CHAPTER III

RESULTS

3.1 Optimization for phenylalanine dehydrogenase production of bacterial strain BC1 and BC2

Thermotolerant bacterial strain BC1 and BC2 from soil, which were screened for NAD^+-dependent phenylalanine dehydrogenase activity by Suriyapanpong *et al.*[73] were studied in the aspect of optimal condition for enzyme production.

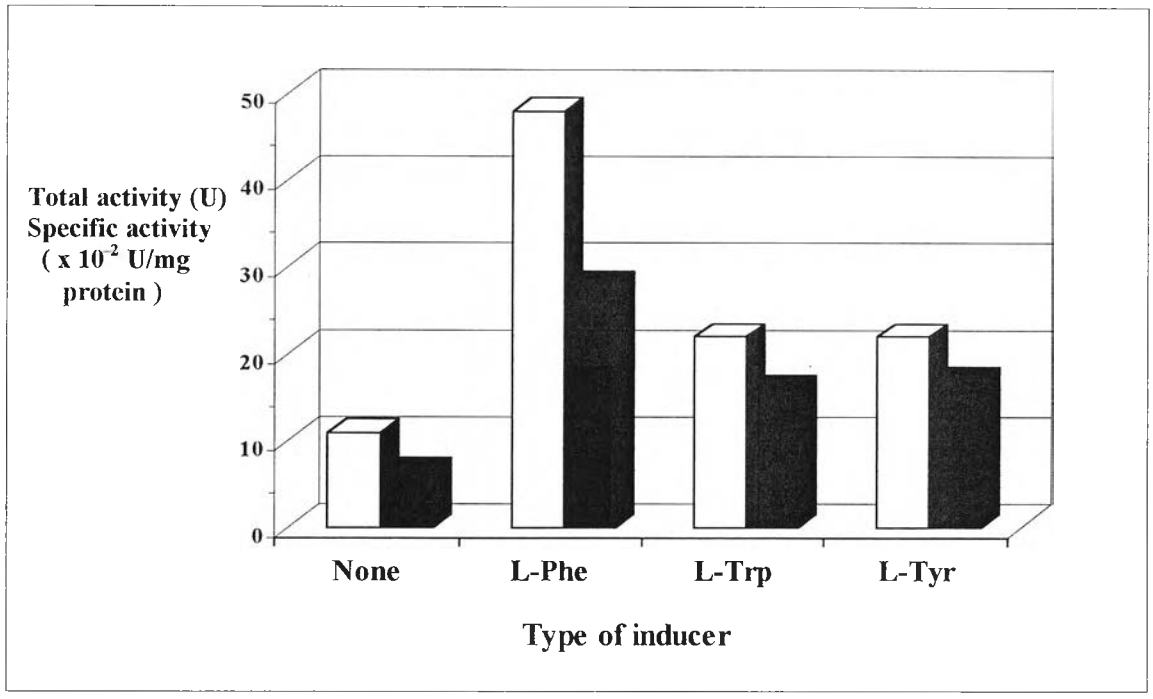
3.1.1 Enzyme induction by amino acids

As a first approach to obtain higher yield of PheDH, the enzyme inducer was studied. Induction of PheDH during growth on different amino acids was carried out as described in section 2.7.1. For strain BC1, Figure 3.1A showed that the formation of PheDH could be induced to 4.4, 2.0 and 2.0 folds by 0.2 % of L-phenylalanine, L-tryptophan and L-tyrosine supplements in 1% peptone medium, pH 7.2, respectively. The highest total activity and specific activity were obtained by 0.2 % L-phenylalanine supplement with 48 units and 0.28 units/mg protein, respectively.

For strain BC2, Figure 3.1B indicated that the production of PheDH could be induced to 3.7 and 1.2 folds by 0.2 % of L-phenylalanine and L-tyrosine supplements in 1% peptone medium, pH 7.2, respectively whereas L-tryptophan could not. The highest total activity and specific activity were obtained by 0.2 % L-phenylalanine supplement with 29.5 units and 0.34 units/mg protein, respectively.

According to the results above, L-phenylalanine showed a notable effect which seemed to be the best inducer for PheDH yield in both strains, BC1 and BC2. Thus, L-phenylalanine was used as enzyme inducer for further experiments.

A



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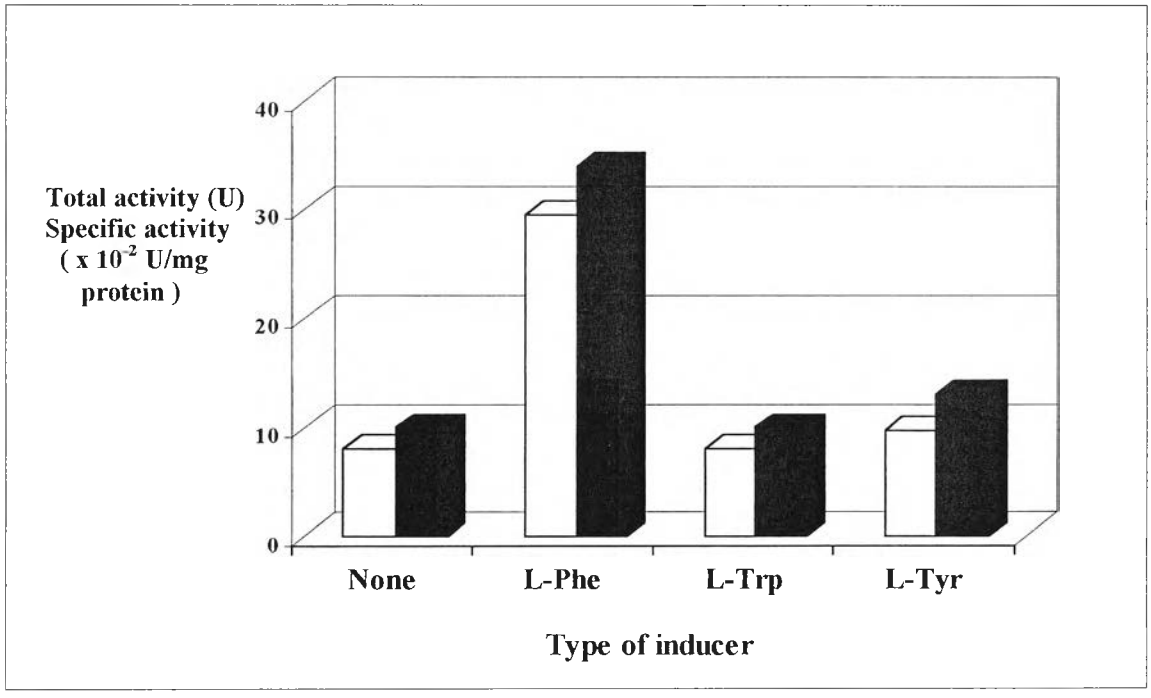


Figure 3.1 Effect of various amino acids as enzyme inducers on phenylalanine dehydrogenase production of bacterial strain BC1 (A) and BC2 (B)

□ Total activity ■ Specific activity

3.1.2 Optimal concentration of inducer

Various concentrations of L-phenylalanine from 0 to 1.4 % were added to culture medium to study its influence on PheDH yield and specific activity as described in section 2.7.2. Similar pattern was obtained for both strains as shown in Figure 3.2 A and 3.2 B. The total activity and specific activity increased corresponding with increasing concentration of the inducer until the concentration reached 0.8 %. At concentrations higher than 0.8 %, total activity and specific activity tended to decrease slowly. However, The cell wet weight seemed to be constant. For strain BC1, the highest enzyme activity could be induced to 7.7 folds by 0.8 % L-phenylalanine supplement with total activity and specific activity of 80 units and 0.39 units/mg protein, respectively. Strain BC2 could be induced to 4.5 folds by 0.8 % L-phenylalanine supplement with total activity and specific activity of 41 units and 0.33 units/mg protein, respectively.

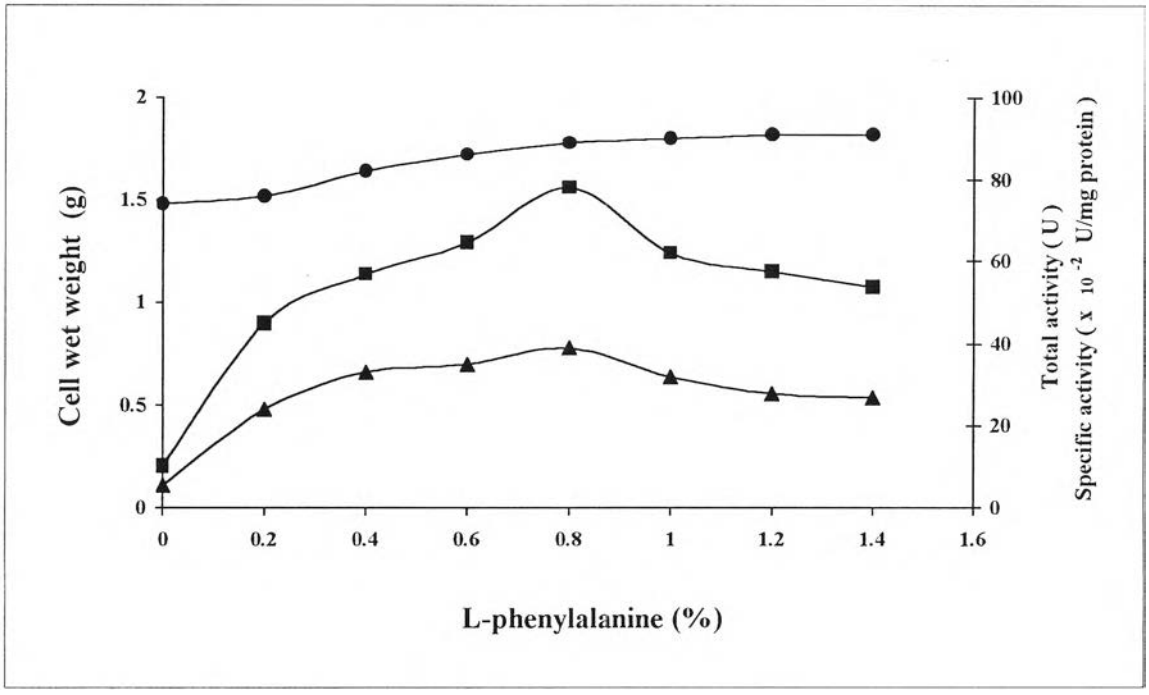
Considering the results above, an optimum was around 0.8 % L-phenylalanine for both strains, BC1 and BC2. Consequently, this concentration was used for further experiments.

3.1.3 Optimal pH of medium

The optimal pH of the culture medium was studied in order to enhance PheDH production as mentioned in section 2.7.3. The effect of pH on enzyme activity and yield is given in Figure 3.3 A and 3.3 B. For strain BC1 (Figure 3.3 A), cell wet weight, total activity and specific activity increased rapidly corresponding with increasing pHs of the medium until the pH reached 6.5 at which the highest total activity (101 units) and specific activity (0.27 units/mg protein) were obtained. At pH above 6.5, cell wet weight was quite constant whereas total activity and specific activity decreased slowly. Total activity at pH 8.5 was about 60 % compared with that at pH 6.5. As a result, an optimum pH of the medium for strain BC1 was 6.5. Hence, this pH was used for further experiments.

