CHAPTER 3

RESULTS

Section I: RBP purification and applications

1. RBP purification and peroxidase activity assay

One hundred grams of mature *Hevea* leaves per batch were used to purified rubber peroxidase (RBP). The purification procedure was started from crude extract by using centrifugal fractionation to remove the leaf debris. The supernatant obtained from this step contained 3,564 mg protein with 33,526 units peroxidase activity (Table 5). The supernatant was further purified by 40% $(NH_4)_2SO_4$ precipitation to separate other high molecular weight proteins from the solution, and then by 80% $(NH_4)SO_4$ to collect the protein pellet. The RBP obtained from 80% ammonium sulfate precipitation contained 382.0 mg protein and 19,604 units peroxidase activity. After passing through a DEAE-Sephacel column, two major protein peaks were detected in the stepwise elution profile (Figure 14). Most of the peroxidase activity (9,000 units) was found in the first peak eluted with 10 mM Tris-HCl, pH 7.5, while the second peak, eluted with 0.3 M NaCl in the same buffer, showed no peroxidase activity (Figure 14).

When size exclusion chromatography of the first peak obtained from DEAE-Sephacel was performed in a Sephadex G-75 column the protein with high peroxidase

activity was found in the first peak (Figure 15). The purity of the pooled RBP was 90.6 fold that of the initial extract and contained 7.8 mg protein with 6,670 units peroxidase activity. The purity of the enzyme was also verified by ND-PAGE with 7-15% gradient gel according to Laemmli (Laemmli, 1970) and the RBP was kept at -20 ^{o}C for RBP-antibody conjugate preparation and other applications.

Table 5. Purification of RBP from 100 g *Hevea* leaves.

Purification step	Total activity (units, ΔA_{460} /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

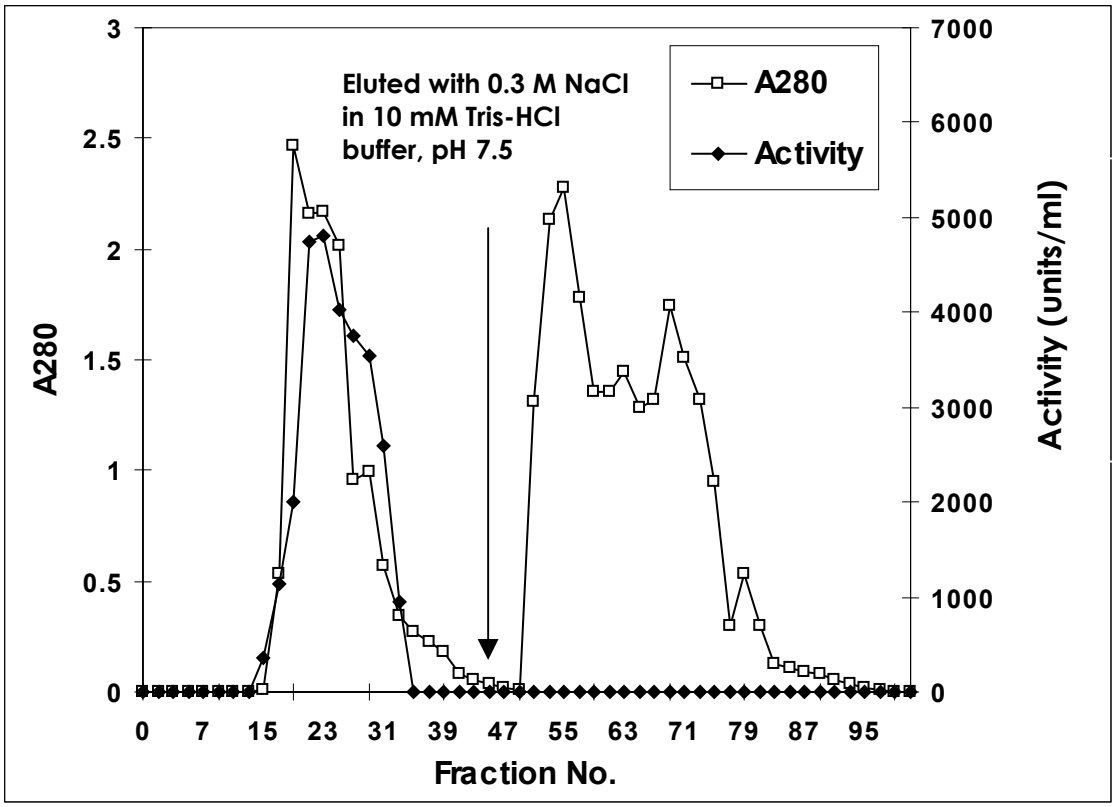


Figure 14. DEAE-Sephacel chromatography of the RBP obtained from 80% sat. $(NH4)_2SO_4$. The column (1.5 × 24 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, followed by 0.3 M NaCl in the same buffer at 4 ^{o}C with a flow rate of 16 ml/hr. Fractions of 2 ml were collected.

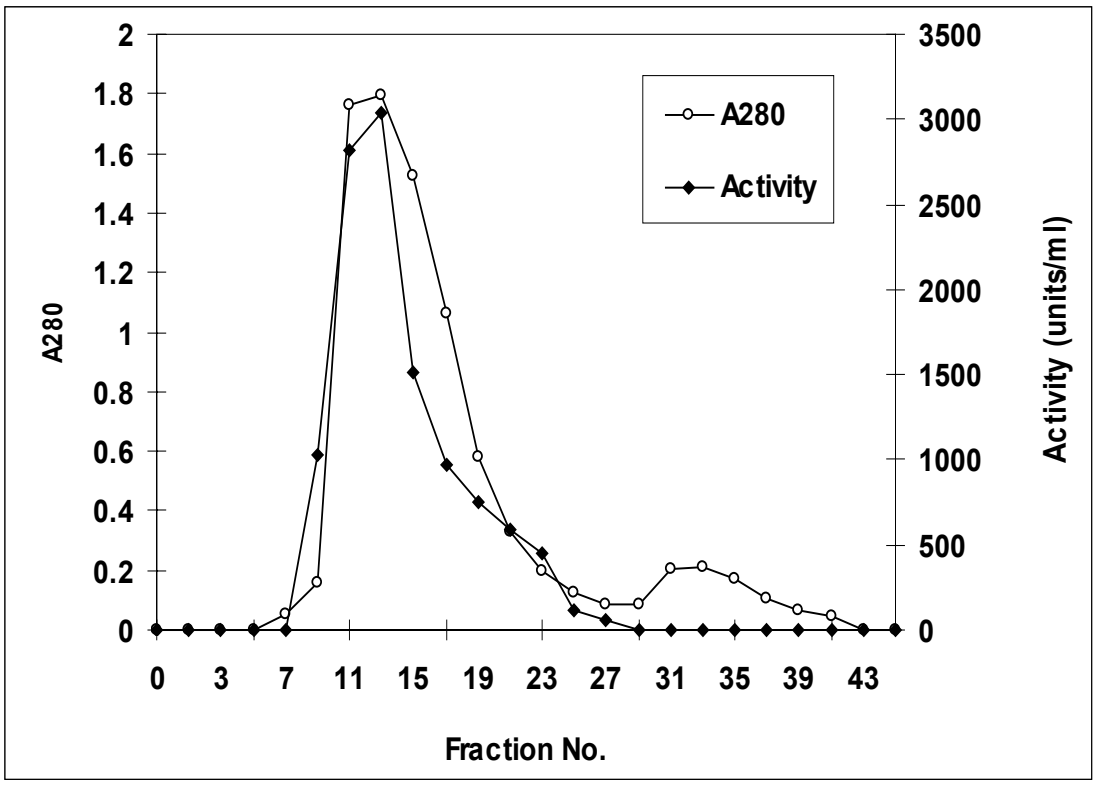


Figure 15. Sephadex G-75 chromatography of the RBP obtained from the DEAE-Sephacel column. The column (0.9 × 30 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5 at 4 ^{o}C with a flow rate of 16 ml/hr. Fractions of 2 ml were collected.

2. Conjugation and characterization of RBP-antibody conjugate

2.1 RBP-antibody conjugate preparation by using glutaraldehyde

When the RBP-antibody conjugate, prepared by using glutaraldehyde was purified, two peaks of protein were eluted from a Sephadex G-200 column. The first peak of RBP-anti-rabbit IgG conjugate was followed by the free RBP according to its peroxidase activity and absorbance at 403 nm (Figure 16). The chromatogram showed good resolution of unconjugated and conjugated RBP, but the conjugate retained only 9.7% of the initial activity.

2.2 RBP-antibody conjugate preparation by using sulfo-SMCC

The RBP-anti-rabbit IgG conjugate prepared using sulfo–SMCC was applied through a Sephadex G-200 column. Only a small amount of the conjugate was formed as shown by the very small first peak and it retained only 5.3% peroxidase activity (Figure 17), much lower than that of the conjugate prepared using glutaraldehyde. The second peak of the A_{280} in Figure 17 may be unconjugated IgG and free sulfo-SMCC, since it does not accompany with either the A_{403} or A_{460}.

2.3 The optimal concentration of $NaIO_4$ for the formation of RBP-antibody conjugate

It has been previously found that the concentration of sodium periodate may affect on the formation of enzyme-antibody conjugate (Tijssen and Kurstak, 1984). The optimal concentration of sodium periodate was established by using half scale RBP-anti-rabbit IgG conjugate preparations with various concentrations of sodium periodate. It was found that 0.06 M periodate gave the highest yield as judged from 48.9% peroxidase activity retained, compared to 40.5% and 33.2% for 0.04 and 0.08 M, respectively (Figure 18).

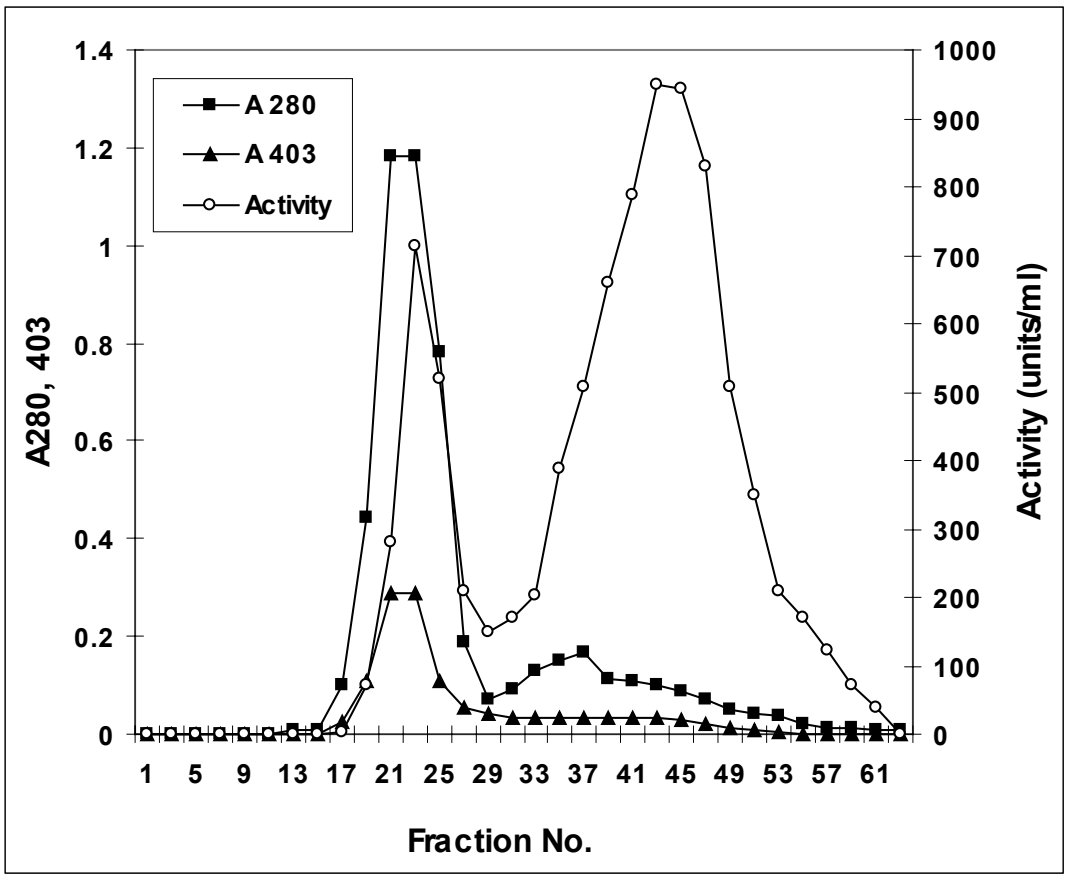


Figure 16. Sephadex G-200 chromatography of RBP-anti-rabbit IgG conjugate prepared using glutaraldehyde. The column (1.3 × 65 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, at 4 ^{o}C with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

