II. LITERATURE REVIEWS

A. Isolation of bilirubin oxidase from *Myrothecium verrucaria*

In 1981 and 1982, Murao and Tanaka reported that bilirubin oxidase could be produced from *Myrothecium verrucaria* strain MT-1. This strain was aerobically cultured at 25 °C in a jar fermentor which contained potato glucose medium. The highest activity was obtained after 62 hr cultivation of microorganism. The crude enzyme was stable in the pH range of 9.2 to 9.7 and catalyzed the oxidation of bilirubin substrate to the colorless biliverdin product.

Bilirubin oxidase has been purified from the culture filtrate of *Myrothecium verrucaria* MT-1 by a procedure involving ammonium sulfate precipitation, charcoal treatment, and QAE-Sephadex A-50 and Sephadex G-100 column chromatographies, respectively (Tanaka and Murao, 1982). The enzyme was found to contain copper and carbohydrate moiety in its molecule. In addition to use bilirubin substrate, the enzyme was inhibited by Fe^{+2} and compounds that complex with copper. For the optimal conditions, the enzyme preferred the reaction temperature approximately 40 °C and at the pH of 8 for its reaction.

The purification and some of the general properties of bilirubin oxidase were also reported by Guo *et al.* (1991). Bilirubin oxidase purified from culture filtrate of *Myrothecium verrucaria* Mv 2, 1089 by DEAE-Cellulose and Sephadex G-100 column chromatographies has a specific activity of 30 U/mg protein. Some of general properties of this bilirubin oxidase were reported as following; The enzyme was stable at pH ranging from 9.0 to 9.5. The molecular weight was calculated to be 61,900-62,700 by SDS-PAGE and gel filtration technique. The optimal enzyme activity was obtained after oxidizing bilirubin substrate at the temperature 50 °C and the pH of the reaction was optimal at 7.5 . Moreover, the activity was greatly reduced by incubation with Fe^{2+}, Hg^{+}, NaN_3, NH_4^{+}, and Zn^{2+} and inhibited by Ca^{2+}, Hg^{+}, Zn^{2+}, Fe^{2+} and BSA.

B. Progress studies on bilirubin oxidase production

In 1993, Satoshi *et al.* purified bilirubin oxidase from the medium of *Myrothecium verrucaria* MT-1 and determined its partial amino acid sequence and isolated cDNA fragment amplified by polymerase chain reaction using oligosaccharide primers designed on the basis of the partial amino acid sequence. The gene for bilirubin oxidase has been cloned from a genomic library using the cDNA fragment as a probe. The bilirubin oxidase gene cloning was shown to be expressed in *Saccharomyces cerevisiae*. The active recombinant bilirubin oxidase was established. This observation would be useful if the high yield of bilirubin oxidase production was achieved from this expression.

Recombinant bilirubin oxidase was also shown to be obtained by using an overexpression system of the bilirubin oxidase gene with *Aspergillus oryzae* harboring an expression vector (Shimizu *et al.*, 1999). After the mutant expression vectors were constructed, the express for bilirubin oxidase mutants were examined. It was found that low level of protein expressed by the mutant was remarkedly obtained. The specific activity of bilirubin oxidase was shown to be 80 % of the authentic enzyme.

C. Measurement of serum bilirubin by the enzymatic method using the isolated bilirubin oxidase

In 1987, Kosaka *et al.* described a method for measuring bilirubin in serum using bilirubin oxidase. Conjugated bilirubin is reported to be rapidly oxidized at pH 10.0 in glycine-NaOH buffer. Total bilirubin was measured at pH 8.0 in Tris buffer containing SDS whereas total conjugated bilirubin was determined in Lactic acid-sodium citrate, pH 3.7, respectively. The decrease in absorbance of the reaction rate caused by the enzyme oxidation was measured at 450 nm. The precision and the accuracy of these methods were compared with a diazo method, an HPLC method and a Kodak Ektachem slide method which good results were obtained. The oxidation rate for δ-bilirubin is at maximum near pH 4.0 and at minimum near pH 10.0. This dependency of the oxidation rates on the pH of the reaction mixture has led to the development of enzymatic method for measuring total bilirubin and bilirubin fractions in serum (Doumas *et al.*, 1987 ; Otsuji *et al.*, 1988).