For strain BC2 (Figure 3.3 B), cell wet weight, total activity and specific activity increased rapidly corresponding with increasing pHs of medium until

A



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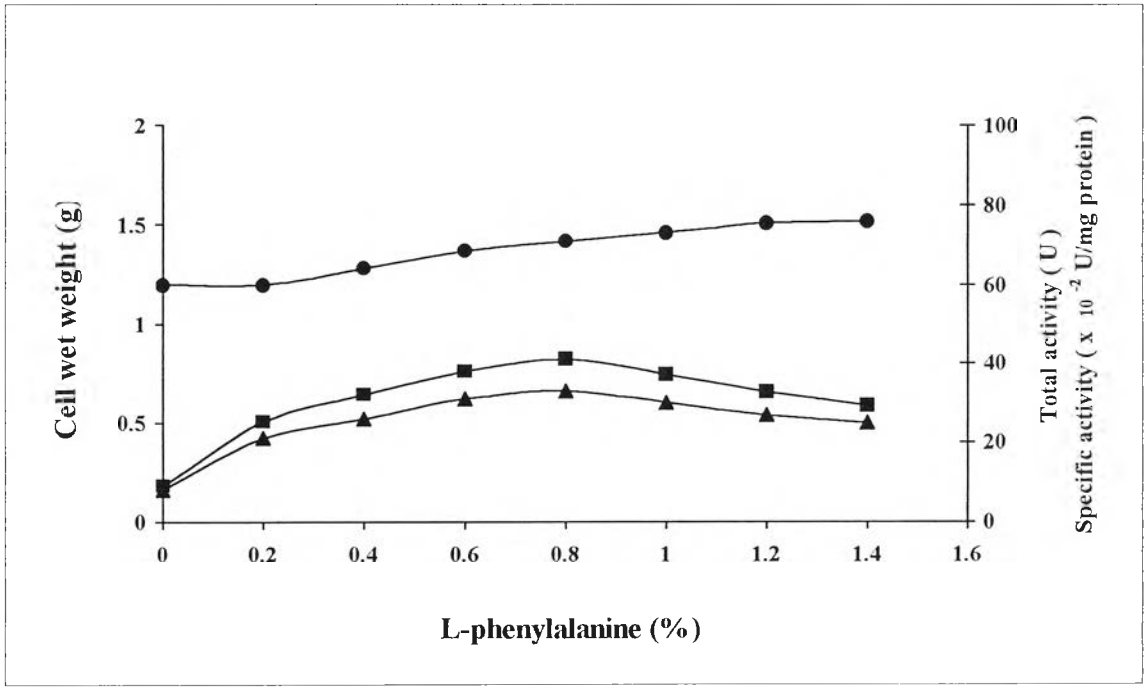
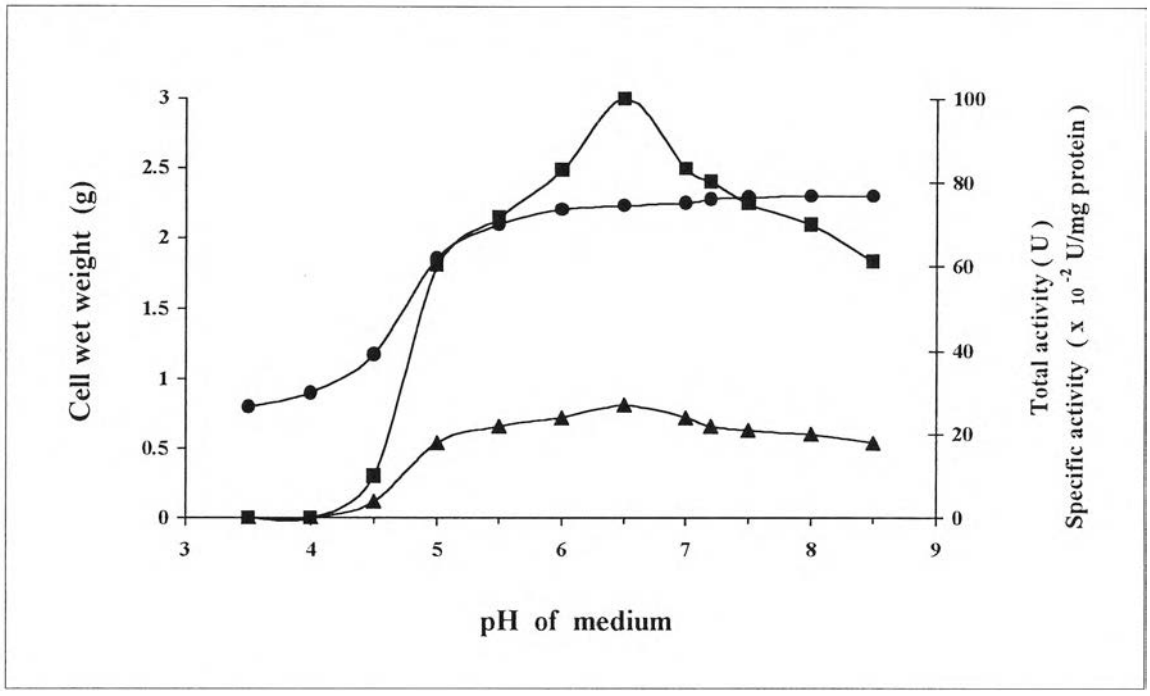


Figure 3.2 Effect of the concentration of L-phenylalanine as enzyme inducer on phenylalanine dehydrogenase production and growth of bacterial strain BC1 (A) and BC2 (B)

● Cell wet weight ■ Total activity ▲ Specific activity

A



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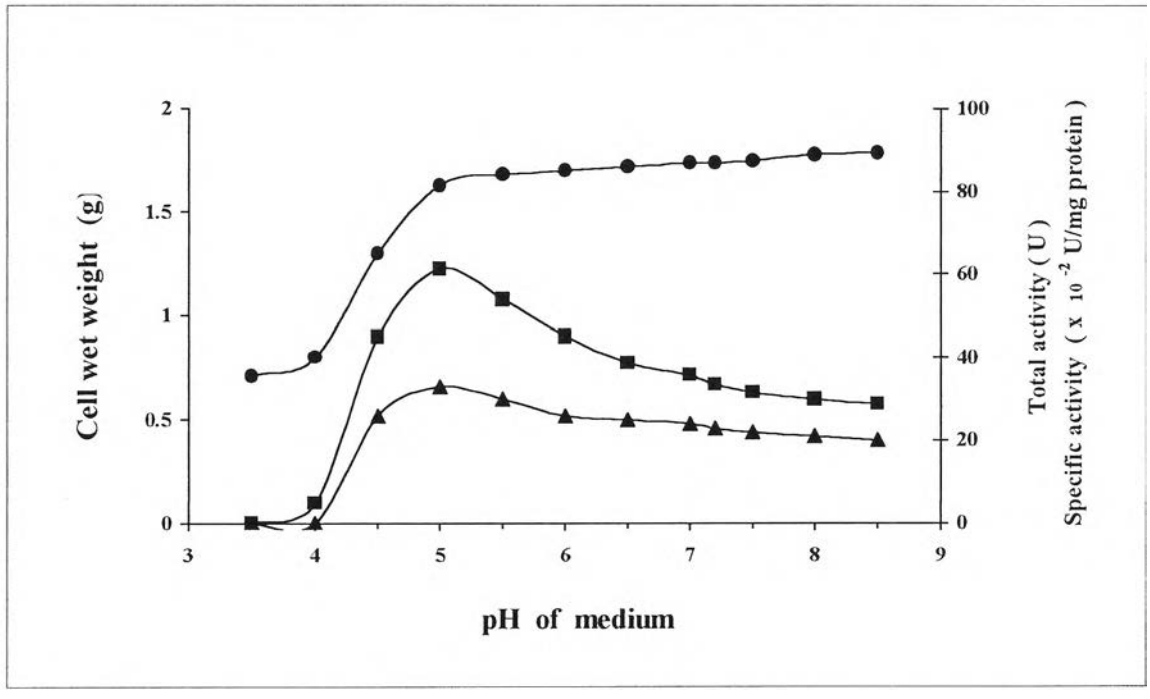


Figure 3.3 Effect of pH of medium on phenylalanine dehydrogenase production and growth of bacterial strain BC1 (A) and BC2 (B)

● Cell wet weight ■ Total activity ▲ Specific activity

the pH reached 5.0 at which the highest total activity (61 units) and specific activity (0.33 units/mg protein) were obtained. At pH above 5.0, cell wet weight increased slowly whereas total activity and specific activity decreased slowly. Total activity at pH 8.5 was about 47 % compared with that at pH 5.0. As a result, an optimum pH of the medium for strain BC2 was 5.0. Hence, this pH was used for further experiments .

3.1.4 Optimal cultivation temperature

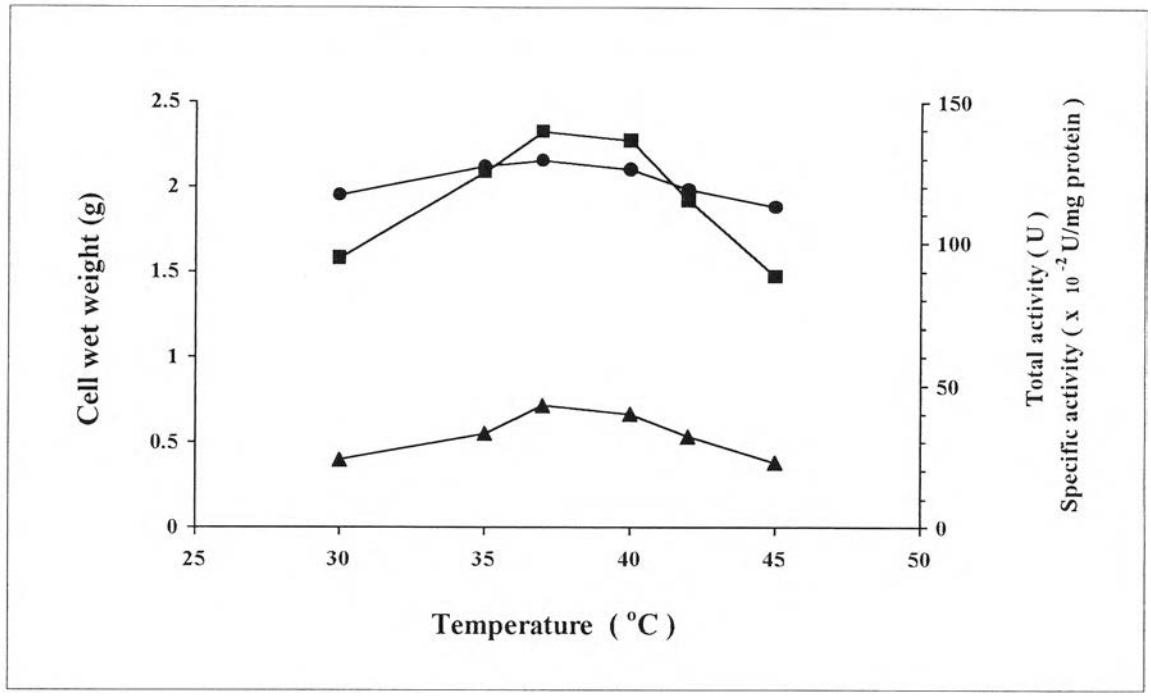
The experiments as described in section 2.7.4 were performed to find out the optimal growth temperature for PheDH production. As shown in Figure 3.4 A for strain BC1 and Figure 3.4 B for strain BC2, cell wet weight, total activity and specific activity tended to be positive direction corresponding with increasing growth temperature from 30 to 37°C. At temperature above 40°C, those were decreasing slowly for strain BC1, but rapidly for strain BC2. At 45°C, total enzyme activity decreased to be 63 % with respect to that of at 37°C for strain BC1 and no activity was observed for strain BC2. The maximum enzyme activity could be obtained by cultivation at 37°C for strain BC1, which total activity and specific activity were 140 units and 0.43 units/mg protein, and strain BC2, which total activity and specific activity were 82.6 units and 0.31 units/mg protein, respectively.

Based on the results, cultivation at 37°C was used as optimal cultivation temperature for further experiments.