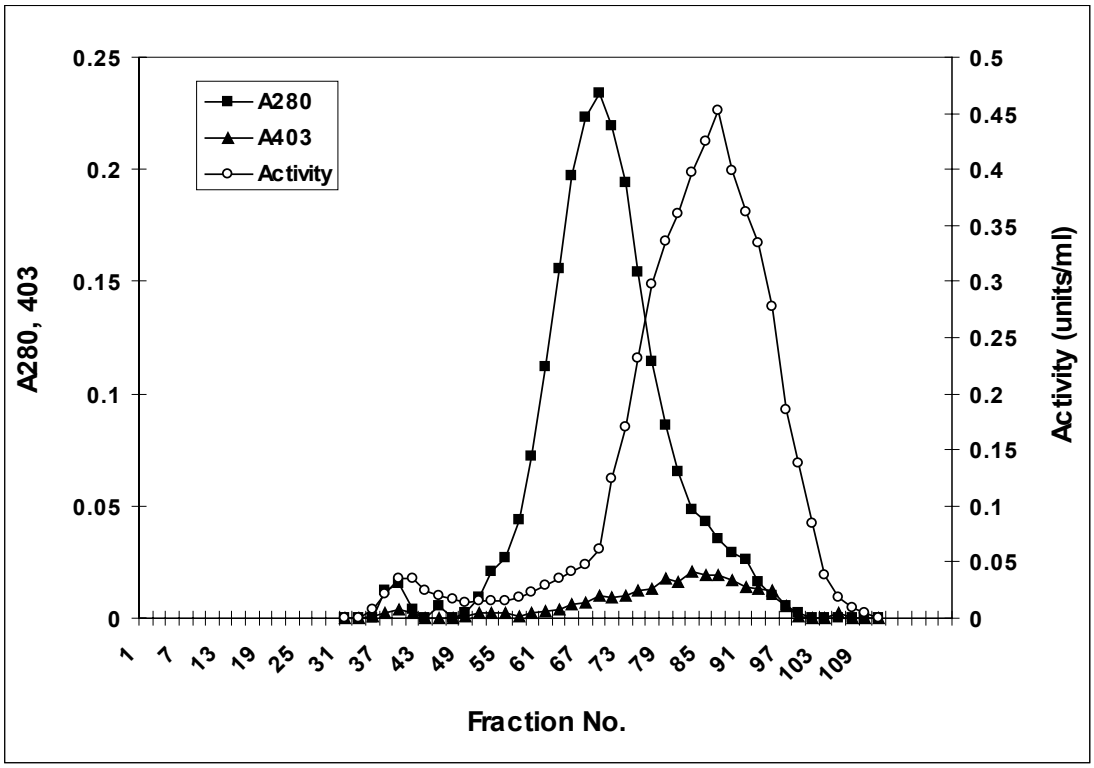


Figure 17. Sephadex G-200 chromatography of RBP-anti-rabbit IgG conjugate prepared using sulfo-SMCC. The column (1.3 × 65 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, at 4 ^{o}C with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

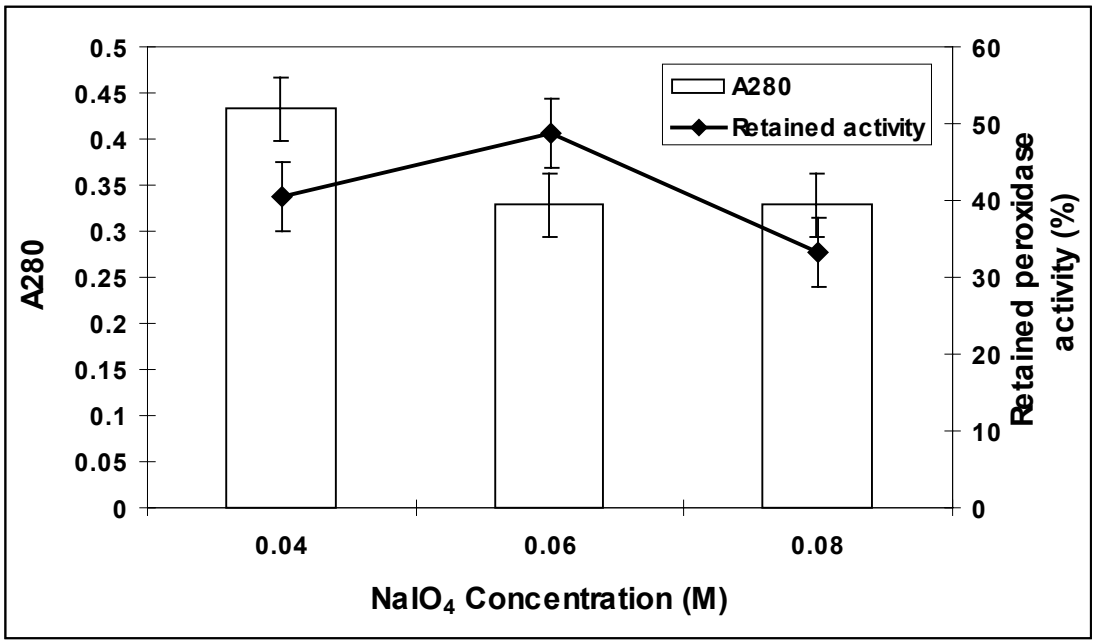


Figure 18. Retained peroxidase activity and the A_{280} of the RBP-anti-rabbit IgG conjugate prepared by using 0.04, 0.06 and 0.08 M sodium periodate, after Sephadex G-200 chromatography (in a 1.3 × 30 cm column) at 4 ^{o}C.

2.4 RBP-antibody conjugate preparation using sodium periodate

A full-scale preparation of RBP-anti-rabbit IgG conjugate using 0.06 M $NaIO_4$ has been performed. The elution profile of purification by gel filtration on a Sephadex G-200 column (Figure 19) showed that at least two not clearly separated peaks of protein were eluted. The first peak was RBP-antibody conjugate and the second was free RBP as found in the conjugate preparation by using glutaraldehyde and sulfo-SMCC. The conjugate retained 68% of its initial activity, which was higher than that prepared by using glutaraldehyde and sulfo-SMCC.

The sodium periodate method with the highest yield for the RBP-anti-rabbit IgG conjugate was also chosen to prepare an RBP-anti-human IgG peroxidase conjugate. Its elution profile (Figure 20) from the Sephadex G-200 column was similar to that of the RBP-anti-rabbit IgG conjugate in Figure 19. Two peaks of protein were eluted, but the second peak was smaller, suggesting that it is free RBP (Figure 20). It also gave high protein yield and high retained peroxidase activity (76.9%).

2.5 RBP–antibody conjugate preparation by modified periodate oxidation method

Using sodium periodate as a cross-linker showed the highest efficiency when compared with glutaraldehyde and sulfo–SMCC. The modified periodate oxidation method was attempted to get clearly separation of protein peaks using 80% ammonium sulfate precipitation before separation by Sephadex G-200 column. The RBP-anti-rabbit IgG conjugate was also found in the first peak, but both its peroxidase activity (29.4%) and its protein yield were much less than those of the preparation without ammonium sulfate precipitation (Figure 21).

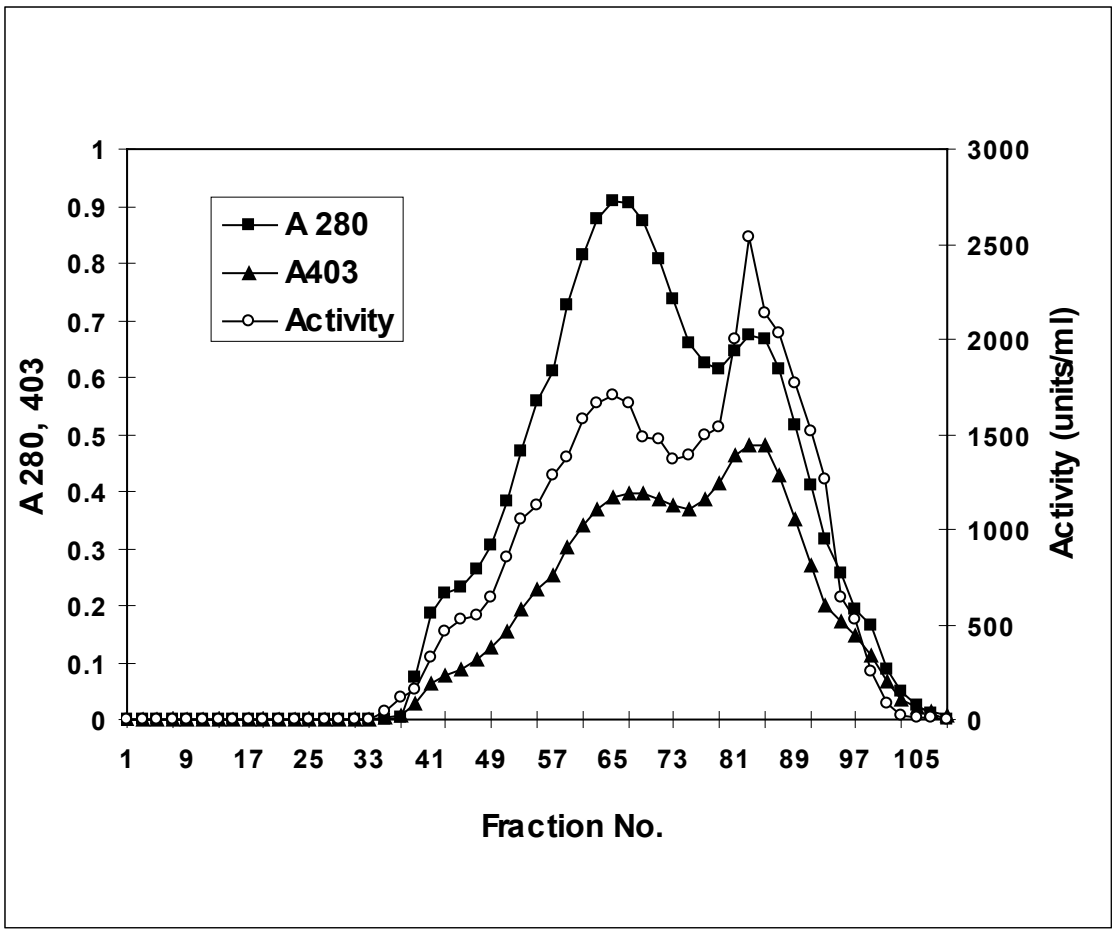


Figure 19. Sephadex G-200 chromatography of RBP-anti-rabbit IgG conjugate prepared with sodium periodate. The column (1.3 × 65 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, at 4 ^{o}C with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

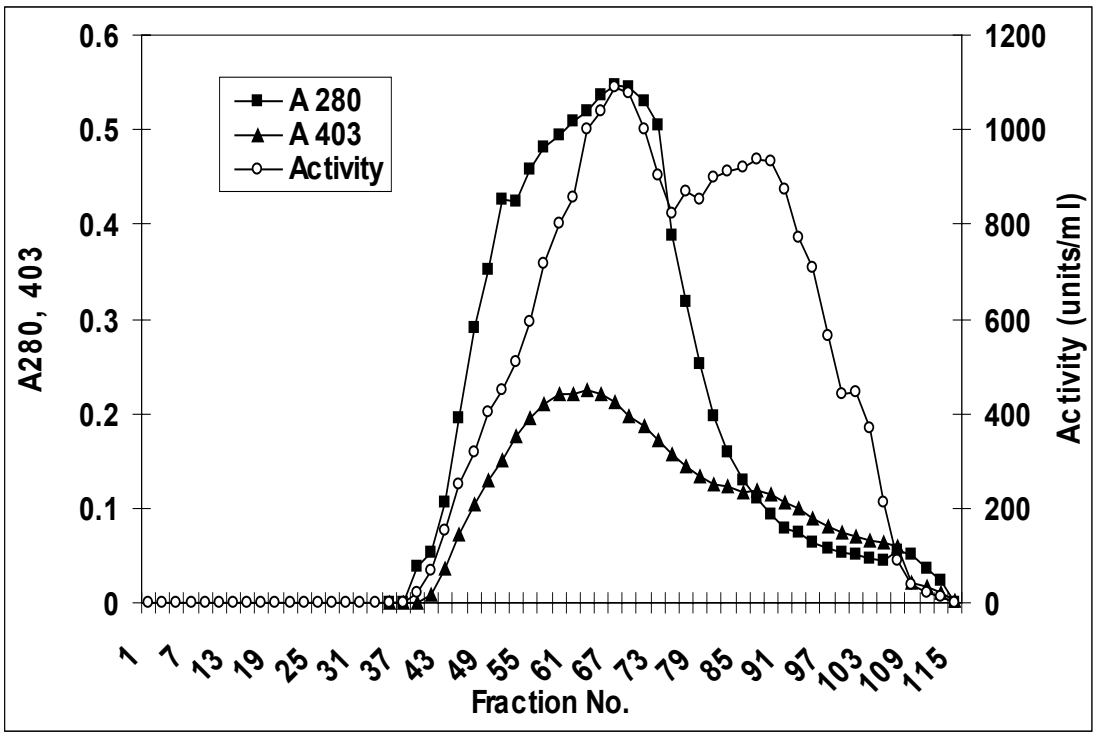


Figure 20. Sephadex G-200 chromatography of RBP-anti-human IgG conjugate prepared with sodium periodate. The column (1.3 × 65 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, at 4 ^{o}C with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

