CHAPTER IV

RESULTS

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

Pseudomonas sp. PE-2 was grown in a nutrient broth and basal medium supplemented with various concentrations (0.7%, 1.5% and 3% (v/v)) of PEG 2000, PEG 4000, PPG 1000 and PPG 2000 as described in section 3.4.6.1. The PEG-DH and PPG-DH activities were measured with cell-free extract prepared from cells grown in each condition towards several types of PEG and PPG (section 3.4.2 and 3.4.3). Growth of the bacterium was monitored spectrophotometrically as culture turbidity (OD_{610}). Bacterial growth and enzymes production in cell-free extract of each condition are shown in Figure 4.1-4.5.

The PEG-DH and PPG-DH activities were found at negligible level in the cell grown on nutrient broth with the maximum specific activity of 2.8 and 0.2 nmol min^{-1} mg $protein^{-1}$ when assayed with PEG 4000 and PPG 1000 at 24 hours, respectively (Figure 4.1).

When cells were grown on basal medium containing various concentrations of PEG 2000, it was found that *Pseudomonas* sp. PE-2 grew rapidly in 2-3 days and was into the stationary phase after 3 days. The PEG-DH and PPG-DH activities were detected in the cell-free extract after 1-2 days. Maximum specific activity of PEG-DH and PPG-DH were found in the late logarithmic to early stationary phase, about 3-4 days. 3% (v/v) PEG 2000-grown cells for 3 days exhibited PEG-DH and PPG-DH of 11.4 and 15.1 nmol min^{-1} mg $protein^{-1}$ when assayed with PEG 2000 and PPG 1000, respectively (Figure 4.2).

When cells were grown on basal medium containing various concentrations of PEG 4000, it was found that the growth rate of *Pseudomonas* sp. PE-2 in 0.7% (v/v) PEG 4000 was constantly increased for 6 days, after that the growth of cell was stable. For 1.5% and 3% (v/v) PEG 4000-grown cells grew rapidly in 2-3 days and were into the stationary phase after 3 days. The PEG-DH and PPG-DH activities were observed and found that 3% PEG 4000-grown cell for 3 days exhibited PEG-DH and PPG-DH of 25.8 and 17.8 nmol min^{-1} mg $protein^{-1}$, when assayed with PEG 2000 and PPG 1000 respectively. These specific activities were found in the late logarithmic phase of cell growth (Figure 4.3).

When compared the basal medium supplemented between PEG 2000 and PEG 4000, it was found that 3% (v/v) PEG 4000 could induce the PEG-DH activity better than 3% (v/v) PEG 2000 about 2.3 fold when assayed with PEG 2000. Thus, the greatest extent of PEG-DH activities was observed with 3% (v/v) PEG 4000 as sole carbon and energy sources for *Pseudomonas* sp. PE-2.

When cells were grown on basal medium containing various concentrations of PPG 1000, it was found that the growth rate of *Pseudomonas* sp. PE-2 in 0.7% (v/v) PPG 1000 was slightly increased for 4 days, after that the cell growth increased on sixth to seventh day and stable on eighth to tenth day. In 1.5% (v/v) PPG 1000, cells slightly grew on the first four days, after that growth was rapid. In the basal medium supplemented with 3% (v/v) PPG 1000, cells did not grow. The PEG-DH and PPG-DH activities with 1.5% PPG 1000-grown cells for 7 days were 7.4 and 21.0 nmol min^{-1} mg $protein^{-1}$ when assayed with PEG 2000 and PPG 1000, respectively. These specific activities were found in the logarithmic phase of cell growth (Figure 4.4).

When cells were grown on basal medium containing various concentrations of PPG 2000, it was found that the growth rate of *Pseudomonas* sp. PE-2 in 0.7% (v/v) PPG 2000 was slightly increased for 5 days, after that the cell growth increased after 5 days and was into the stationary phase on eighth day. In 1.5% (v/v) PPG 2000, cells slightly grew on the first four days, after that growth was rapid. In the basal medium supplemented with 3% (v/v) PPG 2000, cells did not grow. The PEG-DH and PPG-DH activities with 1.5% PPG 2000-grown cells for 7 days were 5.7 and 5.3 nmol min^{-1} mg $protein^{-1}$ when assayed with PEG 2000 and PPG 1000, respectively. These specific activities were found in the logarithmic phase of cell growth (Figure 4.5).

When compared the basal medium supplemented between PPG 1000 and PPG 2000, it was found that 1.5% (v/v) PPG 1000 could induce the PPG-DH activity better than 1.5% (v/v) PEG 2000 about 4 fold when assayed with PPG 1000. Thus, the greatest extent of PPG-DH activities was observed with 1.5% (v/v) PPG 1000 as sole carbon and energy sources for *Pseudomonas* sp. PE-2.

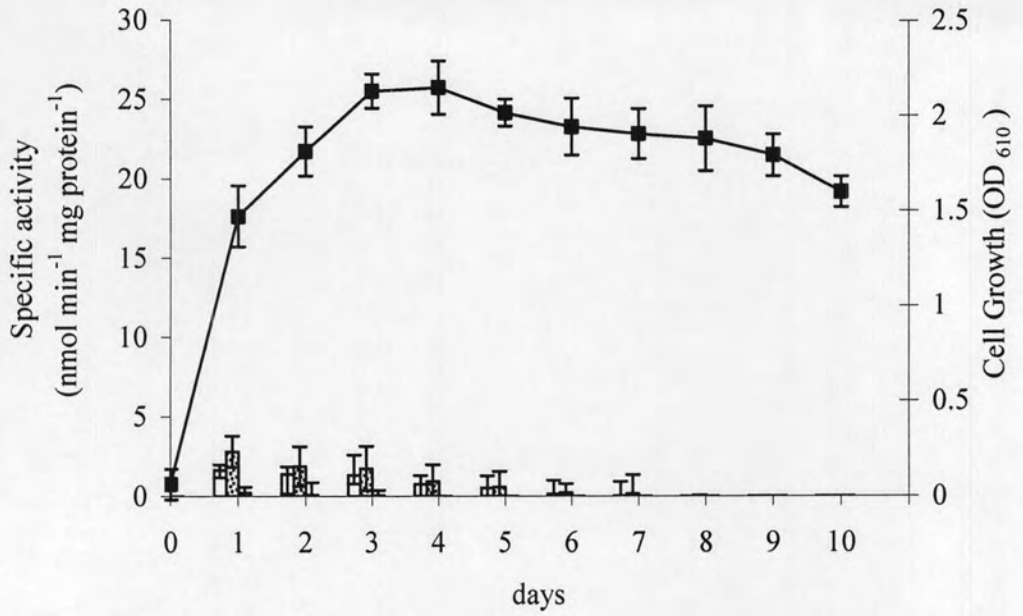


Figure 4.1 Growth of *Pseudomonas* sp. PE-2 and formation of PEG-DH and PPG-DH when grown on nutrient broth (NB)

Cells were grown at 30°C for 10 days with aeration and agitation (300 rpm). Cell growth (—■—) was measured. PEG-DH and PPG-DH activities were assayed with the cell-free extract towards various substrates at 10 mM: PEG-DH activity with substrate PEG 2000 (□), PEG-DH activity with substrate PEG 4000 (▩), PPG-DH activity with substrate PPG 1000 (▨), PPG-DH activity with substrate PPG 2000 (▥).

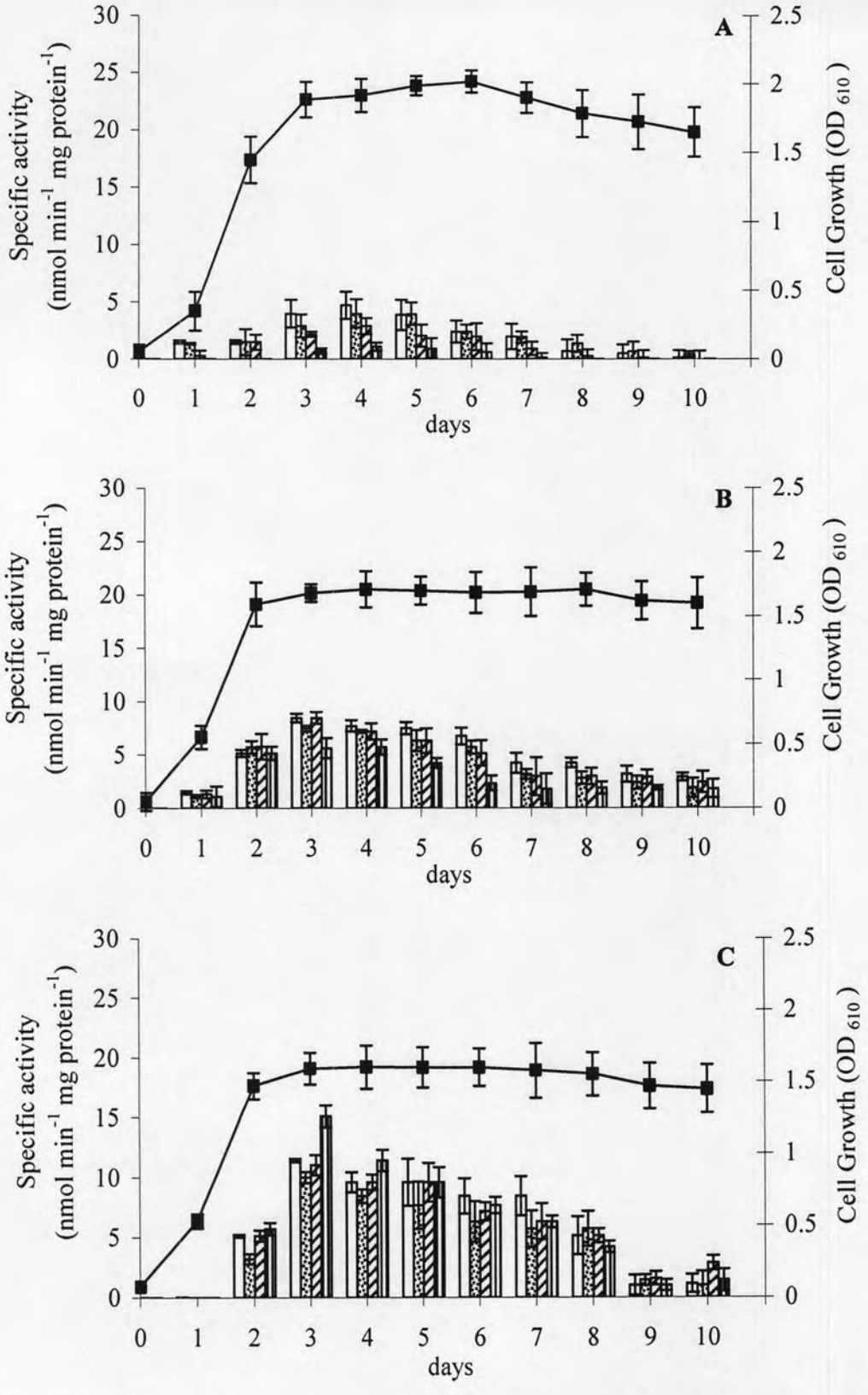


Figure 4.2 Growth of *Pseudomonas* sp. PE-2 and formation of PEG-DH and PPG-DH when grown on PEG 2000

Cells were grown on 200 ml of basal medium containing various concentrations (v/v) of PEG 2000; 0.7% (A), 1.5% (B) and 3% (C) as the sole source of carbon at 30°C for 10 days with aeration and agitation (300 rpm). Cell growth (—■—) was measured. PEG-DH and PPG-DH activities were assayed with the cell-free extract towards various substrates at 10 mM: PEG-DH activity with substrate PEG 2000 (▭), PEG-DH activity with substrate PEG 4000 (▩), PPG-DH activity with substrate PPG 1000 (▨), PPG-DH activity with substrate PPG 2000 (▮▮▮▮).

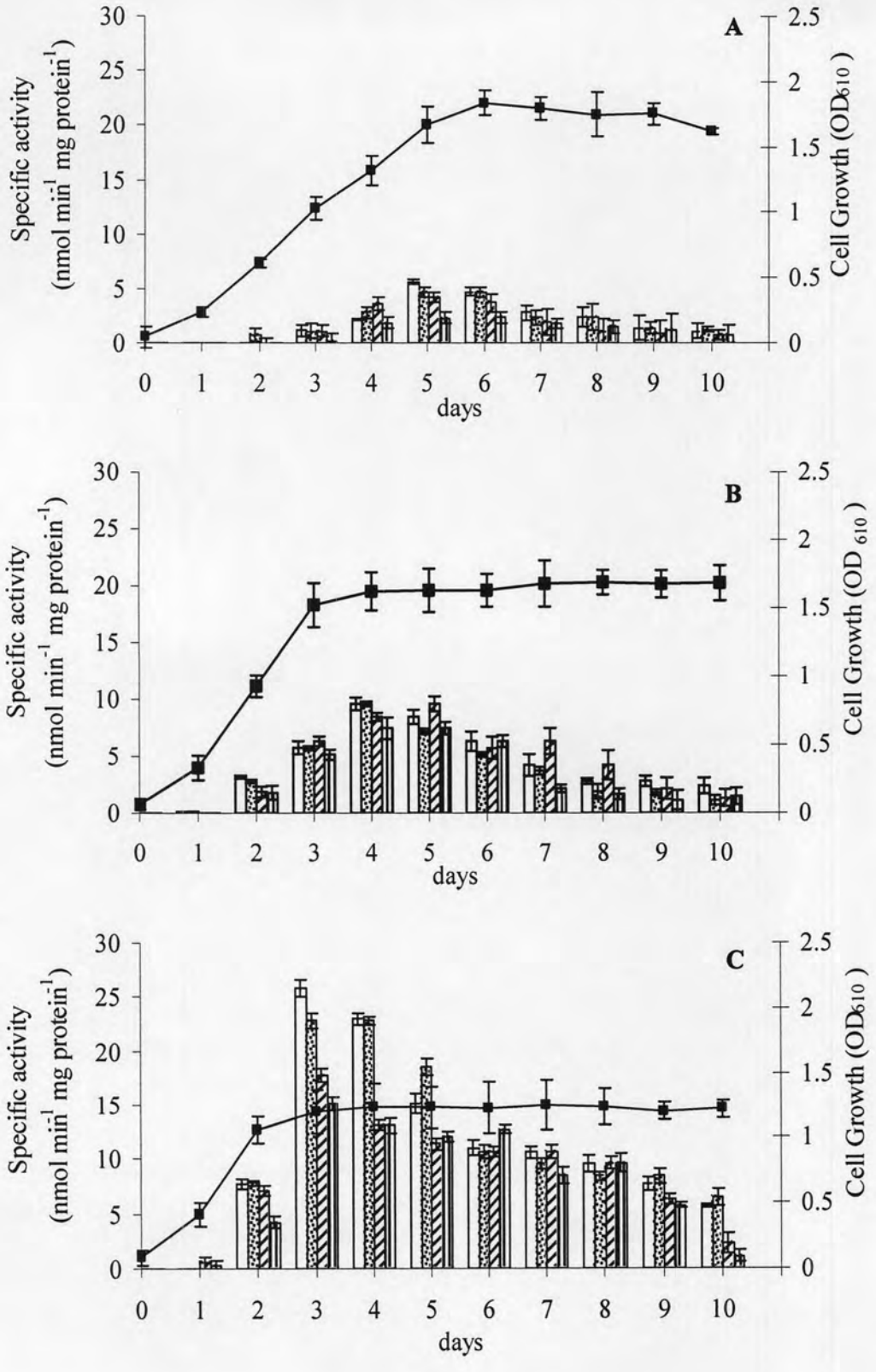


Figure 4.3 Growth of *Pseudomonas* sp. PE-2 and formation of PEG-DH and PPG-DH when grown on PEG 4000

Cells were grown on 200 ml of basal medium containing various concentrations (v/v) of PEG 4000; 0.7% (A), 1.5% (B) and 3% (C) as the sole source of carbon at 30°C for 10 days with aeration and agitation (300 rpm). Cell growth (—■—) was measured. PEG-DH and PPG-DH activities were assayed with the cell-free extract towards various substrates at 10 mM: PEG-DH activity with substrate PEG 2000 (▭), PEG-DH activity with substrate PEG 4000 (▩), PPG-DH activity with substrate PPG 1000 (▨), PPG-DH activity with substrate PPG 2000 (▮▮▮).

