Chapter 4

DISCUSSION

1. RBP purification

This study was focused on potential applications of the rubber peroxidase (RBP) from *Hevea* leaves, especially for clinical and research diagnosis. Horseradish peroxidase is usually used to prepare peroxidase-antibody conjugate, but it is imported from Europe and as such is an expensive substance. RBP has also been found in the bark strips (Wititsuwannakul *et al*., 1997) and in the leaves of the rubber tree and in the C-serum of rubber latex (Suvachittanont and Pongpaiboon, 1994). Interestingly, RBP from leaves showed a rather high peroxidase activity. Moreover, it exhibited a great thermostability as it is stable up to 50 ^{o}C for 24 hr with high activity in a wide range of pH values, namely from 4.5 to 6.9 (Rattanapumee, 2000). These physiological properties of the RBP from rubber leaves and the abandance of rubber plantations in Thailand made it more interesting to study, to find out whether it could become a new source of plant peroxidase, and support related works in such that our country could benefit from the rubber tree once more.

The RBP from rubber leaves was therefore purified. Under the purification procedure, only a small amount of other proteins in the crude extract were removed by 40% ammonium sulfate precipitation (Table 5). The protein removed could be polyphenol oxidase which cause browning (Lópes-Molina *et al*., 2003). The enzyme was purified to 5.4-fold by 80% ammonium sulfate precipitation, and then by ion

exchange chromatography using a DEAE-Sephacel column. The RBP is an acidic enzyme (Rattanapumee, 2000) which should bound to the column, however it was eluted out in the void volume resulting in a more than 50-fold purified RBP. This is possibly due to the high salt concentration [50 mM Tris-HCl, pH 7.5, plus some $(NH_4)_2SO_4$] in the enzyme pellet before applying it to the column. After size exclusion chromatography on a Sephadex G-75 column, the purity of RBP was increased up to 91-fold with 20% yield. Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) showed at least two isozymes of the RBP. The purified RBP was used for RBP-antibody conjugate preparations and in other applications.

2. Conjugation and characterization of RBP-antibody conjugate

The RBP purified from the Sephadex G-75 column was conjugated with either goat-rabbit IgG antibody or goat-human IgG antibody by using three cross-linkers: glutaraldehyde, sulfo-SMCC, and sodium periodate. The efficiency of the conjugation process was determined by the retained peroxidase activity and by the absorbance of a heme group at 403 nm of the conjugate after it was separated from unconjugated substances by passing through a Sephadex G-200 column. It was found that the RBP-anti-rabbit IgG conjugate from the periodate oxidation method had the highest retained peroxidase activity of 68%, whereas those from the glutaraldehyde and sulfo-SMCC yielded only 9.7 and 5.3%, respectively.

The low efficiency of the conjugation by glutaraldehyde may be due to a low stability of the glutaraldehyde activated RBP, leading to less RBP-antibody conjugate formation as shown by the large second peak of free RBP in Figure 16.

A similar result was found in the conjugate formation using sulfo-SMCC as cross-linker. Less activated RBP by NaO_3S^- in an initial step resulted in a low conjugate formation as shown by a very small peak of the conjugate and a large peak of free RBP and anti-rabbit IgG antibody present in the second peak (Figure 17).

In addition, amino groups of antibody and RBP, randomly reacted by glutaraldehyde and sulfo-SMCC in a cross-link reaction, may lead to reduction of the activity of both proteins (Faulstich and Fiume, 1985). On the other hand, the carbohydrate moieties instead of amino group of the RBP react with the cross-linker, $NaIO_4$ in the periodate oxidation method. The three conjugation methods have been reported to be successfully used for conjugation with proteins (Agostini *et al*., 2000; Vierling *et al*., 1999; Liu *et al*., 2000). The periodate oxidation method is generally recommended for conjugation of glycoprotein since the carbohydrate moiety is not required for its activity (O'Shannessy and Quarles, 1987; Tijssen, 1993). RBP is also a glycoprotein (Rattanapumee, 2000). It has been reported that under optimal conditions of the periodate oxidation method, only 68% of the peroxidase activity is retained for horseradish peroxidase (HRP)-antibody conjugate preparation (Nakane and Kawaoi, 1974). The reduction of some enzyme activity compared with its initial activity is probably due to an inhibition of the enzyme by periodate. A similar result was obtained in this study when the RBP-anti-rabbit IgG and RBP-anti-human IgG conjugate prepared by using $NaIO_4$, yielded 68 and 76.9% retained peroxidase activity, respectively (Figure 19 and 20).