3.1.5 Optimal cultivation time

The effect of cultivation time was performed as described in section 2.7.5. Figure 3.5 A and 3.5 B showed that total activity and specific activity increased during growth period. Cell wet weight increased rapidly in the first 6 hours. Then after cultivation up to 9 hours, cell wet weight increased slowly. On the other hand, total activity and specific activity increased from 0 to 24 hours, before decreasing slowly. Highest enzyme activity could be obtained by cultivation for 24 hours for both strains, BC1 and BC2. Total activity and specific activity of strain BC1 were 150 units and 0.48 units/mg protein, respectively, whereas total

A



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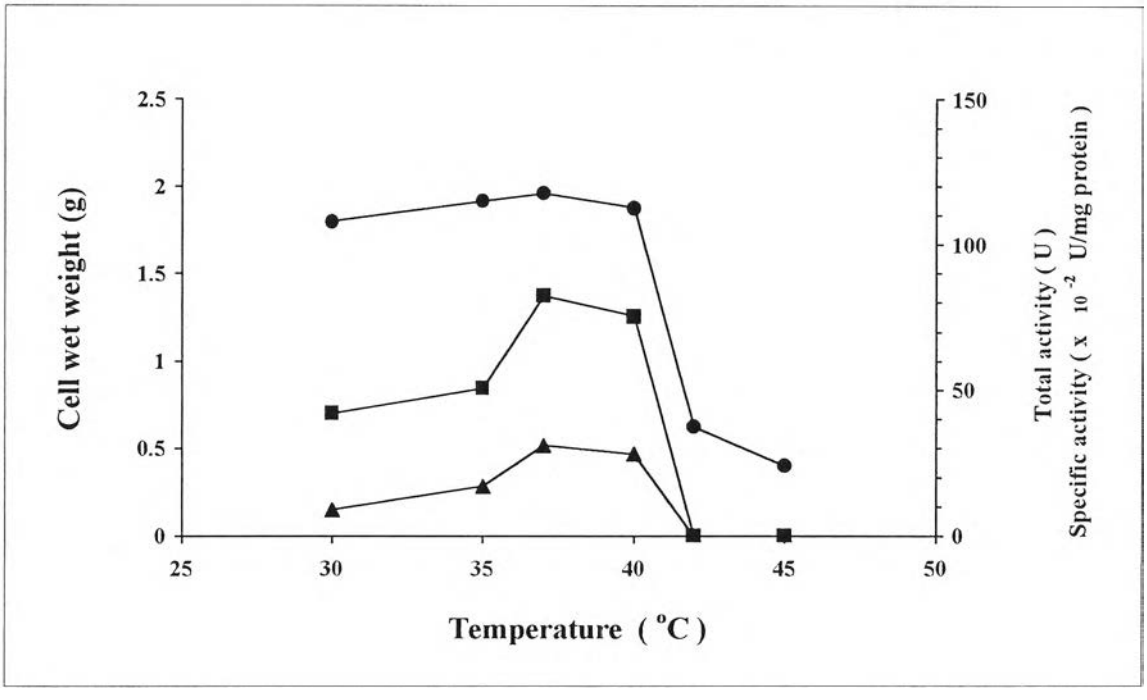
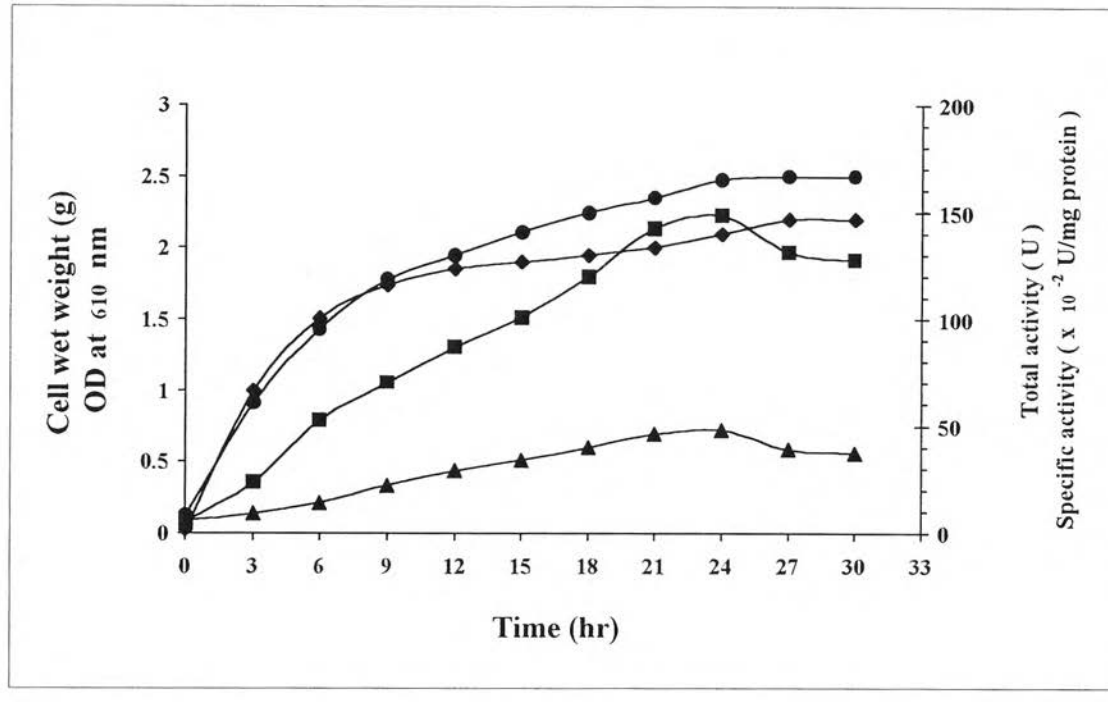


Figure 3.4 Effect of cultivation temperature on phenylalanine dehydrogenase production and growth of bacterial strain BC1 (A) and BC2 (B)

● Cell wet weight ■ Total activity ▲ Specific activity

A



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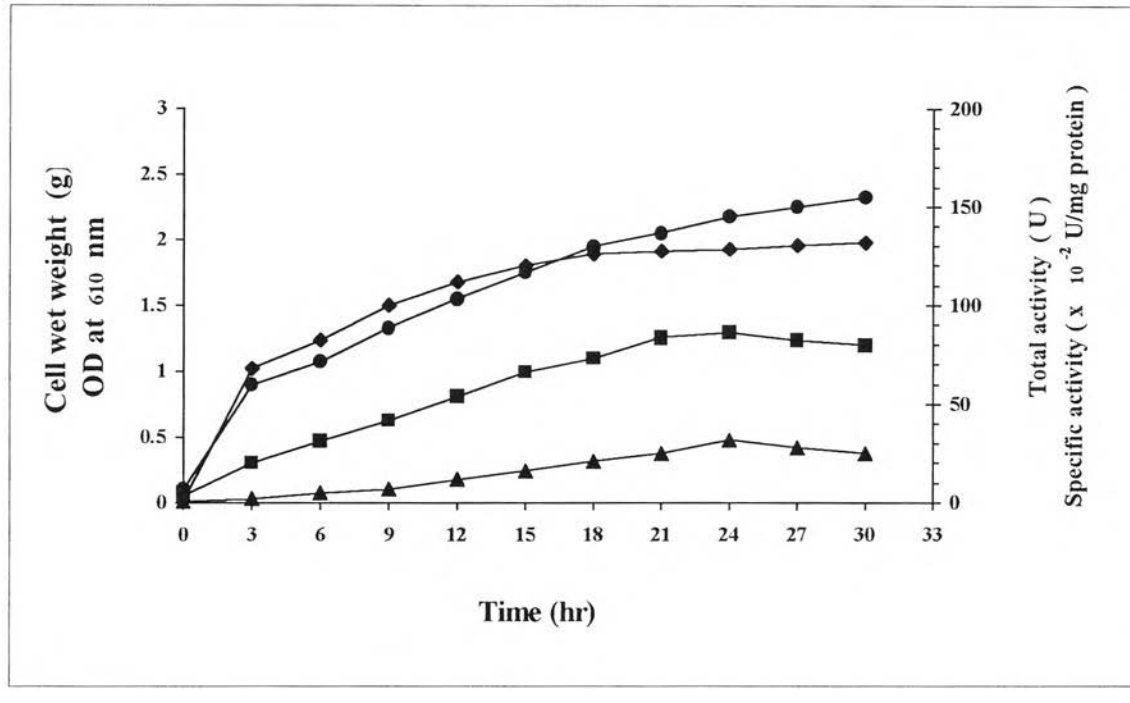


Figure 3.5 Effect of cultivation time on phenylalanine dehydrogenase production and growth of bacterial strain BC1 (A) and BC2 (B)

● Cell wet weight ■ Total activity ▲ Specific activity ◆ OD at 610 nm

activity and specific activity of strain BC2 were 86 units and 0.32 units/mg protein, respectively.

The summary of optimal conditions for PheDH production of thermotorelant bacterial strain BC1 and BC2 is shown in Table 3.1. According to the results, PheDH from strain BC1 was higher in total activity and specific activity than those of strain BC2 in all experiments on optimization. Moreover, strain BC1 was identified as *Bacillus badius* by Chareonpanich *et al* [74]. Therefore, *B. badius* BC1 was chosen for further studies on purification, characterization and kinetic mechanism.

3.2 Purification of phenylalanine dehydrogenase from *Bacillus badius* BC1

3.2.1 Preparation of crude enzyme solution

Crude PheDH was prepared from 45 g of *B. badius* BC1, which was cultivated from 6 litres of medium, as described in section 2.8.1 and 2.8.2. Crude enzyme solution contained 14,478 mg proteins with 4,533 units of PheDH activity in total volume of 100 ml. Thus, the specific activity of the enzyme in the crude preparation was 0.3 units/mg protein.

3.2.2 Ammonium sulfate precipitation

Crude enzyme solution was further purified by ammonium sulfate precipitation as mentioned in section 2.8.3.1. To determine the suitable ammonium sulfate concentration for precipitation of PheDH, preliminary experiment was performed by step-wise increase of ammonium sulfate at 10 % increment from 0 % to 90 %. Most of enzyme activity was determined in the 20-30, 30-40, 40-50 and 50-60 % fractions with the highest activity in 40-50 % fraction. Therefore, to harvest most of the enzyme, protein fractionation was carried out in the range of 40-50 % saturated ammonium sulfate precipitation. The protein remained was 7,095

Table 3.1 Summary of optimal conditions for phenylalanine dehydrogenase production of thermotolerant bacterial strain BC1 and BC2

Optimal conditions	Strain BC1	Strain BC2
Type and concentration of amino acid as an inducer	0.8 % L-phenylalanine	0.8 % L-phenylalanine
pH of medium	6.5	5.0
Cultivation temperature	37 °C	37 °C
Cultivation time	24 hours	24 hours

mg with enzyme activity recovered at 2,981 units (about 65.8 % recovery from crude enzyme). The specific activity of the enzyme from this step was 0.4 units/mg protein (Table 3.2).

3.2.3 DEAE-Toyopearl column chromatography

The enzyme precipitate from 40-50 % saturated ammonium sulfate was dissolved and dialysed against working buffer. The enzyme solution was applied onto DEAE-Toyopearl column as described in section 2.8.3.2. The chromatographic profile is shown in Figure 3.6. The unbound proteins were eluted from DEAE-Toyopearl column with working buffer. The bound proteins were then eluted with linear salt gradient of 0 to 0.5 M potassium chloride solution. The enzyme was eluted at 0.25 M potassium chloride solution as indicated in the profile. The fractions with PheDH activity were pooled, concentrated by aquasorb to reduce enzyme volume and dialyzed against working buffer. The protein remained from this step was 825 mg with 2,030 activity units of enzyme and specific activity of the enzyme was 2.5 units/mg protein (Table 3.2). The enzyme activity was purified for about 8.3 folds and recovery was about 44.8 % compared with crude enzyme.

