CHAPTER V

DISCUSSIONS

Both PEG and PPG are representative polyethers used in large quantities, although their physicochemical properties are quite different from each other. As PEG is hydrophilic and PPG is hydrophobic, their copolymers are used as nonionic surfactants. At present, the production level has been increased due to an expansion in the need for nonionic surfactants and other products including PEGs and PPGs, even though no report has described the serious accumulation of polyethers in nature (their temporary presence has been detected by Rychlowska *et al.* (2003)), microorganisms must eventually metabolize them. Until now, various kinds of PEG-utilizing bacteria have been isolated, which differ in their assimilation limits toward various sizes of PEG (from oligomers to 20,000 Da). On the other hand, only one bacterium (*Stenotrophomonas maltophilia*) has been reported to assimilate PPG, but it cannot grow on PEG (Tachibana *et al.*, 2002). The bacteria reported so far could assimilate only one polymer.

Various sources of microorganisms including soils, activated sludge, and wastewater from industrial plants were screened by enrichment culture techniques for polyalkylene glycol (PAG)-utilizing ability. Activated sludge samples were acclimated step by step with an increased concentration of PAG starting from 0.05 to 5%. From the result of 0.5% PAG medium, two consortia that showed appreciable growth (approximately 0.8-0.9 by absorbance at 610 nm) were selected for isolation of bacteria growing on PEG and PPG. All the consortia, *Pseudomonas* sp.PE-2 was one of them, which could grow well on PEG and PPG. The PEG-DH and PPG-DH activities could be detected in cell-free extract of this microorganism (Hu *et al.*,

2007). *Pseudomonas* sp. PE-2 was used in this study for purification and characterization PEG-DH and PPG-DH.

5.1 Induction of PEG deydrogenase and/or PPG dehydrogenase using various concentrations of either PEG or PPG

Cell-free extracts from *Pseudomonas* sp. PE-2 grown on different molecular weight of PEG and PPG were examined for PEG-DH and PPG-DH activities using PEG 2000, PEG 4000, PPG 1000 and PPG 2000 as substrate (section 3.4.6.1). It was found that 3% (v/v) PEG 4000-grown cells exhibited PEG-DH of 25.8 nmol/min.mg protein (assayed with PEG 2000 at 72 hours) (Figure 4.3C), while they were only 2.8 nmol/min.mg protein (assayed with PEG 4000 at 24 hours) when cells were grown in nutrient broth (Figure 4.1). Therefore, PEG-DH was inducible with PEG. This result was the same as that from Hu *et al.* (2007) which showed that PEG 4000-grown cells had PEG-DH activity (16.7 nmol/min.mg protein), but nutrient broth-grown cells did not have significant activity (4.3 nmol/min.mg protein). Similar result was also found with PEG-DH from *Sphingomonas* sp. N6 (Kawai and Enokibara, 1996), *Sphingopyxis macrogoltabida* EY-1 and *Sphiniomonas* sp. EK-1 (Hu *et al.*, 2007). However, PEG-DH from *Sphingomonas macrogoltabidus* was a constitutive enzyme (Kawai and Yamanaka, 1989; Yamanaka and Kawai, 1989).

For PPG-DH, it was found that 1.5% (v/v) PPG 1000-grown cells exhibited PPG-DH of 21.9 nmol min^{-1} mg $protein^{-1}$ (assayed with PPG 1000 at 168 hours) (Figure 4.4B), while they were only 0.2 nmol min^{-1} mg $protein^{-1}$ (assayed with PPG 1000 at 24 hours) when cells were grown in nutrient broth (Figure 4.1). Therefore, PPG-DH of *Pseudomonas* sp. PE-2 was inducible with PPG. Nonetheless, different finding was reported in PPG-DH of *Pseudomonas* sp. PE-2 (Hu *et al.*, 2007).

Their result showed that PPG-DH activity was found with PPG 1000-grown cells (17.9 nmol min^{-1} mg $protein^{-1}$) and nutrient broth-grown cells (12.1 nmol min^{-1} mg $protein^{-1}$), indicating that PPG-DH was constitutively active. From these discrepancies, we repeated this experiment four times to confirm the results. However, our result still showed that PPG-DH of *Pseudomonas* sp. PE-2 was inducible enzyme. Similar result was also found with PPG-DH from *Stenotrophomonas maltophilia* (Tachibana *et al.*, 2002). However, PPG-DH from *Sphingopyxis macrogoltabida* EY-1 and *Sphiniomonas* sp. EK-1 were constitutive (Hu *et al.*, 2007).

5.2 Cellular localization of PEG/PPG-degrading enzymes from *Pseudomonas* sp. PE-2

Subcellular fractionation with glucose-6-phosphate dehydrogenase as the cytoplasmic marker, alkaline phosphatase as the periplasmic marker, and NADH oxidase as the membrane marker permitted us to locate the PEG-DH in the periplasmic fraction. As much as 76% of PEG-DH was found in the periplasmic fraction, while only 11% was found in the cytoplasmic fraction (Table 4.1). The fact that *Pseudomonas* sp. PE-2 could grow well in PEG 4000, but not PEG 8000 suggested that *Pseudomonas* sp. PE-2 was not able to efficiently metabolize PEGs of high molecular weight due to a permeability barrier. The localization of PEG-DH oxidative activity in the periplasmic space placed this barrier in the outer membrane. The similar result was also found in PEG-DH from *Pseudomonas stutzeri* (Obradors and Aguilar, 1991). Obradors and Aguilar (1991) proposed that a strain of *Pseudomonas stutzeri* exhibited considerably higher rates of biodegradation of PEGs (molecular weight, up to 14,000) via the activity of a periplasmic dehydrogenase. On

the basis of this cellular localization and the upper limit on the size of PEGs capable of supporting growth, Obradors and Aguilar (1991) suggested that porins might facilitate the transport of the PEG across the outer membrane into the periplasmic space (Obradors and Aguilar, 1991).