The conditions used for RBP-antibody conjugate preparation in this report are recommended as the optimal conditions according to O'Sullivan and Marks (1981). Factors which may affect the conjugate formation, like concentration of reagents

(RBP, antibody, and cross-linker), optimal temperature, and incubation time were examined. Only the $NaIO_4$ concentration significantly affected the conjugate formation. The higher concentration of $NaIO_4$ (0.08 M) gave less antibody-peroxidase conjugate than did the lower concentration (0.04 M). A similar result has been reported when HRP-antibody conjugate was prepared by using 0.01- 0.32 M $NaIO_4$ (Tissen and Kurstak, 1984).

Carbohydrate moieties of the RBP contain hydroxyl groups that are susceptible to oxidation by periodic acid or its sodium salts. The carbon-carbon bonds containing hydroxyls in these sugars are oxidized by periodate to aldehydes, which then react with amino groups of antibody added via Schiff's base formation. However, strong oxidation may result in inactivation of enzymes and formation of polymers, since strongly oxidized peroxidase acts as a bridge-molecule between two IgG (O'Sullivan and Marks, 1981). Therefore, a high concentration of $NaIO_4$ resulted in a low retained peroxidase activity and also in the formation of a high molecular weight polymer of the conjugate. The molecular weight of the RBP-anti-rabbit IgG conjugate was 348 kDa as determined by the Sephadex G-200 column (Figure 22). In fact, it was higher than 176 + 59 = 235, which may have been caused by self-polymerization of either RBP or antibody, because both are proteins containing chemically reactive residues that can react with cross-linking reagents. This may be due to the fact that the ratio of RBP:antibody was 3:1, higher than 1, but this remains unclear, as the extent of the cross-linking reaction is often difficult to control (O'Sullivan and Marks, 1981).

The thus prepared RBP-antibody conjugate was used as a secondary antibody to detect proteins of interest by using various immunoassay techniques. The RBP-

antibody conjugate titer was determined to establish an optimal concentration of the conjugate to be used in dot blot, Western blot as well as ELISA techniques by chequerboard titration, using a nitrocellulose membrane and a microtiter plate. From the titer obtained, an optimal dilution at 1:200 was recommended for dot blot and Western blot techniques. The low titer may be due to two reasons 1) the lower activity of the RBP prepared, and or 2) the lower concentration of the conjugate prepared.

3. Applications of RBP and RBP-antibody conjugate

RBP-antibody conjugate was used as a secondary antibody in both dot blot and western blot techniques. If the exact quantity of an interested protein is not required, the dot blot technique provides a rapid, cheap and convenient means for screening a large number of samples. It can be used to quantitatively determine an interested substance by observing the relative intensity of the colour developed from the dots. In such an experiment, using the ability of the RBP-anti-rabbit IgG conjugate prepared as the secondary antibody in detecting the presence of protein, such as HMG-Co A synthase in C-serum of rubber latex was confirmed. The amount of the RBP-anti-rabbit IgG conjugate in various fractions eluted from the column can also be determined. Figure 28 shows that the conjugate from fractions number 85-107, which exhibited peroxidase activity (see Figure 19) was free peroxidase, because only antibody linked to RBP can be detected if conjugation has been formed.

Another technique for confirming the identity of a protein of interest is western blot. This technique is extremely useful, as it can be used to determine not only the presence of protein but also its molecular weight (MW). In this study, the

presence of vitellogenin and HMG-CoA synthase in the samples were clearly indicated by using both RBP-antibody conjugate and commercial HRP-antibody conjugate as shown in Figure 29, with the optimal dilutions of 1:200 and 1:3,000, respectively. It appears that about ten times more RBP-anti-rabbit IgG conjugate is used than HRP-anti-rabbit IgG conjugate. This is due to the fact that the prepared RBP-conjugate has ten times lower protein concentration.

The RBP-anti-human IgG conjugate was also prepared and used as a secondary antibody to analyze infected leptospira, the virus strain that causes leptospirosis disease in humans, by detecting the presence of anti-leptospira antibody in serum samples using the ELISA technique. Figure 30 shows that using either RBP-anti-human IgG conjugate or HRP-anti-human IgG conjugate as a secondary antibody gives similar results. Therefore, the RBP-anti-human IgG conjugate can be used to determine the presence of anti-leptospira. However, the titer of both conjugates were different because the protein concentration of the prepared RBP-anti-rabbit IgG conjugate was lower than that of commercial HRP-anti-rabbit IgG conjugate.