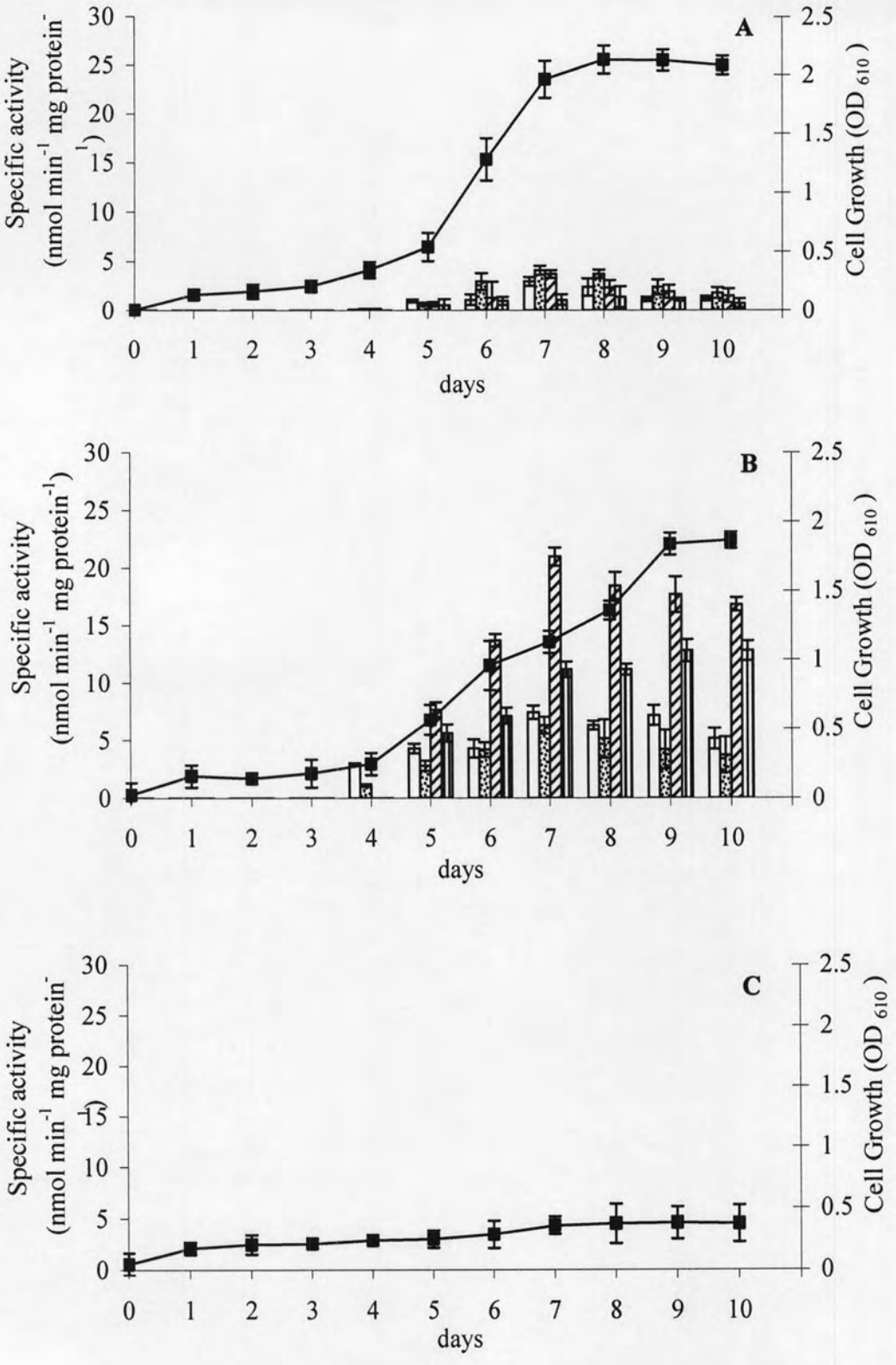


Figure 4.4 Growth of *Pseudomonas* sp. PE-2 and formation of PEG-DH and PPG-DH when grown on PPG 1000

Cells were grown on 200 ml of basal medium containing various concentrations (v/v) of PPG 1000; 0.7% (A), 1.5% (B) and 3% (C) as the sole source of carbon at 30°C for 10 days with aeration and agitation (300 rpm). Cell growth (—■—) was measured. PEG-DH and PPG-DH activities were assayed with the cell-free extract towards various substrates at 10 mM: PEG-DH activity with substrate PEG 2000 (▭), PEG-DH activity with substrate PEG 4000 (▩), PPG-DH activity with substrate PPG 1000 (▨), PPG-DH activity with substrate PPG 2000 (▮).

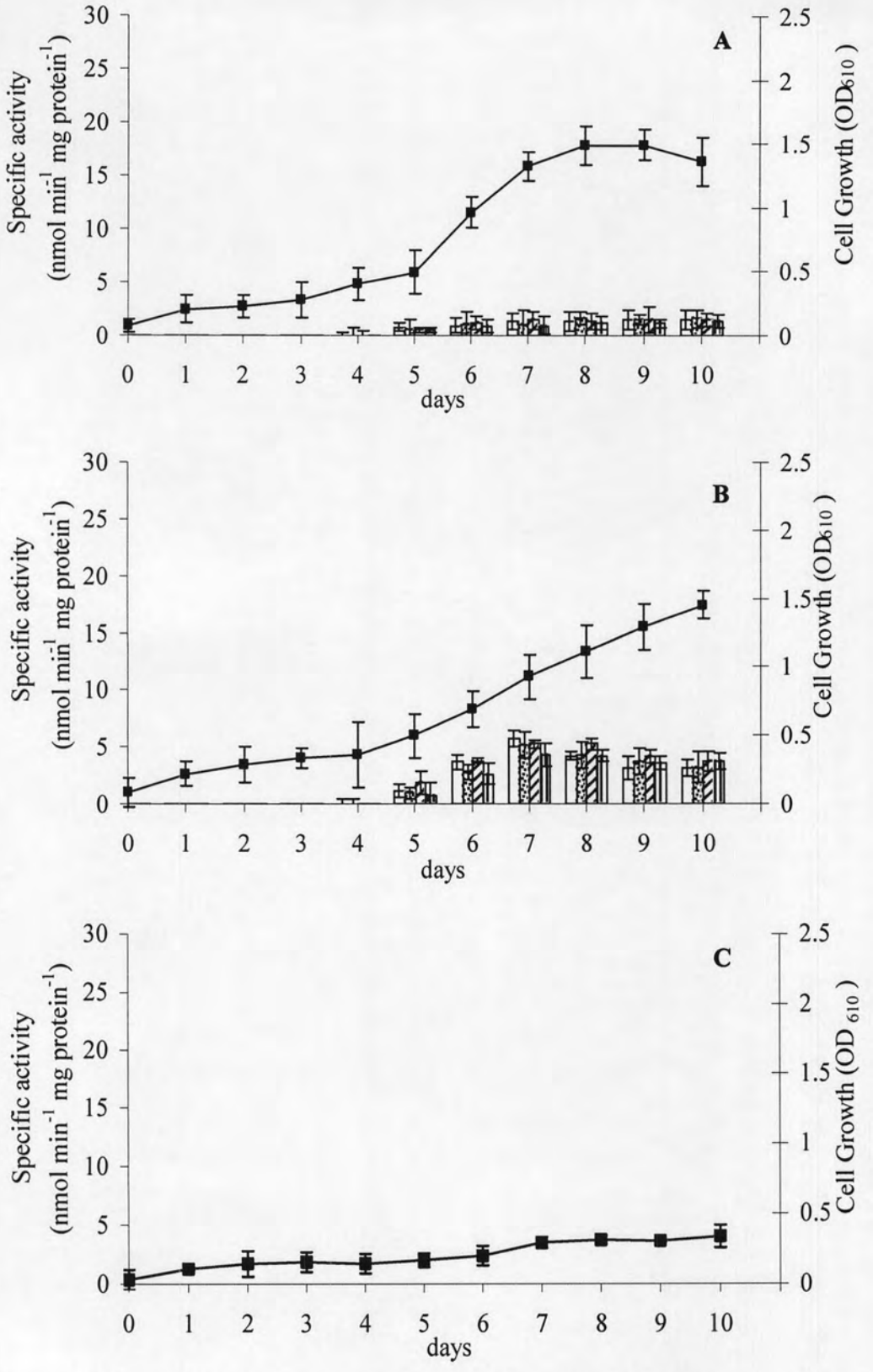


Figure 4.5 Growth of *Pseudomonas* sp. PE-2 and formation of PEG-DH and PPG-DH when grown on PPG 2000

Cells were grown on 200 ml of basal medium containing various concentrations (v/v) of PPG 2000; 0.7% (A), 1.5% (B) and 3% (C) as the sole source of carbon at 30°C for 10 days with aeration and agitation (300 rpm). Cell growth (—■—) was measured. PEG-DH and PPG-DH activities were assayed with the cell-free extract towards various substrates at 10 mM: PEG-DH activity with substrate PEG 2000 (□), PEG-DH activity with substrate PEG 4000 (▩), PPG-DH activity with substrate PPG 1000 (▨), PPG-DH activity with substrate PPG 2000 (▥).

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

The cellular localization of PEG-DH and PPG-DH were determined as described in section 3.4.6.2. The results were summarized in Table 4.1. Most of the PEG-DH activity (76.6±0.29%) was found in the periplasmic fraction, while PPG-DH activity (82±0.71%) was found mainly in the cytoplasmic fraction. The separation efficiency and purity of each cellular fraction were determined by measuring the activity of the marker enzymes: alkaline phosphatase, NADH oxidase and glucose-6-phosphate dehydrogenase as described in section 3.4.6.2.2. The separation efficiency and purity of each cellular fraction were shown in Table 4.1. These results showed that the subcellular fractionation was reliable, as indicated by the absence of cross contamination of markers in each fraction.

Table 4.1 Cellular localizations of PEG-DH and PPG-DH of *Pseudomonas* sp. PE-2[+]

Distribution of enzyme in cellular fraction	Specific activity*									
	PEG-DH		PPG-DH		Alkaline phosphatase		NADH oxidase		Glucose-6-phosphate dehydrogenase	
	nmol min^{-1} mg $protein^{-1}$	(%)	nmol min^{-1} mg $protein^{-1}$	(%)	nmol min^{-1} mg $protein^{-1}$	(%)	nmol min^{-1} mg $protein^{-1}$	(%)	nmol min^{-1} mg $protein^{-1}$	(%)
Periplasmic fraction	13.1	**76.6±0.3**	3.4	13.9±0.5	675.2	**61.2±0.6**	0	0±0.6	41.7	9.3±0.4
Membrane fraction	0.5	2.9±0.4	0.5	2.1±0.4	245.0	22.2±0.8	892.4	**93.3±0.3**	72.7	16.1±0.3
Cytoplasmic fraction	1.9	11.4±0.2	19.9	**82.0±0.7**	163.6	14.8±0.6	54.9	5.7±0.7	328.4	**72.9±0.5**
Cell-free extract	17.1	100	24.4	100	1103.2	100	956.2	100	450.3	100

* The specific activity of the cell-free extract was defined as 100%.

[+] The results are mean of three individual experiments.

4.3 Partial purification PEG dehydrogenase from *Pseudomonas* sp. PE-2

The PEG-DH from *Pseudomonas* sp. PE-2 which could grow on PEG and PPG has not yet been purified and characterized. Three approaches were usually applied for the partial purification of the enzymes: ammonium sulfate precipitation, ion-exchange chromatography and hydrophobic interaction chromatography. Several attempts were carried out to purify PEG-DH from *Pseudomonas* sp. PE-2 to homogeneity by various types of column chromatography as described in section 3.4.6.3.1. However, CM-cellulose column and DEAE-Cellulose column were not suitable for purification of PEG-DH due to the unacceptable great loss of PEG-DH activity. Therefore, PEG-DH from *Pseudomonas* sp. PE-2 was successfully partially purified with a procedure involving: cell extraction, 30-45% ammonium sulfate precipitation, followed by DEAE-650M Toyopearl (with stepwise elution) and Phenyl-Sepharose CL-4B column (with stepwise elution), respectively.

Cell-free extract was prepared from 40 g (wet weight) of *Pseudomonas* sp. PE-2, which was cultivated from 8 liters of the basal medium supplemented with 3% (v/v) PEG 4000 as described in section 3.4.1.1.1. Cell-free extract used in PEG-DH purification contained 651.6 mg protein with 30,186 nmol/min of PEG-DH total activity in total volume of 50 ml. Thus, the specific activity of the enzyme in the cell-free extract was 46.3 nmol min^{-1} mg $protein^{-1}$ (Table 4.3).

4.3.1 Partial purification of PEG dehydrogenase

4.3.1.1 Ammonium sulfate precipitation

Cell-free extract was first purified by ammonium sulfate precipitation as mentioned in section 3.4.6.3.1.1. To determine the suitable ammonium sulfate concentration for precipitation of PEG-DH, preliminary experiment was performed by a stepwise increase at 15% increment from 0 to 60% and 20% increment from 60 to 80%, respectively. The highest PEG-DH activity was found in 30-45% saturation of the ammonium sulfate fraction (Table 4.2). Therefore, to harvest the most of enzyme, protein fractionation was carried out in the range of 30-45% saturated ammonium sulfate precipitation. The protein remained was 422.7 mg with enzyme activity of 28,323 nmol/min (about 93.8% yield from cell-free extract). The specific activity of the enzyme from this step was 67.1 nmol min^{-1} mg $protein^{-1}$ (Table 4.3).

Table 4.2 Ammonium sulfate precipitation in the various concentrations of ammonium sulfate

Step	Volume (ml)	Total protein (mg)	Total activity (nmol/min)		Specific activity (nmol min^{-1} mg $protein^{-1}$)	
			PEG-DH	PPG-DH	PEG-DH	PPG-DH
Cell-free extract	60	293.5	12,969	21,689.4	44.2	73.9
0-15% Ammonium sulfate precipitation	10.8	4.6	142.2	55.9	30.7	13.3
15-30% Ammonium sulfate precipitation	2	0.7	19.1	19.1	26.8	26.8
30-45% Ammonium sulfate precipitation	20	114.6	**11,478**	2,981.4	**100.2**	26
45-60% Ammonium sulfate precipitation	5.5	48.1	424.3	219.3	8.8	4.6
60-80% Ammonium sulfate precipitation	4	21.2	44.7	**2,564**	1.8	**102.4**

4.3.1.2 DEAE-650M Toyopearl column chromatography

The enzyme precipitated from 30-45% saturated ammonium sulfate was dissolved and dialyzed against 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF. The enzyme solution was applied onto DEAE-650M Toyopearl column as described in section 3.4.6.3.1.4. The chromatographic profile was shown in Figure 4.6. The unbound proteins were eluted from the column with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF. The bound proteins were then eluted with a stepwise elution of 0.2 M and 0.5 M of sodium chloride in the same buffer. The enzyme was eluted at 0.2 M sodium chloride solution as indicated in the profile. The fractions number 81-102 with PEG-DH activity were pooled (total of 80 ml), concentrated to 19 ml using Ultracel-50k and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. The protein remained from this step was 137.3 mg with 4,720 nmol/min of enzyme and the specific activity of 34 nmol min^{-1} mg $protein^{-1}$ (Table 4.3). The enzyme was 0.7 fold purified and the yield was about 15.6% compared with the cell-free extract.

In this step, PEG-DH was essentially incubated with PQQ before the enzymatic assay 30 minutes for recovery of activities as described in section 3.4.3.

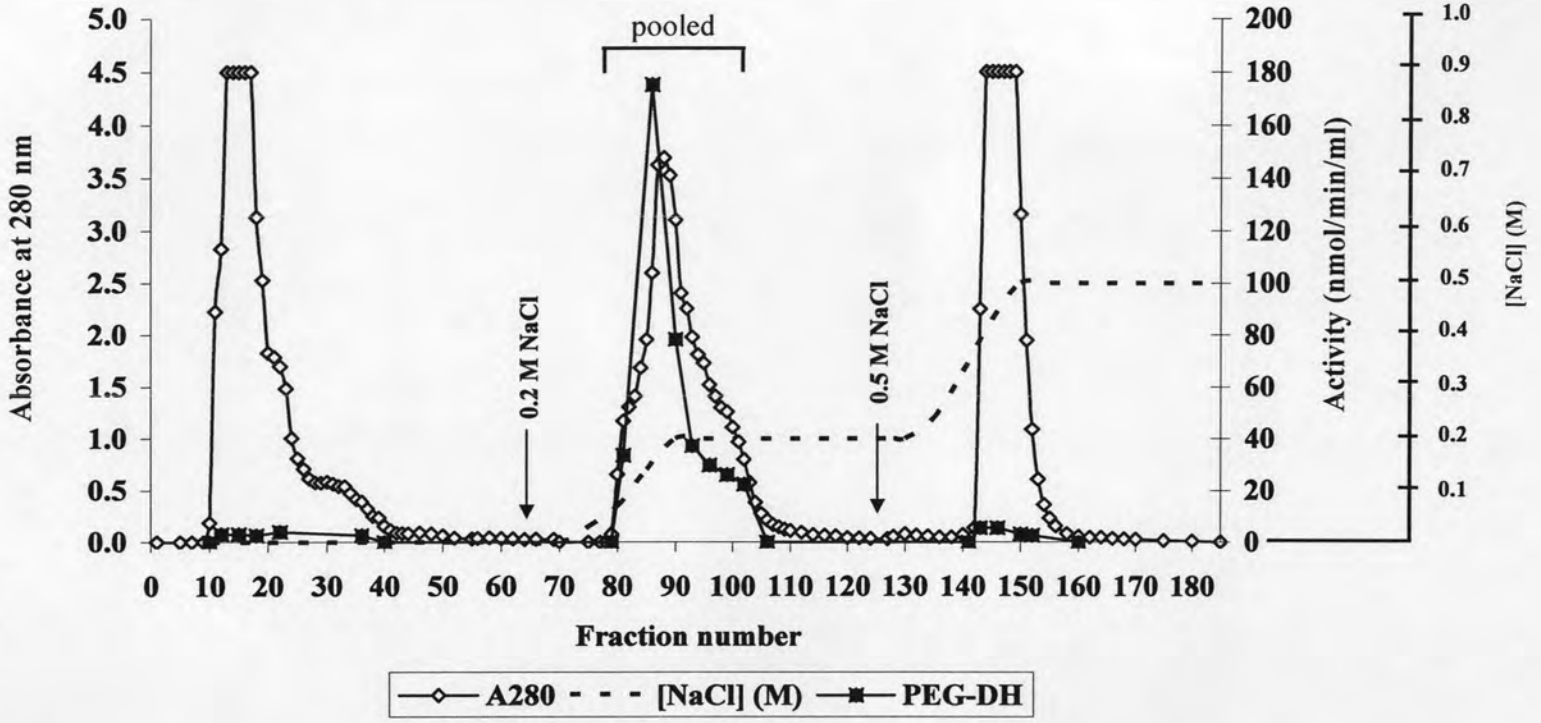


Figure 4.6 Partial purification of PEG dehydrogenase from *Pseudomonas* sp. PE-2 by DEAE-650M Toyopearl column

The enzyme solution was applied to DEAE-650M Toyopearl column (2.5 x 14 cm; 69 ml) and washed with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF until A_{280} decreased to baseline. Elution of bound proteins was performed by 0.2 and 0.5 M NaCl in the same buffer at the flow rate of 60 ml/h. The fractions of 5 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 81 to 102 was pooled.