For PPG-DH, 82% activity was found mainly in the cytoplasmic fraction (Table 4.1). Similar result was shown with dye-linked PPG-DH of *Stenotrophomonas maltophilia* where high activity was found in cytoplasm towards low molecular weight PPG (Tachibana *et al.*, 2002). Nonetheless dissimilar finding was reported in *Pelobacter venetianus* (Frings *et al.*, 1992) where the ether cleavage of high molecular weight PEG was confined in cytoplasm.

Although there have been sereval proposed explanations for PEG-DH and PPG-DH localization and transit of PEG and PPG across the inner membrane, the complete explanation is still lacking and there was some doubt concerning the effectiveness of the outer membrane porins for PEG and PPG passage (Schmid *et al.*, 1991). A point raised by Dwyer and Tiedje (Dwyer and Tiedje, 1986) concerns the structural conformation of PEG molecules. Although the lower molecular weight PEGs display a zig-zag shape, the larger polymers display a helical conformation which could directly influence the uptake and depolymerization processes.

5.3 Partial purification of PEG dehydrogenase and PPG dehydrogenase from *Pseudomonas* sp. PE-2

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration

of the desired protein and its stability for the intended application. The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites (Janson and Ryden, 1998). Most purification protocols require more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriate techniques, to optimize their performances and to combine them in a logical way to maximize yield and minimize the number of steps required (Janson and Ryden, 1998).

5.3.1 Cell-free extract preparation

The first step in protein purification is the preparation of an extract containing the protein in a soluble form and extraction procedures should be selected according to the source of the protein. In this work, PEG-DH and PPG-DH, the intracellular enzymes, were extracted from *Pseudomonas* sp. PE-2, a gram-negative bacterium, which has a thin cell wall covered by a complex outer membrane (Voet and Voet, 1995). The cell wall is responsible for strength, rigidity as well as shape and is the major barrier to release of any intracellular proteins. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification. A French pressure cell was used in this work for cell breakage by placing the sample under high pressure followed by a sudden release to atmospheric pressure. The rapid change in pressure causes cells to burst. Phenylmethylsulfonylfluoride (PMSF) was used in the extraction buffer as serine protease inhibitor because the control of metabolic regulation mechanisms is lost

when the cell is disrupted. Thus, the desired protein may be degraded by intrinsic catabolic enzymes such as proteolytic enzymes. In addition, the protein will encounter an oxidizing environment after disruption that may cause inactivation, denaturation or aggregation (Bollag and Edelstein, 1993). Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation protein. To maximize recovery of active enzyme, the extract and equipment were pre-chilled several times. While maintaining the pressure, adjustment the outlet flow rate to 2-3 ml per minute (about one drop per second) minimized foaming and shearing, thereby minimizing protein denaturation.

5.3.2 Protein separation by precipitation

After cell disintegration, the homogenate was then centrifuged to remove any insoluble materials. The desired protein remains soluble in the solution.

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols. When high concentrations of salt are present, proteins tend to aggregate and precipitate out of the solution. The most effective salt to bring about protein precipitation was ammonium sulfate, because it combines many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag and Edelstein, 1993).

In this study, PEG-DH was precipitated in the presence of 30-45% saturated ammonium sulfate. From this step of purification, one third of the proteins were removed while the yield remained over 90% (Table 4.3). When compared with the precipitation step of PEG-DH from mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) (Kawai *et al.*, 1980), it was found that the active protein fraction was in the range of 0-30% saturated ammonium sulfate. One third of the proteins

were also removed while the yield remained 88%. Moreover, the purification of PEG-DH from *Sphingomonas macrogoltabidus* showed that PEG-DH was precipitated in the range of 0-70% saturated ammonium sulfate. One fourth of the proteins were removed while the yield remained 79%.

For PPG-DH, it was precipitated in the presence of 60-80% saturated ammonium sulfate. From this step of purification, 91% of the proteins were also removed while the yield remained 82% (Table 4.4). No report of other PPG-DH purification by ammonium sulfate precipitation.

In the ammonium sulfate precipitation step, partial activity of PEG-DH and PPG-DH was lost. This may be due to the different surrounding proteins presented in the cell-free extract and affected the salting out procedure. The explanation for the different degree of PEG-DH activity could be the removal of some factors, during the salt precipitation, which are important for stabilizing the enzyme activity. In addition, the speed at which salt was added to an enzyme solution and the efficiency of stirring were also important factors. For many proteins, small amount of salt was added and allowed to dissolve before making further additions. After the last bit of salt has dissolved, stirring should continue for 10-30 minutes to allow complete equilibrium between dissolved and aggregated proteins. Stirring must be regular and gentle because stirring too rapidly will cause protein denaturation as evidenced by foaming (Bollag and Edelstein, 1993). In this step, the specific activity of PEG-DH was not different between the enzyme incubated with and without PQQ. Therefore, the loss of enzyme activity might be happened by the stirring too rapidly of sample or localize concentration when added salt rapidly.

5.3.3 Column chromatography

Most of purification schemes involve chromatography, which has become an essential tool for protein purification. Ion exchange chromatography is probably the most frequently used chromatographic technique for the separation and protein purification. The reasons for the success of ion exchange are its widespread applicability, high resolving power, high capacity, and the simplicity and controllability of the method (Janson and Ryden, 1998). This technique is based on the interaction between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix.