In 1987, Mullon and Langer reported that the maximum absorbance of bilirubin oxidase activity using bilirubin substrate could be measured at 460 nm. Determination of total bilirubin in serum required pH 7.45 sodium cholate phosphate buffer to dissociate albumin-bound bilirubin before reacting with bilirubin oxidase and thus increase the reaction rate, while the conjugated bilirubin are directly oxidized in citric acid buffer at pH 4.5. This enzymatic method showed good correlation for total bilirubin assay with both the Jendrassik-Grof (Sigma 605) and Kodak Ektachem methods. The linearity of total bilirubin determination by this assay was ranged from 30 to 200 mg/L. For determination of conjugated bilirubin in serum, this enzymatic method showed very good agreement with the conjugated Kodak Ektachem assay and the linearity was reported to be 2 to 18 mg/L.

From several reports described above, it was demonstrated that optimal pH of enzymatic reaction varied with different conditions used in each laboratory. In 1987, Doumas *et al.* also reported different optimal pH for determination of serum, total conjugated bilirubin (DBIL) which included mono-conjugated bilirubin, di-conjugated bilirubin, and most of the δ-bilirubin, respectively. The rate of enzyme activity oxidized total conjugated bilirubin was carried out in phosphate buffer, pH 4.5. The resulting decrease in absorbance at 460 nm is linearly related to the concentration of DBIL in serum. The results showed high correlation coefficients for DBIL by this assay with those of Kodak Ektachem and the diazotization method (Jendrassik and Grof, 1938).

The determination of serum bilirubin using bilirubin oxidase method in the automated chemistry analyzer was initially introduced by Heinemann and Vogt in 1988. Total bilirubin method was assayed using the Dri-STAT® bilirubin reagent kit. The change in absorbance at 415 and 546 nm were measured with a Hitachi 704 selective analyzer. Analytical reproducibility was obtained with good correlation with the reference method (Jendrassik and Grof, 1938).

Recently, a new bilirubin oxidase enzymatic method using autoanalyzer for serum conjugated bilirubin determination has been developed (Kurosaka *et al.*, 1998). The conjugated bilirubin was selectively oxidized by bilirubin oxidase at pH 5.5 in the presence of sodium fluoride and N-acetylcysteine which help decrease bilirubin oxidase reactivity to unconjugated and delta bilirubin in the same reaction mixture. The decrease in absorbance at 450 nm was measured by a Hitachi 7070 or 7170 autoanalyzer. The conjugated bilirubin

concentrations, analyzed by this new enzymatic method showed good agreement with the HPLC reference method.

The analysis of bilirubin in serum has frequently been requested by the physicians to help diagnosis of hemolytic and liver diseases. As described earlier that the most widely used method for bilirubin determination in most clinical chemistry laboratories is the diazo coupling method. However, it was reported that the direct diazo reagent also reacts with some unconjugated bilirubin in serum (Killenberg *et al.*, 1980). Furthermore, it has less specificity to estimating the albumin bound conjugated bilirubin (δ-bilirubin) which reacts slowly in the direct reaction determination (Doumas *et al.*, 1987). Therefore an alternative enzymatic method, which proposed to have more specificity than the diazotized method, was suggested for fractional determination of bilirubin in serum. At present, the method is still limited because of the variation of the assay conditions in various instruments (Doumas *et al.*, 1999 ; Mullon and Langer, 1987) and the enzymatic kit is too expensive to be used in the routine clinical chemistry laboratories (Heineman and Vogt, 1988 ; Nakayama, 1995).

Objectives of this study

1. To investigate and develop the conditions for cultivation of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 in order to obtain the highest yield of bilirubin oxidase enzyme production.

2. To develop the purification and identification methods of bilirubin oxidase enzyme isolated from *Myrothecium verrucaria* TISTR 3112 and TISTR 3225.

3. To evaluate and apply the bilirubin oxidase enzyme isolated from *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 for clinical uses.