4.3.1.3 Phenyl-Sepharose CL-4B column chromatography

The pooled active fraction (19 ml; 137.3 mg protein) from DEAE-650M Toyopearl column was further purified by Phenyl-Sepharose CL-4B column as described in section 3.4.6.3.1.5. The chromatographic profile was shown in Figure 4.7. The unbound proteins were eluted from the column with 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF. The other proteins bound to the column were eluted with a stepwise elution of 0.2 M and 0 M ammonium sulfate in the same buffer. The PEG-DH was eluted at 0 M ammonium sulfate concentration as indicated in the profile. The fractions number 84-92 with PEG-DH activity were pooled (total of 27 ml), concentrated to 9 ml using Ultracel-50k and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. This final step yielded the enzyme with 8.2 mg protein, 4,472 nmol/min and the specific activity of 547.4 nmol min^{-1} mg $protein^{-1}$. The enzyme was purified to 11.8 fold with about 14.8% yield (Table 4.3). The enzyme from this step was kept as aliquot at 4°C for further characterization experiments.

In this step, PEG-DH was essentially incubated with PQQ before the enzymatic assay 30 minutes for recovery of activities as described in section 3.4.3.

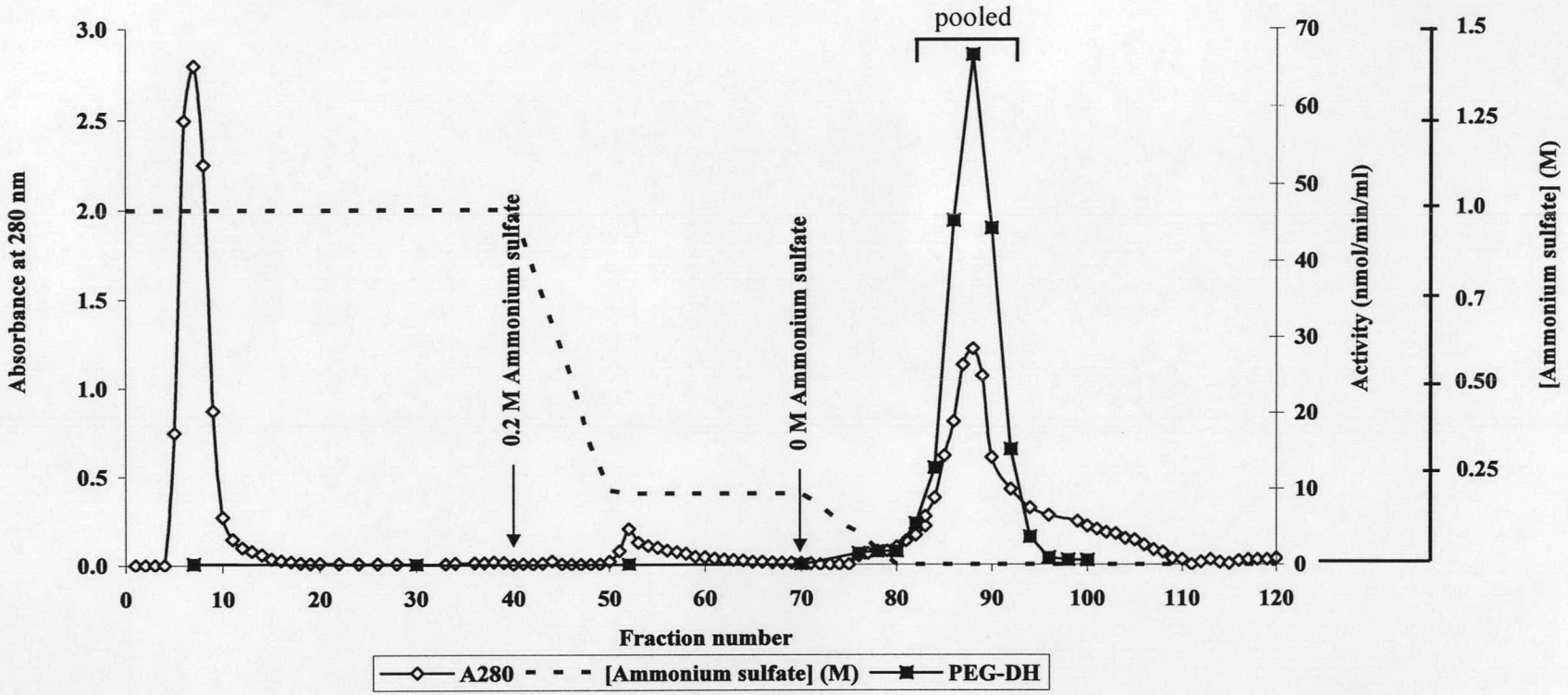


Figure 4.7 Partial purification of PEG dehydrogenase from *Pseudomonas* sp. PE-2 by Phenyl-Sepharose CL-4B column

The enzyme solution was applied to Phenyl-Sepharose CL-4B column (2.5 x 8 cm; 40ml) and washed with 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF until A_{280} decreased to baseline. Elution of bound proteins was performed by a decreasing stepwise elution of ammonium sulfate of 0.2 M and 0 M at the flow rate of 30 ml/h. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 84 to 92 was pooled.

Table 4.3 Partial purification of PEG dehydrogenase from *Pseudomonas* sp. PE-2 grown on 3% (v/v) PEG 4000

Purification step	**Volume (ml)**	**Total activity (nmol/min)**	**Total protein (mg)**	**Specific activity (nmol min^{-1} mg $protein^{-1}$)**	**Purification (fold)**	**Yield (%)**
Cell-free extract	50	30,186	651.6	46.3	1	100
30-45% ammonium sulfate precipitation	30	28,323	422.7	67.1	1.4	93.8
DEAE-650M Toyopearl	19	4,720	137.3	34.4	0.7	15.6
Phenyl-Sepharose CL-4B	9	4,472	8.2	547.4	11.8	14.8

4.3.1.4 Determination of enzyme purity and protein pattern on non-denaturing and SDS-PAGE

The enzyme from each step of purification was analyzed for purity and protein pattern by non-denaturing (Figure 4.8) and SDS-PAGE (Figure 4.9) as described in section 3.4.5.1 and 3.4.5.2. Both non-denaturing and SDS gels were stained for protein with Coomassie blue. With the non-denaturing PAGE, the PEG-DH activity staining was also performed to compare the position of the partially purified enzyme with protein staining. Figure 4.8 shows non-denaturing PAGE analysis with protein staining as well as activity staining. Lane 4 in Figure 4.8 (B) showed the protein band (as indicated by an arrow) with a PEG-DH activity staining. This band is corresponded to the protein band with protein staining (Figure 4.8 (A); lane 4) indicating partial purity of the enzyme from Phenyl-Sepharose CL-4B. The result was then confirmed by SDS-PAGE analysis (Figure 4.9).

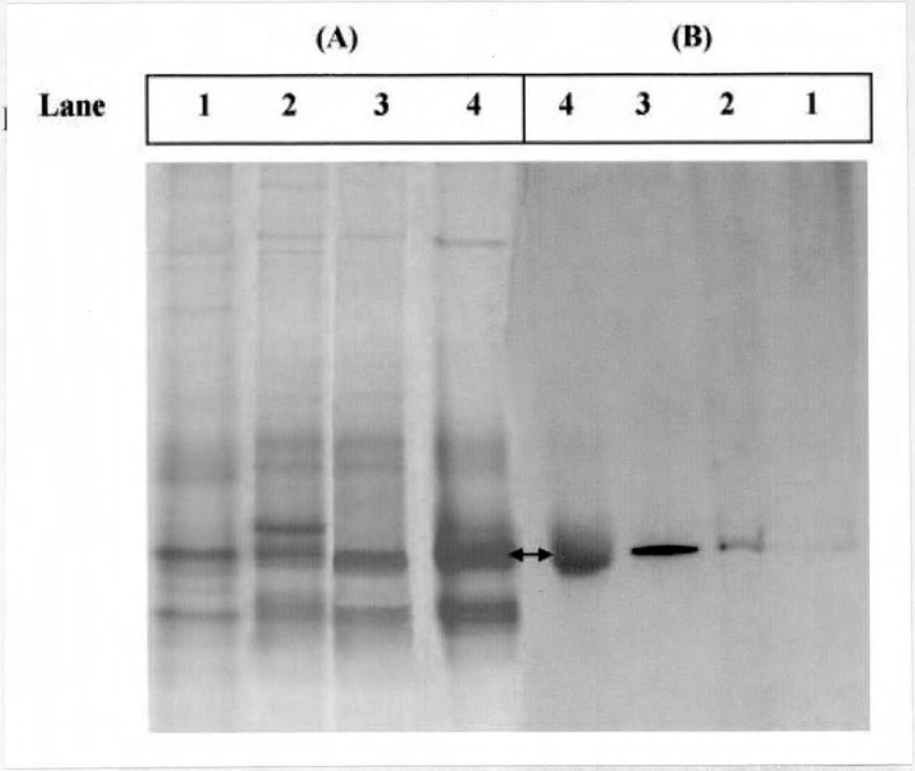


Figure 4.8 Non-denaturing PAGE analysis of PEG dehydrogenase in each step of partial purification from *Pseudomonas* sp. PE-2 on a 7.5% acrylamide gel (A) Coomassie blue staining, (B) PEG dehydrogenase activity staining. Each lane represents 30 μg protein of :

Lane 1: Cell-free extract

Lane 2: 30-45% ammonium sulfate precipitation

Lane 3: DEAE-650M Toyopearl

Lane 4: Phenyl-Sepharose CL-4B

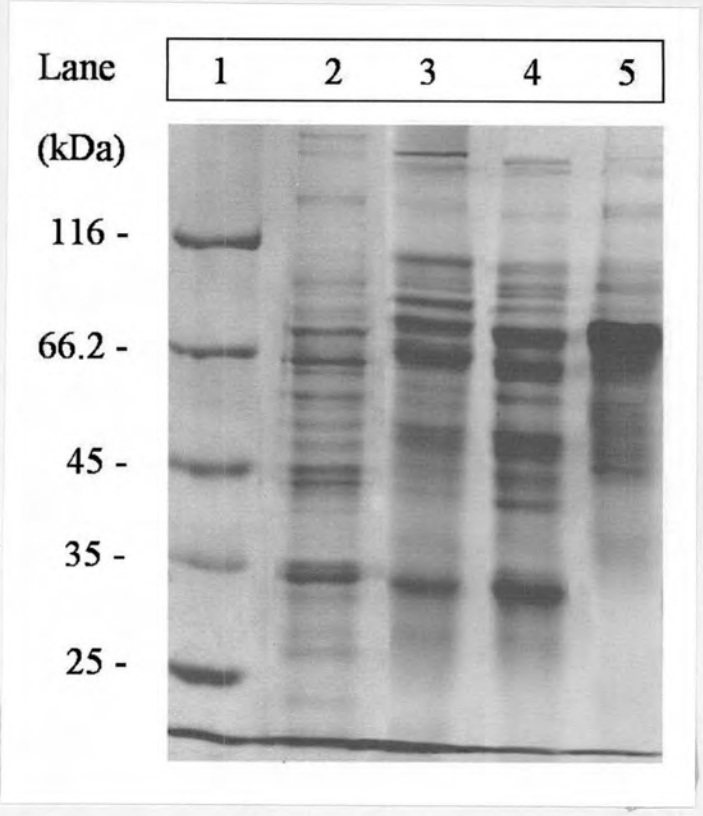


Figure 4.9 SDS-PAGE analysis of PEG dehydrogenase in each step of partial purification from *Pseudomonas* sp. PE-2 on a 10% acrylamide gel

Lane 1: Protein molecular weight marker

(Fermentas, #SM 0431 lot. 1811)

[β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-galactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa)]

Lane 2: Cell-free extract (30 μg protein)

Lane 3: 30-45% ammonium sulfate precipitation (30 μg protein)

Lane 4: DEAE-650M Toyopearl (30 μg protein)

Lane 5: Phenyl-Sepharose CL-4B (30 μg protein)

4.4 Partial purification PPG dehydrogenase from *Pseudomonas* sp. PE-2

The PPG-DH from *Pseudomonas* sp. PE-2 which could grow on PEG and PPG has not yet been purified and characterized. Three approaches were usually applied for the partial purification of the enzymes: ammonium sulfate precipitation, ion-exchange chromatography and hydrophobic interaction chromatography. Several attempts were carried out to purify PPG-DH from *Pseudomonas* sp. PE-2 to homogeneity by various types of column chromatography as described in section 3.4.6.4.1. However, CM-cellulose column and DEAE-Cellulose column were not suitable for purification of PPG-DH due to the unacceptable great loss of PPG-DH activity. Therefore, PPG-DH from *Pseudomonas* sp. PE-2 was successfully partially purified with a procedure involving: cell extraction, 60-80% ammonium sulfate precipitation, followed by DEAE-650M Toyopearl (with stepwise elution) and Butyl-Toyopearl column (with stepwise elution), respectively.

Cell-free extract was prepared from 40 g (wet weight) of *Pseudomonas* sp. PE-2, which was cultivated from 8 liters of the basal medium supplemented with 1.5% (v/v) PPG 1000 as described in section 3.4.1.1.1. Cell-free extract used in PPG-DH purification contained 789.2 mg protein with 7,379 nmol/min of PPG-DH total activity in total volume of 90 ml. Thus, the specific activity of the enzyme in the cell-free extract was 9.3 nmol min^{-1} mg $protein^{-1}$ (Table 4.4).

4.4.1 Partial purification of PPG dehydrogenase

4.4.1.1 Ammonium sulfate precipitation

Cell-free extract was first purified by ammonium sulfate precipitation as mentioned in section 3.4.6.4.1.1. To determine the suitable ammonium sulfate concentration for precipitation of PPG-DH, preliminary experiment was performed by a stepwise increase at 15% increment from 0 to 60% and 20% increment from 60 to 80%, respectively. The highest PPG-DH activity was found in 60-80% saturation of the ammonium sulfate fraction (Table 4.2). Therefore, to harvest the most of enzyme, protein fractionation was carried out in the range of 60-80% saturated ammonium sulfate precipitation. The protein remained was 66.5 mg with enzyme activity of 6,112 nmol/min (about 82.8% yield from cell-free extract). The specific activity of the enzyme from this step was 91.9 nmol min^{-1} mg $protein^{-1}$ (Table 4.4).

4.4.1.2 DEAE-650M Toyopearl column chromatography

The enzyme precipitated from 60-80% saturated ammonium sulfate was dissolved and dialyzed against 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF. The enzyme solution was applied onto DEAE-650M Toyopearl column as described in section 3.4.6.4.1.4. The chromatographic profile was shown in Figure 4.10. The unbound proteins were eluted from the column with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF. The bound proteins were then eluted with a stepwise elution of 0.2 M and 0.5 M of sodium chloride in the same buffer. The enzyme was eluted at 0.2 M sodium chloride solution as indicated in the profile. The fractions numbe 72 to 83 with PPG-DH activity were pooled (total of 60 ml), concentrated to 3 ml using Ultracel-50k and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. The protein remained from this step was 6.2 mg with 1,174 nmol/min of enzyme and the specific activity of 188.2 nmol min^{-1} mg $protein^{-1}$ (Table 4.4). The enzyme was 20.1 fold purified and the yield was about 15.9% compared with the cell-free extract.

In this step, PPG-DH was essentially incubated with PQQ before the enzymatic assay 30 minutes for recovery of activities as described in section 3.4.3.