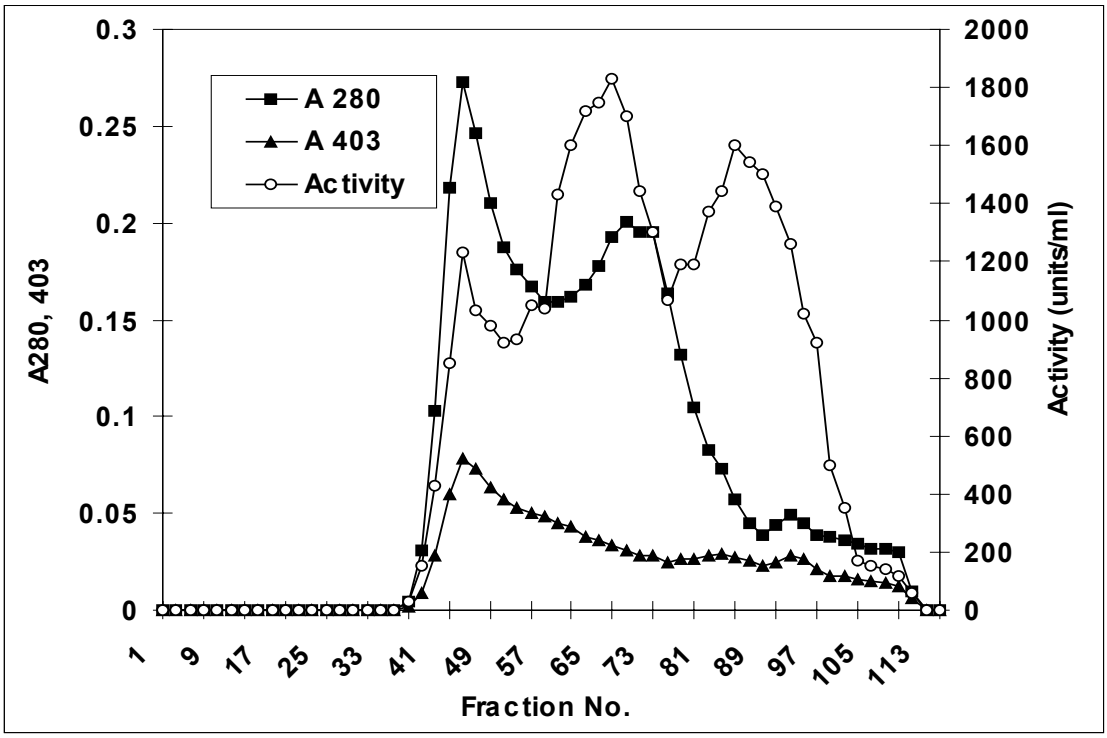


Figure 21. Sephadex G-200 chromatography of RBP-anti-rabbit IgG conjugate prepared by the modified periodate oxidation method. The column (1.3 × 65 cm) was equilibrated and eluted with 10 mM Tris-HCl, pH 7.5, at 4 ^{o}C with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

2.7 Molecular weight determination of RBP-antibody conjugate

The RBP-anti-rabbit IgG conjugate, RBP, and anti-rabbit IgG, as well as standard markers, were separately applied onto the Sephadex G-200 column. Their elution profiles are shown in Figure 22 and the K_{av} and molecular weights were calculated from the elution volume of the standard markers. The molecular weight of the conjugate, RBP and anti-rabbit IgG were estimated from a plot of log MW vs K_{av} to be 348, 59 and 176 kDa, respectively (Figure 23).

2.8 Antigen-antibody interaction of RBP-antibody

The ability of either RBP-anti-rabbit IgG conjugate or RBP-anti-human IgG conjugate to react with the specific antigen, rabbit IgG or human IgG were confirmed by an Ouchterlony double diffusion test. The pooled fractions of both conjugates (Figure 19 or 20) were applied to wells of the agarose gel to react with rabbit or human IgG in serum. A precipitation line of anti IgG-IgG was formed as seen by Coomassie Blue staining (Figure 24 a, b) and by peroxidase activity staining (Figure 24 c, d).

2.9 Determination of RBP-antibody conjugate titer

The titer of RBP-anti-rabbit IgG conjugate was examined in a simple direct test against rabbit IgG. Serially diluted concentrations (1:25 to 1:800) of the pooled fractions (a pool of fractions 49-66 containing 2 mg/ml protein) from the Sephadex G-200 column were compared, based on the colour developed after peroxidase activity staining. The colour was also developed at 1:400 (Figure 25), however, optimal dilution at 1:200 was recommended for dot blot and Western blot techniques.

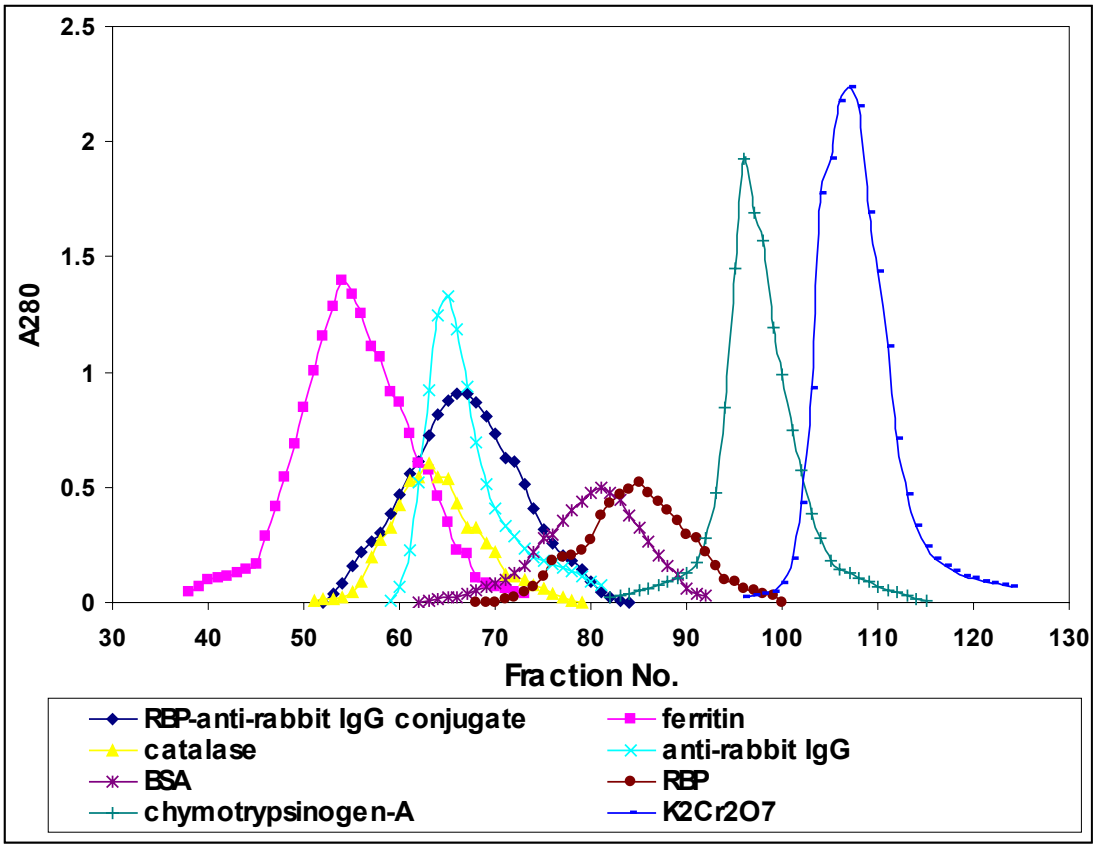


Figure 22. Molecular weight determination of RBP-anti-rabbit IgG conjugate, RBP and anti-rabbit IgG by gel filtration on a Sephadex G-200 column. The column (1.3 × 65 cm) was equilibrated and eluted at 4 ^{o}C with 10 mM Tris-HCl, pH 7.5, with a flow rate of 6 ml/hr. Fractions of 1 ml were collected.

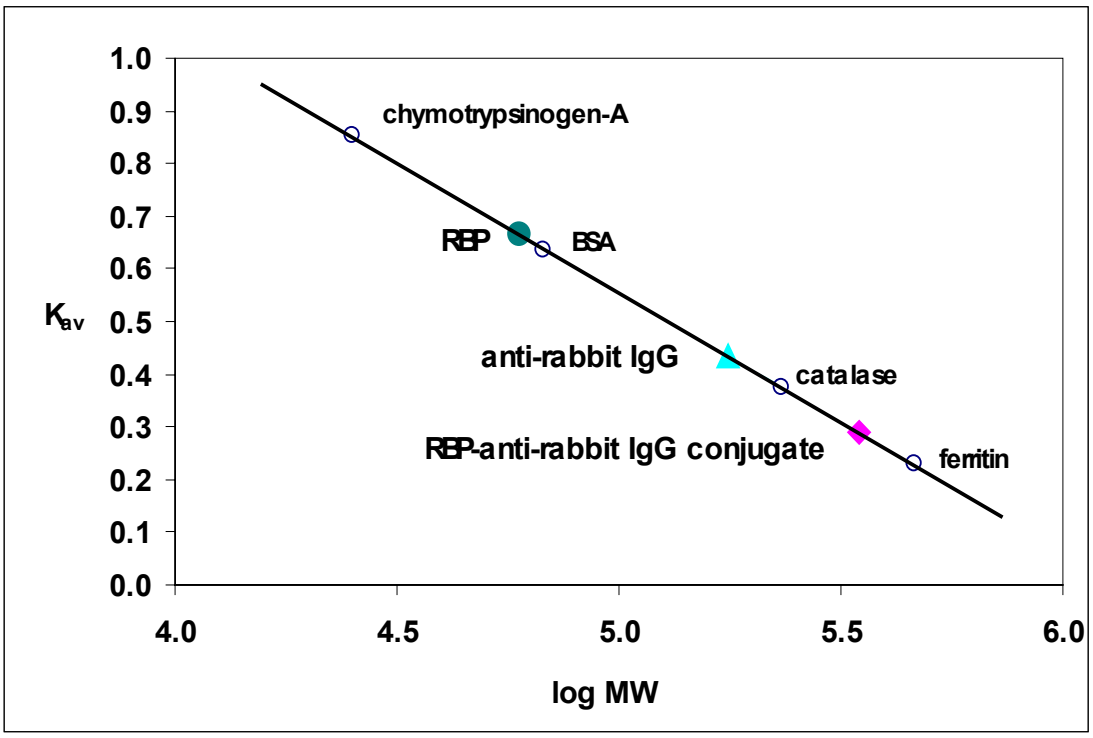


Figure 23. A standard curve (log MW vs K_{av}) for MW estimation of RBP-anti-rabbit IgG conjugate, RBP, and anti-rabbit IgG.