3.2.4 First Butyl-Toyopearl column chromatography

The pooled active fraction from DEAE-Toyopearl column was further purified by the first Butyl-Toyopearl column as described in section 2.8.3.3. The chromatographic profile is shown in Figure 3.7. The unbound proteins were eluted from the column with working buffer containing 25 % saturated ammonium sulfate. The other proteins, which were bound to the column, were eluted by negative salt step-wise method with the buffer containing 25, 20, 15, 10, 5 and 0 % saturated ammonium sulfate, respectively. The enzyme bound to Butyl-Toyopearl column was eluted with the buffer containing 20 % salt saturation. The fractions with phenylalanine dehydrogenase activity were pooled, concentrated by aquasorb and dialyzed against working buffer. The protein remained from this step was 61.8 mg with 1,104 activity units of enzyme and specific activity of the enzyme was 17.9

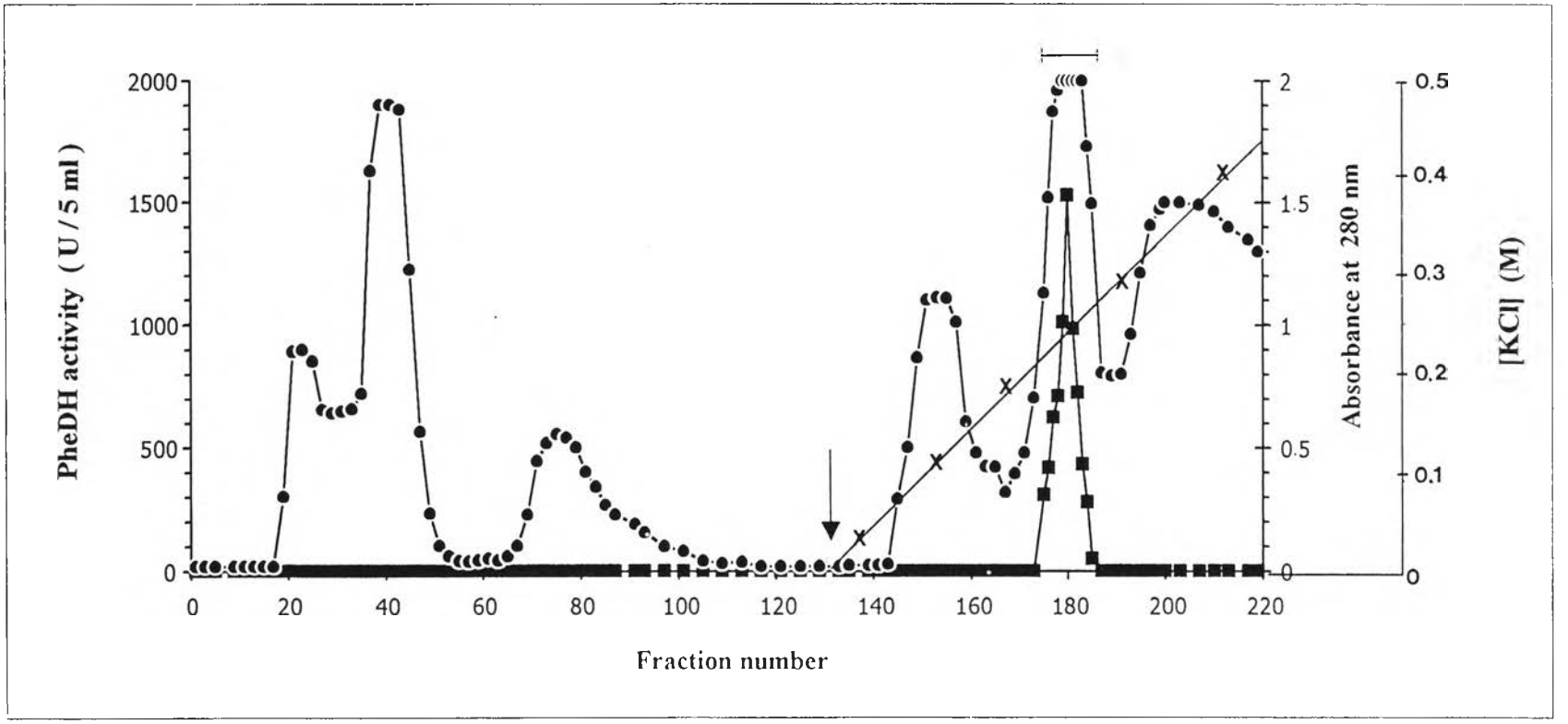


Figure 3.6 Purification of phenylalanine dehydrogenase from *B. badius* BC1 by DEAE-Toyopearl column

The enzyme solution was applied to DEAE-Toyopearl column (2.5 x 22 cm) and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol, 1 mM EDTA and 10% glycerol until A_{280} decreased to base line. Elution of bound proteins was made by 0-0.5 KCl in the same buffer at the flow rate of 1 ml/min. The fractions of 5 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 175 to 184 were pooled.

● A_{280} ■ PheDH activity [KCl] |——| the pool fractions (fraction no. 175-184)

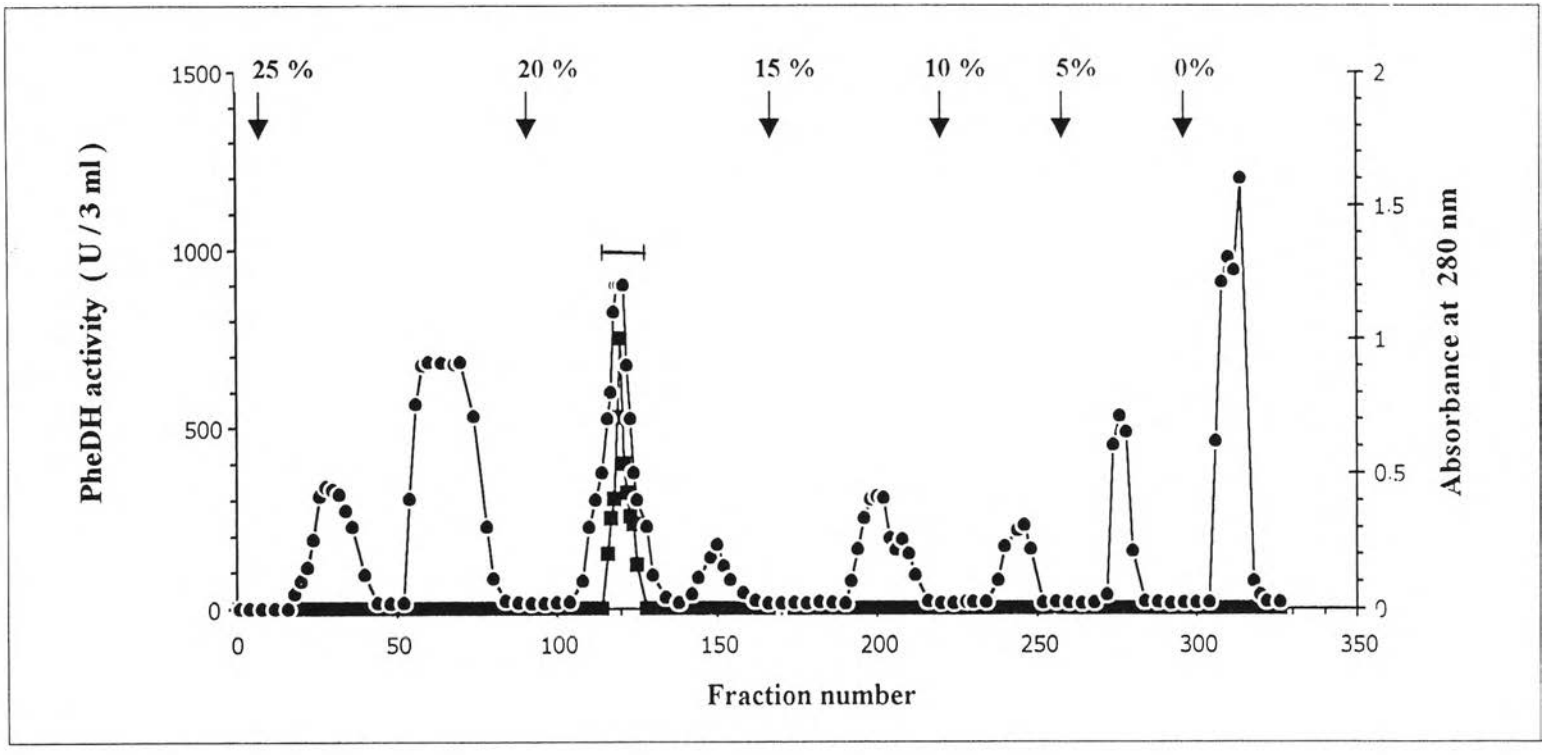


Figure 3.7 Purification of phenylalanine dehydrogenase from *B. badius* BC1 by the first Butyl-Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column (2.2 x 20 cm) and washed with 25% saturation with ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol, 1 mM EDTA and 10% glycerol until A_{280} decreased to base line. Stepwise elution of bound proteins was made by 20, 15, 10, 5 and 0 % saturation ammonium sulfate in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicates where each stepwise started. The protein peak from fraction number 116 to 125 were pooled.

● A_{280} ■ PheDH activity |——| the pool fractions (fraction no. 116-125)

unit/mg protein. From this step, the enzyme was purified to 59.7 folds with about 24.4 % recovery with respect to crude enzyme (Table 3.2).

3.2.5 Second Butyl-Toyopearl column chromatography

The pooled active fraction from the first Butyl-Toyopearl column was loaded into the second Butyl-Toyopearl column as mentioned in section 2.8.3.4. The chromatographic profile is shown in Figure 3.8. The enzyme bound to the Butyl-Toyopearl coumn was eluted with a negative salt gradient, from 25 to 18 % saturated ammonium sulfate in working buffer. The enzyme was eluted at about 19.8 % saturation as indicated in the profile. The active fractions were pooled, concentrated by aquasorb and dialyzed against working buffer. This operation obtained the enzyme with 18.8 mg proteins and 907 activity units. The specific activity of the enzyme from this step was 48.2 units/mg protein. The enzyme was purified to 160.7 folds with about 20 % recovery. The enzyme from this step was kept as aliquot at 4°C for further characterization experiments.

3.2.6 Summary of phenylalanine dehydrogenase purification

PheDH from *B. badius* BC1 was purified by ammonium sulfate precipitation and column chromatographies as described previously. The summary of purification of this enzyme is shown in Table 3.2. At the final step, PheDH was purified to homogeneity with 160.7 purification folds and 20 % yield.

3.2.7 Determination of enzyme purity and protein pattern on non-denaturing polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed for purity and protein pattern by non-denaturing polyacrylamide gel electrophoresis as described in section 2.8.3.5. The activity staining was performed to compare with protein staining. The result is shown in Figure 3.9. The enzyme in lane 5 showed a single protein band on native-PAGE, which coincided with its activity by activity staining, indicating that PheDH from the second Butyl-Toyopearl column should be pure enzyme.