The PEG-DH was partially purified by DEAE-650M Toyopearl which is an anion exchanger as shown in section 4.3.1.2, 68% of proteins from the precipitation step were eliminated with 51% recovered activity (Table 4.3). Yamanaka and Kawai (1989) also used DEAE-650M Toyopearl column as the first column chromatography to purify PEG-DH from *Sphingomonas macrogoltabidus*, resulting a minimum loss of enzyme activity while eliminating about 65% of other proteins. The result from chromatogram (Figure 4.6) also indicated that net charge of the PEG-DH in 50 mM Tris-HCl buffer pH 8.5 was negative because the enzyme could bind to the column. This result implies that the pI of PEG-DH was less than 8.5 which corresponded to that of the PEG-DH from *Sphingomonas macrogoltabidus*, having pI less than 8.0 (Yamanaka and Kawai, 1989).

We also used the DEAE-650M Toyopearl column as the first column chromatography to purify PPG-DH from *Pseudomonas* sp. PE-2 (section 4.4.1.2). Using this column, other proteins were eliminated by 91% and PPG-DH recovery was about 20% (Table 4.4). The result from chromatogram (Figure 4.10) also indicates that net charge of the PPG-DH in 50 mM Tris-HCl buffer pH 8.5 was negative

because the enzyme could bind to the column. This result also confirms that the pI of PPG-DH was less than 8.5. The pI of PPG-DH from *Stenotrophomonas maltophilia* was 5.9. So, the enzyme was acidic protein (Tachibana *et al.*, 2003).

For the second step of column chromatography, the hydrophobic interaction chromatography (HIC) was employed. HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar regions on the surface of proteins (Queiroz *et al.*, 2001). Phenyl-Sepharose CL-4B is the resin with the chemically bonding phenyl groups on the surface of Sepharose. Prior to HIC column, the sample ionic strength should be adjusted with salt to increase hydrophobicity of protein (Queiroz *et al.*, 2001). Thus, care must be taken in the aspect of the salt concentration used for adsorption. It should be lower than the concentration used in precipitation of our enzyme and the salt concentration of 1 M was used for adsorption in this work. The elution of proteins from the column is achieved by decreasing the salt concentration in order to increasing hydrophobicity. Types of salts give rise to differences in the strength between proteins and the HIC adsorbent. Ammonium sulfate was chosen in this study because of its effective property in promoting hydrophobic interactions and it was widely used in HIC (Queiroz *et al.*, 2001).

In purification of PEG-DH, using a Phenyl-Sepharose CL-4B column, a stepwise elution by decreasing concentration of ammonium sulfate from 1 M to 0 M was successfully used for specific separation of PEG-DH from unwanted proteins. The success in using this column was judged by the near homogeneity of PEG-DH according to gel electrophoresis (Figure 4.8). With this column, PEG-DH was eluted

by 50 mM Tris-HCl buffer, pH 8.0 without ammonium sulfate. The result suggested that this enzyme is a relatively strong hydrophobic protein. It was also found that even though some of PEG-DH activity was lost, we could remove other proteins by 67% (Table 4.3). In conclusion, effective purification of PEG-DH from *Pseudomonas* sp. PE-2 was by cell extraction, ammonium sulfate precipitation, followed by DEAE-650M Toyopearl and Phenyl-Sepharose CL-4B column, respectively. These procedures gave acceptable yield (14.8%) and purification fold (11.8 fold) (Table 4.3).

Some purification guidebooks suggest that HIC is ideal for used immediately after salt precipitation where the ionic strength of the sample will enhance hydrophobic interaction and also for avoiding the desalting step (Janson and Ryden, 1998; Queiroz *et al.*, 2001). Preliminary experiment was performed by direct application of the enzyme solution from ammonium sulfate precipitation step to Phenyl-Sepharose CL-4B column. It was found that even though great removal of other proteins was observed but the elution time was too long causing the unacceptable great loss of PEG-DH activity. The purification of PEG-DH using ammonium sulfate precipitation followed by Phenyl-Sepharose CL-4B and DEAE-650M Toyopearl column also did not lead to the desired level of enzyme activity.

For the purification of PPG-DH, we used Butyl-Toyopearl column as the second step of column chromatography. We didn't use Phenyl-Sepharose CL-4B column because of the unacceptable great loss of PPG-DH activity. With Butyl-Toyopearl column, the PPG-DH was eluted with a stepwise elution of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF without ammonium sulfate (Figure 4.11). According to Table 4.4, 64% of other proteins were removed whereas 11% of total activity was also lost. High protein removal and a loss of

enzyme activity were also found at this purification step. The result suggested that this enzyme is a relatively strong hydrophobic protein. Thus, the PPG-DH was partially purified using salt precipitation, ion exchange (DEAE-650M Toyopearl column) and hydrophobic interaction (Butyl-Toyopearl column) chromatography. These procedures gave acceptable yield (14.1%) and purification fold (51.4 fold) (Table 4.4).

The loss of the PEG-DH and PPG-DH activities when column chromatography was used may be caused by the removal of some factors important for stabilizing the enzyme activity. In these cases, PEG-DH and PPG-DH activities of the fractions were assayed after preincubation with and without PQQ. There was only negligible or slight PEG-DH and PPG-DH activity in the case of preincubation without PQQ whereas enhancement of the enzyme activities was observed when preincubated with PQQ. Thus both enzymes show PQQ-dependent activity. Moreover, these results indicated that PQQ was lost during the purification. It has been reported that PQQ is non-covalently bound to quinohaemoprotein-type enzymes and can be dissociated by dialysis overnight in Tris-HCl buffer pH 8.0 containing 2 mM EDTA (Zarnt *et al.*, 1997) or by heat treatment (Toyama *et al.*, 1995). Therefore, it was possible that PQQ can be dissociated from the enzymes when dialyzed after ammonium sulfate precipitation and by column chromatography.

PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 have not been completely purified (if more purification step is used, the enzyme activities will be greatly reduced). Partially purified enzymes might be good enough for their applicable use in the environmental and industrial sites.

