldehyde, the purified RBP (5 mg protein in 200 µl) was added to 200 µl 1.25% glutaraldehyde in 0.1 M potassium phosphate, pH 6.8. The solution was incubated at 30 °C for 18 hr. The excess glutaraldehyde was removed in a Sephadex G-25 column (0.9 x 30 cm), equilibrated and eluted with 0.15 M NaCl at a flow rate of 8 ml/hr. Fractions of 1 ml were collected and the absorbances at 280 nm were measured. The glutaraldehyde activated RBP in the first peak (brown fractions) were pooled and concentrated to 1 ml using a CF-25 centriflo membrane. Anti-rabbit IgG (5 mg in 1 ml of 0.15 M NaCl) was added to the activated RBP, followed by 0.1 ml of 1 M carbonate / bicarbonate buffer pH 9.5, and then the mixture was incubated at 4 °C for 24 hr, after which lysine (0.2 M, 0.1 ml) was added and the whole mixture was left to settle for 2 hr. The RBP-antibody conjugate was then dialyzed three times against 1 liter of phosphate buffered saline (PBS; 0.1 M potassium phosphate, 0.14 M NaCl), pH 7.2 at 4 °C overnight. The RBP-antibody conjugate after dialysis was precipitated with an equal amount of sat. $(NH_4)_2SO_4$ solution at 4°C. The precipitated RBP-antibody conjugate was washed twice with 50 % sat. $(NH_4)_2SO_4$ solution, dissolved in distilled water and dialyzed overnight three times against 0.1 M PBS, pH 7.2 at 4 °C. The dialyzed RBP-antibody conjugate was centrifuged at 1,000 × *g* for 10 min to remove insoluble materials. The RBP-antibody conjugate in the supernatant was further purified.

Formation of RBP-antibody conjugate by the sulfo-SMCC method was done by first activating of RBP as follows. Sulfo-SMCC (1 mg) was added to the RBP (5 mg in 0.1 M sodium phosphate buffer, 0.14 M NaCl and 0.05 M EDTA, pH 7.2), mixed well for 1 hr and left for 30 min at 30 °C. The activated RBP was separated from excess sulfo-SMCC by passing through a Sephadex G-25 column (0.9 x 30 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2. The column was then eluted with the same buffer at a flow rate of 6 ml/hr. Fractions of 1 ml were collected and the absorbance at 280 nm was monitored. The fractions with A_{280} were then pooled and concentrated to 500 µl using a CF-25 centriflo membrane. Anti-rabbit IgG (4 mg) was reduced by adding in 500 µl of 0.5 M β-mercaptoethylamine in 10 mM sodium phosphate buffer containing 0.14 M NaCl, 5 mM EDTA and incubated at 37 °C for 90 min. Excess reducing agent was removed using a Sephadex G-25 column as described above. The reduced anti-rabbit IgG collected (4 fractions of the first peak) were pooled and concentrated to 500 µl using a CF-25 centriflo membrane. The activated RBP (500 µl) was mixed with the reduced anti-rabbit IgG, incubated at 30 °C for 30 min and left at 4 °C overnight. Finally, the RBP-anti-rabbit IgG conjugate formed was purified.

Purification of RBP-Antibody Conjugate

The RBP-antibody conjugates obtained by the three methods were further purified using a Sephadex G-200 column (1.3 x 65 cm), equilibrated and eluted with an appropriate buffer according to the method of preparation. Fractions collected were monitored measuring the absorbance for protein at 280 nm, for the heme group at 403 nm, and for peroxidase activity using *o*-dianisidine at 460 nm. The efficiency of each conjugation method was compared on the basis of the absorbance at 403 nm and the retained peroxidase activity of RBP-antibody conjugate.