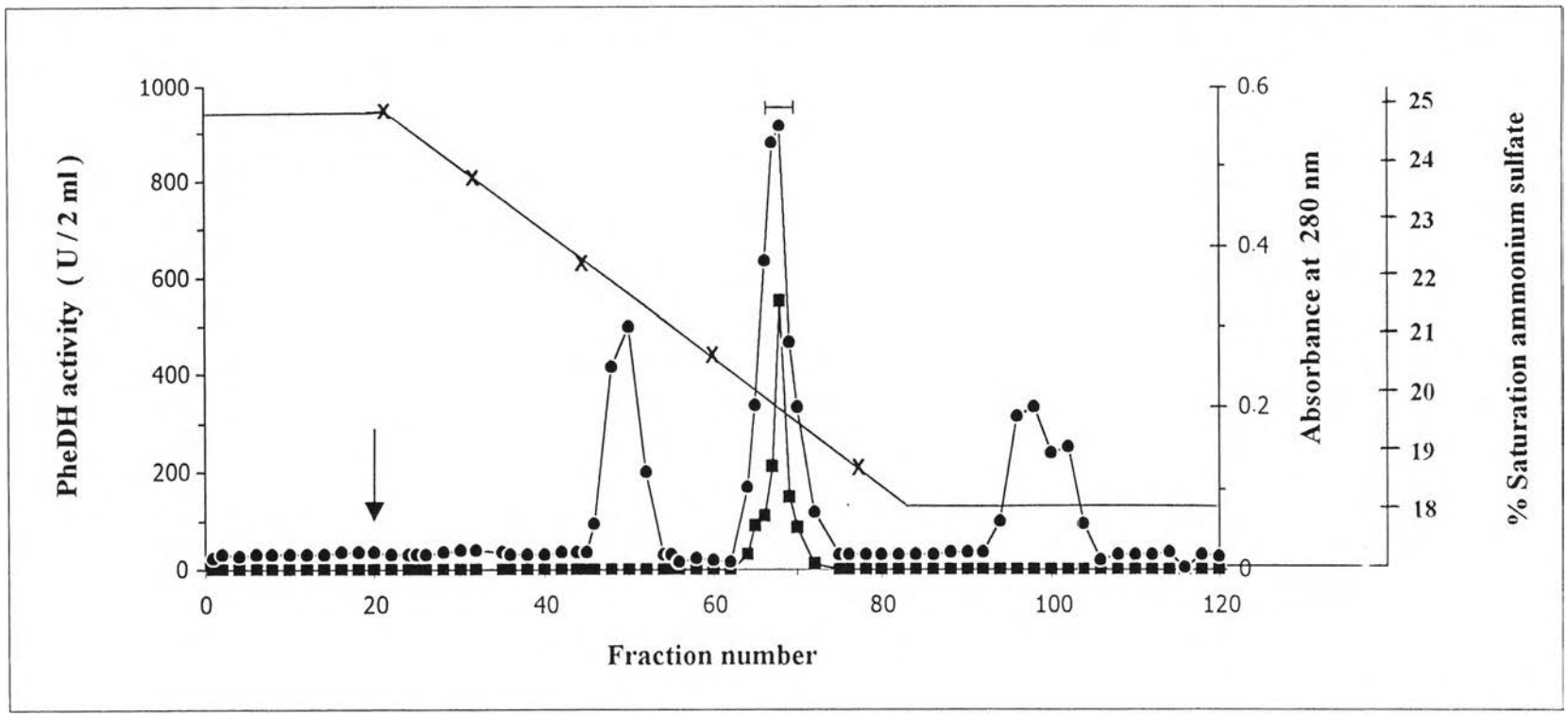


Figure 3.8 Purification of phenylalanine dehydrogenase from *B. badius* BC1 by the second Butyl-Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column (2.2 x 9.5) and washed with 25% saturation with ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol, 1 mM EDTA and 10% glycerol until A_{280} decreased to base line. Gradient elution of bound proteins was made by 25-18 % saturation ammonium sulfate in the same buffer at the flow rate of 1 ml/min. The fractions of 2 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 65 to 70 were pooled.

● A_{280} ■ PheDH activity |——| the pool fractions (fraction no. 65-70)

Table 3.2 Purification of phenylalanine dehydrogenase from *B. badius* BC1

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit / mg protein)	% Recovery	Purification fold
Crude enzyme	4,533.0	14,478.0	0.3	100.0	1.0
40-50 % Ammonium sulfate precipitation	2,981.0	7,095.0	0.4	65.8	1.3
DEAE-Toyopearl column	2,030.0	825.0	2.5	44.8	8.3
First Butyl-Toyopearl column	1,104.0	61.8	17.9	24.4	59.7
Second Butyl-Toyopearl column	907.0	18.8	48.2	20.0	160.7

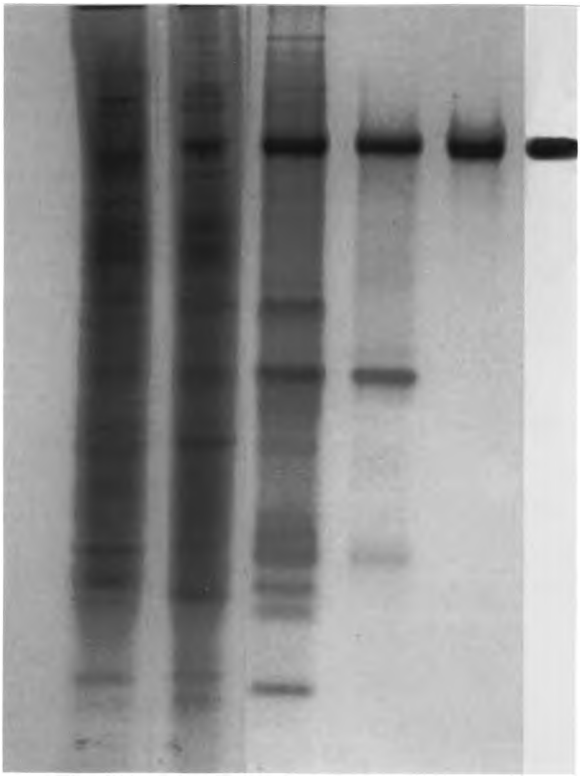


Figure 3.9 Non-denaturing PAGE of the *B. badius* BC1 phenylalanine dehydrogenase from each step of purification

A. Protein staining

Lane 1	crude enzyme	75	μg protein
Lane 2	40-50 % ammonium sulfate precipitation	65	μg protein
Lane 3	DEAE-Toyopearl column	30	μg protein
Lane 4	first Butyl-Toyopearl column	17	μg protein
Lane 5	second Butyl-Toyopearl column	7.5	μg protein

B. Activity staining

Lane 6	second Butyl-Toyopearl column	7.5	μg protein

3.3 Characterization of phenylalanine dehydrogenase from *Bacillus badius* BC1

3.3.1 Molecular weight determination of phenylalanine dehydrogenase

The native molecular weight of PheDH was determined from molecular weight calibration curve (Figure 3.10) obtained from chromatography of standard proteins on Sephadex G-200 column as mentioned in section 2.9.1.1. The enzyme was found to have the native molecular weight of 358,000. The molecular weight of PheDH subunit was also determined by SDS-polyacrylamide gel electrophoresis (Figure 3.11 and 3.12) which included a series of standard proteins in the run. From the mobility in SDS-PAGE, the molecular weight of the enzyme protein monomer was calculated to be 44,500.

3.3.2 Substrate specificity of phenylalanine dehydrogenase

An action of PheDH on substrates in the deamination and amination directions was studied in this work as mentioned in section 2.9.2. Substrate specificity of PheDH in the direction of oxidative deamination is illustrated in Table 3.3. The highest activity was observed with L-phenylalanine as substrate. No activity was observed with *o*-fluoro-DL-phenylalanine, *p*-hydroxyphenylacetate, DL-β-phenyllactate, *N*-methyl-L-phenylalanine, α-methyl-DL-phenylalanine, hydrocinnamate, D-alanine, D-aspartate, D-arginine, D-glutamate, D-histidine, D-leucine, D-methionine, D-phenylalanine, D-serine, D-threonine, D-tryptophan, D-valine, L-alanine, L-asparagine, L-aspartate, L-arginine, L-cysteine, L-glutamate, L-glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-serine, L-threonine and L-tyrosine.

In the reductive amination, the enzyme acted exclusively on phenylpyruvate as shown in the same table. The following substrates were inert: *p*-hydroxyphenylpyruvate, indole-β-pyruvate, α-keto-n-butyrate, α-ketoglutarate, α-keto-γ-methylthiobutyrate and α-keto-β-methyl-n-valerate.

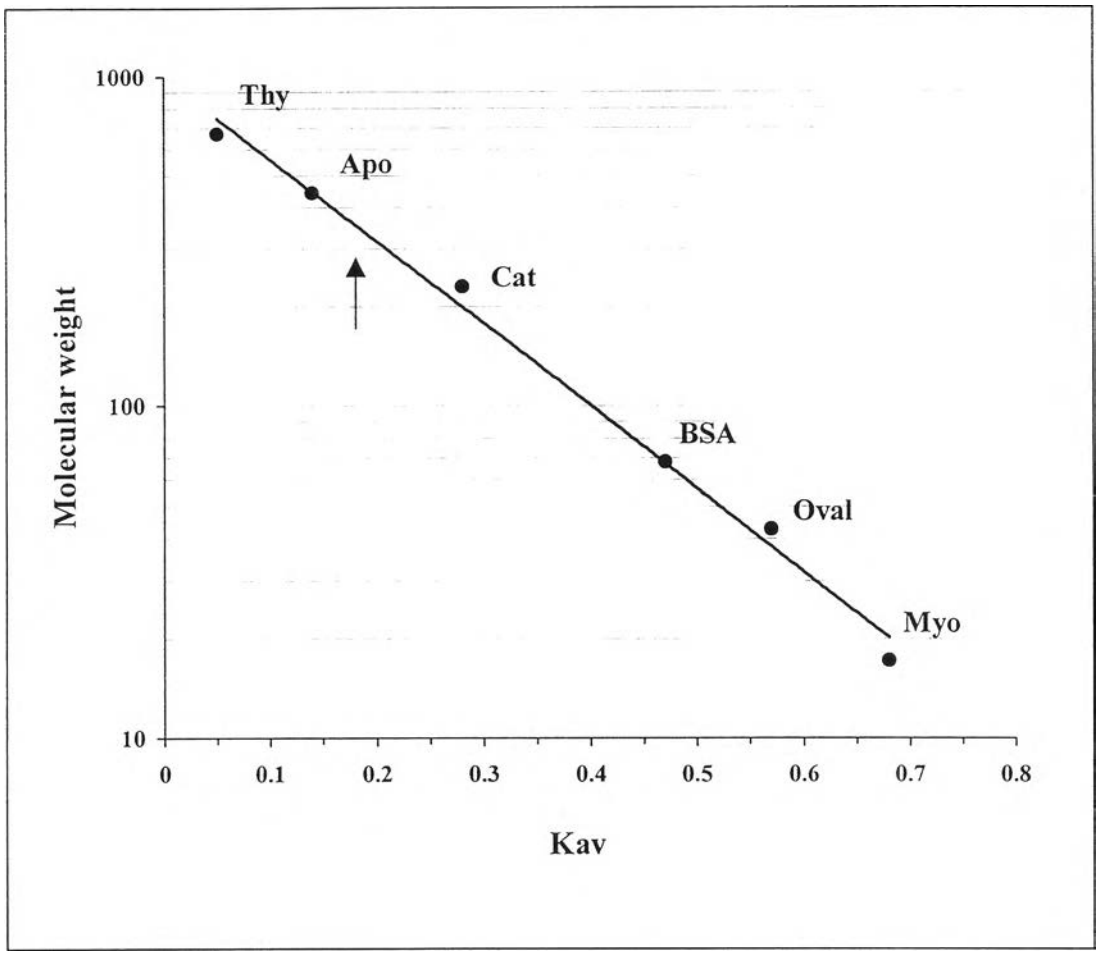


Figure 3.10 Calibration curve for native molecular weight of phenylalanine dehydrogenase from *B. badius* BC1 determined by gel filtration chromatography on Sephadex G-200 column

Thy	=	Thyroglobin	(MW 669,000)
Apo	=	Apoferritin	(MW 443,000)
Cat	=	Catalase	(MW 230,000)
BSA	=	Bovine serum albumin	(MW 68,000)
Oval	=	Ovalbumin	(MW 43,000)
Myo	=	Myoglobin	(MW 17,200)

Arrow indicates the K_{av} of PheDH

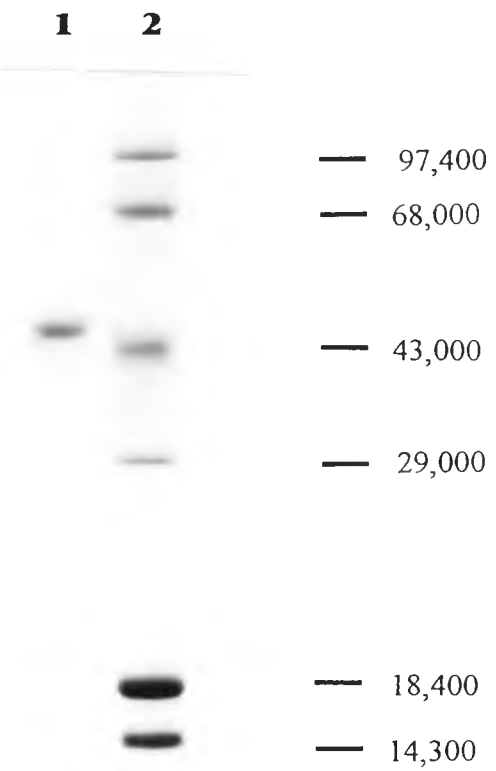


Figure 3.11 SDS-polyacrylamide gel electrophoresis of phenylalanine dehydrogenase from *B. badius* BC1