In this study, several attempts had been carried out to purify PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 to homogeneity. However, when more purification steps were used, the enzyme activities were lost. Several types of chromatography column were tried, but they caused complete lost of enzyme activity. Therefore, the purification steps and procedures reported here were the suitable ones to obtain partially purified PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2.

According to the non-denaturing PAGE with activity staining (Figure 4.8 and Figure 4.12), other contaminated proteins showed no activities towards PEG or PPG substrates; therefore, they did not affect the characterization of PEG-DH and PPG-DH which is described below.

5.4 Characterization of PEG dehydrogenase and PPG dehydrogenase from *Pseudomonas* sp. PE-2

5.4.1 Molecular weight determination of PEG dehydrogenase and PPG dehydrogenase

The molecular weight of the enzyme was determined in the native and denatured conditions by non-denaturing PAGE and SDS-PAGE, respectively. The SDS-PAGE separates proteins based primarily on their molecular weights. SDS binds along polypeptide chain, and the length of the reduced SDS-protein complex is proportional to its molecular weight (Bollag and Edelstein, 1993).

Partially purified PEG-DH was determined for its molecular weight by SDS-PAGE. From the calibration curves in Figure 4.18, the molecular weight of PEG-DH from *Pseudomonas* sp. PE-2 was 73.4 kDa. The non-denaturing PAGE gave the same molecular weight for PEG-DH (73.6 kDa) suggesting that the enzyme is a monomeric protein. The molecular weight of this enzyme was closely in agreement

with PEG-DH from *Rhodopseudomonas acidophila* (a monomeric of 72 kDa) (Yasuda *et al.*, 1996). However, there were reported otherwise. For instance, PEG-DH from *Sphingopyxis terrae* is homodimeric protein with molecular weight 120 kDa (Sugimoto *et al.*, 2001), while the PEG-DH from *Sphingomonas macrogoltabidus* and mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) are homotetrameric proteins with molecular weights 220 and 240 kDa, respectively (Kawai *et al.*, 1980; Kawai and Yamanaka, 1989; Yamanaka and Kawai, 1989). The molecular weight of PEG-DH from other microorganisms was summarized in Table 5.1.

The molecular weight of partially purified PPG-DH from *Pseudomonas* sp. PE-2 was determined to be about 66.2 kDa by non-denaturing PAGE and 36.1 kDa by SDS-PAGE (Figure 4.15 and 4.18). This result suggests that the PPG-DH is a homodimeric protein of two identical subunit size of 36.1 kDa. Only one previous report of Tachibana *et al.* (2003) showed that PPG-DH from *Stenotrophomonas maltophilia* is a homodimeric protein (123 kDa) of two identical subunit of 60 kDa (Tachibana *et al.*, 2003). The PPG-DH from *Stenotrophomonas maltophilia* is twice larger than our PPG-DH (Table 5.2).

Table 5.1 Properties of PEG dehydrogenases from different microorganisms

Stain	Molecular weight	Optimum pH	pH Stability	Optimum temperature	Temperature stability	References
Pseudomonas sp. PE-2	73.6 kDa (monomer)	9.0	8.0-9.5	25°C	25-30°C	This study
Mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.)	240 kDa (homotetramer with subunits about 60-62 kDa)	8.0	7.5-9.0	60°C	below 35°C	Kawai *et al.*, 1980
Sphingomonas macrogoltabidus	220 kDa (homotetramer with subunits about 57 kDa)	7.5-8.0	7.0-8.0	40°C	below 35°C	Kawai and Yamanaka, 1989 Yamanaka and Kawai, 1989
Rhodopseudomonas acidophila	72 kDa (monomer)	ND	ND	ND	ND	Yasuda *et al.*, 1996
Sphingopyxis terrae	120 kDa (homodimer with subunits about 58 kDa)	9.0	ND	ND	ND	Sugimoto *et al.*, 2001

ND = not detected.

Table 5.2 Properties of PPG dehydrogenases from different microorganisms

Stain	Molecular weight	Optimum pH	pH Stability	Optimum temperature	Temperature stability	References
Pseudomonas sp. PE-2	66.2 kDa (homodimer with subunits about 36 kDa)	7.5	7.0-8.0	25°C	25-40°C	This study
Stenotrophomonas maltophilia	123 kDa (homodimer with subunits about 60 kDa)	8.5	6.5-9.0	50°C	up to 60°C	Tachibana *et al.*, 2003

5.4.2 Effect of pH and temperature on PEG dehydrogenase and PPG dehydrogenase activities

A velocity of an enzyme-catalyzed reaction depends on pH of the reaction. Optimal pH of each enzyme is one of its characteristic to be concern because slightly shift in the pH from the optimum value leads to a decrease in the reaction rate, whereas the large pH shift may cause a servere damage in protein structures (Segal, 1976).

PEG-DH from *Pseudomonas* sp. PE-2 showed the maximum relative activity at pH 9.0, corresponding with the optimum pH of other PEG-DH from *Sphingopyxis terrae* (pH 9.0, Table 5.1) (Sugimoto *et al.*, 2001). From our result, the buffer effect was observed. The Tris-HCl buffer was suitable for the PEG-DH from *Pseudomonas* sp. PE-2 since high enzyme activity was observed (Figure 4.19A). So, The Tris-HCl buffer had been chosen to use as an assay buffer and as a buffer for PEG-DH purification.

PPG-DH from *Pseudomonas* sp. PE-2 showed the optimum pH at 7.5. This is different from that of *Stenotrophomonas maltophilia* which had higher optimum pH at 8.5 (Tachibana *et al.*, 2003). From the result, Tris-HCl buffer was a suitable one and had been used as a buffer for the enzymatic assay and purification steps.