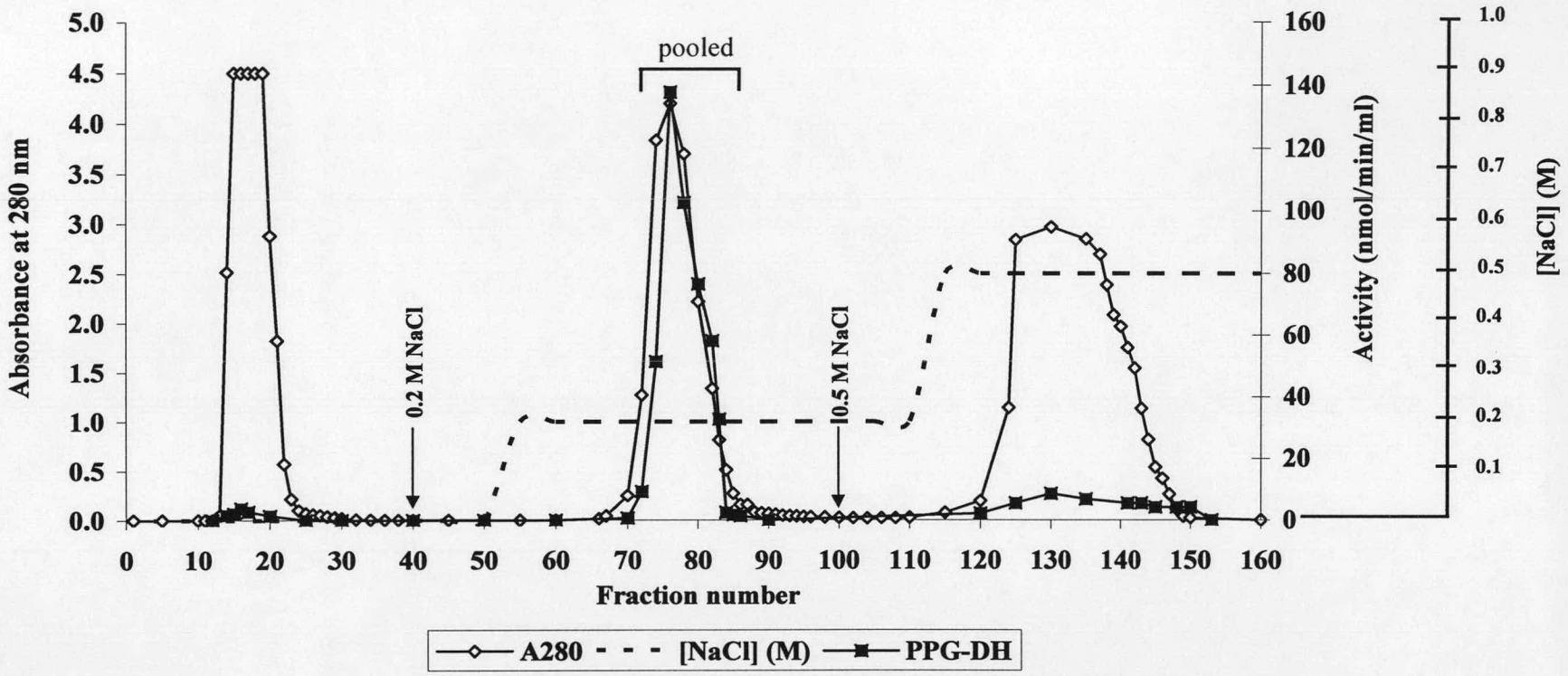


Figure 4.10 Partial purification of PPG dehydrogenase from *Pseudomonas* sp. PE-2 by DEAE-650M Toyopearl column

The enzyme solution was applied to DEAE-650M Toyopearl column (2.5 x 14 cm; 69 ml) and washed with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF until A_{280} decreased to baseline. Elution of bound proteins was performed by 0.2 and 0.5 M NaCl in the same buffer at the flow rate of 60 ml/h. The fractions of 5 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 72 to 83 was pooled.

4.4.1.3 Butyl-Toyopearl column chromatography

The pooled active fraction (3 ml; 6.2 mg protein) from DEAE-650M Toyopearl column was further purified by Butyl-Toyopearl column as described in section 3.4.6.4.1.5. The chromatographic profile was shown in Figure 4.11. The unbound proteins were eluted from the column with 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF. The other proteins bound to the column were eluted with a stepwise elution of 0.6 M and 0 M ammonium sulfate in the same buffer. The PPG-DH was eluted at 0 M ammonium sulfate concentration as indicated in the profile. The fractions number 99-105 with PPG-DH activity were pooled (total of 21 ml), concentrated to 2 ml using Ultracel-50k and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. This final step yielded the enzyme with 2.2 mg protein, 1,043 nmol/min and the specific activity of 480.2 nmol min^{-1} mg $protein^{-1}$. The enzyme was purified to 51.4 fold with about 14.1% yield (Table 4.4). The enzyme from this step was kept as aliquot at 4°C for further characterization experiments.

In this step, PPG-DH was essentially incubated with PQQ before the enzymatic assay 30 minutes for recovery of activities as described in section 3.4.3.

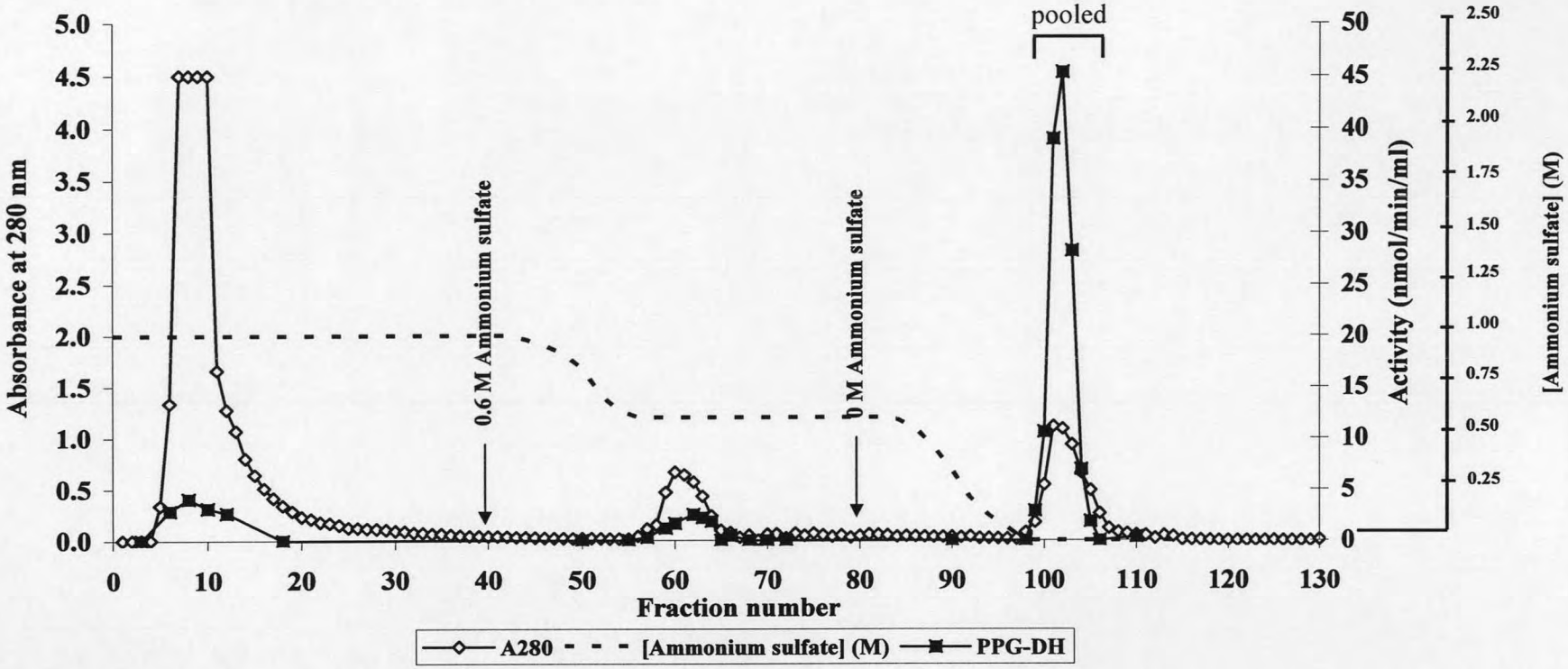


Figure 4.11 Partial purification of PPG dehydrogenase from *Pseudomonas* sp. PE-2 by Butyl-Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column (2.5 x 3 cm; 15 ml) and washed with 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF until A_{280} decreased to baseline. Elution of bound proteins was performed by a decreasing stepwise elution of ammonium sulfate of 0.6 M and 0 M at the flow rate of 30 ml/h. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 99 to 105 was pooled.

Table 4.4 Partial purification of PPG dehydrogenase from *Pseudomonas* sp. PE-2 grown on 1.5% PPG 1000

Purification step	Volume (ml)	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol min^{-1} mg $protein^{-1}$)	Purification (fold)	Yield (%)
Cell-free extract	90	7,379	789.2	9.3	1	100
60-80% ammonium sulfate precipitation	4	6,112	66.5	91.9	9.8	82.8
DEAE-650M Toyopearl	3	1,174	6.2	188.2	20.1	15.9
Butyl-Toyopearl	2	1,043	2.2	480.2	51.4	14.1

4.4.1.4 Determination of enzyme purity and protein pattern on non-denaturing and SDS-PAGE

The enzyme from each step of purification was analyzed for purity and protein pattern by non-denaturing (Figure 4.12) and SDS-PAGE (Figure 4.13) as described in section 3.4.5.1 and 3.4.5.2. Both non-denaturing and SDS gels were stained for protein with Coomassie blue. With the non-denaturing PAGE, the PEG-DH activity staining was also performed to compare the position of the partially purified enzyme with protein staining. Figure 4.12 shows non-denaturing PAGE analysis with protein staining as well as activity staining. Lane 4 in Figure 4.12 (B) showed the protein band (as indicated by an arrow) with a PPG-DH activity staining. This band is corresponded to the protein band with protein staining (Figure 4.12 (A); lane 4) indicating partial purity of the enzyme from Butyl-Toyopearl. The result was then confirmed by SDS-PAGE analysis (Figure 4.13).

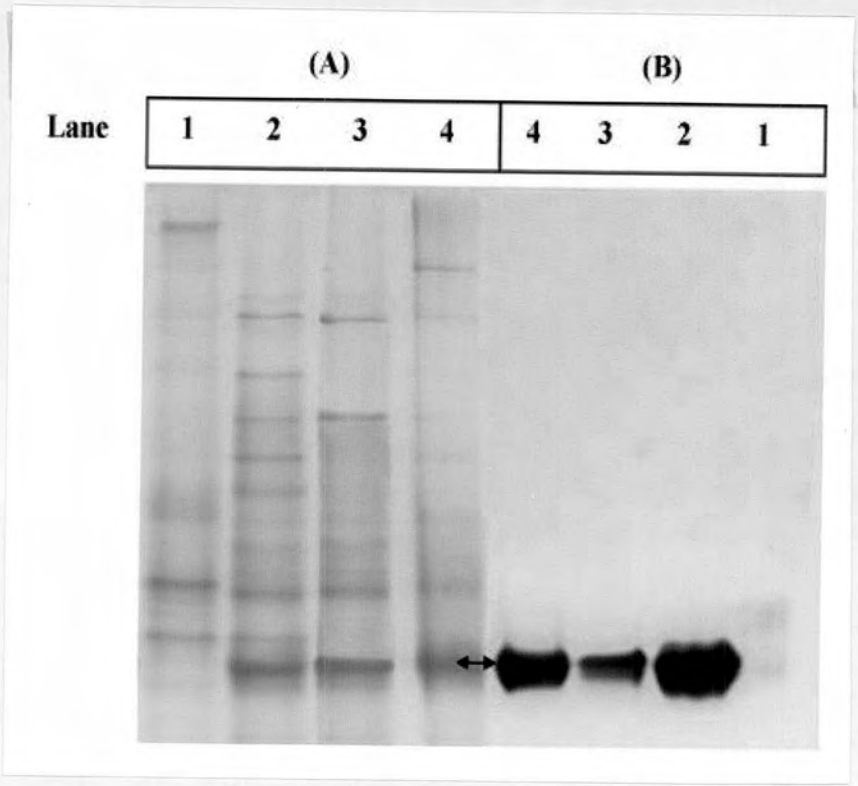


Figure 4.12 Non-denaturing PAGE analysis of PPG dehydrogenase in each step of partial purification from *Pseudomonas* sp. PE-2 on a 7.5% acrylamide gel (A) Coomassie blue staining, (B) PEG dehydrogenase activity staining. Each lane represents 30 μg protein of :

Lane 1: Cell-free extract

Lane 2: 60-80% ammonium sulfate precipitation

Lane 3: DEAE-650M Toyopearl

Lane 4: Butyl-Toyopearl

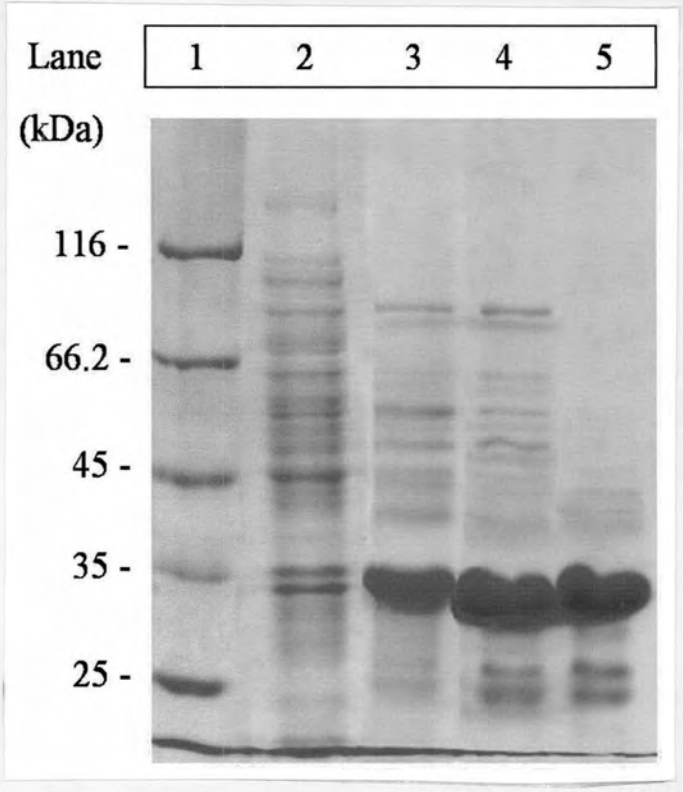


Figure 4.13 SDS-PAGE analysis of PPG dehydrogenase in each step of partial purification from *Pseudomonas* sp. PE-2 on a 10% acrylamide gel

Lane 1: Protein molecular weight marker
(Fermentas, #SM 0431 lot. 1811)
[β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-galactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa)]

Lane 2: Cell-free extract (30 μg protein)

Lane 3: 60-80% ammonium sulfate precipitation (30 μg protein)

Lane 4: DEAE-650M Toyopearl (30 μg protein)

Lane 5: Butyl-Toyopearl (30 μg protein)

4.5 Characterization of partially purified PEG dehydrogenase and PPG dehydrogenase

4.5.1 Molecular weight determination of native PEG dehydrogenase and PPG dehydrogenase by polyacrylamide gel electrophoresis (PAGE)

The native molecular weight of PEG-DH and PPG-DH were determined from molecular weight calibration curve obtained from a logarithmic function of the relative standard protein mobility on PAGE as mentioned in section 3.4.6.5.1. The PEG-DH and PPG-DH were found to have the native molecular weight of 73,600 and 66,200 Da, respectively (Figure 4.14 and 4.15 and Appendix G). The molecular weight of PEG-DH and PPG-DH subunits were also determined by SDS-PAGE which included a series of standard proteins in each run (Figure 4.16, 4.17 and 4.18). From the mobility on SDS-PAGE, the molecular weight of the PEG-DH was 73,400 Da which coincided with the native molecular weight from PAGE. These results indicated that the PEG-DH might be a monomeric protein. The molecular weight of the PPG-DH from the mobility on SDS-PAGE was 36,100 Da which was about half of the native molecular weight from PAGE. These results showed that the PPG-DH might be a homodimeric protein.