Molecular Weight Determination of RBP-Anti - Rabbit IgG Conjugate

The MW of the RBP-anti-rabbit IgG conjugate prepared by periodate oxidation was determined by gel filtration chromatography using high MW proteins (ferritin : MW = 460,000; catalase : MW = 232,000; BSA : MW = 67,000; chymotrypsinogen A : MW = 25,000) as molecular weight standards. RBP-anti-rabbit IgG conjugate, RBP, anti-rabbit-IgG and standard markers were separately applied onto a Sephadex G-200 column (1.3 x 65 cm), equilibrated and eluted with 50 mM Tris-HCl, buffer pH 7.5, at a flow rate of 6 ml/hr. The MW of the RBP-anti-rabbit IgG conjugate was estimated from a plot of log MW vs K_{av}, calculated from the elution volume of the markers.

Detection of RBP-Antibody Conjugate Using Dot Blot Technique

The ability of the RBP-anti-rabbit IgG conjugate as a secondary antibody to detect target protein was performed as follows. A dot of 5 µl of C-serum of rubber latex (1.13 mg/ml protein) containing HMG-CoA synthase, a protein of interest, was placed on the center of one of the grid squares and dried at 30 °C. The membrane was washed three times for 5 min each with Tris-HCl buffered saline (TBS; 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.5) then blocked with 3 % BSA in TBS containing 0.05 % tween-20 (TTBS) and kept at 4 °C overnight. Then the membrane was removed and placed on a sheet of parafilm. A dot of 1 µl of rabbit serum providing anti-HMG-CoA synthase (17.1 mg/ml protein, dilution 1 : 1,000) was placed on the center of one of the grid squares. The membrane was dried at 30 °C, washed as described above, and then blocked with 3 % BSA for 30 min. After washing with TBS, a dot of 3 µl of RBP-anti-rabbit IgG conjugate from each fractions of first peak (approximately 0.23-0.45 mg/ml protein), purified by the Sephadex G-200 column was placed on the center of the grid squares on top of the rabbit serum. The membrane was left to stand at 30 °C for 10 min, blocked with 3 % BSA for 2 hr, and washed with TBS. Peroxidase activity staining was done using 0.05

% *o*-dianisidine, 16.8 % methanol, 50 mM sodium acetate, pH 5.4, and 0.1 M H_2O_2. The reaction was stopped by adding distilled water.

Detection of Vitellogenin and HMG-Co A Synthase in Samples Using RBP-Anti-Rabbit IgG Conjugate by Means of the Western Blot Analysis

Proteins of interest (HMG-CoA synthase in C-serum (11 μg) or vitellogenin in mullet plasma (23 μg)) were separated by SDS-PAGE with 6 to 18 % gradient gel according to Laemmli. [24] The proteins separated on the gel were then electrophoretically transferred to a nitrocellulose membrane using a tank transfer system at 100 volt (450 mA) for 100 min with cooling. The gel was stained with 0.2 % Coomassie blue to verify a complete transfer of proteins to the membrane. Proteins in the membranes were stained with 0.2 % Ponceau-S in 10 % acetic acid for 10 min. The background was removed by washing with distilled water and then photographed. After destaining the membrane was immersed in a blocking solution (3 % BSA in TTBS) with shaking at 30 °C for 3 hr, then incubated at 4 °C overnight. The membrane was incubated with primary antibody, rabbit anti-HMG-CoA synthase (1:1,000) or anti-vitellogenin (1:300), in 1 % BSA in TTBS with shaking at 30 °C for 2 hr. The blots were washed with four changes of TTBS for 15 min each followed by two changes of TBS for 10 min to remove non-specific binding proteins. The membranes were then incubated with 1:200 RBP-anti-rabbit IgG conjugate (37,900 units/ml peroxidase activity, 0.33 mg/ml) in 1 % BSA in TTBS, which acts as a secondary antibody directed against primary antibody, at 30 °C for 1 hr, then washed as described above. A commercial HRP-anti-rabbit IgG conjugate at 1:3,000 dilution in the same buffer was also used as a positive control. The proteins of interest were identified by peroxidase activity staining as described in 2.4.