The optimum temperature of PEG-DH from *Pseudomonas* sp. PE-2 was 25°C, while the optimum temperature of PEG-DH from *Sphingomonas macrogoltabidus* (Kawai and Yamanaka, 1989; Yamanaka and Kawai, 1989) and mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) were 40°C and 60°C,

respectively. At higher temperatures, 40°C and 60°C, the activity of PEG-DH remained 83% and 66%, respectively.

The highest relative activity for our PPG-DH was observed around 25°C, whereas the optimum temperature of PPG-DH from *Stenotrophomonas maltophilia* (Tachibana *et al.*, 2003) was at 50°C. At 50°C, PPG-DH activity of *Pseudomonas* sp. PE-2 remained 73%.

Table 5.1 and 5.2 summarize the comparative data of the optimum pH and temperature on PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 to the enzymes from other microorganisms.

5.4.3 Stabilities of pH and temperature on PEG dehydrogenase and PPG dehydrogenase activities

Enzyme stability was one of the important factors in considering the suitability of any enzyme to be used in the environmental and industrial sites. The stability curve of pH and temperature can be obtained by preincubating the enzyme at the indicated pH and temperature for a time at least as long as the usual assay time (Segal, 1976).

The PEG-DH from *Pseudomonas* sp. PE-2 was stable in a wide pH range of 8 to 9.5 upon incubation at 4°C for 30 minutes which was similar to PEG-DH from mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) but different from that of *Sphingomonas macrogoltabidus* which was only stable in the pH range of 7.0 to 8.0 (Kawai *et al.*, 1980; Kawai and Yamanaka, 1989; Yamanaka and Kawai, 1989).

Our PPG-DH was stable over a pH range of 7.0-8.0 upon incubation at 4°C for 30 minutes. The PPG-DH from *Stenotrophomonas maltophilia* was most stable over a wide pH range of 6.5-9.0 (Tachibana *et al.*, 2003).

The PEG-DH retained 100% relative activity at 25°C in 50 mM Tris-HCl buffer, pH 8.0 when incubated for 30 minutes. The activity was decreased with increase in temperature and was abruptly abolished at 40°C. Our enzyme showed similar thermostability to PEG-DH from mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) (Kawai *et al.*, 1980) and *Sphingomonas macrogoltabidus* (Kawai and Yamanaka, 1989; Yamanaka and Kawai, 1989) which retained a 100% relative activity below 35°C.

The temperature stability of PPG-DH was in the range of 25°C to 40°C. The relative activity was constantly decreased with increase in temperature. Our PPG-DH showed lower thermostability than PPG-DH from *Stenotrophomonas maltophilia* of which the activity was retained up to 60°C (Tachibana *et al.*, 2003).

Table 5.1 and 5.2 summarize the comparative data of the pH and temperature stability of PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 to the enzymes from other microorganisms.

5.4.4 Effect of divalent metal ions and cofactors on PEG dehydrogenase and PPG dehydrogenase activities

Metal ions have been reported to affect enzyme activity by either activating or inhibiting the activity. In our experiment, Co^{2+}, Cu^{2+} and Hg^{2+} at all concentration completely inhibited PEG-DH activity when compared with Ca^{2+} (100%). Mg^{2+} was not a potent inhibitor of the PEG-DH activity, while Mn^{2+} and Ni^{2+} was barely complete inhibited the PEG-DH with 10-22% and 5-13% remaining activity, respectively (Table 4.6A). In mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.), Cu^{2+} and Hg^{2+} at final concentration 1 mM completely inhibited PEG-DH activity. Ca^{2+}, Co^{2+}, Mg^{2+}, $Mn^{2+}$and Ni^{2+} at 1 mM were potent inhibitors of

PEG-DH (Kawai *et al.*, 1980). The effect of Ca^{2+} and Mg^{2+} on PEG-DH had also been previously reported. Ca^{2+} and Mg^{2+} were effective to enhance the PEG-DH from *Rhodopseudomonas acidophila* (Yasuda *et al.*, 1996). With PEG-DH of *Sphingomonas macrogoltabidus*, Ca^{2+} and Mg^{2+} were barely inhibited the PEG-DH and Cu^{2+} and Hg^{2+} activated the PEG-DH activity (Yamanaka and Kawai, 1989). Table 5.3 shows effect of metal ions on PEG dehydrogenases from different microorganisms.

The effect of metal ions on PPG-DH had not been previously reported. From our results, Cu^{2+}, Hg^{2+} and Mn^{2+} significantly inhibited the PPG-DH. Co^{2+} and Mg^{2+} were less potent inhibitors, while Ca^{2+} and Ni^{2+} were the enzyme stimulators. The results showed that Ni^{2+} at all concentration is inhibitor of PEG-DH (Table 4.6).

For PEG-DH and PPG-DH, PQQ played a role of the cofactor. It has been reported that Ca^{2+} and Mg^{2+} are essential to maintain PQQ in the active site of quinoprotein alcohol dehydrogenases (Anthony, 1996; Yamada *et al.*, 2003) and also proposed that Ca^{2+} acts as a Lewis acid by way of its coordination to the C-5 carbonyl oxygen of PQQ, thus stabilizing the electrophilic C-5 for attack by the hydride. The conserved arginine (Arg331) is also likely to be important in increasing the nucleophilicity of the C5 atom of PQQ (Figure 5.1 to Figure 5.5). Ca^{2+} was reported to have an important role in the catalytic mechanism of quinoprotein-type methanol dehydrogenase (Zheng and Bruice, 1997). The possible roles of Ca^{2+} complexed with PQQ in methanol oxidation include: (i) modest reduction of the pK_a of the substrate and facilitating the association of substrate to active site, (ii) polarizing the oxygen at the C-5 position of PQQ, and (iii) placing the reaction components in the right positions to react, therefore contributing to the formation of enzyme-substrate complex (Zheng and Bruice, 1997).