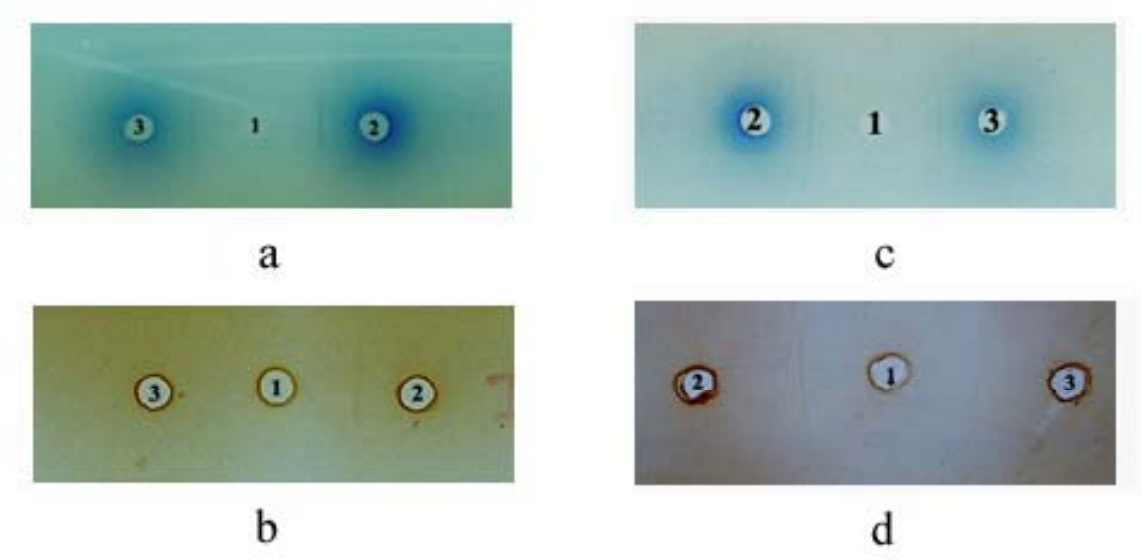


Figure 24. Reaction of RBP-anti-rabbit IgG conjugate with rabbit serum (a; Coomassie Blue staining, b; peroxidase activity staining and RBP-anti-human IgG conjugate with human serum, c; Coomassie Blue staining, d; peroxidase activity staining) in an Ouchterlony double diffusion test. 1= 10 µl of rabbit or human serum, 2 = 20 µl of RBP-anti rabbit IgG or anti-human IgG conjugate, 3 = 10 µl of RBP-anti rabbit IgG or anti-human IgG conjugate.

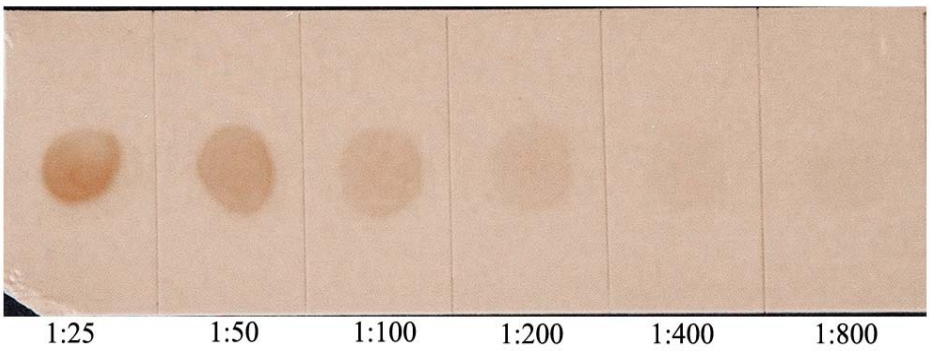


Figure 25. Determination of the RBP-anti-rabbit IgG conjugate titer. The Sephadex G-200 column peak pool (49-66) (Figure 19) was diluted by serial dilution (1:25, 1:50, 1:100, 1:200, 1:400 and 1:800) then tested against rabbit serum.

2.10 Retension of peroxidase activity and immunological stability of the RBP-antibody conjugate

The stability over storage time at -20 ^{o}C of the RBP-anti-rabbit IgG conjugate and RBP-anti-human IgG conjugate were determined. Both conjugates retained peroxidase activity, as demonstrated by measuring their activity with *o*-dianisidine as substrate, for 0, 2, 4, 6 and 8 months (Figure 26). This result suggests that the conjugates were stable for at least eight months at -20 ^{o}C.

The immunological stability of both RBP-anti-rabbit IgG conjugate and RBP-anti-human IgG conjugate were also tested at 2, 4, 6 and 8 months after conjugation. The result in Figure 27A and 27B with the same absorbance was always obtained in all tests. Therefore, the conjugates have a high immunological stability for at least 8 months.

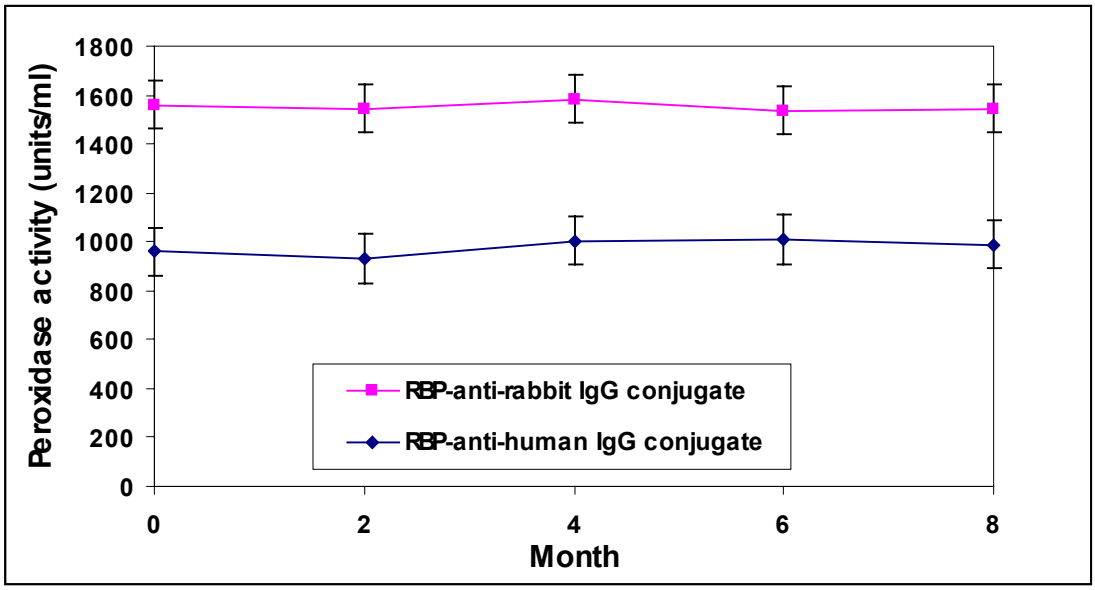


Figure 26. Determination of peroxidase activity of the RBP-anti-rabbit IgG and RBP-anti-human IgG conjugate kept for 8 months at -20 ^{o}C using *o*-dianisidine as substrate. Error bar = the mean of 3 replications.

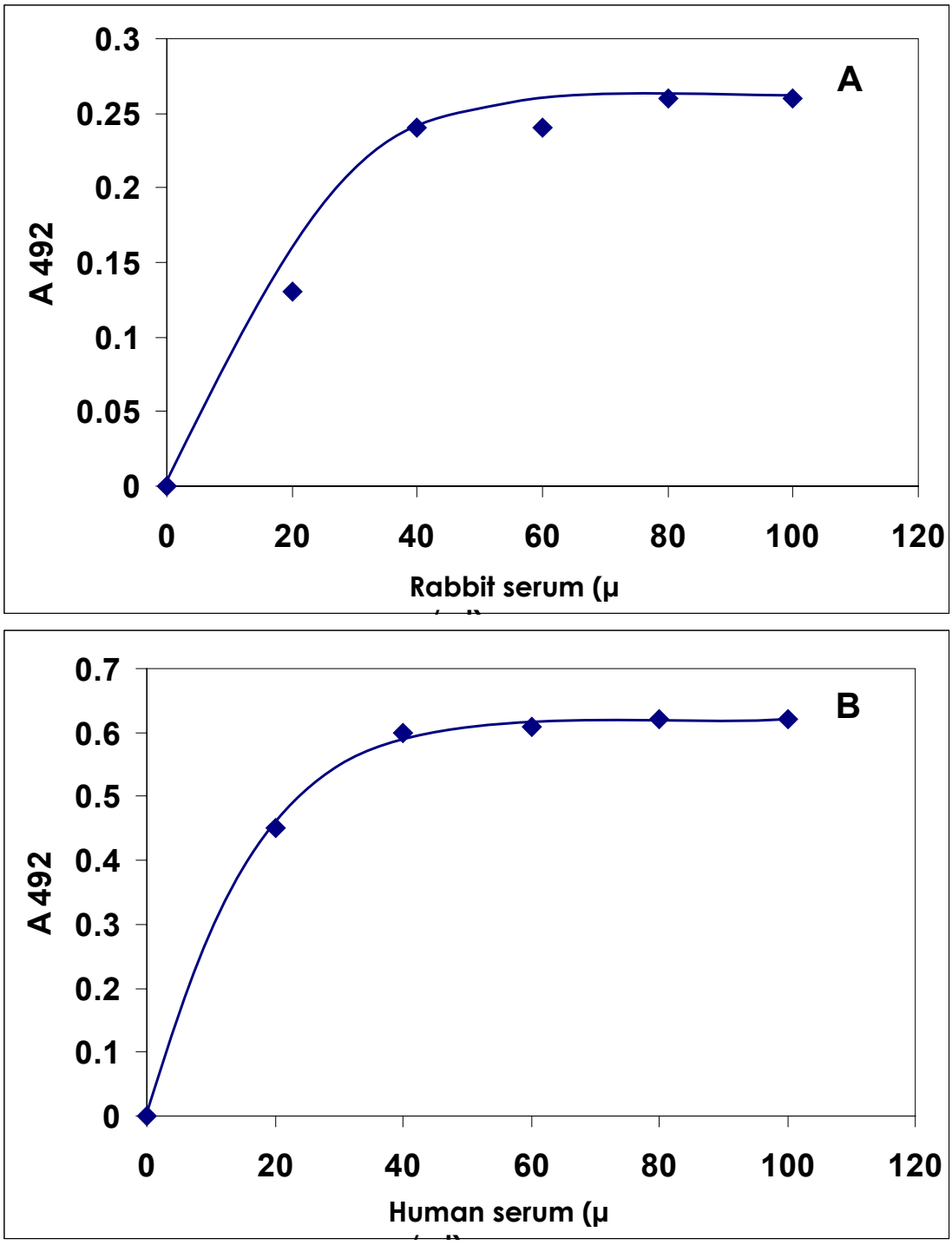


Figure 27. Immunological stability of RBP-anti rabbit IgG conjugate against rabbit serum (rabbit IgG, A) and RBP-anti human IgG conjugate against human serum (human IgG, B) tested in a microtiter plate. The same result was obtained for all the tests, in which the conjugates had been kept at -20 ^{o}C for 2, 4, 6, and 8 months after preparation.

3. Applications of purified RBP and RBP-antibody conjugate

3.1 Dot blot technique

The basis of the dot blot method is that protein samples are bound to a nitrocellulose membrane. Non-specific binding sites on the membrane are blocked, and then a primary antibody against a specific protein is applied to the membrane. A labeled secondary antibody (enzyme-antibody conjugate) is then used to detect the expected binding of the primary antibody.

In this report, HMG-CoA synthase in C-serum of rubber latex was used as the antigen which was detected. Rabbit-anti HMG-CoA synthase and RBP-anti-rabbit IgG conjugate were used as primary and secondary antibody, respectively. After 3 µl of the RBP-anti-rabbit IgG conjugate fractions 45, 49, 53, 57, 61, 67, 69, 81, 85, 87, 105 and 107 from the Sephadex G-200 column were dotted onto the nitrocellulose membrane, and peroxidase activity was then detected as shown in Figure 28. Fractions 45-81 showed peroxidase activity, which is consistent with the absorbance at 403 and 406 nm in Figure 19, whereas no colour developed in fractions 85-107. This result confirmed that the fractions which showed peroxidase activity in Figure 19 (85-107) were free peroxidase.

3.2 Western blot technique

RBP-anti rabbit IgG conjugate can be used as a secondary antibody to detect vitellogenin, a phosphoglycoprotein in mullet plasma and HMG-CoA synthase in C-serum of rubber latex. After polyacrylamide gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane. There are several protein bands in both C-serum and mullet plasma as seen by Ponceau-S staining (Figure 29 a, b). When the proteins on the membrane were allowed to react with either anti-vitellogenin or anti-

HMG-CoA synthase, the membrane was washed and RBP-anti-rabbit IgG or commercial HRP-anti-rabbit IgG conjugate were added at the optimal dilution at 1:200 and 1:3,000, respectively. Only vitellogenin and HMG-CoA synthase bands were detected by peroxidase activity staining. The presence of vitellogenin and HMG-CoA synthase in the samples is indicated by both conjugates in Figure 29 (lane 4, 5 and 9, 10).