Determination of Cholesterol in Human Serum Using Purified RBP

Reagents used for cholesterol determination according to the instruction manual of CPT Diagnostic, Barcelona, Spain were as the following: >0.2 units/ml cholesterol esterase, >0.1 units/ml cholesterol oxidase, RBP or HRP >0.8 units/ml, 0.5 mM sodium cholate, 28 mM phenol, and 4-aminoantipyrine 0.5 mM in 35 mM Pipes buffer, pH 7.0, kept at 2-8 °C. Cholesterol in samples was determined by mixing 10 μl of human serum and 1.0 ml of reagent prepared by using either RBP or HRP, incubated at 25 °C for 10 min. The absorbance of quinoneimine, the reaction product, was measured at 500 nm. Cholesterol concentration in the serum was calculated from its A_{500} and the cholesterol standard curve.

Results and Discussion

RBP Purification

The purification of RBP is summarized in Table 1. Small amount of other proteins in crude extract were removed by 40% ammonium sulfate precipitation. This step should be performed to remove polyphenol oxidases, which cause browning of crude extract, by precipitation with lower salt concentrations. [25] When RBP from 80% ammonium sulfate fractionation containing 382.0 mg protein (19,604 unit peroxidase activity) was applied onto a DEAE-Sephacel ion exchange column, two peaks were detected in the elution profile. Most of the peroxidase was found in the first peak eluted with 10 mM Tris-HCl, pH 7.5. The second peak eluted with 0.3 M NaCl in the same buffer showed no peroxidase activity (Fig 1). High peroxidase activity fractions from a DEAE-Sephacel column were pooled and concentrated, and the RBP obtained was over 50 folds purified. The RBP was further purified by a Sephadex G-75 column. The enzyme was found in the first peak (Fig 2). Only fractions with high peroxidase activity were pooled and concentrated. The purified RBP contained 7.8 mg protein with 6,670 units of peroxidase activity (Table 1).

Characterization of the RBP-Anti-Rabbit IgG Conjugate and the RBP-Anti-Human IgG Conjugate

The RBP-anti-rabbit IgG conjugate prepared by periodate, glutaraldehyde, or sulfo-SMCC was separately purified through gel filtration on a Sephadex G-200 column, two major peaks of proteins were eluted,

Table 1. Purification of RBP from 100 g *Hevea* leaves.

Step	Total activity (units, μmol/min)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude extract	33,526	3,564	9.4	100	1
40%$(NH_4)_2SO_4$	33,110	2,279	14.5	98.7	1.5
80%$(NH_4)_2SO_4$	19,604	382.0	51.3	58.5	5.4
DEAE-Sephacel	9,000	18.8	479.7	26.8	51.0
Sephadex G-75	6,670	7.8	852.9	19.9	90.6

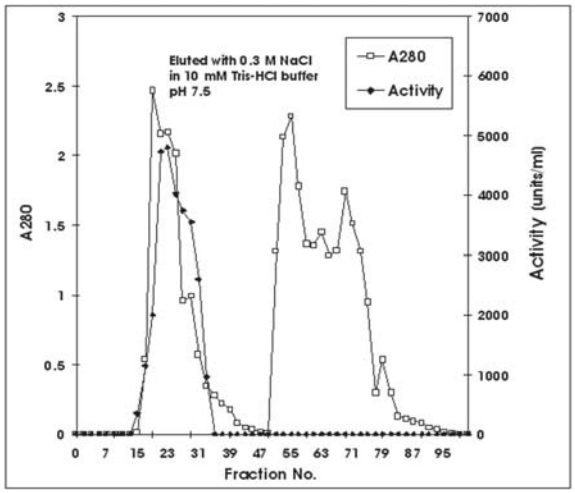

Fig 1. DEAE-Sephacel chromatography of RBP. Peroxidase obtained from 80 % sat. $(NH_4)_2SO_4$ was applied to the column (1.5 × 24 cm) at 4 °C. The column was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5 followed by 0.3 M NaCl in the same buffer with a flow rate of 16 ml/hr. Fractions of 2 ml were collected.