Table 5.3 Effect of metal ions on PEG dehydrogenases from different microorganisms

Metal ions	**Relative activity (%)**			
	***Pseudomonas* sp. PE-2**	**Mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.)**	***Sphingomonas macrogoltabidus***	***Rhodopseudomonas acidophila***
No addition	ND	ND	ND	**100**
Ca^{2+}	**100**	89	9	128
Co^{2+}	0.15	72	50	ND
Cu^{2+}	0	0	**100**	ND
Hg^{2+}	0	0	**100**	ND
Mg^{2+}	93	84	6	133
Mn^{2+}	10	76	33	ND
Ni^{2+}	13	71	27	ND
Sr^{2+}	ND	**100**	ND	ND
Final concentration	2 mM	1 mM	1 mM	1 mM
References	This study	Kawai *et al.*, 1980	Kawai and Yamanaka, 1989 Yamanaka and Kawai, 1989	Yasuda *et al.*, 1996

ND = not detected.

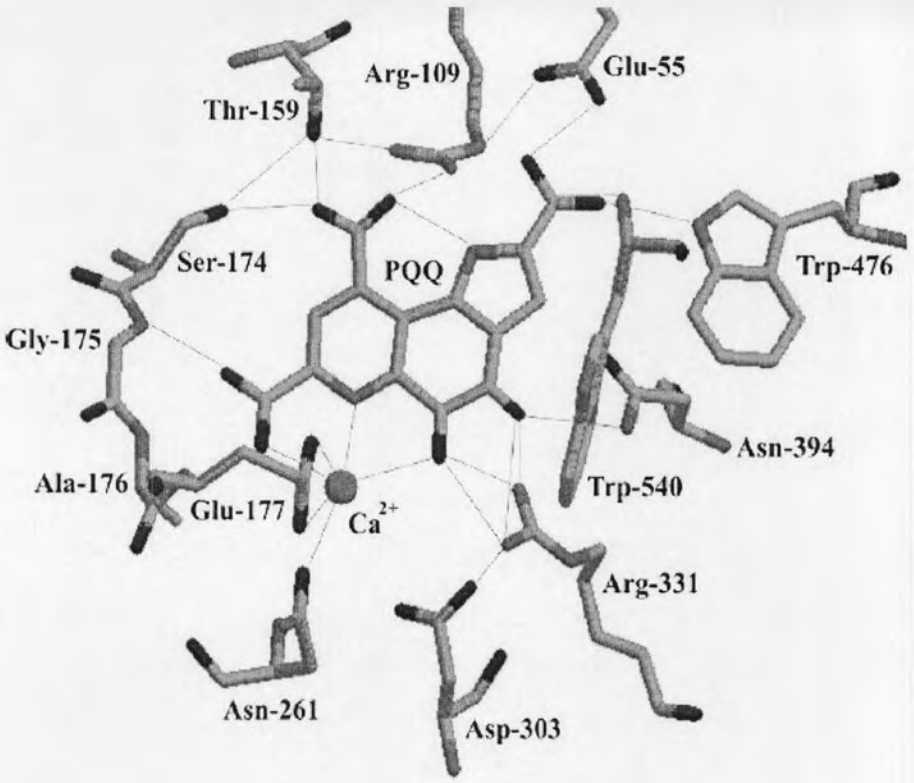

Figure 5.1 Coordination of the Ca^{2+} and PQQ at the active site of methanol dehydrogenase from *Methylobacterium extorquens*

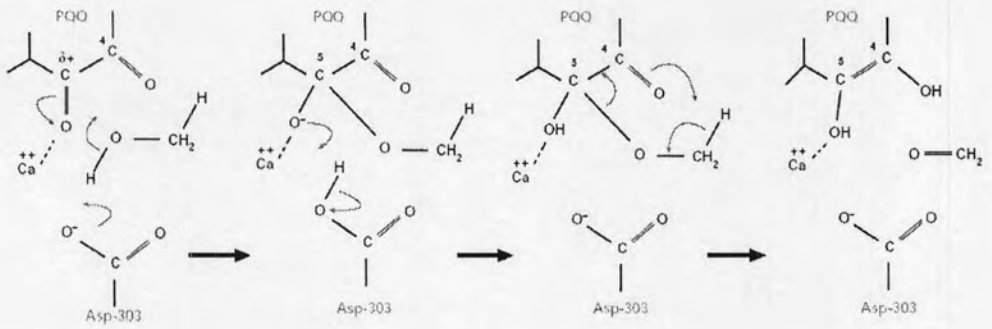

Figure 5.2 Reaction mechanism for methanol dehydrogenase involving a hemiketal intermediate

Proton abstraction by the base leads to an oxyanion form of the substrate which attacks the electrophilic C-5, giving the hemiketal intermediate from which the methyl proton is abstracted; this in turn gives the quinone and product formaldehyde.

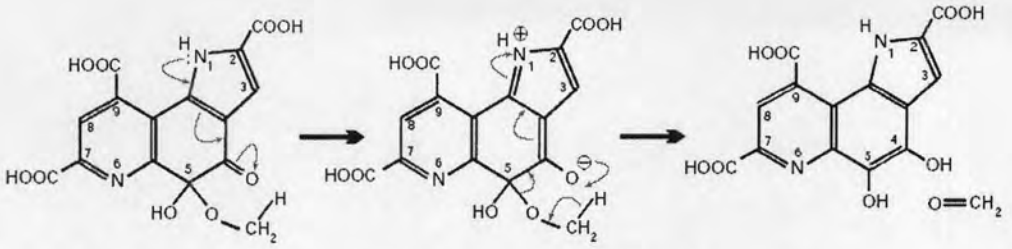

Figure 5.3 Possible involvement of the pyrrole nitrogen in the reaction mechanism of methanol dehydrogenase

This is a modification of the mechanism shown in Figure 5.2. In this variation the difficult proton abstraction from the methyl group is facilitated by the ionization of the C-4 carbonyl oxygen, which is made possible by the presence of the pyrrole nitrogen atom.