3.3 ELISA technique

The conjugate prepared was also used in detecting anti-leptospira in human serum by an ELISA technique. Wells in the microtiter plate coated directly with leptospira, then human serum, positive (PC) and negative (NC) control and human serum containing anti-leptospira which bound specifically to leptospira were detected by incubation with RBP-anti-human IgG conjugate prepared at 1:1,200 dilution. A commercial HRP-anti-rabbit IgG conjugate was used at 1:12,000 dilution as a control. Their peroxidase activities determined at 492 nm were not significantly different (Figure 30).

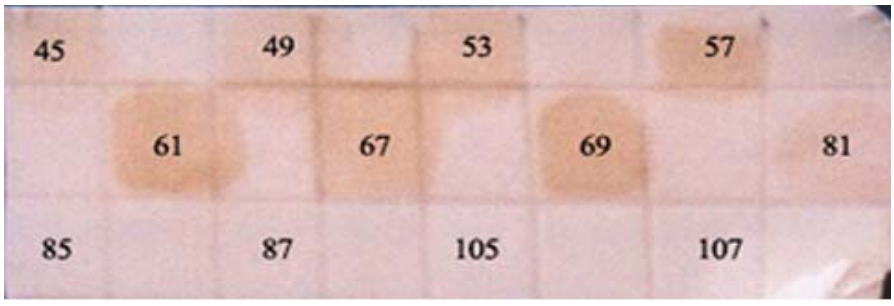


Figure 28. Dot blot assay of HMG-CoA synthase in C-serum of rubber latex. Rabbit serum was used as the first antibody. Fractions of RBP-anti-rabbit IgG conjugate from a Sephadex G-200 column (Figure 19, number 45-107) were spotted onto a nitrocellulose membrane as a secondary antibody.

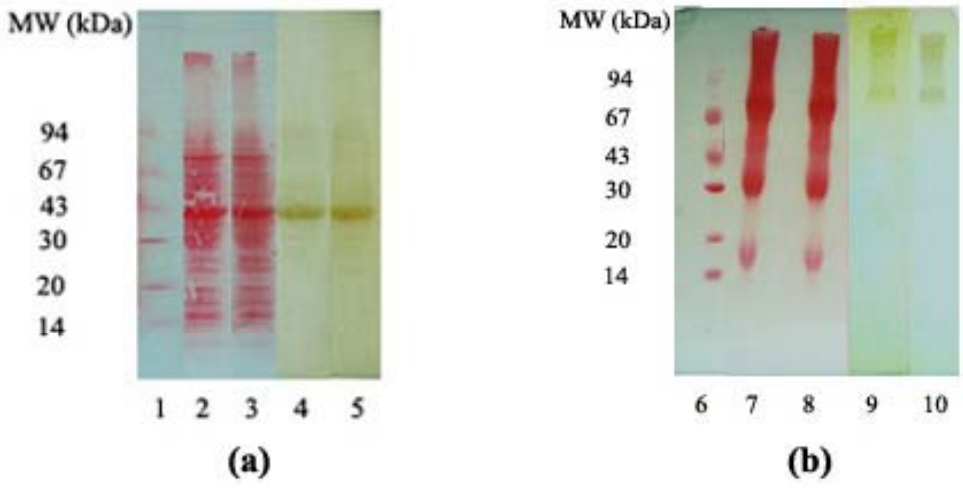


Figure 29. Western blotting of HMG-CoA synthase (a), and of vitellogenin (b), lanes 1 and 6, low MW marker proteins stained with Ponceau-S; lanes 2 and 3, and 7 and 8 were C-serum and samples containing vitellogenin, respectively, stained with Ponceau-S; lane 4 and 5, and 9 and 10 were immunological detection using HRP-anti-rabbit IgG conjugate and RBP-anti-rabbit IgG conjugate as secondary antibodies in lane 4 and 9, and lane 5 and 10, respectively.

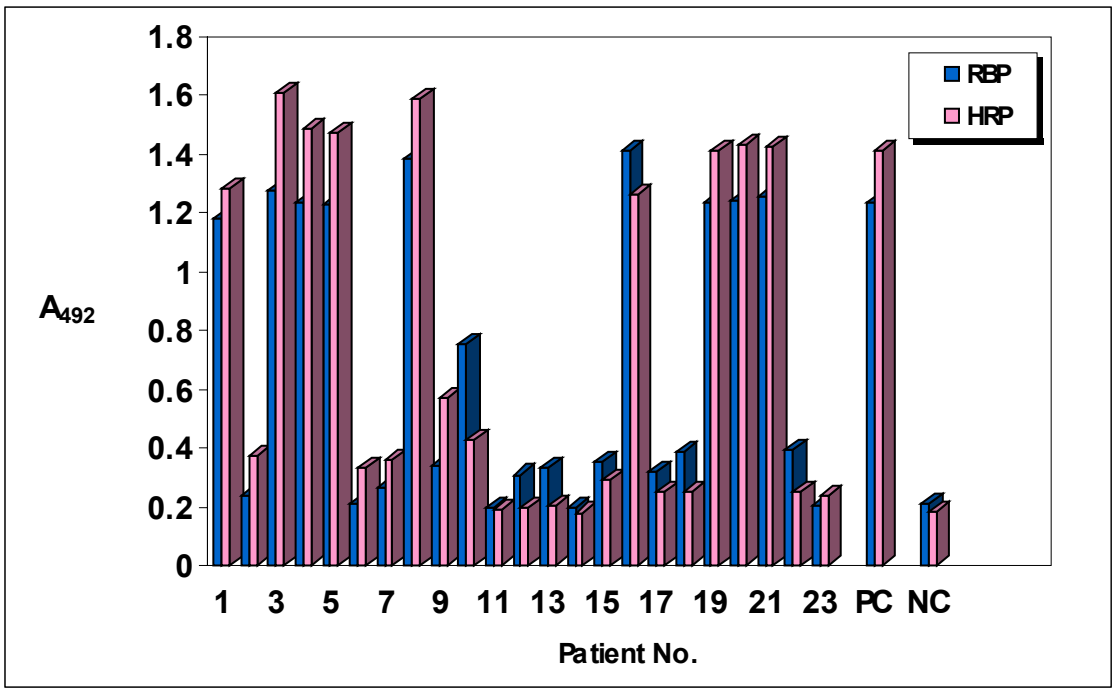


Figure 30. Detection of anti-leptospira in human serum using RBP-anti-human IgG conjugate (blue) and HRP-anti-human IgG conjugate (pink). Peroxidase activity was measured at 492 nm using OPD as substrate.

3.4 Cholesterol determination

RBP could also be used for the determination of serum metabolites such as glucose, uric acid and cholesterol. In this study it was used with cholesterol esterase/oxidase as a reagent in a diagnostic kit to determine the total cholesterol in human serum. The test was based on the colour formation of the quinoneimine dye produced by the reaction of 4-aminoantipyrine and phenol with the hydrogen peroxide generated by the oxidation of cholesterol catalyzed by cholesterol oxidase in the present of peroxidase (Allain *et al*., 1974)

Calibration curves of cholesterol concentration were prepared and developed with 4-aminoantipyrine as a substrate for both RBP and HRP. The optimal concentration of prepared RBP (3.65 units/ml reaction) gave the same calibration curve as that of HRP (3.14 units/ml reaction) (Figure 31).

Analysis of cholesterol content in human serum from 22 different samples using both RBP and HRP gave similar results (Figure 32).

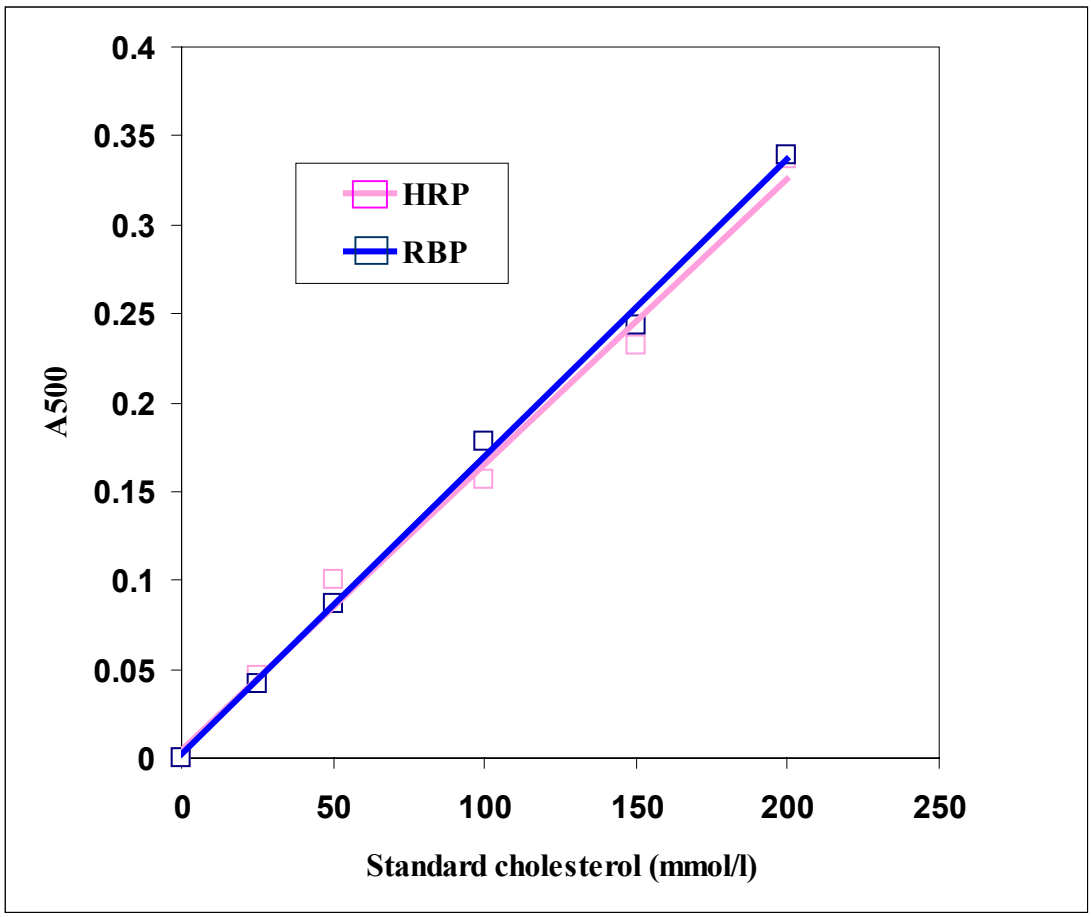


Figure 31. Calibration curves for cholesterol determination, using RBP (pink square) and commercial HRP (blue square).

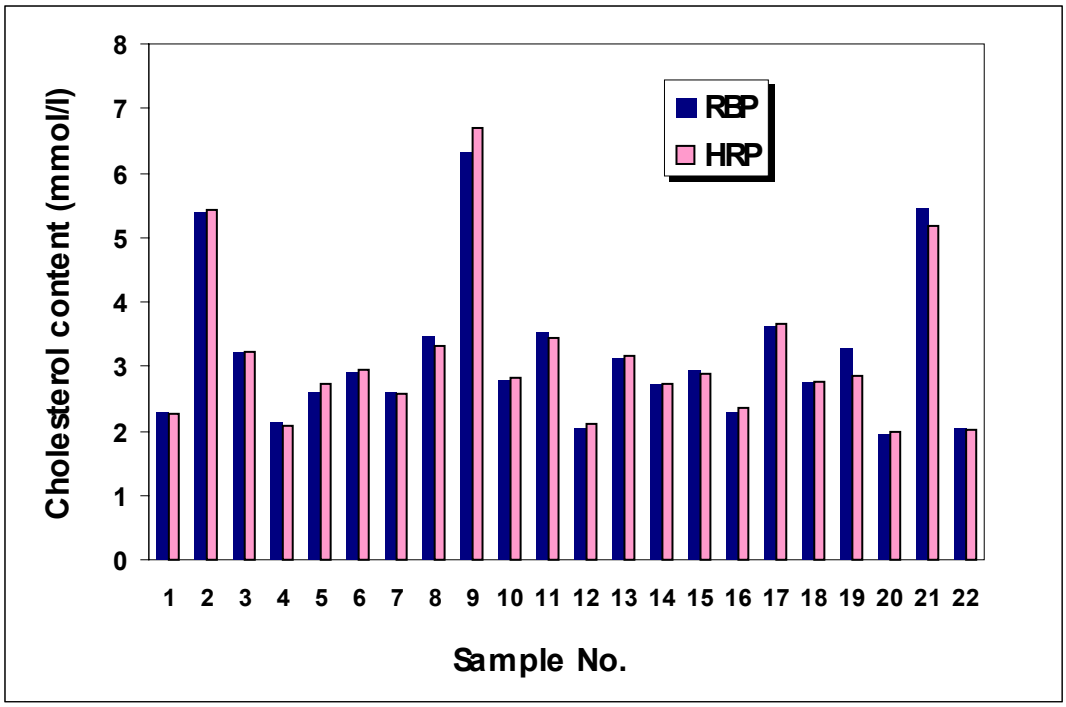


Figure 32. Cholesterol in human serum samples using RBP (blue) compared with commercial HRP (pink). The small differences are statistically insignificant.