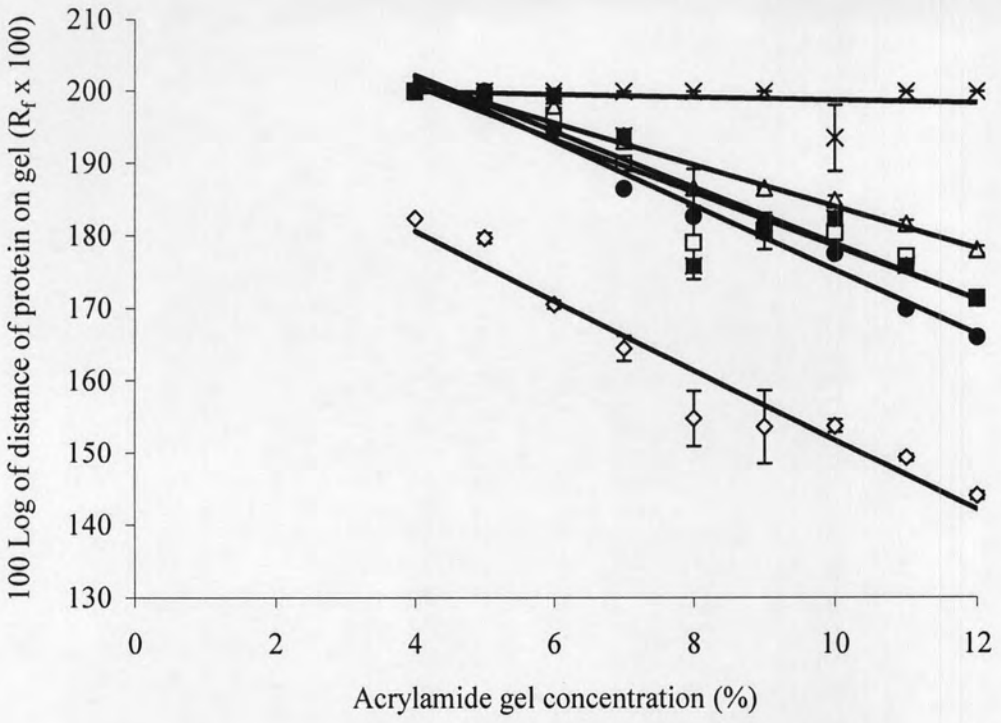


Figure 4.14 Logarithmic curves of the protein mobility in various concentrations of acrylamide gel

Standard protein	Molecular weight (Da)	Slope	R^2
holo-Transferrin (—◇—)	80,000	-4.81	0.94
Bovine serum albumin (—□—)	66,000	-3.69	0.91
Ovalbumin (—△—)	45,000	-2.89	0.96
Trypsin inhibitor (—✕—)	21,000	-0.19	0.91

Enzyme	Molecular weight (Da)	Slope	R^2
PEG dehydrogenase (—●—)	73,600	-4.44	0.98
PPG dehydrogenase (—■—)	66,200	-3.88	0.90

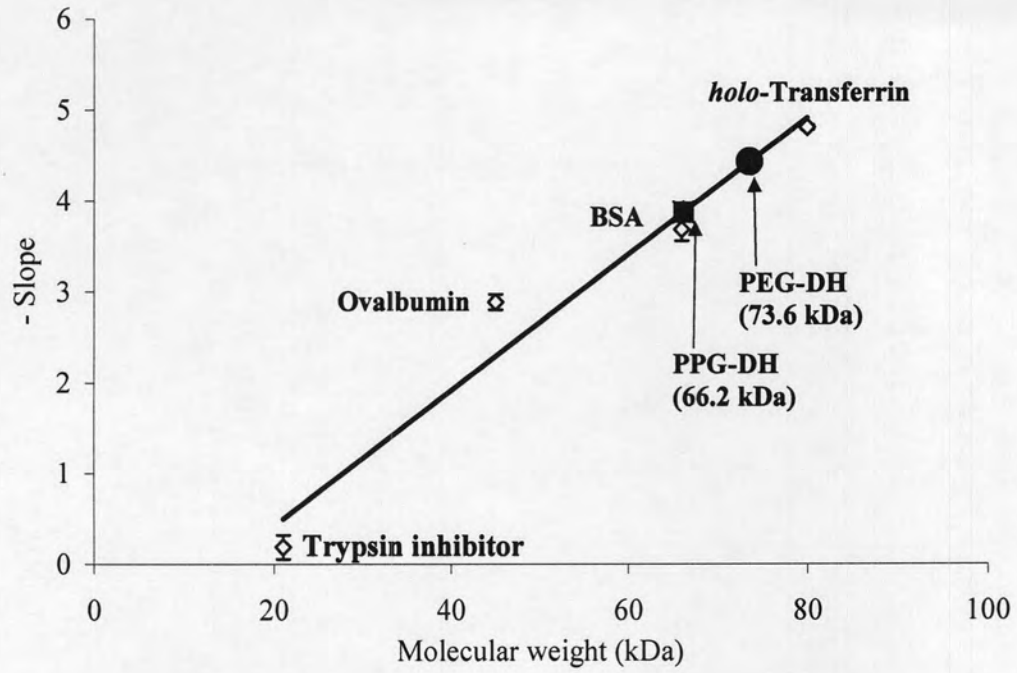


Figure 4.15 Calibration curve for native molecular weight of PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 determined by polyacrylamide gel electrophoresis (PAGE)

The calibration curve was plotted between –slope obtained from Figure 4.14 and molecular weight of each standard protein as shown below. The molecular weight of PEG-DH and PPG-DH were determined as indicated by arrows.

Standard protein	**Molecular weight (Da)**
holo-Transferrin	80,000
Bovine serum albumin	66,000
Ovalbumin	45,000
Trypsin inhibitor	21,000

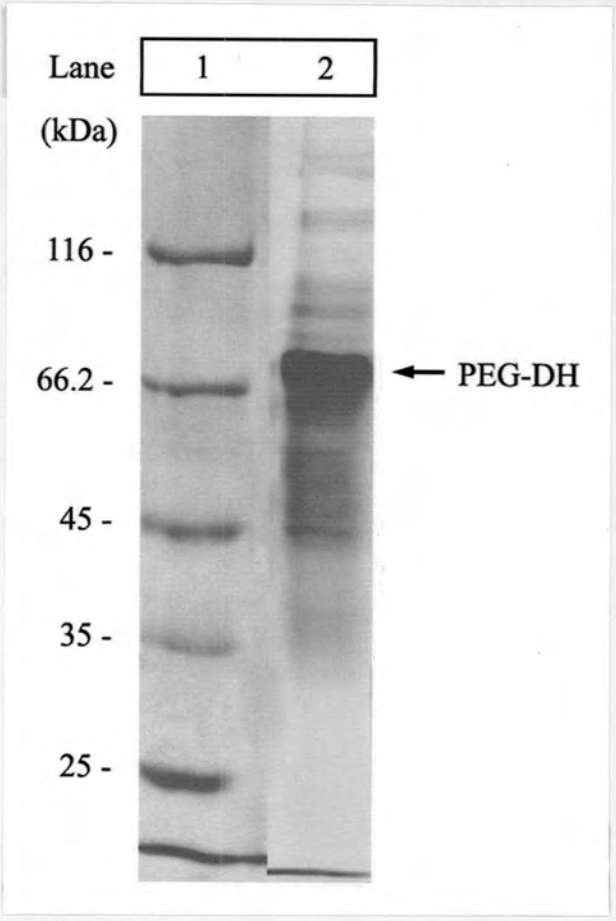


Figure 4.16 SDS-polyacrylamide gel electrophoresis of PEG-DH from *Pseudomonas* sp. PE-2

Lane 1: Protein molecular weight marker

(Fermentas, #SM 0431 lot. 1811)

[β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-galactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa)]

Lane 2: Partially purified PEG-DH (30 μg protein)

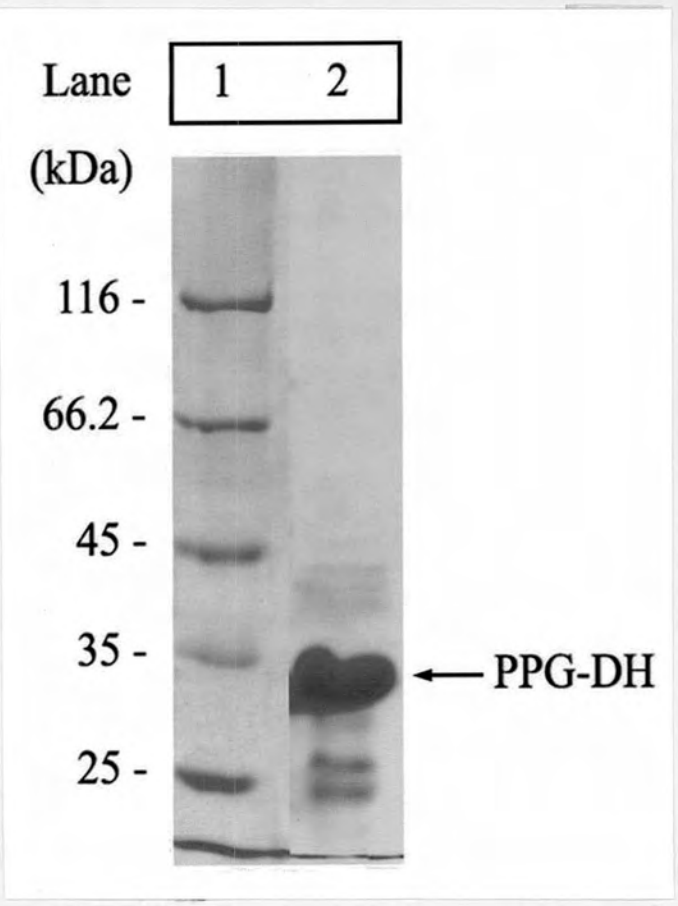


Figure 4.17 SDS-polyacrylamide gel electrophoresis of PPG-DH from *Pseudomonas* sp. PE-2

Lane 1: Protein molecular weight marker

(Fermentas, #SM 0431 lot. 1811)

[β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-galactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa)]

Lane 2: Partially purified PPG-DH (30 μg protein)

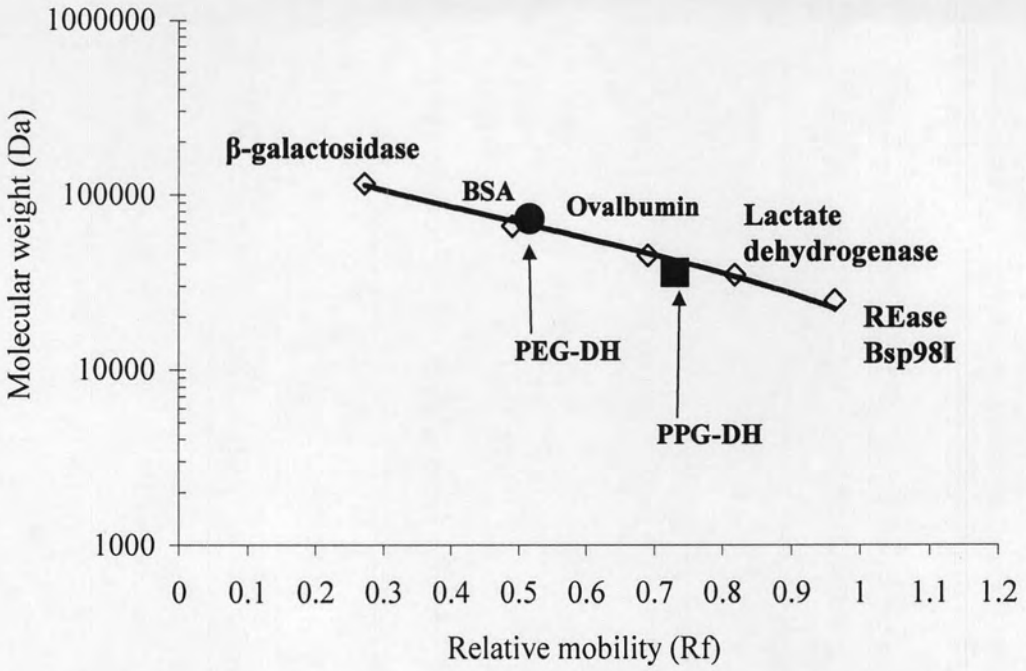


Figure 4.18 Calibration curve for molecular weight of PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2 on SDS-polyacrylamide gel electrophoresis

Standard protein	Molecular weight (Da)
β-galactosidase	116,000
Bovine serum albumin	66,200
Ovalbumin	45,000
Lactate dehydrogenase	35,000
REase Bsp98I	25,000

Arrow indicates the R_f of PEG-DH and PPG-DH.

4.5.2 Effect of pH on PEG dehydrogenase and PPG dehydrogenase activities

The optimum pH of PEG-DH and PPG-DH were determined as mentioned in section 3.4.6.5.2. Activities of the enzyme at different pHs was shown in Figure 4.19. In this study, the 0.1 M of acetate, phosphate, Tris-HCl and glycine-NaOH were used as reaction buffers for pH 4-5.5, 5.5-8.0, 8.0-9.0 and 9.0-11.0, respectively. The optimal pH of the PEG-DH and PPG-DH were pH 9.0 and pH 7.5, respectively. The PEG-DH showed more than 100% of the activity at pH 8.5-9.0, 40-90% of the activity at pH 7.5-8.0 and 9.5-10.0 while the activity was barely observed at pH below 6.5 or above 10.5. The PPG-DH showed more than 100% of the activity at pH 7.5-8.0, 40-90% of the activity at pH 6.5-7.0 and 8.0-9.0 while the activity was observed within 0-18% at pH below 6.0 or above 9.5.

4.5.3 Effect of temperature on PEG dehydrogenase and PPG dehydrogenase activities

The optimum temperature of the PEG-DH and PPG-DH were investigated by incubating the reaction mixture at various temperatures for 3 minutes (section 3.4.6.5.3). Both enzymes showed the highest activity at 25°C which was defined as 100%. The PEG-DH activity at 60°C still remained 66%. For the PPG-DH activity at 60°C still remained 62% (Figure 4.20).

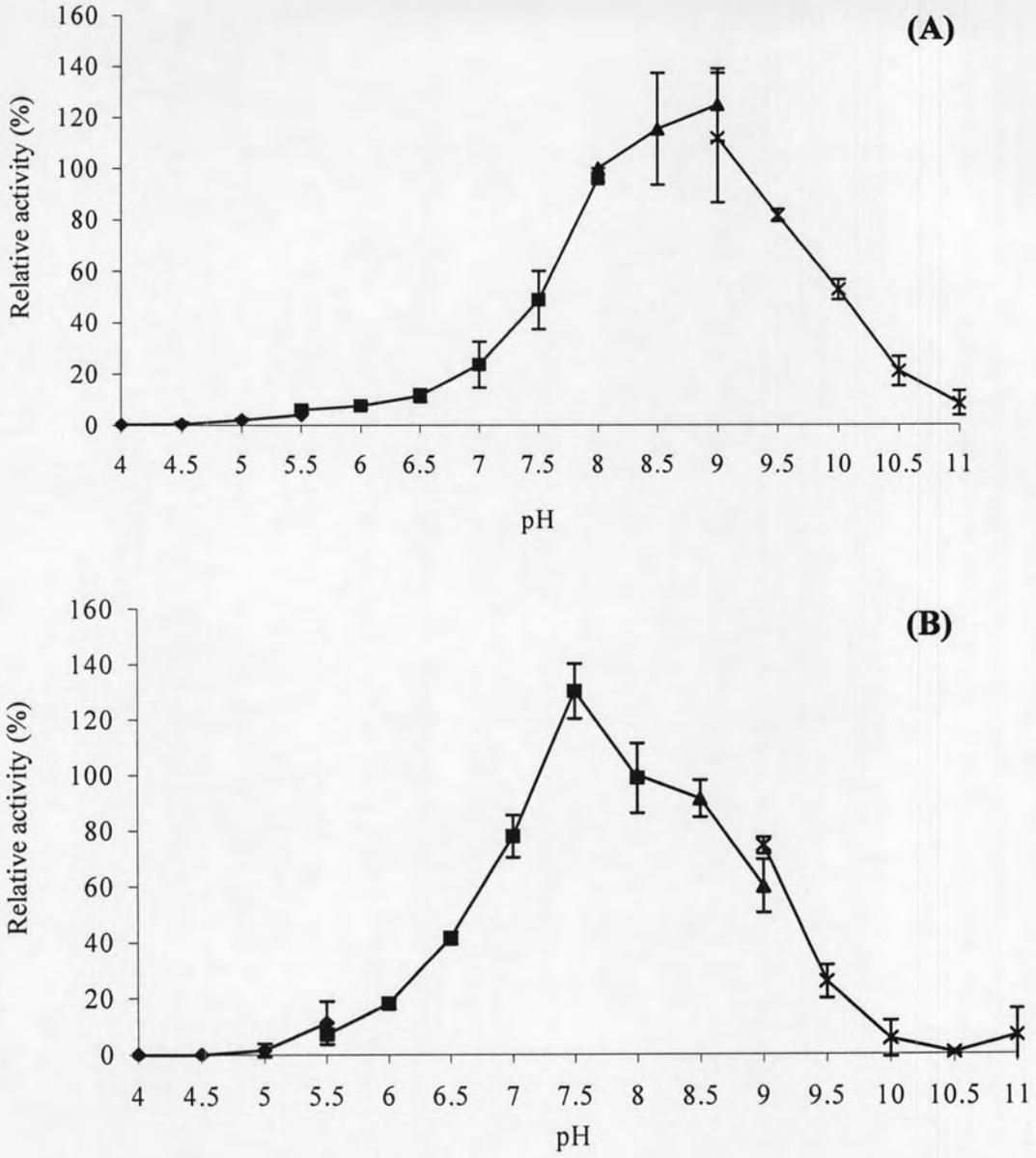


Figure 4.19 Effect of pH on PEG dehydrogenase (A) and PPG dehydrogenase (B) activities

Buffers used: 0.1 M of acetate buffer, pH 4.0-5.5 (—◆—); 0.1 M of potassium phosphate buffer, pH 5.5-8.0 (—■—); 0.1 M of Tris-HCl buffer, pH 8.0-9.0 (—▲—) and 0.1 M glycine-NaOH buffer, pH 9.0-11.0 (—✕—). The activity obtained in Tris-HCl buffer, pH 8.0, at room temperature, was used as 100% activity.