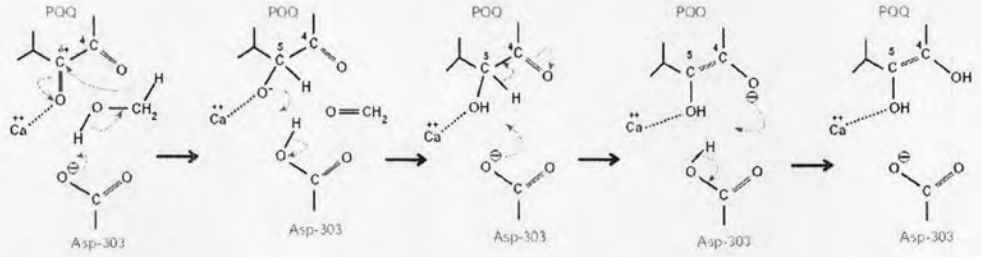

Figure 5.4 Reaction mechanism for methanol dehydrogenase involving hydride transfer

This mechanism is suggested as an alternative to that shown in Figure 5.2. The key difference is that there is no covalent bonding of substrate. In this case the initial proton abstraction is the same, but the electrophilic C-5 is involved directly in removal of the methyl hydrogen as a hydride. The active-site base (Asp-303) acts twice in the mechanism.

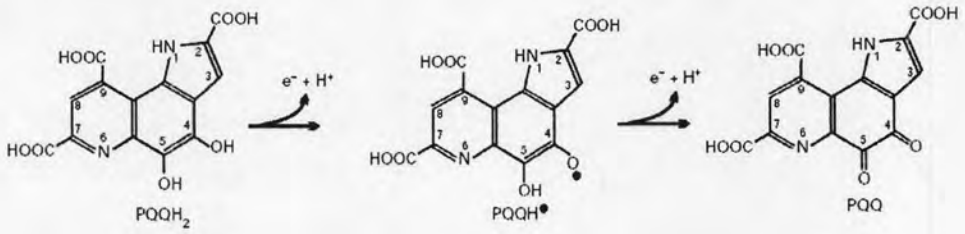

Figure 5.5 Oxidative half-reaction of methanol dehydrogenase

The free-radical semiquinone (PQQH*) is indicated with the unpaired electron on the C-4 carbonyl oxygen, which is perhaps consistent with the fact that this oxygen is seen to be out of the plane of the rest of the PQQ. The electron acceptor is either a dye such as phenazine ethosulphate or the natural electron acceptor cytochrome *c*.

5.4.5 Substrate specificity of PEG dehydrogenase and PPG dehydrogenase activities

The enzymatic catalysis takes place in specific region of the enzyme referred as the active center or catalytic cavity. In general, the substrate 'fits' into the active site of the enzyme in a precise geometric alignment. Moreover, the amino acid residues that form the binding site arrange to interact specifically with the substrate in an attractive manner. Molecules that differ in shape or functional group distribution from the substrate cannot productively bind to the enzyme which means they cannot form enzyme-substrate complexes that lead to the formation of products (Voet and Voet, 1995).

Various primary alcohols were determined whether they are preferred substrates for PEG-DH. PEG-DH was highly active towards 1-pentanol, 1-hexanol and 1-heptanol. Similar result was also found from PEG-DH in mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) (Kawai *et al.*, 1980) and *Sphingomonas macrogoltabidus* (Yamanaka and Kawai, 1989) which preferred relatively longer chain primary alcohols (C_3 to C_6) than the shorter ones (C_1 to C_2). Among diol-type and triol-type compounds tested, our PEG-DH slightly oxidized ethylene glycol and diethylene glycol while glycerol was not a substrate for the enzyme. These results were similar to PEG-DH from mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) (Kawai *et al.*, 1980) and *Sphingomonas macrogoltabidus* (Yamanaka and Kawai, 1989). The test was extended to various PEGs with different molecular weight. The result showed that our PEG-DH preferred lower molecular weight of PEG as the substrate. The pattern was similar to the action of the PEG-DH from *Rhodopseudomonas acidophila*, *Comamonas testosteroni* and *Sphingomonas*

macrogoltabidus (Yamanaka and Kawai, 1989; Yasuda *et al.*, 1996). Table 5.4 shows substrate specificity of PEG dehydrogenase from different microorganisms.

The investigation on the substrate specificity of PPG-DH showed that the response of PPG-DH from *Pseudomonas* sp. PE-2 to primary alcohols seemed to follow a similar pattern of PEG-DH in which medium-chain primary alcohols were preferred substrate. PPG-DH from *Stenotrophomonas maltophilia;* however, preferred relatively shorter-chain primary alcohols than the longer ones (Tachibana *et al.*, 2003). Our PPG-DH could oxidize secondary alcohols (2-propanol and 2-butanol) with relatively low activity (25% and 14%, respectively), while it did not exhibit a preference for polyols (compare ethanol with ethyleneglycol, and 1-propanol with glycerol). However, dissimilar results were obtained from PPG-DH of *Stenotrophomonas maltophilia* where it could not oxidize secondary alcohols and exihibited a preference for polyols (Tachibana *et al.*, 2003). According to the results, it appears that the absence of a second free OH group at a short distance from the first one strongly increased the activity. In line with this, low molecular weight of PPGs (polymers with a less variety of OH groups in the chain) was good substrates for our enzyme. Table 5.5 shows substrate specificity of PPG dehydrogenases from different microorganisms.

Thus, PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 appeared to require the presence of a certain carbon chain length for their activities.