Lane 1	:	Purified PheDH	
Lane 2	:	Phosphorylase B	(MW 97,400)
		Bovine serum albumin	(MW 68,000)
		Ovalbumin	(MW 43,000)
		Carbonic anhydrase	(MW 29,000)
		β-Lactoglobulin	(MW 18,400)
		Lysozyme	(MW 14,300)

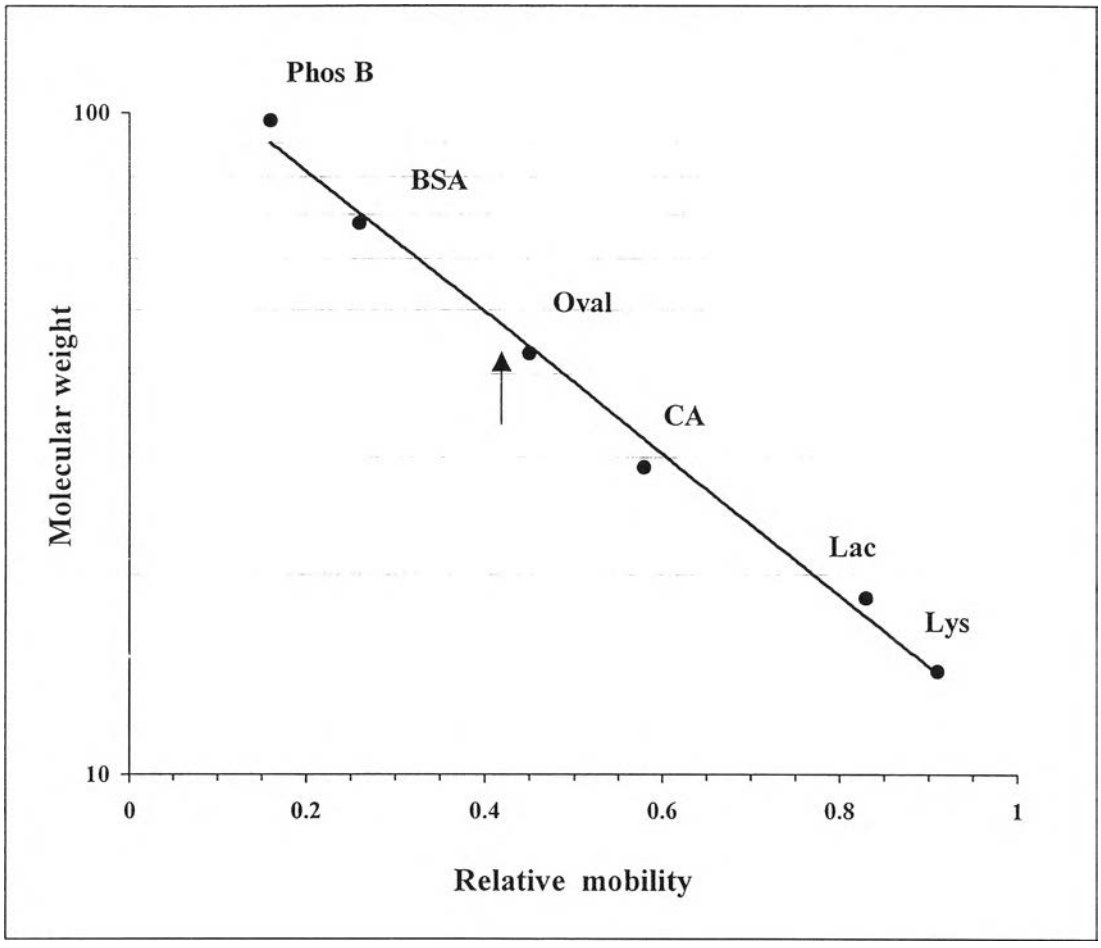


Figure 3.12 Calibration curve for molecular weight of phenylalanine dehydrogenase subunit from *B. badius* BC1 on SDS-polyacrylaminde gel electrophoresis

Phos B	=	Phosphorylase B	(MW 97,400)
BSA	=	Bovine serum albumin	(MW 68,000)
Oval	=	Ovalbumin	(MW 43,000)
CA	=	Carbonic anhydrase	(MW 29,000)
Lac	=	β-Lactoglobulin	(MW 18,400)
Lys	=	Lysozyme	(MW 14,300)

Arrow indicates the K_{av} of PheDH

Table 3.3 Substrate specificity of phenylalanine dehydrogenase from *B. badius* BC1 [a]

Process and substrate	Relative activity (%)
Oxidative deamination [b]	
L-phenylalanine	100
p-fluoro-DL-phenylalanine	11
α-amino-β-phenylbutanoate	8
m-fluoro-DL-phenylalanine	5
L-methionine	4
L-tryptophan	3
L-valine	2
Reductive amination [c]	
phenylpyruvate	100
α-ketocaproate	12
α-ketoisovalerate	5
α-ketoisocaproate	4
α-ketovalerate	3

[a] The data represent the mean values of three independent experiments.

[b] Final concentration of each substrate was 20 mM. The followings were inert: *o*-fluoro-DL-phenyl alanine, *p*-hydroxyphenylacetate, DL-β-phenyllactate, *N*-methyl-L-phenylalanine, α-methyl-DL-phenylalanine, hydrocinnamate, D-alanine, D-aspartate, D-arginine, D-glutamate, D-glycine, D-histidine, D-leucine, D-lysine, D-methionine, D-phenylalanine, D-serine, D-threonine, D-tryptophan, D-valine, L-alanine, L-asparagine, L-aspartate, L-arginine, L-cysteine (5 mM) , L-glutamate , L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-serine, L-threonine and L-tyrosine (1.2 mM).

[c] Final concentration of each substrate was 10 mM. The followings were inert: *p*-hydroxyphenylpyruvate (2.5 mM) , indole-β-pyruvate (1 mM) , α-keto-n-butyrate , α-ketoglutarate , α-keto-γ-methylthiobutyrate , α-keto-β-methyl-n-valerate and pyruvate.

3.3.3 Coenzyme specificity of phenylalanine dehydrogenase

Coenzyme specificity of PheDH was investigated as described in section 2.9.3. PheDH required NAD^+ as a natural coenzyme for oxidative deamination whereas no activity was observed with $NADP^+$. In addition, some analogs of NAD^+ served as a coenzyme as shown in Table 3.4. 3-Acetylpyridine-NAD^+ was much better coenzyme than NAD^+. Nicotinamide hypoxanthine dinucleotide (deamino-NAD^+) was similar to NAD^+ in cofactor activity.

3.3.4 Effect of pH on phenylalanine dehydrogenase activity

The optimum pH of the enzyme was performed at various pHs as mentioned in section 2.9.4. Activities at differents pH are shown in Figure 3.13. Preliminary experiments also performed with potassium phosphate buffer (pH 6-8.5), $NaHPO_4$-NaOH buffer (11-12) and KCl-NaOH (11-13) but no significant data was observed. Therefore, in this work, Tris-HCl and glycine-KCl-KOH buffers (200 mM final concentration) were used for pH range 7.5-9.0 and 8.5-12.0, respectively. The pH of each reaction mixture was measured with a pH meter after the reaction. The enzyme exhibited the maximum activity for oxidative deamination at pH 10.7 which was the actual pH of the reaction mixture. In reductive amination, the enzyme showed the highest activity at pH 8.3 which was the actual pH of the reaction mixture.

3.3.5 Effect of temperature on phenylalanine dehydrogenase activity

The optimum temperature of the enzyme was investigated by incubating of the reaction mixture at various temperatures as mentioned in section 2.9.5. The result is shown in Figure 3.14. The enzyme showed the highest activity at 50°C and 45°C for the oxidative deamination and reductive amination, respectively which were defined as 100 % activity.

Table 3.4 Coenzyme specificity of phenylalanine dehydrogenase from *B. badius* BC1 [a]

Coenzyme [b]	Relative activity (%)
β-Nicotinamide adenine dinucleotide (NAD^+)	100
3-Acetylpyridine adenine dinucleotide	166
Nicotinamide hypoxanthine dinucleotide (Deamino-NAD^+)	96
Nicotinamide guanine dinucleotide	70
Nicotinamide 1, N^6-ethenoadenine dinucleotide	51
Thionicotinamide adenine dinucleotide	43
β-Nicotinamide adenine dinucleotidephosphate ($NADP^+$)	0
Nicotinic acid adenine dinucleotide (Deamido-NAD^+)	0
3-Pyridinealdehyde adenine dinucleotide	0

[a] The data represent the mean values of three independent experiments.

[b] Final concentration of each coenzyme analog was 2.0 mM. The assay was conducted at the following wavelenghts: 3-acetylpyridine adenine dinucleotide, 363 nm ($\varepsilon = 9.1 \times 10^3$); β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 340 nm ($\varepsilon = 6.2 \times 10^3$); Nicotinamide 1,$N^6$-ethenoadenine dinucleotide, 334 nm ($\varepsilon = 6.9 \times 10^3$); Nicotinamide guanine dinucleotide, 340 nm ($\varepsilon = 6.2 \times 10^3$); Nicotinamide hypoxanthine dinucleotide, 338 nm ($\varepsilon = 6.2 \times 10^3$); Nicotinic acid adenine dinucleotide, 338 nm ($\varepsilon = 6.2 \times 10^3$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3 \times 10^3$) and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3 \times 10^3$). The reaction was carried out at pH 9.5 to avoid degradation of NAD^+ analogs at a more alkaline pH

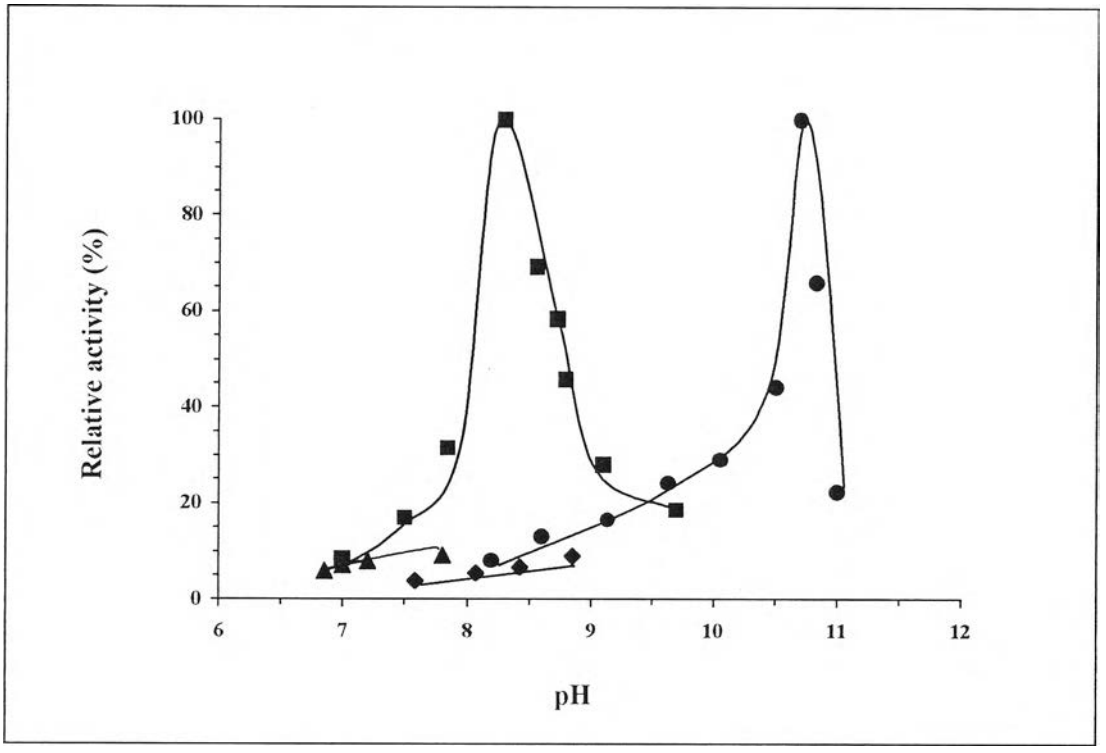


Figure 3.13 Effect of pH on phenylalanine dehydrogenase activity

The oxidative deamination and reductive amination activities of the enzyme were measured at different pHs with 200 mM Tris-HCl buffer or glycine-KCl-KOH buffer. The pH of each reaction mixture was measured with a pH meter at room temperature after the reaction. The relative activities of three independent experiments were averaged.