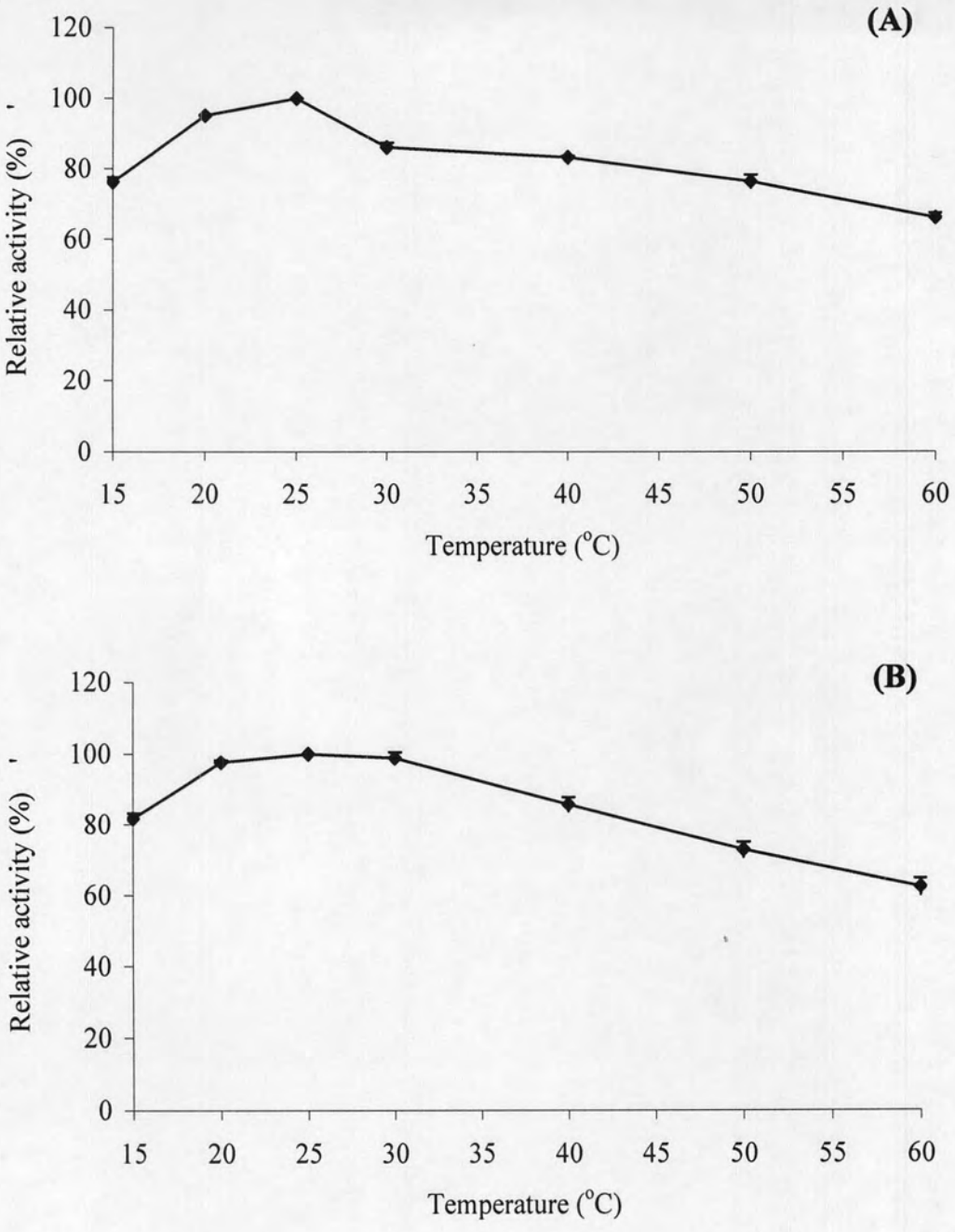


Figure 4.20 Effect of temperature on PEG dehydrogenase (A) and PPG dehydrogenase (B) activities

The PEG-DH and PPG-DH activities were measured at various temperatures as described in section 3.4.6.5.3. The activity obtained in Tris-HCl buffer, pH 8.0, at 25°C was used as 100% activity.

4.5.4 pH Stability of PEG dehydrogenase and PPG dehydrogenase activities

The pH stability of the PEG-DH and PPG-DH were measured by incubating the enzyme at 4°C for 30 minutes in 50 mM buffers, pHs from 4.0 to 11.0 (the total volume of 1 ml) before the residual enzyme activity was measured as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). Both enzymes were stable in the pH range of 8 to 9.5 and 7 to 8, respectively (Figure 4.21).

4.5.5 Temperature stability of PEG dehydrogenase and PPG dehydrogenase activities

The temperature stability of PEG-DH and PPG-DH were investigated by preincubation at 25, 30, 40, 50 and 60°C for 30 minutes followed by the measurement of residual PEG-DH and PPG-DH activities under the assay conditions as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). The temperature stability of both enzymes was in the range of 25°C to 30°C (Figure 4.22). The result showed that PPG-DH was more stable than PEG-DH.

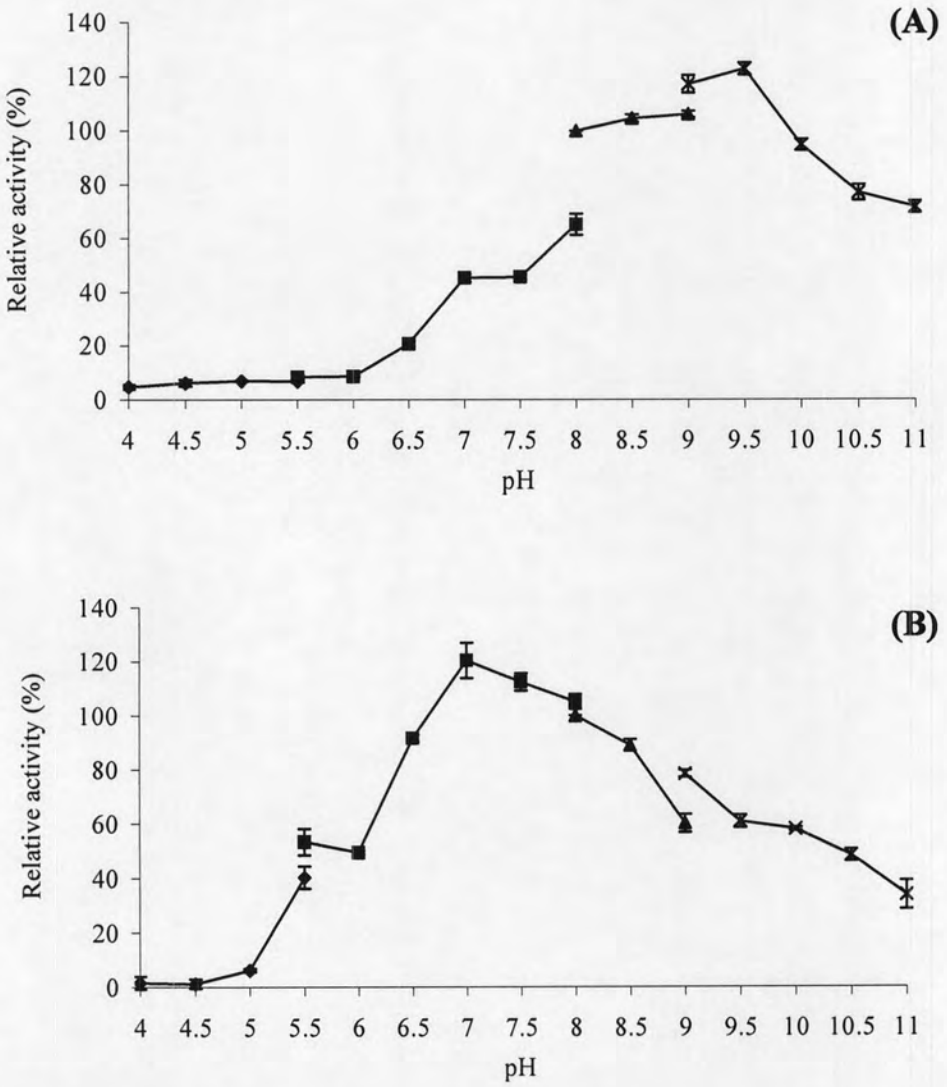


Figure 4.21 pH Stability of PEG dehydrogenase (A) and PPG dehydrogenase (B)

The PEG-DH and PPG-DH were incubated in 50 mM acetate buffer, pH 4.0-5.5 (—◆—); 50 mM potassium phosphate buffer, pH 5.5-8.0 (—■—); 50 mM of Tris-HCl buffer, pH 8.0-9.0 (—▲—) and 50 mM glycine-NaOH buffer, pH 9.0-11.0 (—✕—) at 4°C for 30 minutes. The residual enzyme activity was measured as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively. Both enzyme activities incubated in Tris-HCl buffer, pH 8.0, was used as 100% activity.

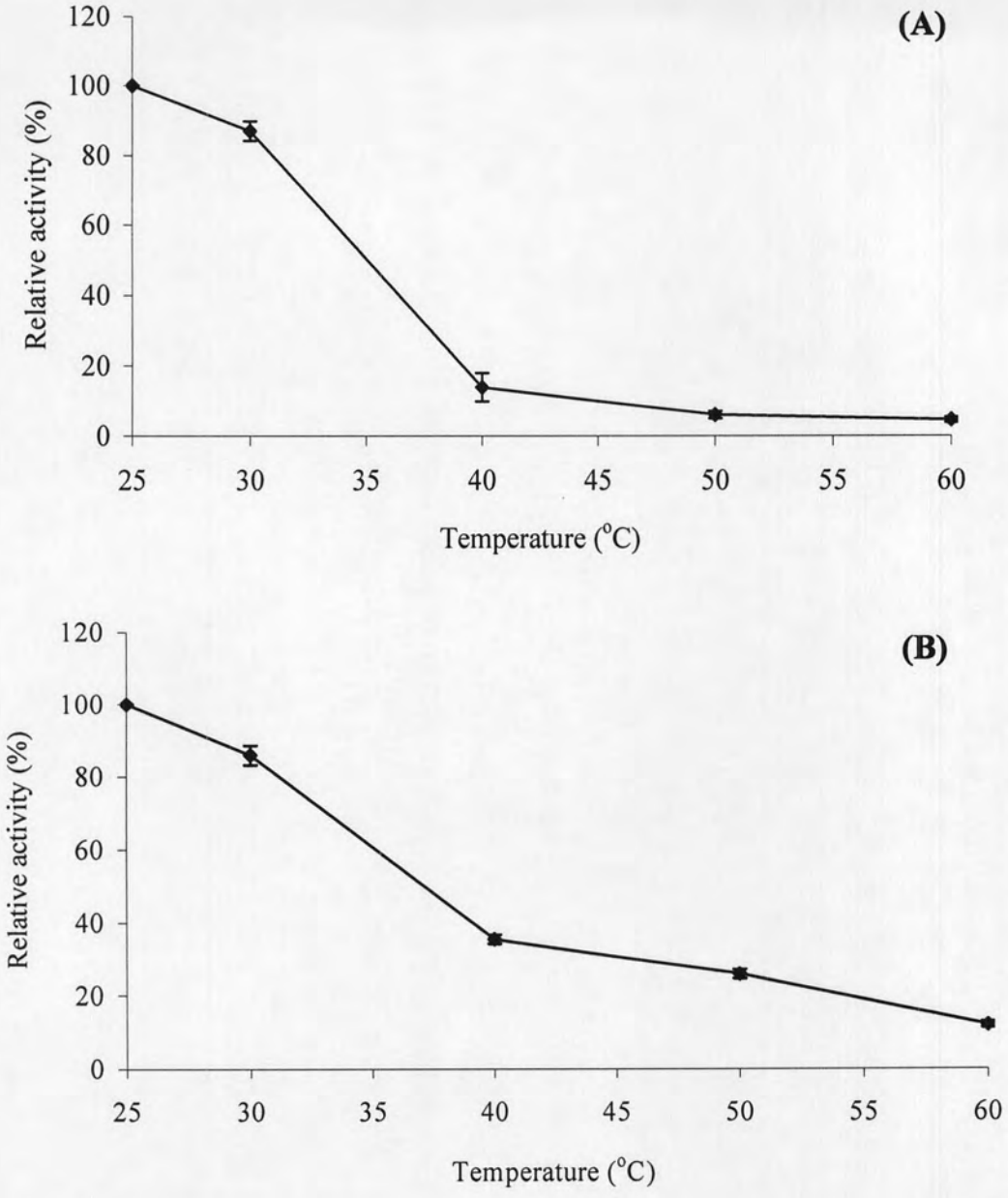


Figure 4.22 Temperature stability of PEG dehydrogenase (A) and PPG dehydrogenase (B)

The PEG-DH and PPG-DH were preincubated at temperature 25, 30, 40, 50 and 60°C for 30 minutes followed by the measurement of residual PEG-DH and PPG-DH activities under the assay conditions as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). Both enzyme activities preincubated at 25°C were used as 100% activity.

4.5.6 Effect of various electron acceptors on PEG dehydrogenase and PPG dehydrogenase activities

The PEG-DH and PPG-DH activities were assayed in the presence of various electron acceptors (DCIP, NAD and FAD) as described in section 3.4.6.5.6. The effect of various electron acceptors on PEG-DH and PPG-DH activities were summarized in Table 4.5. The PEG-DH and PPG-DH reacted readily with various electron acceptors such as DCIP and FAD in the presence of PMS and also with NAD. However, the PEG-DH activities decreased when used only FAD as the electron acceptor. Moreover, PQQ, NAD and FAD acted as the cofactor of the enzyme. For PEG-DH and PPG-DH, PQQ was the best cofactor (Table 4.5).

Table 4.5 Effect of various electron acceptors on the activity of the partially purified PEG dehydrogenase (A) and PPG dehydrogenase (B)

(A)

Electron acceptor	Specific activity (nmol min^{-1} mg $protein^{-1}$)	Specific activity (fold)
PMS+DCIP	7.4±3.1	1.0
PQQ/PMS+DCIP	**196.9±9.5**	**26.4**
NAD	24.7±1.7	3.3
FAD	4.2±1.5	0.6
FAD+PMS	11.2±2.1	1.5

(B)

Electron acceptor	Specific activity (nmol min^{-1} mg $protein^{-1}$)	Specific activity (fold)
PMS+DCIP	4.1±1.7	1.0
PQQ/PMS+DCIP	**211.0±12.0**	**50.9**
NAD	36.7±2.2	8.9
FAD	8.0±2.7	1.9
FAD+PMS	17.3±2.9	4.2

4.5.7 Effect of divalent metal ions on PEG dehydrogenase and PPG dehydrogenase activities

The PEG-DH and PPG-DH activities were assayed in the presence of various concentrations of divalent metal ions (2 mM, 5 mM and 10 mM) as described in section 3.4.6.5.7. The effect of divalent metal ions on PEG-DH and PPG-DH activities were summarized in Table 4.6. The PEG-DH activity was completely inhibited by Co^{2+}, Cu^{2+} and Hg^{2+} at all concentrations. Mg^{2+} was a less potent inhibitor of the PEG-DH with 7-18% inhibition, while Mn^{2+} inhibited the PEG-DH about 78-90%. Moreover, Ni^{2+} was barely complete inhibited the PEG-DH with 90-95%.

The PPG-DH activity was completely inhibited by Cu^{2+} at all concentrations. Co^{2+}, Hg^{2+} and Mn^{2+} inhibited the PPG-DH activity in the range of 31-86%, 87-94% and 80-96%, respectively. Mg^{2+} was a less potent inhibitor of the PPG-DH with a 3-8% inhibition at all concentrations. On the other hand, Ni^{2+} was shown to be an enzyme stimulator. Ni^{2+} could enhance the PPG-DH activity by 31-54% at 2-10 mM concentrations.

Table 4.6 Effect of divalent metal ions on the activity of the partially purified PEG dehydrogenase (A) and PPG dehydrogenase (B)

(A)

Divalent metal ions	Relative activity (%)		
	2 mM	5 mM	10 mM
Ca^{2+}	100	100	100
Co^{2+}	0.2±0.1	0	0
Cu^{2+}	0	0	0
Hg^{2+}	0	0	0
Mg^{2+}	93.2±0.2	92.4±0.6	82.8±4.2
Mn^{2+}	10.9±1.3	13.5±5.2	22.6±10.9
Ni^{2+}	13.4±2.3	5.8±0.6	12.0±2.6

(B)

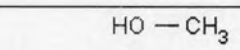
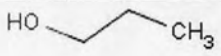
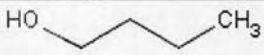
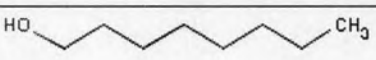
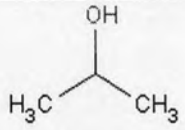
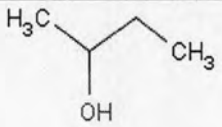
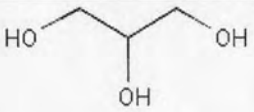
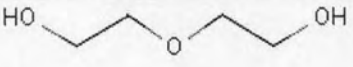
Divalent metal ions	Relative activity (%)		
	2 mM	5 mM	10 mM
Ca^{2+}	100	100	100
Co^{2+}	69.2±6.2	62.0±3.3	14.9±6.1
Cu^{2+}	0.9±1.3	0.6±0.9	0
Hg^{2+}	13.1±2.6	13.9±2.8	6.9±4.0
Mg^{2+}	97.6±9.1	93.2±3.6	93.2±4.0
Mn^{2+}	20.7±2.7	14.4±7.1	4.4±2.4
Ni^{2+}	**139.0±7.0**	**154.0±12.3**	**131.3±9.3**

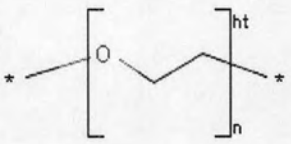
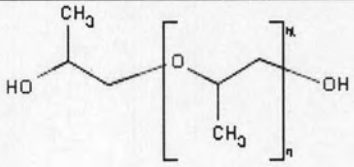
4.5.8 Substrate specificity of PEG dehydrogenase and PPG dehydrogenase activities

The determination of substrate specificity of PEG-DH and PPG-DH towards various hydroxyl compounds was performed. Among the alcohol substrates tested, both enzymes preferred relatively longer chain primary alcohols than the shorter ones. For example, PEG-DH showed much higher relative activity (215.85±12.37%) with 1-pentanol while 7.85±1.20% was obtained with 1-butanol.