Table 5.4 Substrate specificity of PEG dehydrogenases from different microorganisms

	Substrate	Relative activity (%)				
		***Pseudomonas* sp. PE-2**	**Mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.)**	***Sphingomonas macrogoltabidus***	***Rhodopseudomonas acidophila***	***Comamonas testosteroni***
Short-chain alcohols length	Methanol	7	0	0	ND	ND
	Ethanol	8	1	0	72	ND
	1-Propanol	5	18	72	143	ND
	1-Butanol	7	87	227	125	82
Medium-chain primary to long alcohol	1-Pentanol	215	111	267	ND	ND
	1-Hexanol	183	115	280	16	ND
	1-Heptanol	154	114	ND	ND	ND
	1-Octanol	47	ND	187	17	ND
Secondary alcohol	2-Propanol	5	0	0	0	ND
	2-Butanol	6	0	0	ND	ND
Triol-type compound	Glycerol	0	0	0	ND	ND
Diol-type compound	Ethylene glycol	6	0	0	ND	ND
	Diethylene glycol	28	4	27	ND	ND
	Tetraethylene glycol	ND	ND	ND	**100**	**100**

	Substrate	Relative activity (%)				
		***Pseudomonas* sp. PE-2**	**Mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.)**	***Sphingomonas macrogoltabidus***	***Rhodopseudomonas acidophila***	***Comamonas testosteroni***
Diol-type compound	PEG 200	ND	ND	145	ND	ND
	PEG 300	ND	ND	170	ND	ND
	PEG 400	ND	49	169	113	83
	PEG 600	176	ND	ND	ND	ND
	PEG 1000	ND	26	131	87	58
	PEG 2000	**100**	ND	ND	ND	ND
	PEG 4000	60	20	**100**	45	63
	PEG 6000	47	**100**	95	78	69
	PEG 8000	52	ND	ND	ND	ND
	PEG 20000	ND	10	27	ND	ND
	Final concentration	10 mM	5 mM	-	5 mM	5 mM
	References	This study	Kawai *et al.*, 1980	Yamanaka and Kawai, 1989	Yasuda *et al.*, 1996	Yasuda *et al.*, 1996

ND = not detected.

Table 5.5 Substrate specificity of PPG dehydrogenase from different microorganisms

	Substrate	**Relative activity (%)**	
		***Pseudomonas* sp. PE-2**	***Stenotrophomonas maltophilia***
Short-chain alcohols length	Methanol	5	1
	Ethanol	5	2
	1-Propanol	22	25
	1-Butanol	87	38
Medium-chain primary to long alcohol	1-Pentanol	45	11
	1-Hexanol	94	16
	1-Heptanol	87	ND
	1-Octanol	87	ND
Secondary alcohol	2-Propanol	25	0
	2-Butanol	14	0
Triol-type compound	Glycerol	0	177
Diol-type compound	Ethylene glycol	8	192
	Diethylene glycol	14	3
	PPG 400	ND	39
	PPG 700	ND	**100**
	PPG 725	126	ND
	PPG 1000	**100**	148
	PPG 2000	95	193
	PPG 3000	ND	35
	Final concentration	10 mM	10 mM
	References	This study	Tachibana *et al.*, 2003

ND = not detected.

5.4.6 Kinetic parameters of PEG dehydrogenase and PPG dehydrogenase activities

Kinetic studies were carried out to determine the Michaelis constant (K_m). To determine the kinetic parameters of PEG-DH, The various molecular weight of PEG was used as substrate. Table 5.6 summarizes kinetic parameters of partially purified PEG-DH from *Pseudomonas* sp. PE-2 obtained in this study and compared with PEG-DH from mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.), *Sphingomonas macrogoltabidus* and *Sphingomonas macrogoltabidus* (Kawai *et al.*, 1980; Yamanaka and Kawai, 1989; Sugimoto *et al.*, 2001). The low molecular weight of PEGs, PEG 600, PEG 2000 and PEG 4000, were preferred substrates as the enzyme exhibited low K_m values towards them than the larger ones, PEG 6000 and PEG 8000 (Table 4.8). These results suggest a size restriction of substrate to the enzyme. The results were similar to PEG-DH from *Sphingomonas macrogoltabidus* (Yamanaka and Kawai, 1989) and *Sphingopyxis terrae* (Sugimoto *et al.*, 2001) but differed from PEG-DH of mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.) (Kawai *et al.*, 1980) in which low apparent K_m value was obtained with PEG 6000 (Table 5.6).

Kinetic studies on PPG-DH had not been previously reported. The K_m values of PPG-DH from *Pseudomonas* sp. PE-2 showed that the enzyme has the highest preference to PPG 1000, followed by PPG 725 and PPG 2000, respectively (Table 4.8). The results indicated that PPG-DH preferred the smaller substrate as well.

Table 5.6 Kinetic parameters of PEG dehydrogenases from different microorganisms

Substrate	K_m (mM)			
	***Pseudomonas* sp. PE-2**	**Mixed culture (*Sphingopyxis terrae* and *Pseudomonas* sp.)**	***Sphingomonas macrogoltabidus***	***Sphingopyxis terrae***
Tetraethylene glycol	ND	10.0	ND	14
PEG 300	ND	ND	ND	0.8
PEG 400	ND	ND	**1.0**	**0.7**
PEG 600	**0.7**	ND	ND	ND
PEG 1000	ND	ND	1.7	1.6
PEG 2000	3.3	ND	ND	ND
PEG 4000	4.6	ND	2.8	ND
PEG 6000	30.5	**3.0**	5.9	2.4
PEG 8000	28.3	ND	ND	ND
References	This study	Kawai *et al.*, 1980	Yamanaka and Kawai, 1989	Sugimoto *et al.*, 2001

ND = not detected.