4. Amino acid sequence analysis of purified RBP

4.1 RBP further purification

Affinity chromatography of the RBP obtained from Sephadex G-75 was performed in a Con-A agarose column. The protein in the column was eluted by using Tris-HCl buffer containing $CaCl_2$ (TC buffer) (fractions no. 1-21), EDTA (TE buffer) (fractions no. 22-45) and TE buffer containing methyl mannoside (fractions no. 46-66). The A_{280} was monitored for protein content and peroxidase activity was determined. Two peaks of protein were found in TC buffer fractions, but only the small protein peak showed high peroxidase activity (A). After that a single protein peak with high peroxidase activity was found in the TE buffer fractions (B) and another peak (C) in the methyl mannoside fractions, respectively (Figure 33). Peroxidase activity fractions no. 18-24, 28-50 and 53-60 were pooled and named A, B, and C, respectively.

Some high peroxidase activity fractions were analyzed on 15% gel native-PAGE using the original RBP from the Sephadex G-75 column as a marker. Gels were stained first for peroxidase activity using bentidin as an enzyme substrate. Two RBP bands (I and II) were observed in lanes 4-7, one band (II) in lanes 8-17 and two bands (II and III) in lanes 18-20 as seen in Figure 34. Thereafter the same gels were stained with 0.02% Coomassie Brilliant Blue R-250. Those protein bands (I, II and III) which corresponded to RBP bands in Figure 34 are shown in Figure 35.

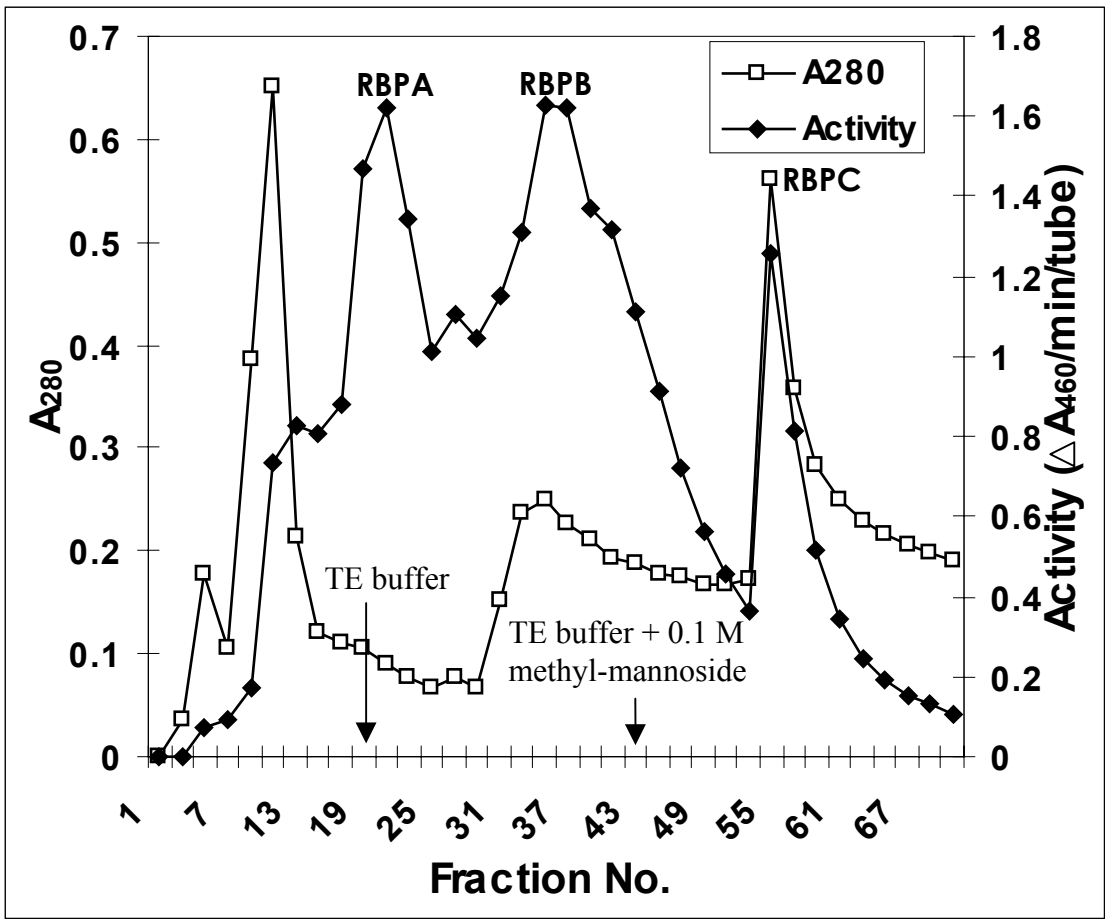
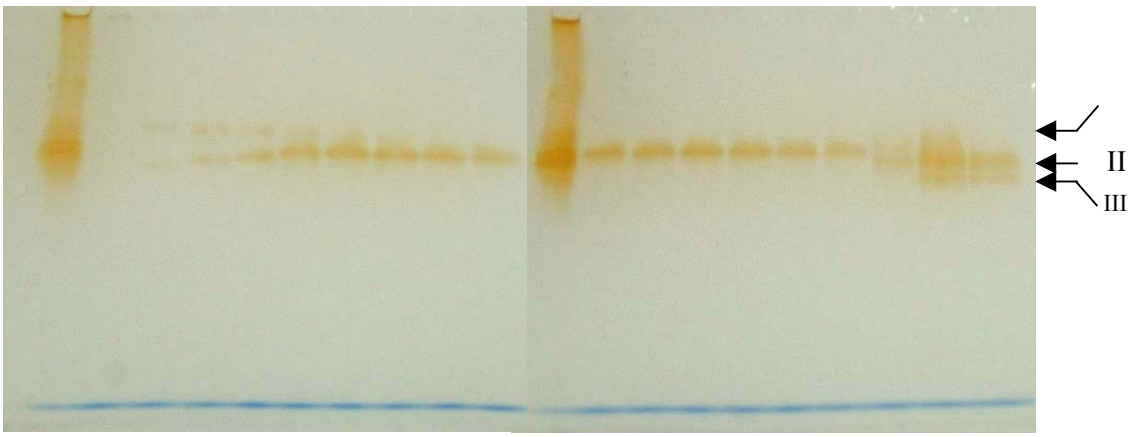


Figure 33. Con-A agarose chromatography of RBP. Peroxidase obtained from the Sephadex G-75 column was applied to the column (2 x 10 cm) at 23-25 ^{o}C. The column was equilibrated with Tris-HCl buffer containing $CaCl_2$ buffer and eluted with the same buffer (fractons no. 1-21) followed by Tris-HCl buffer containing EDTA (TE) buffer (fractions no. 22-45) and TE buffer containing 0.1 M methyl-mannoside (fractions no. 46-66), respectively. Fractions of 60 drops/tube were collected.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 34. Non-denaturing polyacrylamide gel electrophoresis of RBP fractions from Con-A agarose column. Gels were stained for peroxidase activity in 0.6 mM bentidin, 3 mM $CaCl_2$ and 5 mM H_2O_2 in 30 mM Tris-HCl, pH 7.4.

Lane 1, 11: RBP from Sephadex G-75 column

Lane 2: Fraction No. 5, lane 3: fraction No. 9, lane 4: fraction No. 12

Lane 5: Fraction No. 14, lane 6: fraction No. 16, lane 7: fraction No. 18

Lane 8: Fraction No. 20, lane 9: fraction No. 22, lane 10: fraction No. 24

Lane 12: Fraction No.30, lane 13: fraction No. 32, lane 14: fraction No. 34

Lane 15: Fraction No. 36, lane 16: fraction No. 38, lane 17: fraction No. 40

Lane 18: Fraction No. 53, lane 19: fraction No. 54, lane 20: fraction No. 56

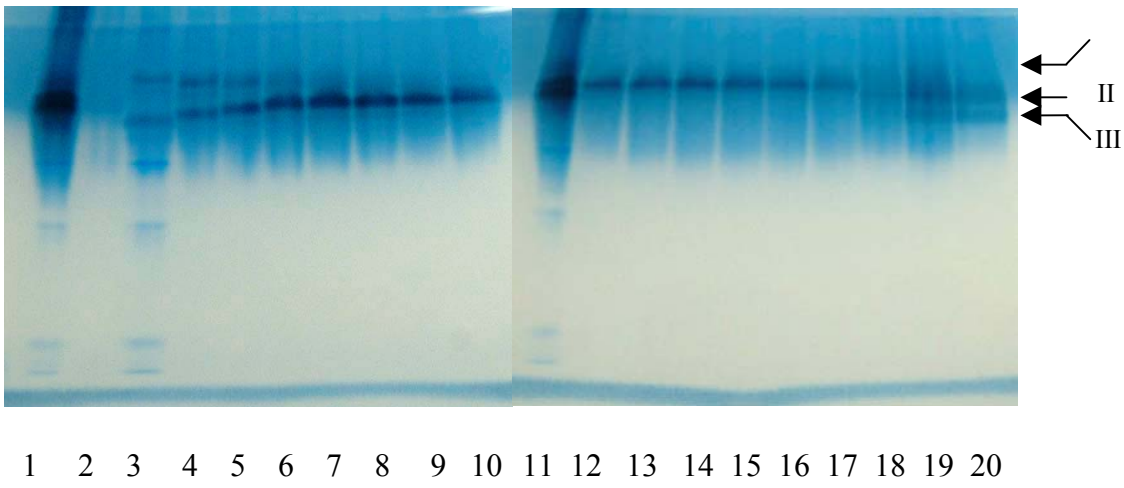


Figure 35. Non-denaturing polyacrylamide gel electrophoresis of RBP fractions from Con-A agarose column. Gels were stained in 0.02% Coomassie Brilliant Blue R-250.

Lane 1, 11: RBP from Sephadex G-75 column

Lane 2: Fraction No. 5, lane 3: fraction No. 9, lane 4: fraction No. 12

Lane 5: Fraction No. 14, lane 6: fraction No. 16, lane 7: fraction No. 18

Lane 8: Fraction No. 20, lane 9: fraction No. 22, lane 10: fraction No. 24

Lane 12: Fraction No. 30, lane 13: fraction No. 32, lane 14: fraction No. 34

Lane 15: Fraction No. 36, lane 16: fraction No. 38, lane 17: fraction No. 40

Lane 18: Fraction No. 53, lane 19: fraction No. 54, lane 20: fraction No. 56

4.2 Amino acid sequence analysis

To determine the amino acid sequence, each fraction of A, B and C was digested with either CNBr (CN) or LEP (lysyl endopeptidase) (K) to cleave the enzymes. The resulting peptides were purified by reverse phase HPLC and then sequenced. Their sequences were obtained by automated amino acid sequencing and the peptide maps are shown in the Appendix.

The RBP amino acid sequence obtained was used as a query for searching in the DNA Databank of Japan (DDBJ) with FASTA to determine its homology with known peroxidase sequences in other organisms and any possible related proteins. Many peroxidase sequences and related sequences were found in DNA databank. Peptide RBPB_K35, RBPA_K25, RBPA_K28, RBPB_K32, RBPA_CN19, and RBPA_K24 exhibited the highest scores of similarity with an *Arabidopsis thaliana* peroxidase sequence, as shown in Figure 36 (A), whereas peptide RBPB_CN20, RBPB_K17, RBPB_K30, and RBPB_K34 and RBPC_K24 were strongly revealed as Concanavalin A and β-glycosidase, respectively (Figure 36 (B) and (C)).