When various PEGs and PPGs with different molecular weight were used as substrate, it was found that PEG and PPG at lower molecular weight was preferred. Ethylene glycol, diethylene glycol and secondary alcohols such as 2-propanol and 2-butanol were oxidized to some extent, while glycerol was not a substrate for both enzymes (Table 4.7).

Table 4.7 Relative activity of PEG-DH and PPG-DH towards various hydroxyl compounds

Substrate[a]	Structure	Relative activity[b] (%)	
		PEG-DH	PPG-DH
Methanol		7.2±0.3	5.4±2.8
Ethanol		8.1±1.0	5.3±2.3
1-Propanol		5.5±0.3	22.4±4.9
1-Butanol		7.8±1.2	88.0±9.7
1-Pentanol		**215.8±12.4**	45.5±1.6
1-Hexanol		**183.3±8.8**	94.6±0.3
1-Heptanol		**154.2±12.2**	87.4±0.6
1-Octanol		47.6±0.8	87.0±3.6
2-Propanol		5.5±0.3	25.0±1.3
2-Butanol		6.0±3.6	14.3±0.7
Glycerol		0	0
Ethylene glycol		6.8±2.2	8.0±0.8
Diethylene glycol		28.8±3.6	14.0±9.4

Substrate[a]	Structure	Relative activity[b] (%)	
		PEG-DH	PPG-DH
PEG 600		**177.0±7.8**	13.2±8.2
PEG 2000		100	8.0±0.8
PEG 4000		60.0±8.0	7.2±0.4
PEG 6000		47.5±7.6	6.3±1.6
PEG 8000		52.8±10.1	4.4±1.1
PPG 725		8.1±3.4	**126.8±3.9**
PPG 1000		9.8±5.9	100
PPG 2000		0	95.8±0.6

[a]Final concentration, 10 mM

[b]The PEG-DH and PPG-DH activities towards PEG 2000 and PPG 1000 were defined as 100%, respectively.

4.5.9 Determination of kinetic parameters of PEG dehydrogenase and PPG dehydrogenase activities

The typical Lineweaver-Burk plot was shown for PEG 600, PEG 2000, PEG 4000, PEG 6000 and PEG 8000 as the substrate of PEG-DH (Figure 4.23, 4.24, 4.25, 4.26 and 4.27, respectively) and PPG 725, PPG 1000 and PPG 2000 as the substrate of PPG-DH (Figure 4.28, 4.29 and 4.30, respectively). Kinetic parameters of PEG-DH and PPG-DH with various substrates were summarized in Table 4.8. The V_{max}/K_m values of PEG-DH for PEG 600, PEG 2000 PEG 4000 PEG 6000 and PEG 8000 were calculated to be 133.7, 22.7, 16.9, 6.0 and 4.5 nmol min^{-1} mg $protein^{-1}$ mM^{-1}, respectively, whereas V_{max}/K_m values of PPG-DH for PPG 725, PPG 1000 and PPG 2000 were about 7.6, 10.9 and 2.6 nmol min^{-1} mg $protein^{-1}$ mM^{-1}, respectively. This result indicated that PEG-DH was more active towards PEG 600, PEG 2000 and PEG 4000 than PEG 6000 and PEG 8000, while PPG-DH was more active towards PPG 725 and PPG 1000 than PPG 2000.

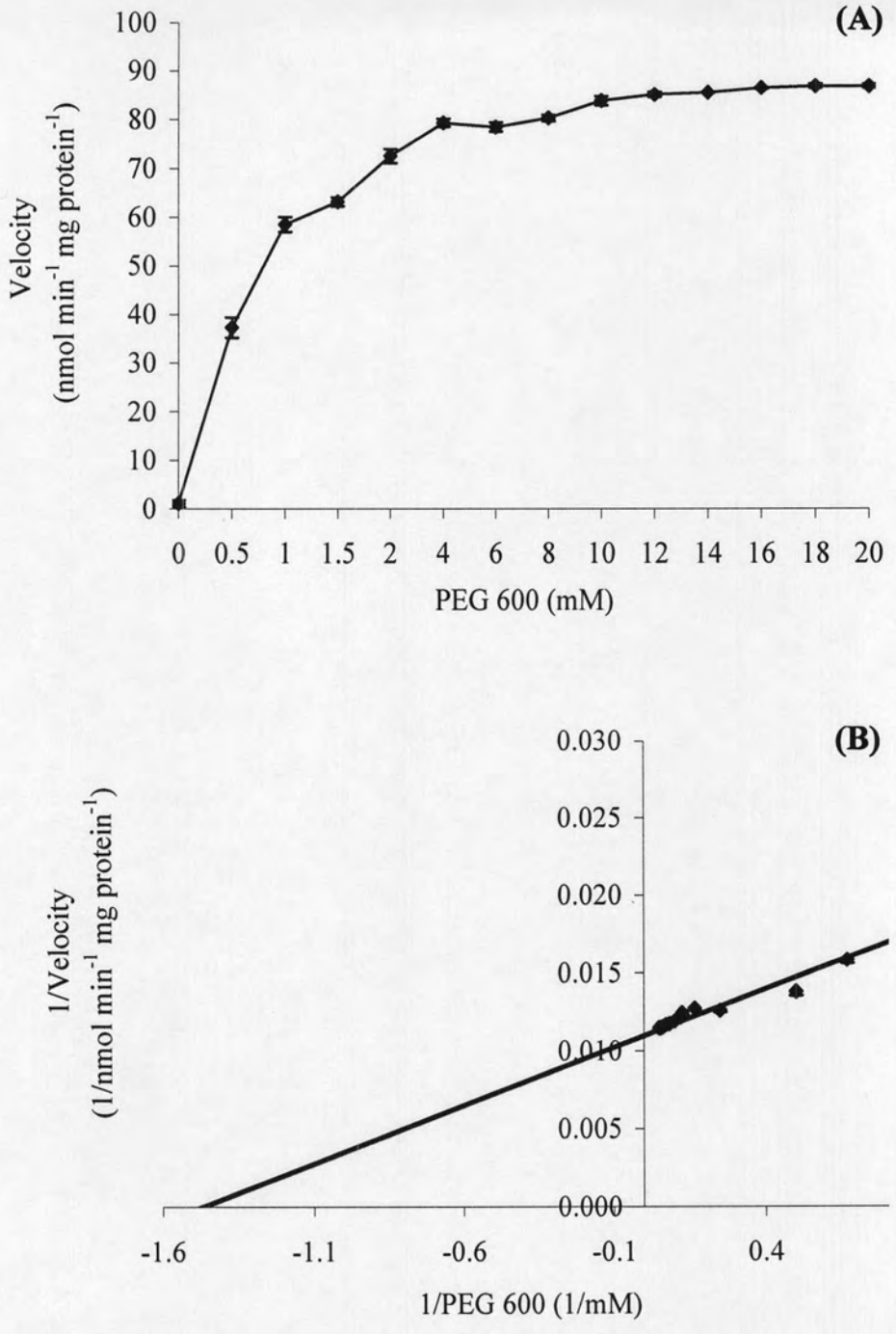


Figure 4.23 Kinetic studies of PEG-DH with PEG 600 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

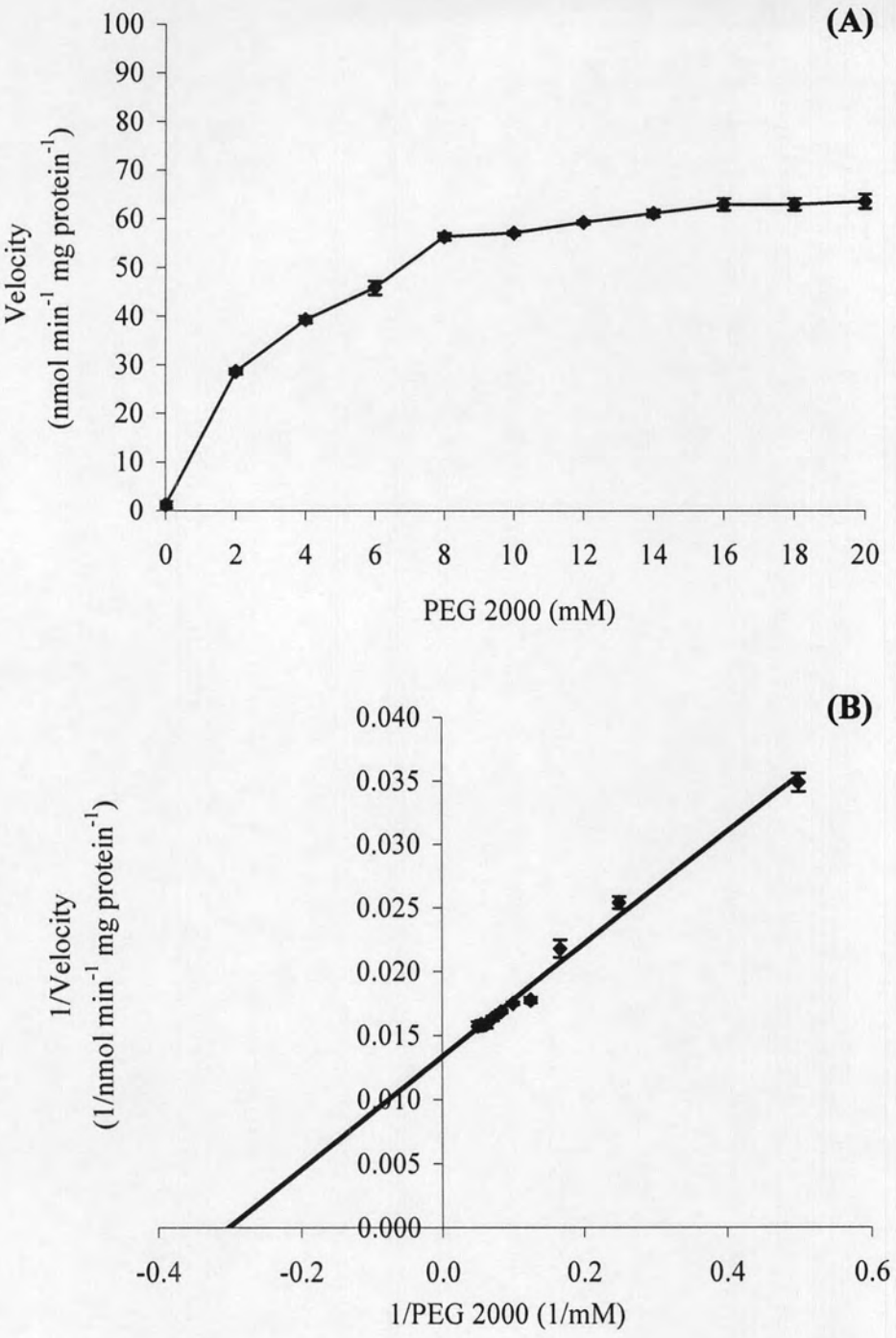


Figure 4.24 Kinetic studies of PEG-DH with PEG 2000 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

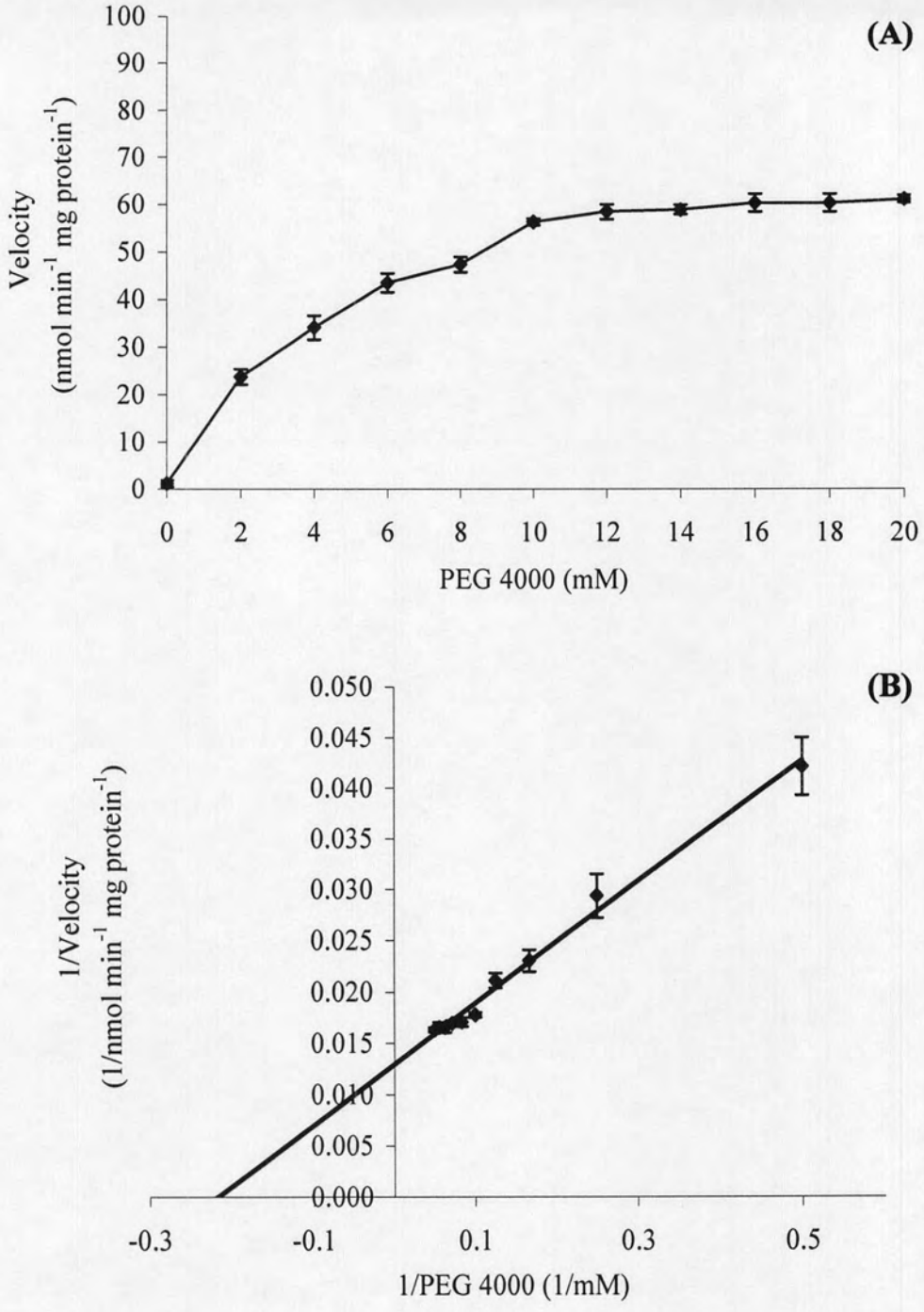


Figure 4.25 Kinetic studies of PEG-DH with PEG 4000 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

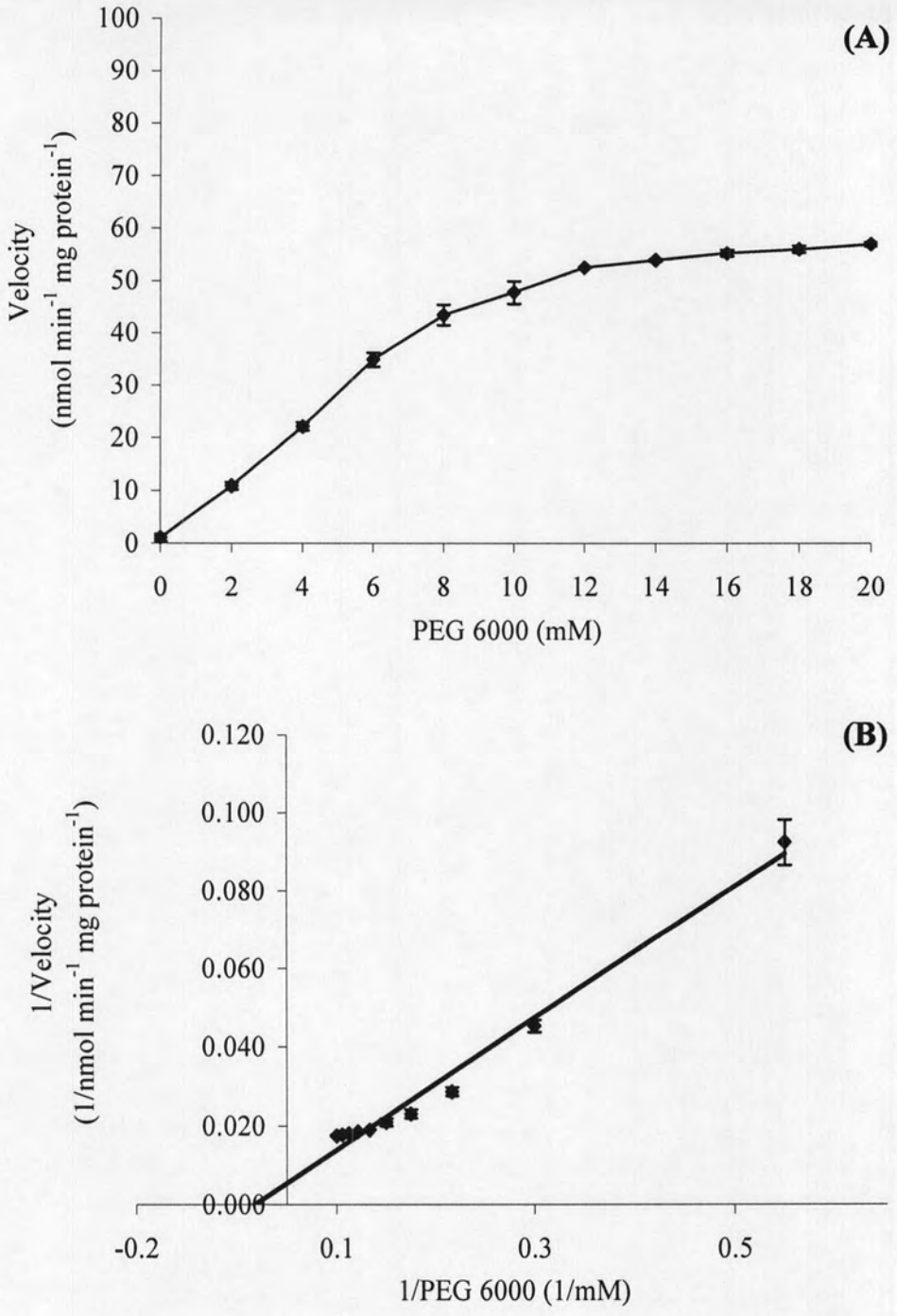


Figure 4.26 Kinetic studies of PEG-DH with PEG 6000 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