Nowadays, peroxidase is not only used as secondary antibody in clinical diagnosis, but also as a coupled in various test kits for serum metabolite determination such as glucose, uric acid and cholesterol. We also used purified RBP in cholesterol esterase/oxidase as a reagent to determine the total cholesterol in 22 different human serum samples. Determination of cholesterol using the RBP and by means of a commercial HRP in the reaction gave similar results.

Although RBP and HRP can be used equally well, the amount of peroxidase from rubber leaves is only 38.6% of that from horseradish roots (215 vs 557 units/g) (Rattanapumee, 2000). Lower yield of peroxidase was found in *Hevea* leaves, but as

the leaves are available as by-product on rubber plantations, 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 benefit from a lower price and a regular supply. The prepared RBP-anti-rabbit IgG conjugate had a peroxidase/antibody ratio of 3:1, higher than that of 0.8-1.5 of the commercial HRP-anti-rabbit IgG conjugate (Sigma). However, it is thus likely that the RBP-anti-rabbit IgG conjugate, like the commercial HRP-anti-rabbit IgG conjugate, could be used in various applications such as Western blot or dot blot.

4. RBP isozymes

Affinity chromatography of the RBP was performed in a Con-A agarose column using Tris-HCl buffer containing 10 mM $CaCl_2$ as an equilibrating and binding buffer that allowed some proteins to bind to agarose in the presence of calcium. Unbound protein with lower peroxidase activity was eluted in the first protein peak (fractions No. 1-21) (Figure 33), and was confirmed by peroxidase activity staining of fraction No. 5 and 9 in native-PAGE, which showed no peroxidase activity in Figure 34, whereas its staining in Coomassie Blue shows several bands of protein (Figure 35). The agarose-bound proteins were eluted by Tris-HCl buffer containing 10 mM EDTA. An elution profile of these fractions demonstrated a large amount of a single protein peak with high peroxidase activity. Their native-PAGE also showed only one RBP band, which corresponded to the lower band of RBP in lane 4-7 in Figure 34. So they ought to be the same RBP. At the end of the eluting step, the proteins bound to Con-A were eluted by using TE buffer containing methyl-mannoside. According to the fact that RBP is a glycoprotein (Rattanapumee, 2000), it

could bind to Con-A and be eluted with 0.1 M methyl-mannoside, which was quite a high concentration glycoside. Methyl-mannoside fractions were expected to contain RBP. This is confirmed by the presence of two RBP bands in lane 18-20 in Figure 34, which also shows two protein bands in Figure 35. From the elution profile of RBPA, RBPB and RBPC in Figure 33 plus the peroxidase activity staining of native-PAGE in Figure 34, it was concluded that there are at least three RBPs.

5. Amino acid sequence analysis of the RBP

To determine the amino acid sequences of the RBPA, RBPB and RBPC fractions from the Con-A agarose column, used RBP peptides were treated with cyanogen bromide and separated by reverse phase HPLC, but then only two pure peptides, namely RBPA_CN19 and RBPB_CN20, were isolated. The three RBPs were, therefore, also treated with lysyl endopeptidase and the products were separated again in HPLC. Nine pure peptides RBPB_K35, RBPA_K25, RBPA_K28, RBPB_K32, RBPA_K24, RBPB_K17, RBPB_K30, RBPB_K34 and RBPC_K24 were then obtained, as shown in Figure 36. Most isolated peptides showed homology with known peroxidases by searching in DDBJ with FASTA. In particular, RBPA_K24 showed the highest score of similarity with *A. thaliana* peroxidase (Figure 36 (A)). Four isolated peptides RBPB_CN20, RBPB_K17, RBPB_K30 and RBPB_K34 from RBPB fractions had homology with concanavalin A (Figure 36 (B)). These fractions may contaminated by Concanavalin A which packed in the Con-A column. It is surprising that the peptide RBPC_K24, which exhibited peroxidase activity by its absorbance at 460 nm and by its peroxidase staining with bentidin and H_2O_2 in native- PAGE (Figure 34), had homology with β-glycosidase

(Figure 36 (C)). This is probably more than one protein in each fractions from the Con-A agarose column. The peptide RBPA_K28 had the same identical residues with the deduced amino acid sequence of 3́ RACE RBP1 and 3́ RACE RBP2 in Figure 41. This result is also indicated in the alignment of complete RBP1 in Figure 43. The difference of other isolated peptides in Figure 36 (A) from the RBP1 in Figure 43 could be explained that these peptides probably exist in other regions of RBP2 or other RBP that were not determined in this study.