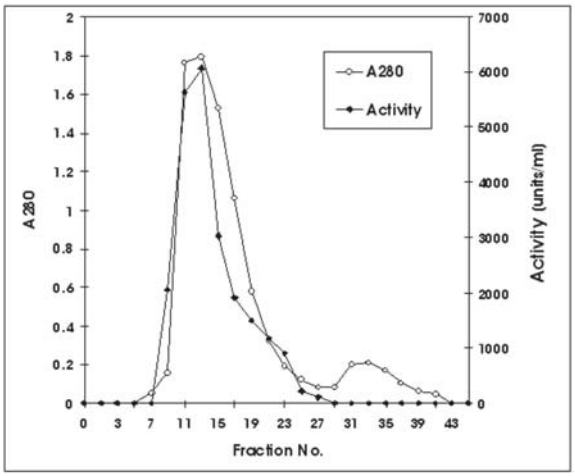

Fig 2. Sephadex G-75 chromatography of RBP. Peroxidase obtained from the DEAE-Sephacel column was applied to a Sephadex G-75 column (0.9 × 30 cm) at 4 °C. The column was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5 with a flow rate of 16 ml/hr. Fractions of 2 ml were collected.

the first peak was RBP-anti-rabbit IgG conjugate and the second was free peroxidase as shown in Fig 3. The efficiencies of the three conjugation methods were compared on the basis of the absorbance at 403 nm and on the retained peroxidase activity of the conjugate. It was found that periodate oxidation is the optimal method. The RBP-anti-rabbit IgG conjugate prepared by using periodate possessed the highest retained peroxidase activity of 68 %, whereas the glutaraldehyde and sulfo-SMCC yielded only 9.7 and 5.3 % of peroxidase activity, respectively. The low efficiency of the conjugation by glutaraldehyde or sulfo-SMCC was probably due to unsuitable conditions for the formation of protein-protein conjugate of anti-IgG with RBP. Several factors may affect the conjugation process, such as concentrations of reactants (antibody, enzyme and cross-linker), pH, temperature, incubation time and purity of the enzyme.[26] In addition, amine groups of antibody and RBP randomly activated by glutaraldehyde and sulfo-SMCC in cross-linking reaction may lead to reduction of antibody and enzyme activity.[27,28] The periodate oxidation method is generally recommended for conjugation of glycoprotein as the carbohydrate moieties of antibody and enzyme are generally not required for their activities. As RBP is a glycoprotein,[18] conjugation of RBP through the carbohydrate moieties should not affect its activities.[29,30] Under optimal condition 68% of the peroxidase activity was also reported for periodate oxidation of the horseradish peroxidase. The loss of activity is probably due to inhibition of the enzyme by periodate.[31] A similar result was obtained when the RBP-anti-human IgG conjugate was prepared by using periodate, but with a higher retained peroxidase activity (76.9%).

The MW of the RBP-anti-rabbit IgG conjugate, anti-rabbit-IgG and RBP determined by gel filtration through a Sephadex G-200 column were 348, 176 and 59 kDa, respectively. The higher MW of the conjugate, i.e. 348 > 176 + 59 = 235 kDa, may have been caused by self-polymerization of either RBP or antibody, since both proteins contain chemically reactive residues which can react with cross-linking reagents. This may be due to the fact that the ratio of RBP/antibody was higher than 1, but this remains unclear, as the extent of the cross-linking reaction is often difficult to control.[22]

Ability of RBP-Anti-Rabbit IgG Conjugate to Detect the Presence of Specific Substance Using the Dot Blot Technique