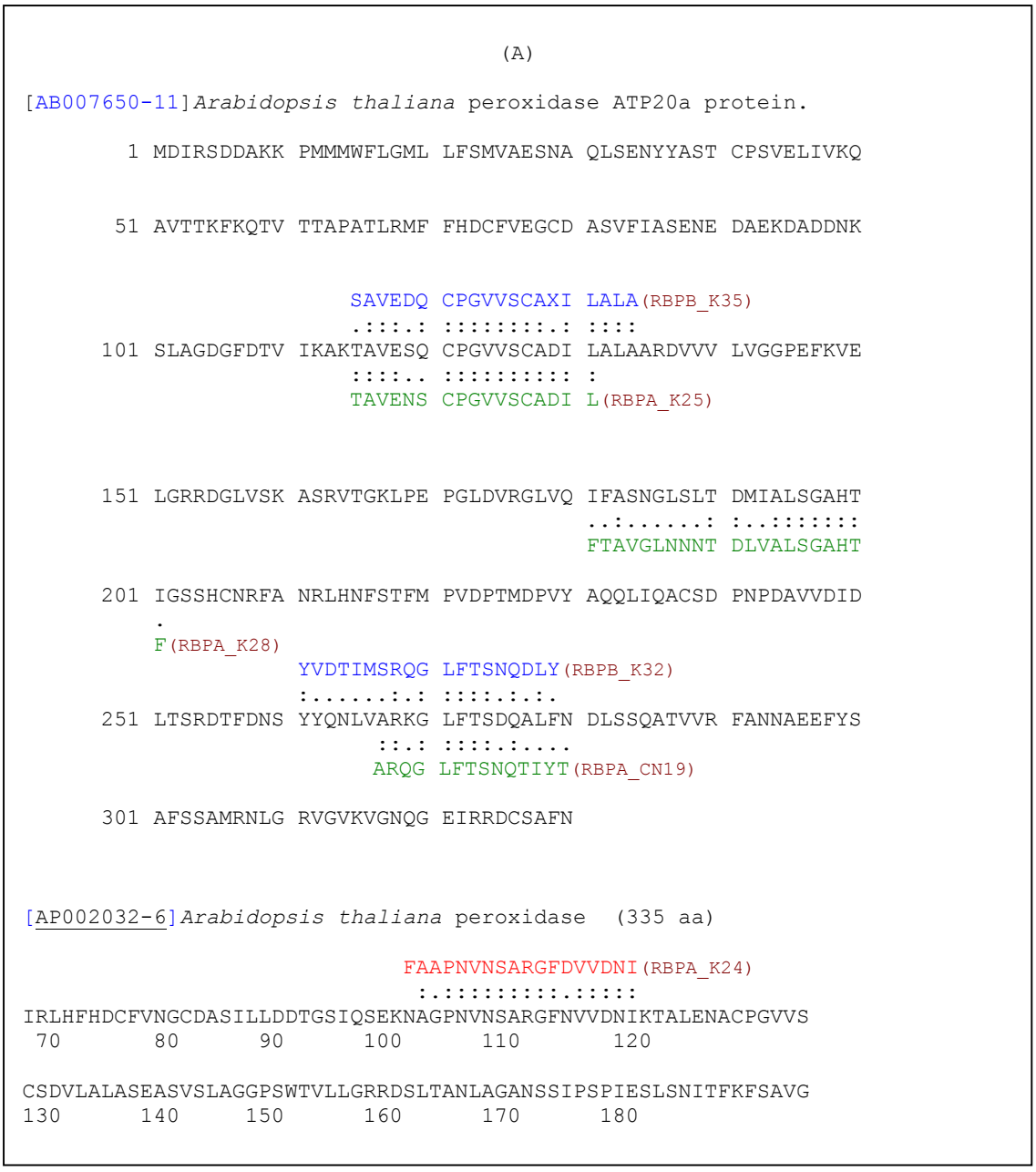


Figure 36. Amino acid homology between RBP, *Arabidopsis thaliana* peroxidase (A), and two related proteins, Concanavalin A (B) and *Manihot esculenta* β-glycosidase (C). The sequenced regions of the isolated peptides are shown and the homology is scored by dots, : for identical and . for similar.

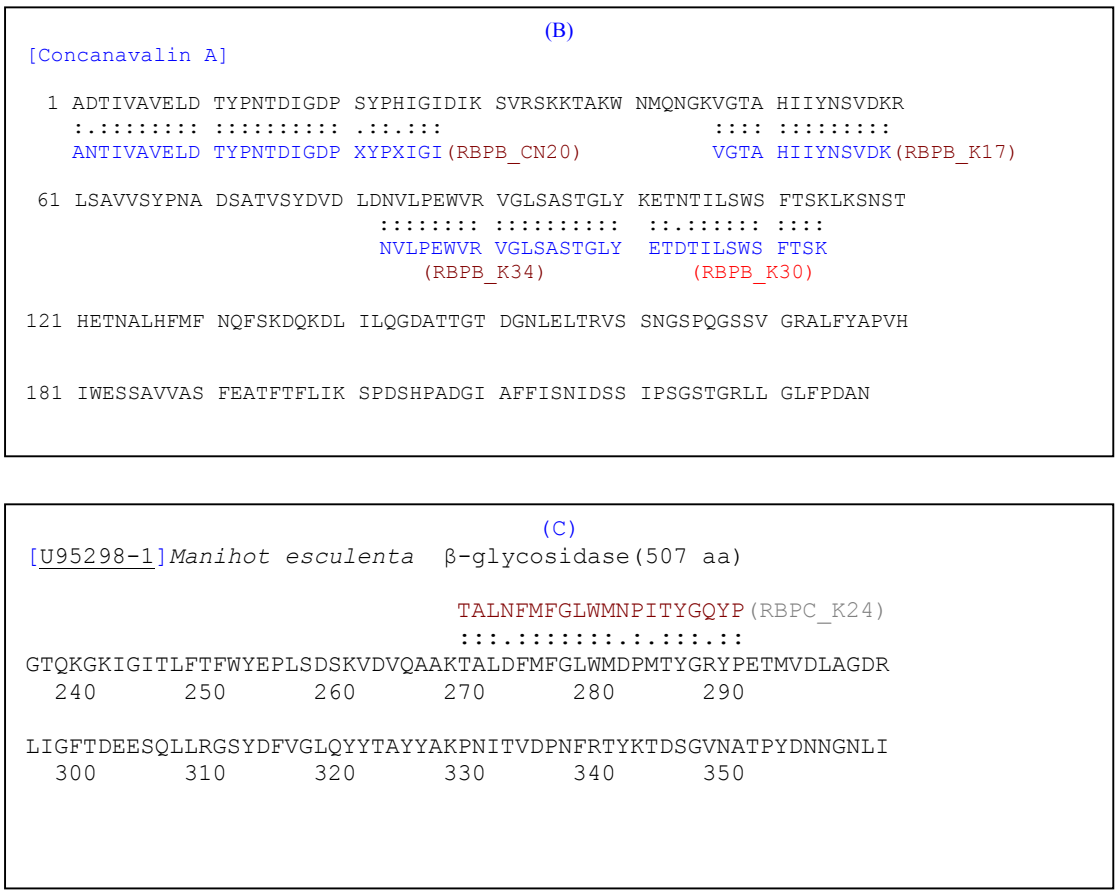


Figure 36. (continued).

Section II. Molecular cloning and sequencing of cDNA encoding RBP

Three isoforms of the RBP were obtained after Con-A agarose column chromatography in section I. In order to confirm this result, cloning and sequencing of cDNA encoding RBP were performed. In a previous study by Rattanapumee (2000), the RBP has a great thermostability that is suitable for commercial applications. Therefore, characterization of the gene encoding RBP would lead to more understanding of the presence of several isozymes.

1. First PCR for *rbp* cDNA

The cDNA encoding RBP was amplified by PCR. The peptide sequences of RBP from Figure 36 (A) were compared with 4 sequences of other plant peroxidases by using the ClustalX 1.81 program. The conserved regions SARGFDVVDN and SGAHTF that exist in the RBPA_K24 and RBPA_K28, respectively, were represent by GeneDoc program in the conservation mode (Figure 37). GAHTF and another conserved region from others plants in Figure 37, HFHDCF were used to design degenerated primers, RRBP1 (reverse) and FRBP1 (forward), respectively, as shown in Table 4. The PCR product analyzed on 1.5% agarose gel electrophoresis shows only one band of about 400 bp (Figure 38). Its sequence was obtained by automated DNA sequencing, as shown in the Appendix. From the DNA sequencing, the partial *H. brasiliensis* cDNA encoding an RBP was 399 bp in length. It had high nucleotide sequence similarity to other plant peroxidases (Figure 39). Gene specific primers, GSP1 and GSP2 (Table 4) were designed from the sequence and used for 3′ and 5′ RACE.

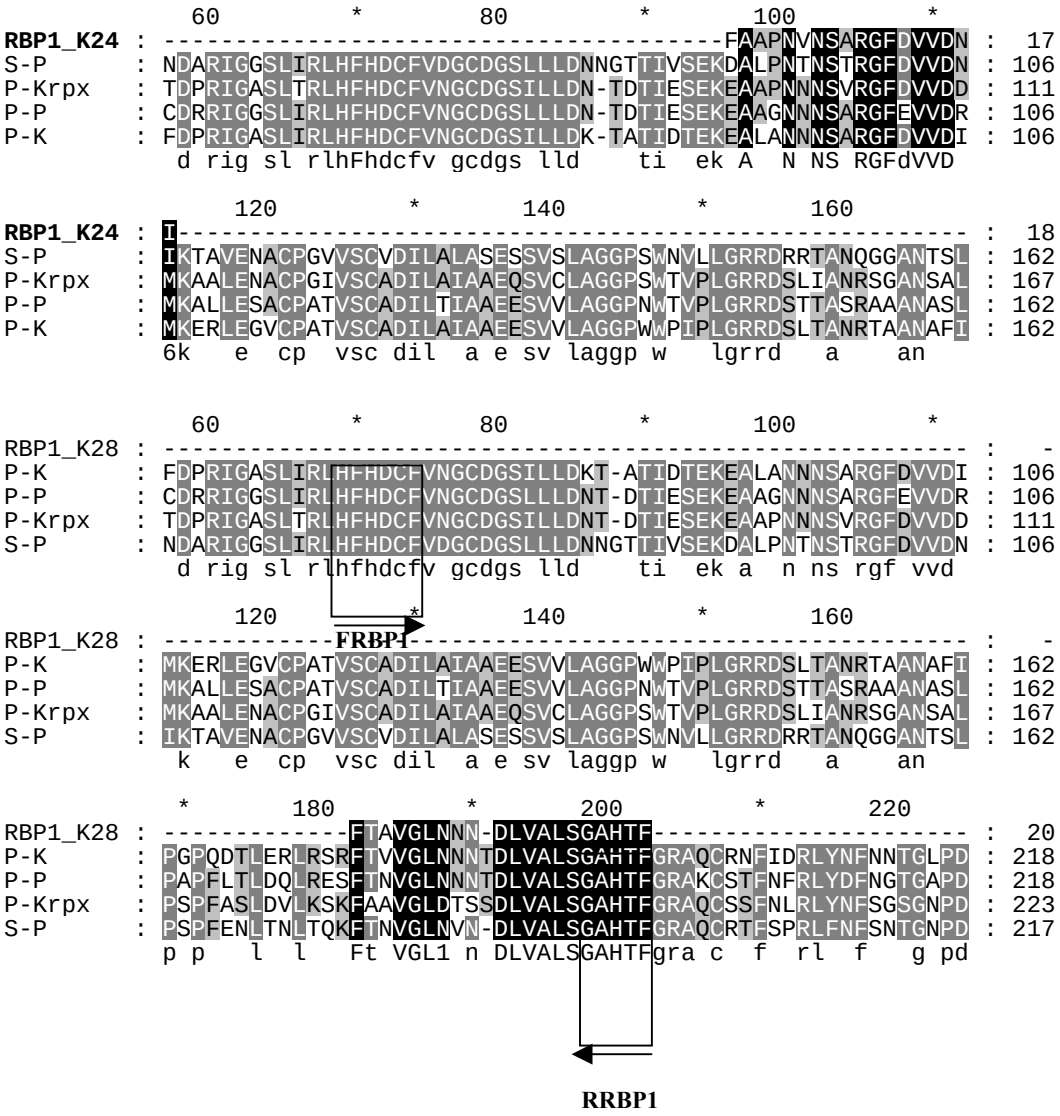
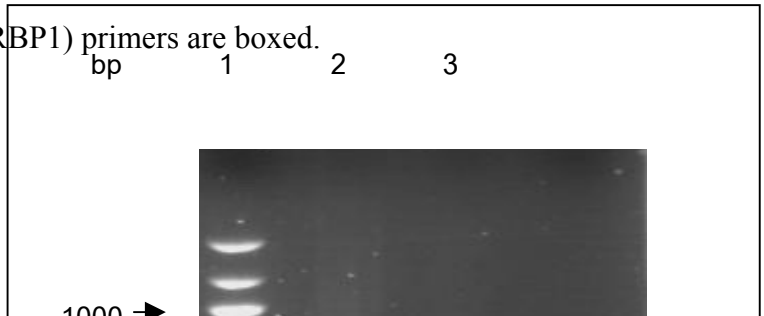
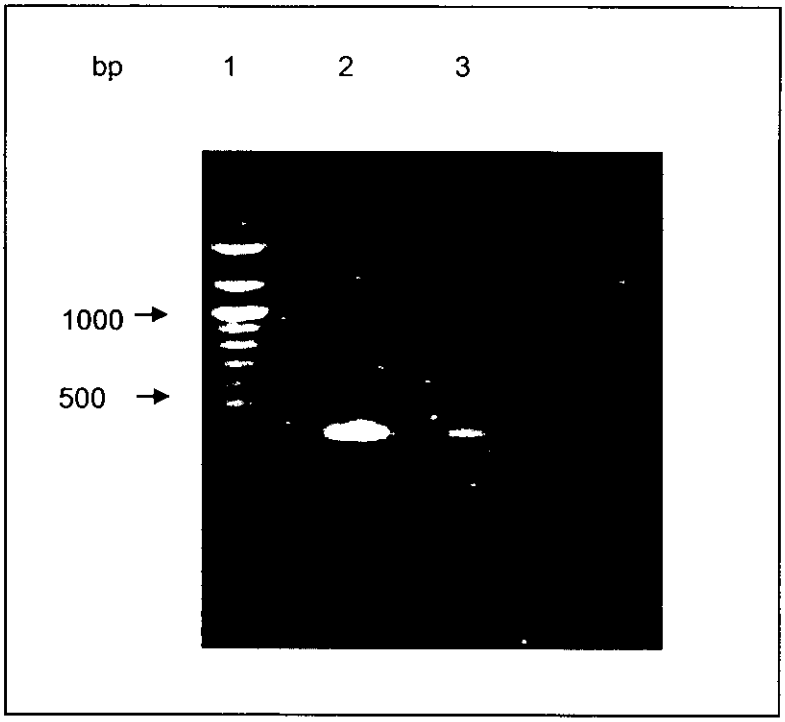