The oxidative deamination : (◆) Tris-HCl buffer, (●) Glycine-KCl-KOH buffer. The reductive amination : (Δ) Tris-HCl buffer, (■) Glycine-KCl-KOH buffer

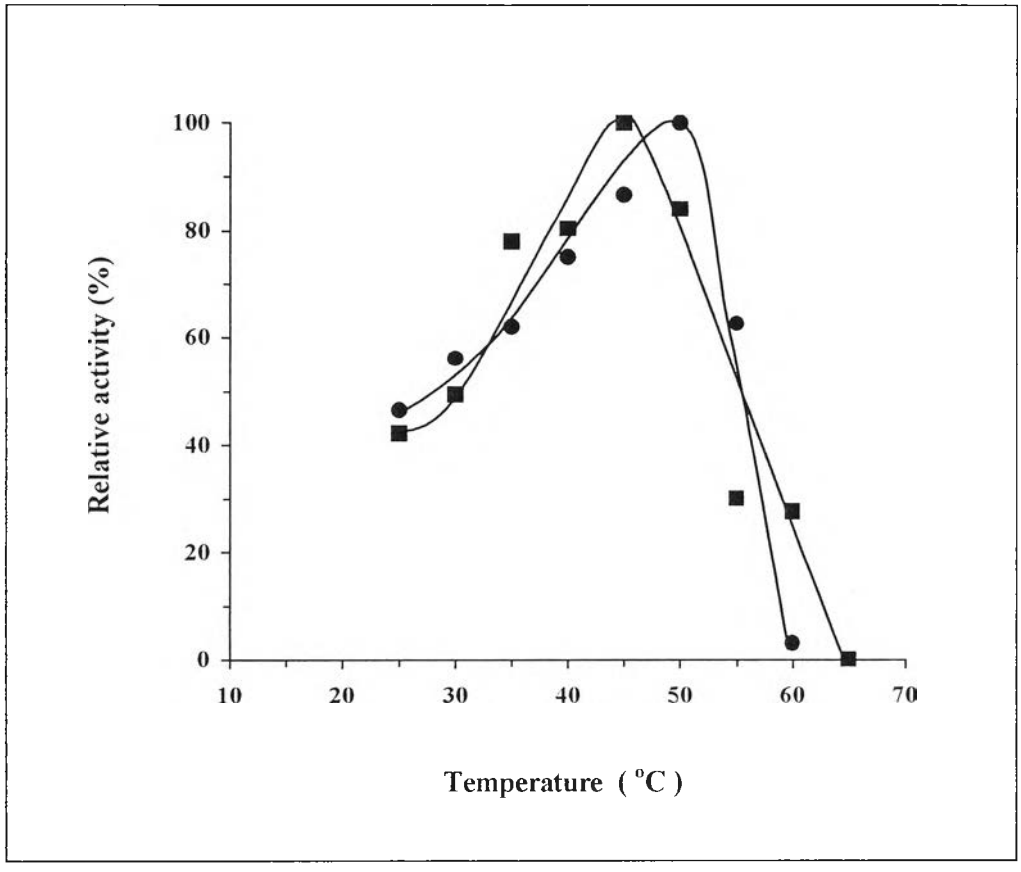


Figure 3.14 Effect of temperature on phenylalanine dehydrogenase activity

The oxidative deamination (•) and reductive amination (■) activities of the enzyme were measured at various temperatures. The relative activities of three independent experiments were averaged.

3.3.6 Effect of pH on phenylalanine dehydrogenase stability

The pH stability of the enzyme was measured by incubating the enzyme for 20 minutes at 30°C in various 10 mM buffers, pHs from 4.0 to 12.0 before the residual enzyme activity was measured as described in section 2.9.6. The enzyme showed high stability over a pH range of 6.0 to 11.0 as shown in Figure 3.15.

3.3.7 Effect of temperature on phenylalanine dehydrogenase stability

PheDH was preincubated at various temperatures for 10 minutes before its activity was assayed as described in section 2.9.7. The enzyme activity of non-preincubated enzyme was defined as 100 % relative activity. The result is shown in Figure 3.16 A. The enzyme retained its full activity up to 40°C, but lost about half of the activity at 45°C and lost all activity at 60°C. The experiment was further continued by incubating the enzyme at 40°C and checking for its activity at every 3 hours. The remained deamination activities were expressed as the percentages of the original activity. The result is shown in Figure 3.16 B. No significant loss of activity was observed when incubated the enzyme for 2 hours. However after that, the enzyme activity was relatively decreased with increasing of incubation time and approximately half of the enzyme activity was lost after incubation for 24-30 hours. The enzyme activity was entirely abolished after incubation at 40°C for 57 hours.

3.3.8 Effect of metal ions and chemical reagents on phenylalanine dehydrogenase activity

The effect of metal ions and some chemical reagents on PheDH activity are summarized in Table 3.5. The enzyme was incubated with each compound at 30°C (final concentration 10 mM unless otherwise noted) before starting the reaction with the coenzyme as described in section 2.9.8. Reducing agents (β-mercaptoethanol and DTT) and chelating or carbonyl reagents (EDTA,

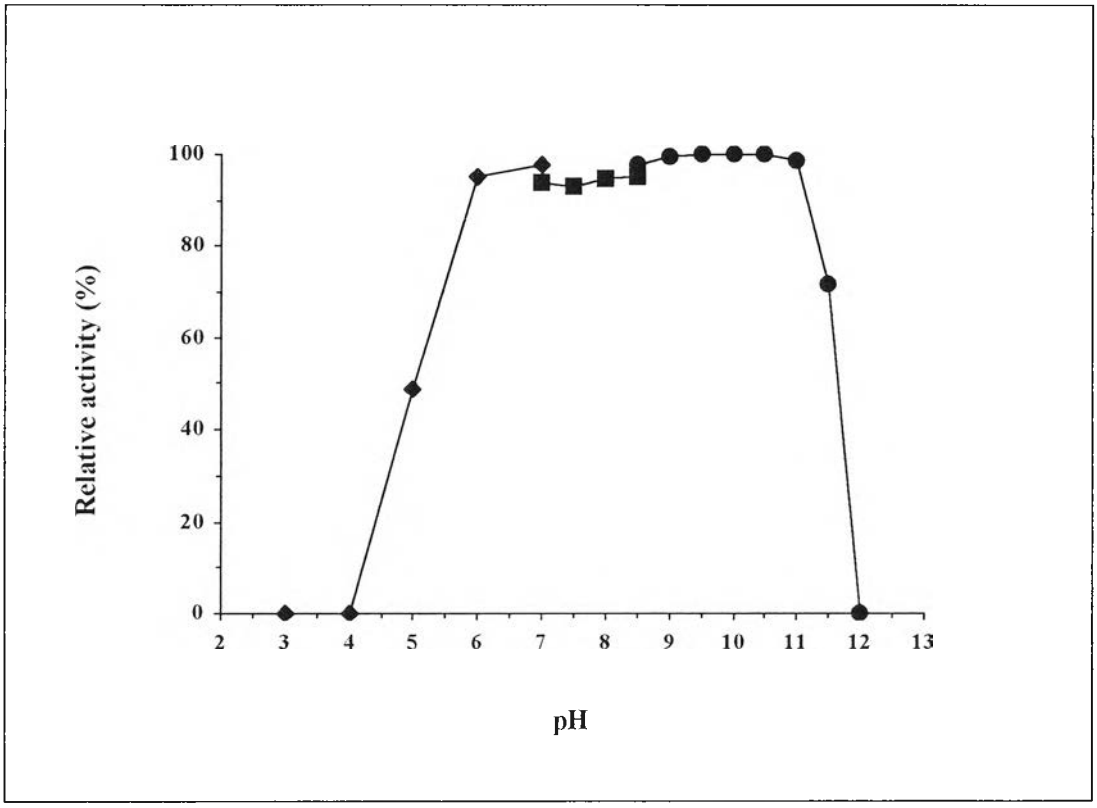
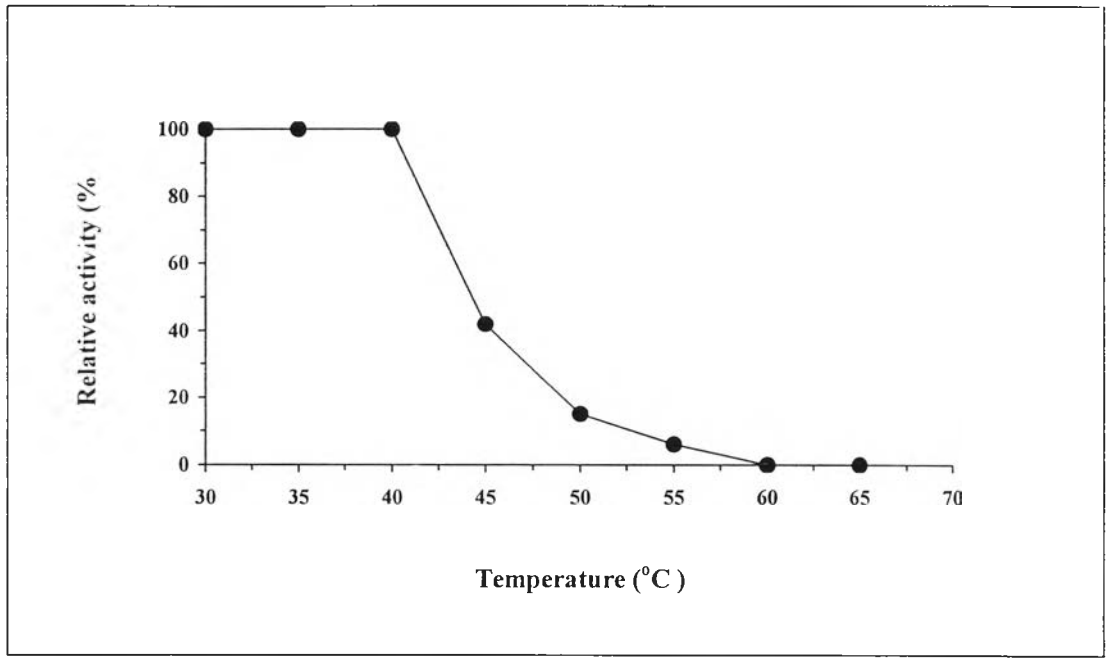


Figure 3.15 Effect of pH on phenylalanine dehydrogenase stability

The enzyme in buffer of various pHs was incubated at 30°C for 20 min and then the relative activity was assayed for the oxidative deamination of L-phenylalanine at 30°C. The buffers (10 mM) used were citrate buffer (◆), potassium phosphate buffer (■) and glycine-KCl-KOH buffer (●).
The relative activities were averaged based on three independent experiments.