This dot blot technique provides a rapid and convenient means for screening large number of samples, as it can be used to determine an interesting substance quantitatively by observing the relative intensity of the dots that result from colour development. In this experiment, both the ability of the RBP-anti-rabbit IgG conjugate prepared as secondary antibody in detecting the presence of protein of interest and its amount in various fractions from purification step were also determined. Rabbit anti-HMG-CoA synthase and RBP-anti-rabbit IgG conjugate prepared by periodate oxidation were used as a primary and secondary antibodies, respectively. The RBP-anti-rabbit IgG conjugate fractions (3 μl) purified by Sephadex G-

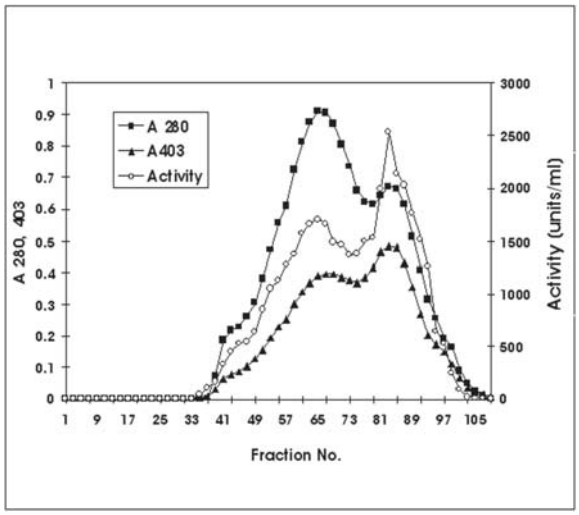

Fig 3. Sephadex G-200 chromatography of RBP-antibody conjugate prepared by periodate oxidation method in a column (1.3 × 65 cm) at 4 °C . The column was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5 with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

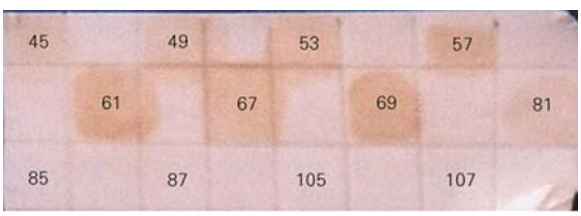

Fig 4. Detection of RBP-anti-rabbit IgG conjugate using dot blot assay. Nitrocellulose membrane was spotted with C-serum containing HMG-CoA synthase . HMG-CoA synthase on the membrane was allowed to react with rabbit anti-HMG-CoA synthase as a primary antibody. Fractions of RBP-anti-rabbit IgG from a Sephadex G-200 column (Fig 3, number 45-107) were spotted onto the nitrocellulose membrane as a secondary antibody.

200 (Fig 3) were dotted onto the nitrocellulose membrane with HMG-CoA synthase and allowed to bind with primary antibody (anti-HMG-CoA synthase), peroxidase activity were detected as shown in Fig 4. The fraction numbered 45-81 in Fig 4 show peroxidase activity, which is consistent with the absorbance at 403 and 460 nm as shown in Fig 3. These results show the high selectivity of antibody for target protein, suggesting that this technique can be used to verify the formation of the conjugate as well as to detect HMG-CoA synthase in C-serum.

RBP-Anti-Rabbit IgG Conjugate as a Secondary Antibody in Western Blot Technique

An important characteristic of an enzyme-antibody conjugate is its ability to be a detector in an enzyme immunoassay such as ELISA or western blot. The prepared RBP-anti-rabbit IgG conjugate was used as a secondary antibody in western blot to detect vitellogenin, a phosphoglycoprotein in mullet plasma, and HMG-CoA synthase, an enzyme in C-serum of rubber latex, after polyacrylamide gel electrophoresis. There were several protein bands in both C-serum and mullet plasma as seen by Ponceau-S staining (Fig 5 a, b). When the proteins on the membrane were allowed to react with either anti-vitellogenin or with anti-HMG-CoA synthase from rabbit, the membrane was washed and our RBP-anti-rabbit IgG conjugate or commercial HRP-anti-rabbit IgG conjugate was added. The presence of vitellogenin and HMG-CoA synthase in the samples was clearly indicated by both conjugates as seen in Fig 5.