Figure 37. Amino acid sequence alignment of RBP1_K24 and RBP1_K28 with 4 plant species: *Populus kitakamiensis P. trichocarpa*, *Lupinus albus and Ipomoea batatas*.

Positions where the identity of the residues is conserved in 100 %, 80 % and 60 % of sequences are highlighted in black, dark gray, and light gray respectively. The amino acid sequences for forward (FRBP1) and reverse (RRBP1) primers are boxed.





igure 38. The first PCR product of FRBP1 (forward) and RRBP1 (reverse) on the 1.5% agarose gel electrophoresis.

Lane 1: 100 bp DNA marker

Lane 2: 10 µl PCR product

Lane 3: 5 µl PCR product

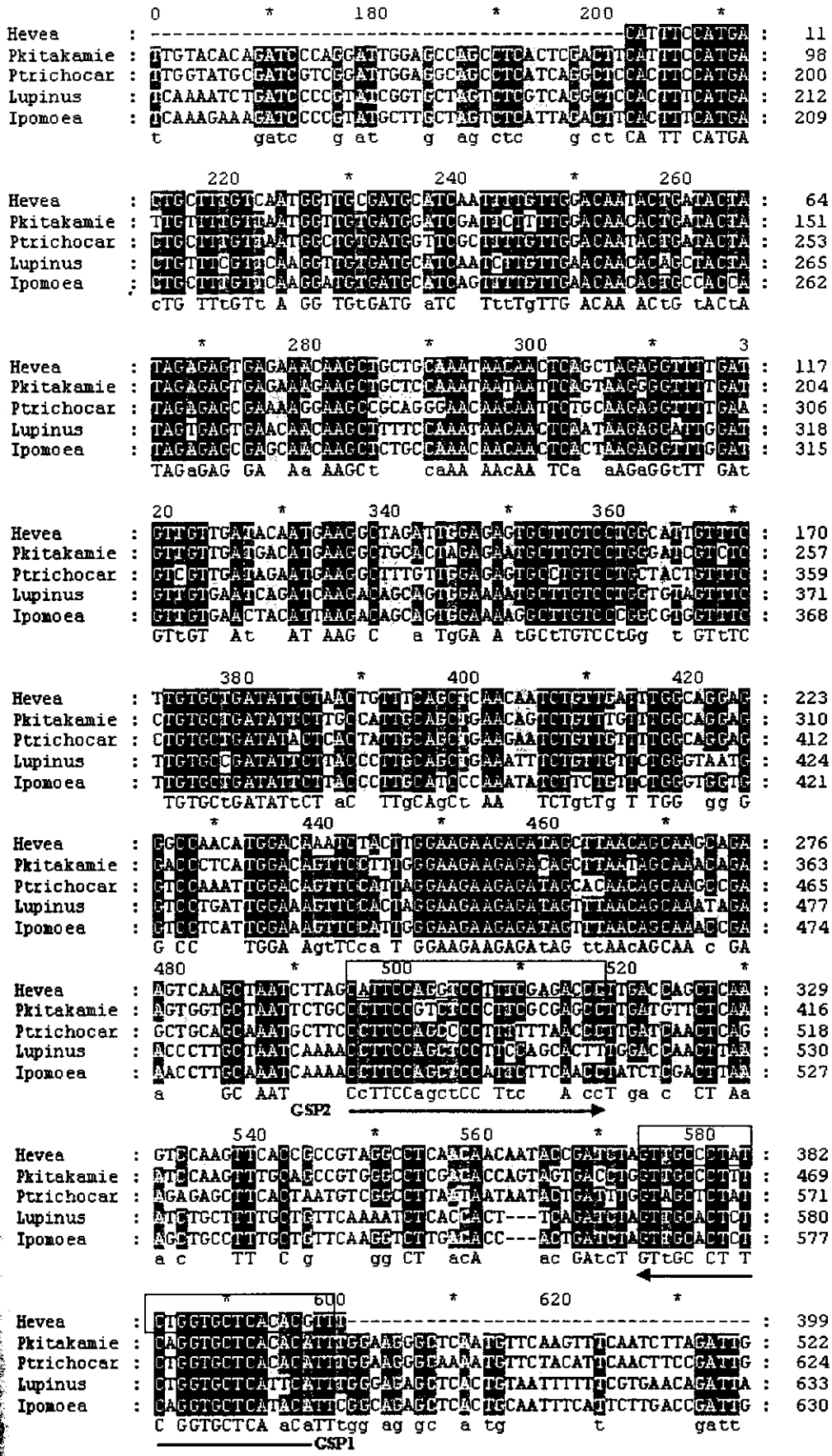

Hevea
Pkitakamie
Ptrichocar
Lupinus
Ipomoea
GSP2
GSP1

Figure 39. Alignment of deduced nucleotide sequences for *Hevea brasiliensis* RBP with peroxidase genes from 4 plant species: *Populus kitakamiensis P. trichocarpa*, *Lupinus albus and Ipomoea batatas*. Positions where the identity of the residues is conserved in 100%, 80% and 60% of sequences are highlighted in black, dark gray, and light gray respectively. The arrows indicate the nucleotide sequence for reverse (GSP1) and forward (GSP2) primers, respectively.

2. Generating the 3′and 5′cDNA encoding RBP.

In an attempt to isolate the 3′ and 5′ cDNA encoding RBP, the 3′, 5′ RACE procedure was performed using GeneRacer™ 3′primer, GeneRacer™ 5′primer and gene-specific primer GSP1 (reverse) and GSP2 (forward) as shown in Table 4 (also indicated in the sequenogram of nucleotide sequence in the Appendix) for the second PCR. The 3′ RACE and 5′ RACE cDNA were obtained and detected by 1.5 % agarose gel electrophoresis. Both 3′ RACE and 5′ RACE PCR products shows only one band of about 700 bp (Figure 40). From the DNA sequencing, two clones encoding RBP of 3′RACE were obtained whereas 5′ RACE gave only one clone. After sequencing the amino acid sequence of 3′RACE *rbp1* and *rbp2* encoding RBP1 and RBP2 were deduced and their 7 different positions are shown in Figure 41. The full-length of *rbp1* was generated from the nucleotide sequence of 3′RACE *rbp1* and 5′RACE *rbp1*.

The *rbp1* has 1145 bp in length. This sequence has an open reading frame of 1041 bp with deduced peptide of 346 amino acids (Figure 42). Its nucleotide sequence has ATG as the start codon and TAG as the stop codon. The computed MW

searching in GenBank and Protein Database (NCBI) with blastp, the sequence showed high similarity to 100 known peroxidase sequences with 85% identity with *Populus trichocarpa*. The conserved regions for peroxidase active site and proximal heme-ligand , and the secretory signal peptide at N-terminus (predicted with SignalP version 3.0) are indicated in Figure 42. Its alignment with 8 plant peroxidase sequences is shown in Figure 43. The conserved regions LSGAHTF found from amino acid sequence analysis are also indicated in Figure 43.

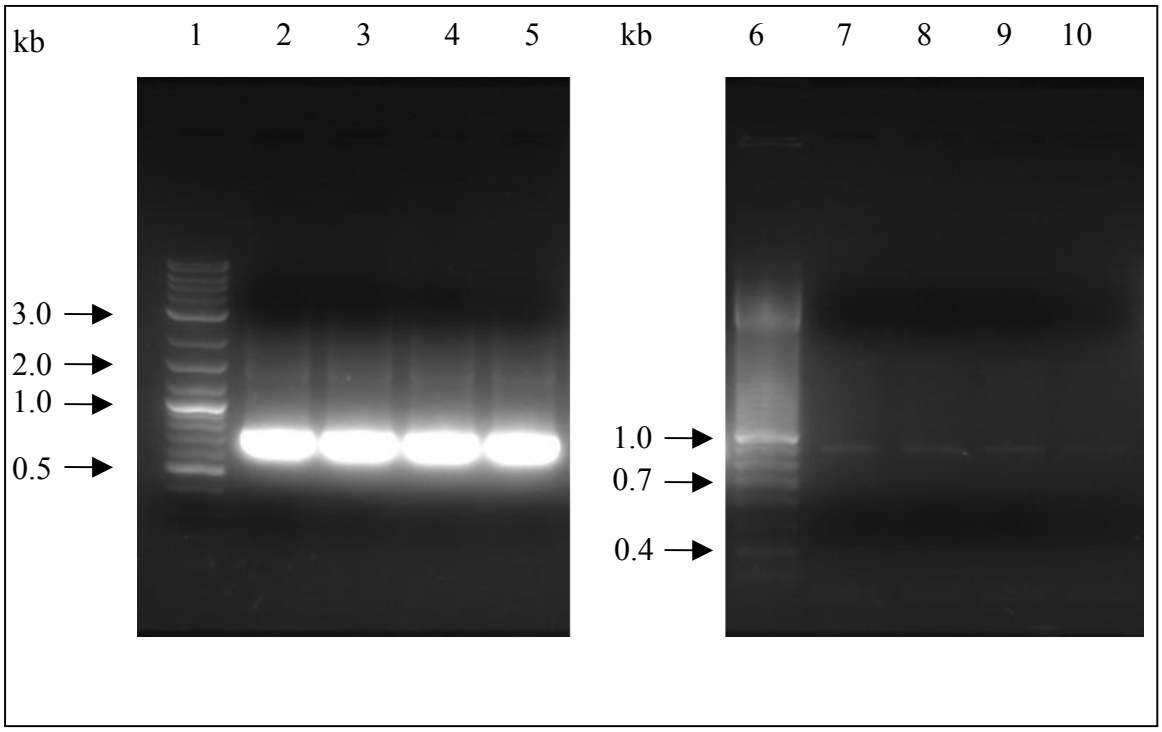


Figure 40. ***rbp*** **cDNA after 5′and 3′RACE on 1.5 % agarose gel electrophoresis.**

Lane 1: 2 log DNA marker

Lane 2-5: PCR of 3′RACE

Lane 6: 100 bp DNA marker

Lane 7-10: PCR of 5′RACE

```
RBP1:  I P G P F E T L D Q L K S K F T A V G L N   :21
       : : : : : : : : : : : : : : : : : : : : :
RBP2:  I P G P F E T L D Q L K S K F T A V G L N   :21

RBP1:  N N T D L V A L S G A H T F G R A Q C R T   :42
       : : : : : : : : : : : : : : : : : : : : :
RBP2:  N N T D L V A L S G A H T F G R A Q C R T   :42
```