6. The cDNA encoding RBP

After the PCR reactions, the cDNA encoding RBP was cloned and sequenced. The sequence analysis after 3́, 5́ RACE PCR showed that there are at least two genes encoding RBP in the rubber tree, since there are two different clones from 3́ RACE (Figure 41). This agrees with the reports that most plant peroxidase genes are multiple genes (Yoshida *et al*., 2002). The 3́ RACE gave two products with different nucleotide sequences, namely RBP1 and RBP2 while only one product was obtained from the 5́ RACE. Comparison of the deduced amino acid sequences of 3´RACE RBP1 and 3́ RACE RBP2 in Figure 41 shows that there are 31 different residues in the C-terminal region. The complete cDNA of *rbp1* gene was generated following the 3́, 5́ RACE protocol. The *rbp1* proved to be 1145 nucleotides, starting with an initiation codon ATG at position 46 and ending with a termination codon TAG at position 1084 of the first nucleotides. In addition, the coding region is flanked by 5́ and 3́ untranslated sequences of 45 and 59 bp. The nucleotide sequences extended at the N-terminal region encoded MHISPLMLVAIALYAILVGSSRP LAYAQ with predicted cleavage site between AYA- and –Q, that is supposed to acts as a signal peptide to target the protein toward the vacuole as found in other plant peroxidases

(Kis *et al*., 2004). The deduced amino acid sequence of RBP1 is shown in Figure 42. The protein encoded by this cDNA has 346 amino acid residues with a computed MW of 37.076 kDa and a deduced isoelectric point (pI) of 4.6, whereas it was previously determined to be approximately 3.6 by isoelectric focusing (IEF) (Rattanapumee, 2000). The difference could be explained that the pI of 3.6 was estimated from the mature protein consistent with no signal sequence. The amino acid residues of signal peptide consist of 23 neutral-, 1 acidic- and 3 basic-residues, that is its pI could be higher than that of the mature protein. Alignment of the deduced amino acid sequence of RBP1 from *Hevea* leaves with the amino acid sequences of 8 representative peroxidases from other plants presented in Figure 43 shows that most differences were found in the N- and also the C-terminal regions. Despite the differences, the sequence comparison reveals high similarity with 85% identity between RBP1 and peroxidase from *Populus tricocarpa*. The sequences of peroxidase from *P. kitakamiensis*, *Ipomoea batatas, Arabidopsis thaliana, Pisum sativum*, *Glycine max*, *Amoracia rusticana* and *Nicotiana tabacum* and that of *Hevea brasiliensis* share identities between 79-83%.

The conserved regions were also found from amino acid sequence alignment. By similarity, peroxidase active site RLHFHD and proximal heme-ligand LSGAHTF, conserved in all members of the plant peroxidase superfamily (Veitch, 2004) are found at residues R65-D70 and L193-F199, respectively. Plant peroxidases contain glycosylation sites N-X-S/or T (Welinder and Laren, 2003), although, according to the isozyme the number varies greatly, from one in turnip to eight in horseradish (Bufford *et al*., 1990). Highly glycosylated character was also found in barley peroxidase (Saeki, 1986). RBP1 has thirteen potential glycosylation sites at residues

N40, N98, N162, N186, N195, N205, N221, N239, N251, N255, N272, N286, and N293 (Figure 43). This feature agrees with the high molecular weight of 59 kDa of the native RBP determined by the Sephadex G-200 gel filtration (Figure 22). There are four predicted disulphide bridges between cysteine residues 28-118, 71-76, 124-327 and 204-236. Two potential calcium binding sites are also predicted at positions including residues 70, 73, 75 and 77, and 198, 249, 252 and 257.

In order to confirm the full-length *rbp1*, its cloning and sequencing will be performed in a future study. Forward and reverse gene-specific primers will be designed from 5′ and 3′ ends of *rbp1* obtained from 5′and 3′ RACE PCR and used to amplify full coding region cDNA with RNA from *Hevea* leaves as template.

When the full-length *rbp* cDNAs are confirmed, the way is open for a semi-industrial study to establish the economic and technical feasibility of using the leaves of *Hevea brasiliensis* for commercial production of peroxidase. On the other hand, the level of RBP from bark strip revealed positive correlations with rubber latex yield and dried rubber content (Wititsuwannakul, *et al*., 1997), therefore, its DNA sequences will open the possibility of characterization of the expression patterns of individual peroxidase isoforms during normal plant development.