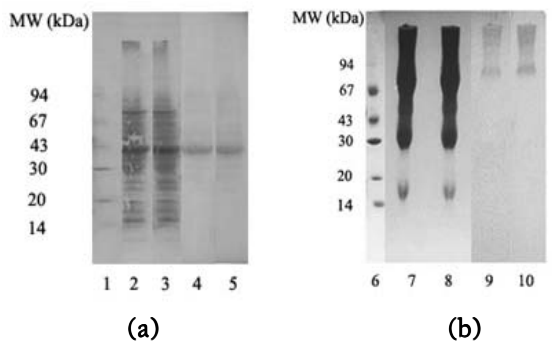

Fig 5. Western blotting of HMG-CoA synthase (a), and of vitellogenin (b), lane 1, 6; low MW marker proteins stained with Ponceau-S, lane 2, 3; C-serum stained with Ponceau-S, lane 4; detected HMG-CoA synthase using HRP-anti-rabbit IgG conjugate as a secondary antibody, lane 5; detected HMG-CoA synthase using RBP-anti-rabbit IgG conjugate as a secondary antibody, lane 7, 8; samples containing vitellogenin stained with Ponceau-S, lane 9; detected vitellogenin using HRP-anti-rabbit IgG conjugate as a secondary antibody, and lane 10; detected vitellogenin using RBP-anti-rabbit IgG conjugate as a secondary antibody.

RBP as an Enzyme in Cholesterol Determination

RBP could also be used in the determination of serum metabolites such as glucose, uric acid and cholesterol. We used it in combination with cholesterol esterase/oxidase as a reagent in a diagnostic kit to determine the total cholesterol in human serum, based on the colour formation of the quinoneimine dye which was produced by the reaction of 4-aminoantipyrine and phenol with hydrogen peroxide. Hydrogen peroxide was generated by the oxidation of cholesterol in a reaction catalyzed by cholesterol oxidase, [32] as shown by the following reactions:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Free fatty acid} \quad (1)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone} + H_2O \quad (2)$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{\text{Peroxidas}} \text{Quinoneimine} + H_2O \quad (3)$$

In these reactions, sufficient amounts of the three enzymes must be present, to ensure that the whole amount of cholesterol, produced in the reaction is converted stoichiometrically to a coloured substance by using 4-aminoantipyrine as peroxidase's substrate. Analysis of cholesterol in human serum from 22 different samples using both RBP and HRP gave similar results (Fig 6).

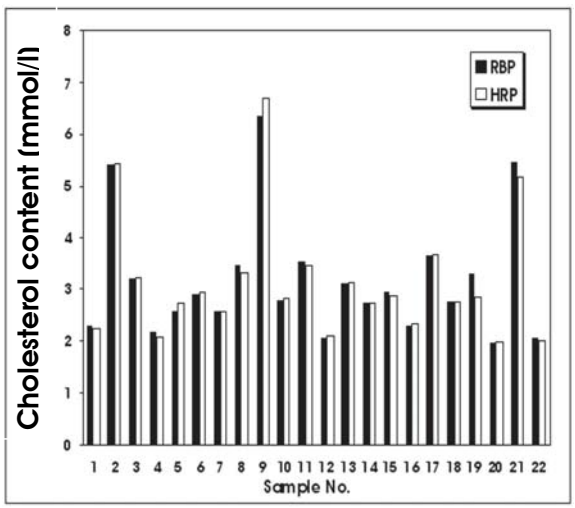

Fig 6. Determination of cholesterol in 22 different human serum samples using reagents prepared with RBP (solid bar) compared with the commercial HRP (open bar). The small differences are statistically insignificant.