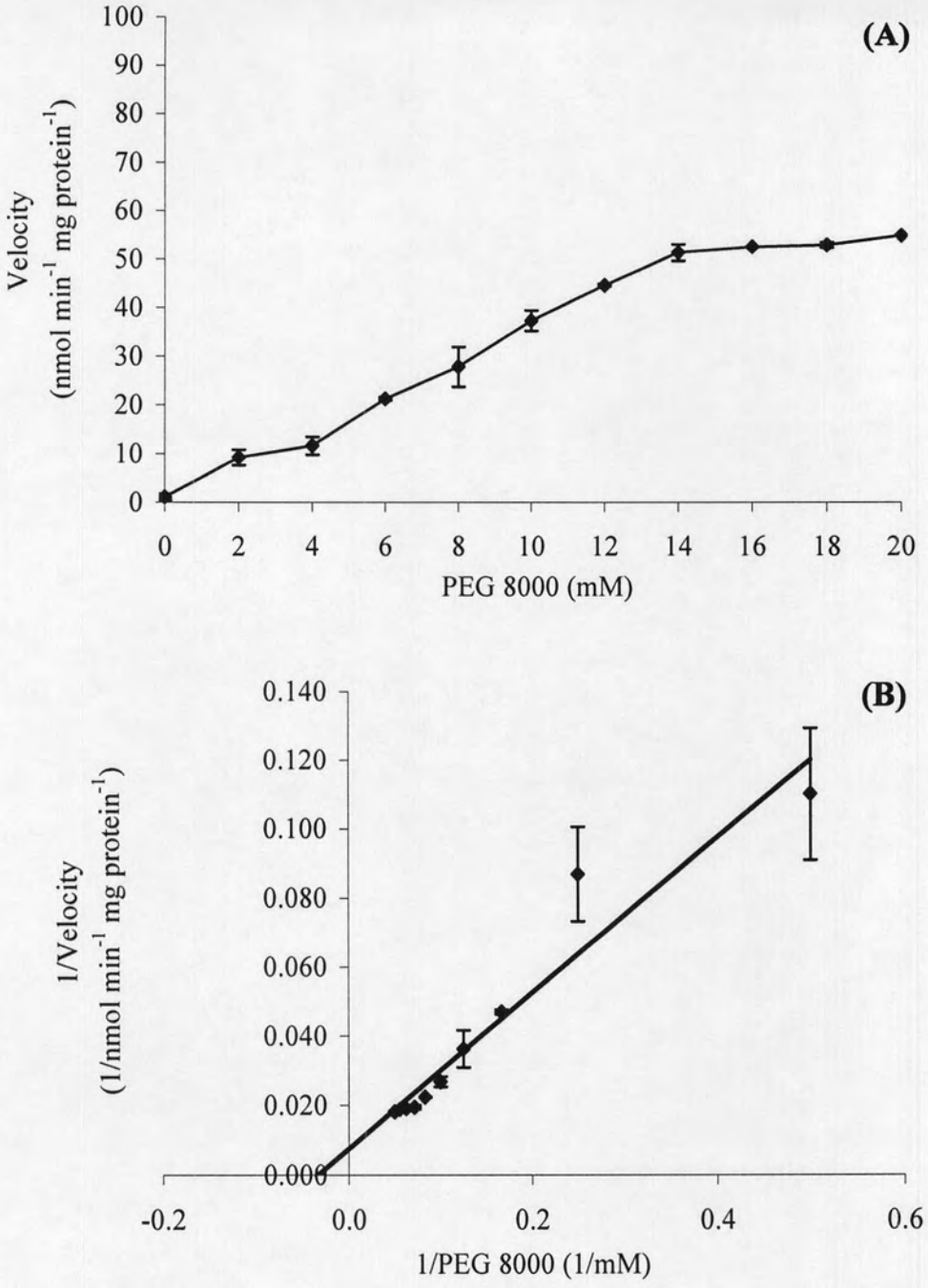


Figure 4.27 Kinetic studies of PEG-DH with PEG 8000 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

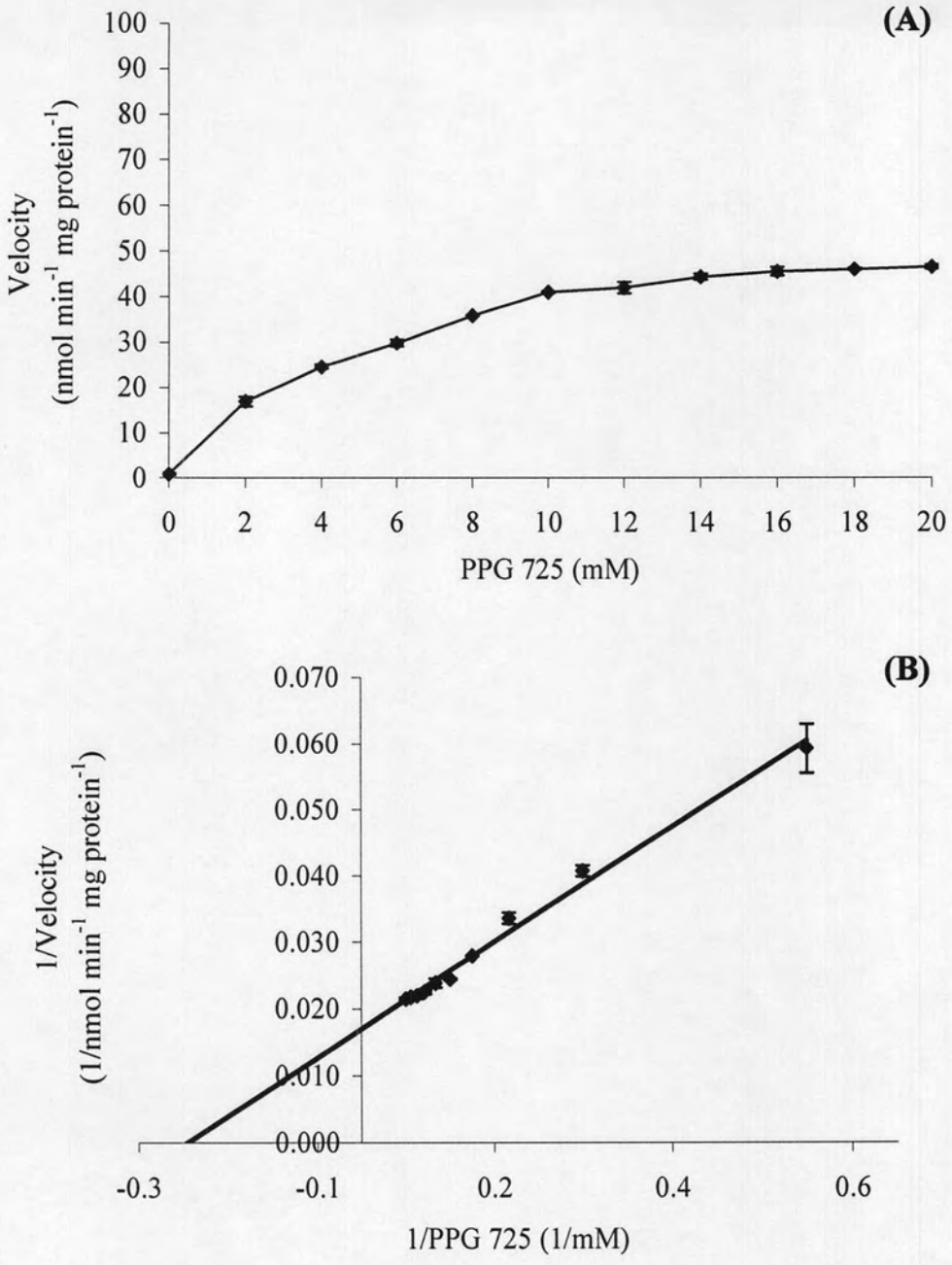


Figure 4.28 Kinetic studies of PPG-DH with PPG 725 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

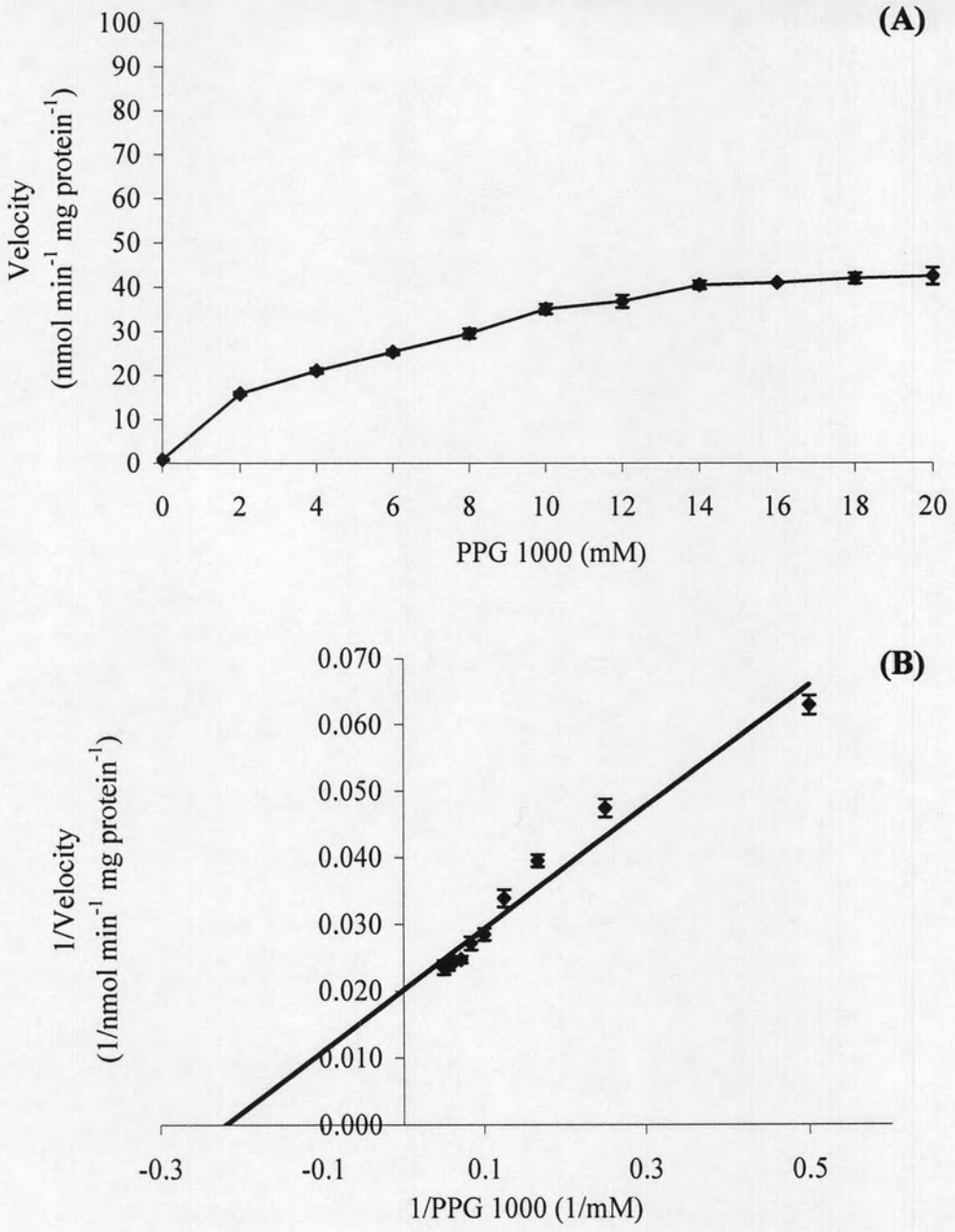


Figure 4.29 Kinetic studies of PPG-DH with PPG 1000 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

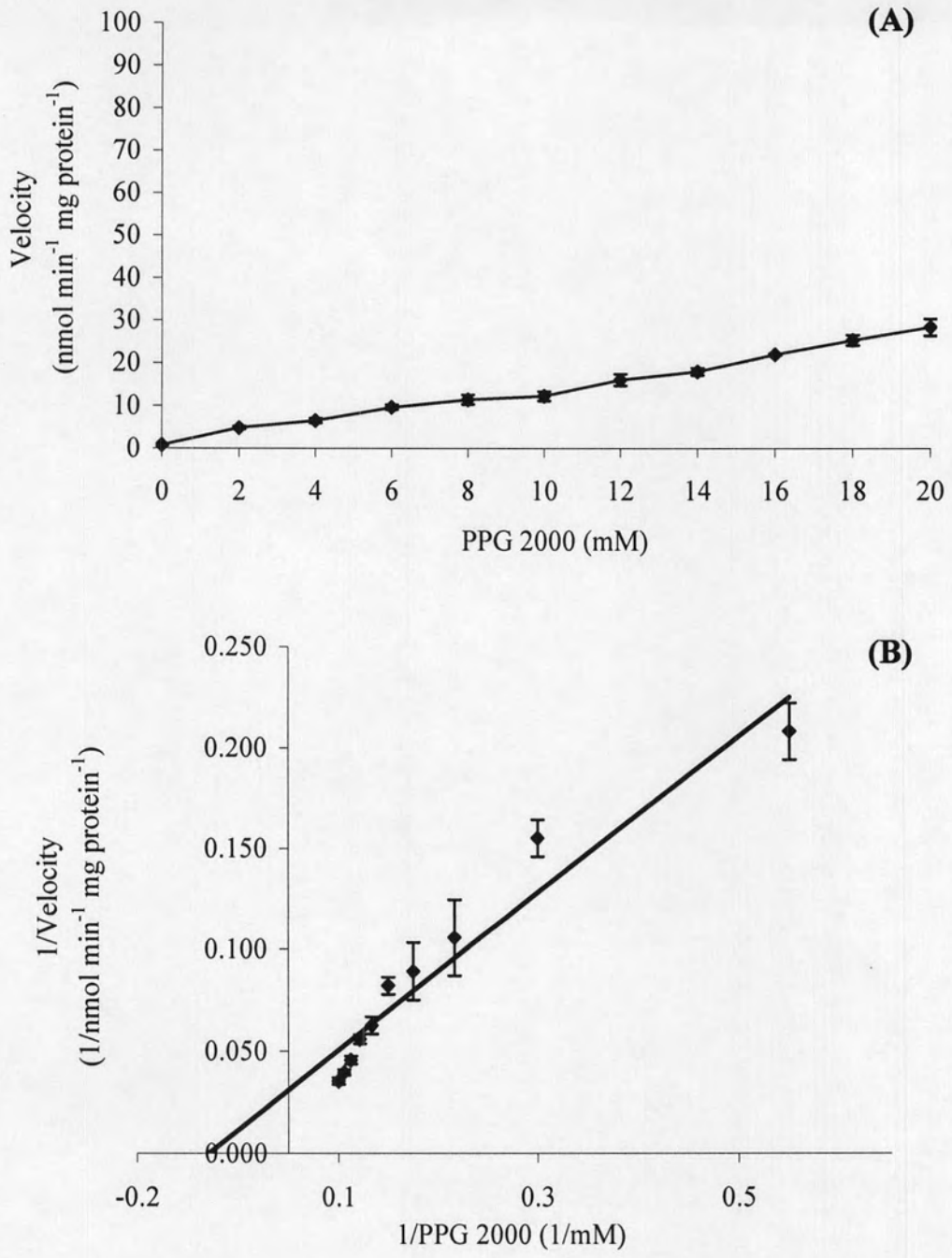


Figure 4.30 Kinetic studies of PPG-DH with PPG 2000 as a substrate

A) Saturation curve

B) Lineweaver-Burk plot

Table 4.8 Kinetic parameters of PEG-DH and PPG-DH from *Pseudomonas* sp. PE-2

Enzyme	Substrate	V_{max} (nmol min^{-1} mg $protein^{-1}$)	K_m (mM)	V_{max}/K_m (nmol min^{-1} mg $protein^{-1}$ mM^{-1})
PEG-DH	PEG 600	90.9	0.7	133.7
	PEG 2000	75.2	3.3	22.7
	PEG 4000	76.9	4.6	16.9
	PEG 6000	181.8	30.5	6.0
	PEG 8000	128.2	28.3	4.5
PPG-DH	PPG 725	38.8	5.1	7.6
	PPG 1000	49.8	4.6	10.9
	PPG 2000	32.6	12.6	2.6