Potential Applications of RBP

Although RBP and HRP can be used equally well, we have also explored how much peroxidase can be extracted from rubber-tree leaves, and found that the amount of peroxidase from rubber leaves is only 38.6% of that from horseradish roots (215 vs 557 units/g). Lower yield of peroxidase was found in *Hevea* leaves, but as the leaves are available as by-product on rubber plantation, the cost of *Hevea* leaves as a source of peroxidase might be lower than that of horseradish. Preparation of RBP for conjugation with anti-IgG and the use of RBP in clinical diagnosis would justify further investigations. The RBP-anti-rabbit IgG conjugate we prepared had a peroxidase/antibody ratio of more than one, similar to the 0.8-1.5 ratio of the HRP-anti-rabbit IgG conjugate commercially prepared by Sigma. It is thus likely that the RBP-anti-rabbit IgG conjugate and the commercial HRP-anti-rabbit IgG conjugate are interchangeable. The RBP-anti-rabbit IgG conjugate can be applied as secondary antibody in detecting interesting compounds in both dot blot and western blot analysis. The optimal dilution of RBP-anti-rabbit IgG conjugate was at 1:200, while that of commercial HRP-anti-rabbit IgG conjugate is 1:3,000. As the RBP-anti-rabbit IgG conjugate was prepared and pooled from purified fractions at a small scale, the protein concentration (total protein of 0.33 mg/ml) was lower

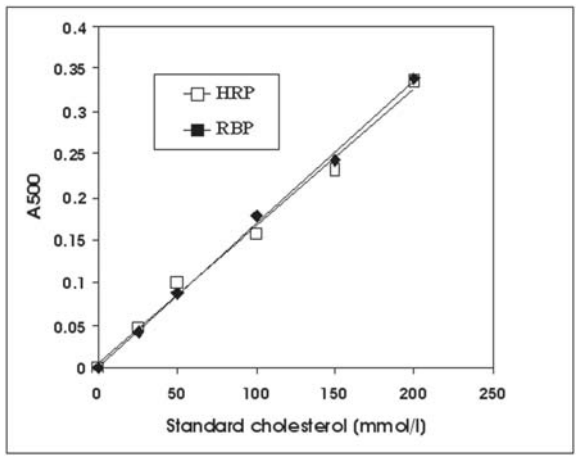

Fig 7. Calibration curves for cholesterol determination, using RBP (solid square) and the commercial HRP (open square).

than that of commercial HRP-anti-rabbit IgG, in which the minimal protein concentration is 4 mg/ml for affinity isolated antibodies. In our study, an optimal concentration of prepared RBP (3.65 units/ml reaction) gave the same calibration curve of cholesterol concentration as that of commercial HRP (3.14 units/ml reaction) (Fig 7).

In conclusion, RBP and RBP-antibody conjugate may offer economic and scientific benefits for Thailand, because RBP can be obtained as a by-product from rubber plantations, depending on whether the cost of RBP and RBP-antibody conjugate preparation at a large scale is competitive with that of the industrial production from horseradish. It is therefore of paramount benefit to develop an efficient industrial extraction method.

Conclusions

In this study, we reported that rubber trees, the most important agro-economic plantation tree of Thailand, could possibly become a new source of plant peroxidase. Rubber peroxidase (RBP) was purified and its applications for clinical diagnostic was investigated. RBP-antibody conjugate was successfully prepared by using periodate, a recommended cross-linker for

conjugation of glycoprotein. The RBP-antibody conjugates prepared using anti-rabbit IgG and anti-human IgG retained 68 % and 76 % peroxidase activity, respectively. RBP-anti-rabbit IgG conjugate (37,900 units/ml, dilution 1:200) can be used as a secondary antibody for indirect immunoassay to detect vitellogenin in mullet plasma and HMG-CoA synthase in C-serum of rubber latex equally well as the commercial horseradish peroxidase (HRP) -anti-rabbit IgG conjugate.

Another application of RBP could well be in medical analytical laboratories, because peroxidase is one of the enzymes used in a reaction mixture to determine human metabolites. Cholesterol assay reagents using either RBP or HRP prepared to determine cholesterol in 22 serum samples gave nearly the same result.

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