A



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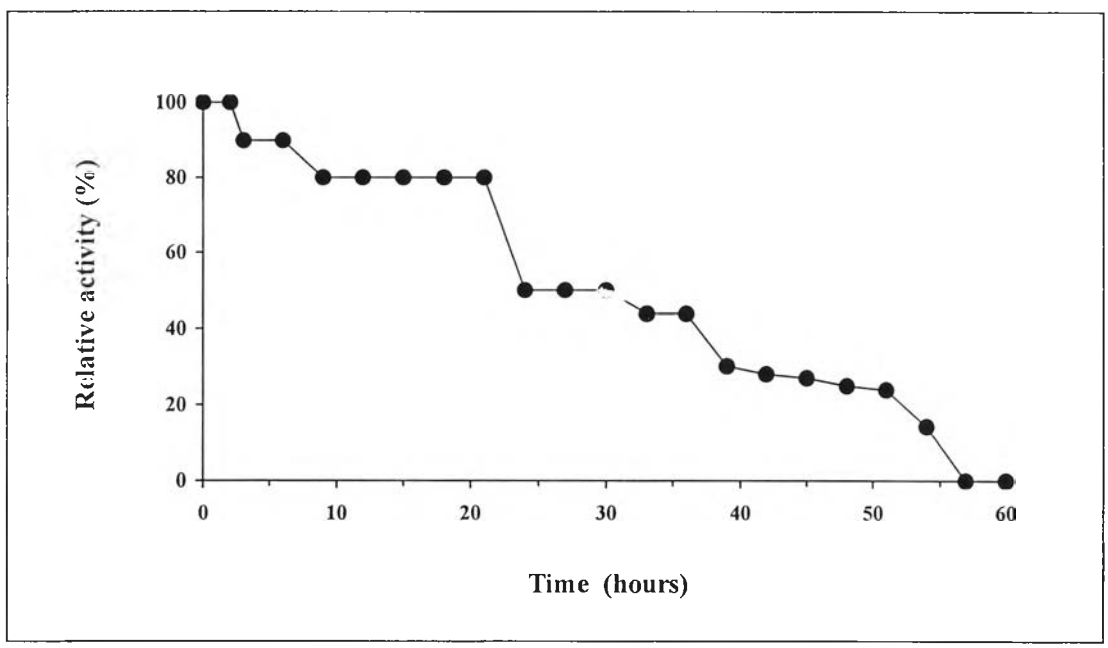


Figure 3.16 Effect of temperature on phenylalanine dehydrogenase stability

(A) Thermostability of the enzyme. After enzyme treatment at various temperatures for 10 min and then chilled on ice. The relative activity was assayed for the oxidative deamination of L- phenylalanine at 30°C.

(B) Enzyme stability at 40°C. The enzyme was incubated at 40°C and the relative activity was assayed for the oxidative deamination of L- phenylalanine at 30°C at intervals.

Table 3.5 Effect of metal ions and chemical reagents on phenylalanine dehydrogenase activity [a]

Compounds [b]	Final concentration (mM)	Relative activity (%)
None	-	100
LiCl	10	93
NaCl	10	100
KCl	10	99
RbCl	10	92
$MgCl_2$	10	99
$CaCl_2$	10	92
$BaCl_2$	10	96
$MnCl_2$	10	36
$FeCl_3$	1	23
$CoCl_2$	10	84
$ZnCl_2$	10	84
$MgSO_4$	10	100
$FeSO_4$	1	0
$CuSO_4$	10	88
$ZnSO_4$	10	79
KBr	10	100
K_2CO_3	10	92
$NaNO_3$	10	92
$AgNO_3$	1	0
$HgCl_2$	1	0
β-Mercaptoethanol	10	100
DTT	10	100

Table 3.5 Effect of metal ions and chemical reagents on phenylalanine dehydrogenase activity [a] (continue)

Compounds [b]	Final concentration (mM)	Relative activity (%)
EDTA	10	100
Sodium azide	10	98
Hydroxylamine	10	96

[a] The data represent the mean values of three independent experiments.

[b] Abbreviations : PMSF, phenylmethylsulfonyl fluoride ; DTT, dithiothreitol ; EDTA, ethylenediamine tetraacetic acid.

sodium azide and hydroxylamine) had almost no effect on the enzyme activity. On the other hand, the enzyme was strongly inhibited by the presence of sulfhydryl reagents (1 mM $AgNO_3$ and $HgCl_2$) suggesting the presence of essential thiol group for enzyme activity. In addition, $FeSO_4$, $FeCl_3$ and $MnCl_2$ were found to have influence on enzyme activity. None of the following compounds exhibited a significant effect on enzyme activity: LiCl, NaCl, KCl, RbCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, $CoCl_2$, $ZnCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, KBr, K_2CO_3 and $NaNO_3$.

3.3.9 Inhibitory effect of various amino acids and keto acids on phenylalanine dehydrogenase activity

Inhibitory effect of various amino acids and keto acids, which were not the substrates of PheDH, on the oxidative deamination of L-phenylalanine and reductive amination of phenylpyruvate, respectively, were investigated as described in section 2.9.9. The result is shown in Table 3.6. Among various nonsubstrate amino acids examined for inhibitory effects on the direction of oxidative deamination, D- and L- forms of amino acids with non-polar side chain (isoleucine, leucine, methionine, tryptophan and valine) except for alanine and glycine, and acidic side chain (aspartate and glutamate) exhibited significant inhibition against L-phenylalanine. In addition, among phenylalanine analogs tested, only *o*-fluoro-DL-phenylalanine was found to be inhibited enzyme activity. For the inhibitory effect of reductive amination of phenylpyruvate, no significant inhibition was found upon the addition of various keto acids, except for α-ketoglutarate which slighty inhibited the reaction.

3.3.10 Effect of group-specific reagents on phenylalanine dehydrogenase activity

PheDH from *B. badius* BC1 was chemically modified with a series of group-specific reagents to identify essential amino acid residues by incubating purified enzyme with 10 mM of each modifying reagent at 30°C for 20

Table 3.6 Inhibitory effect of various amino acids and keto acids on phenylalanine dehydrogenase activity [a]

Process and amino acids / keto acids	Relative activity (%)
Oxidative deamination [b]	
None	100
D-aspartate	30
D-glutamate	32
D-isoleucine	50
D-leucine	47
D-methionine	19
D-phenylalanine	6
D-tryptophan	13
D-valine	46
L-aspartate	47
L-glutamate	51
L-isoleucine	32
L-leucine	35
L-tyrosine	86
o-fluoro-DL-phenylalanine	10
Reductive amination [c]	
None	100
α-ketoglutarate	87
pyruvate	99

[a] The data represent the mean values of three independent experiments.

[b] Amino acid concentration was 20 mM except for L-tyrosine (1.2 mM) and *o*-fluoro-DL-phenylalanine (10 mM). *p*-Hydroxyphenylacetic acid, DL-β-phenyllactate, *N*-methyl-L-phenylalanine, α-methyl-DL-phenylalanine, hydrocinnamate, D-alanine, D-arginine, D-glycine, D-histidine, D-lysine, D-serine, D-threonine, L-alanine, L-asparagine, L-arginine, L-cysteine, L-glycine, L-histidine, L-lysine, L-serine and L-threonine did not inhibit the reaction.

[c] Keto acid concentration was 10 mM. *p*-Hydroxyphenylpyruvate (2.5 mM), indole-β-pyruvate (1 mM), α-keto-n-butyrate, α-keto-γ-methylthiobutyrate and α-keto-β-methyl-n-valerate did not inhibit the reaction.

minutes under the selective condition for each reagent as described in section 2.9.10. The residual activity for oxidative deamination and reductive amination were then determined and compared with those of untreated enzyme as shown in Table 3.7. PheDH activity was completely inhibited by 10 mM of NBS, CT, DEPC and TNBS in both of oxidative deamination and reductive amination. Hence, tryptophan, methionine, histidine and lysine residues may play an important role in the active site of the enzyme. The significant loss of activity was observed when enzyme was modified with 10 mM of PG which was specific for arginine. In contrast, NAI, DTT and PMSF, the modifiers of tyrosine, cysteine and serine, respectively, had no effect on the enzyme activity since almost all activities retained after incubation with these reagents.

3.4 Kinetic mechanism studies of phenylalanine dehydrogenase from *Bacillus badius* BC1

3.4.1 Initial velocity studies for oxidative deamination

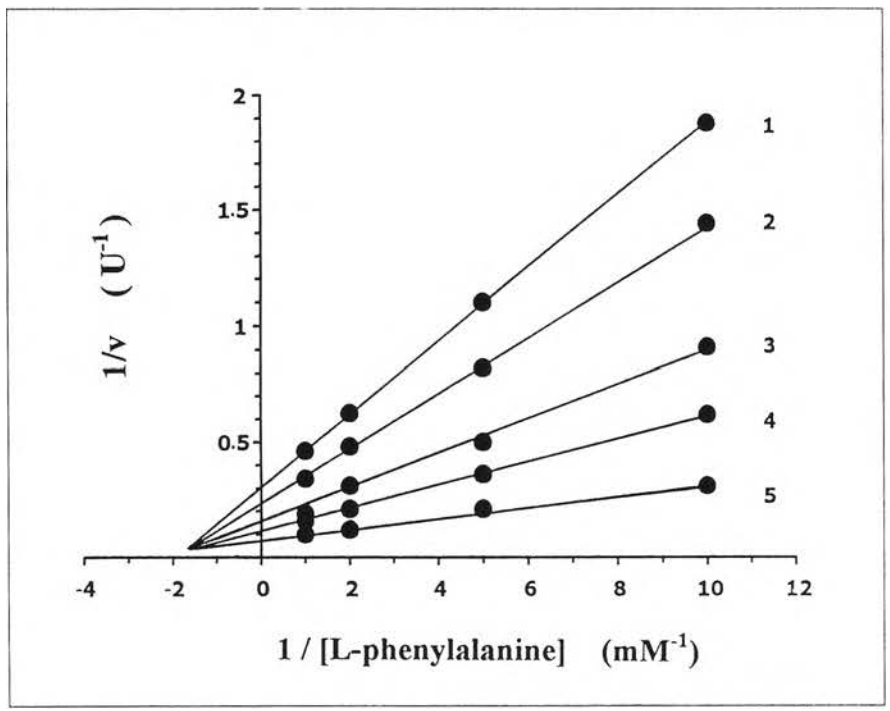
A series of steady state kinetic analyses was carried out to investigate the reaction mechanism. First, initial velocity studies for oxidative deamination were performed with L-phenylalanine as a variable substrate in the presence of several fixed concentrations of NAD^+. Plots of reciprocals of initial velocities versus reciprocals of L-phenylalanine concentrations gave a family of straight lines, which intersected in the upper left quadrant (Figure 3.17 A). These results show that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD^+ and L-phenylalanine[85]. The apparent K_m value for L-phenylalanine was calculated to be 0.59 mM from Figure 3.17 A. The apparent K_m value for NAD^+ was calculated to be 0.28 mM from the secondary plots of the intercepts at the ordinate versus reciprocal concentrations of NAD^+ (Figure 3.17 B).

Table 3.7 Effect of various group-specific reagents on phenylalanine dehydrogenase from *B. badius* BC1 [a]

Group-specific reagent (10 mM)	Amino acid involved	Residual activity (%)	
		Oxidative deamination	Reductive amination
None	-	100	100
N-Acetylimidazole (NAI)	Tyrosine	100	100
N-Bromosuccinimide (NBS)	Tryptophan	0	0
Chloramine T (CT)	Methionine	0	0
Diethylpyrocarbonate (DEPC)	Histidine	0	0
Dithiothreitol (DTT)	Cysteine	100	100
Phenylglyoxal (PG)	Arginine	12.3	10.5
Phenylmethylsulfonyl fluoride (PMSF)	Serine	100	100
2,4,6-Trinitrobenzene sulfonic acid (TNBS)	Lysine	0	0

[a] The enzyme was treated at 30°C for 20 minutes under the condition of pH specified for each respective modifiers and the residual activity was then assayed.

A



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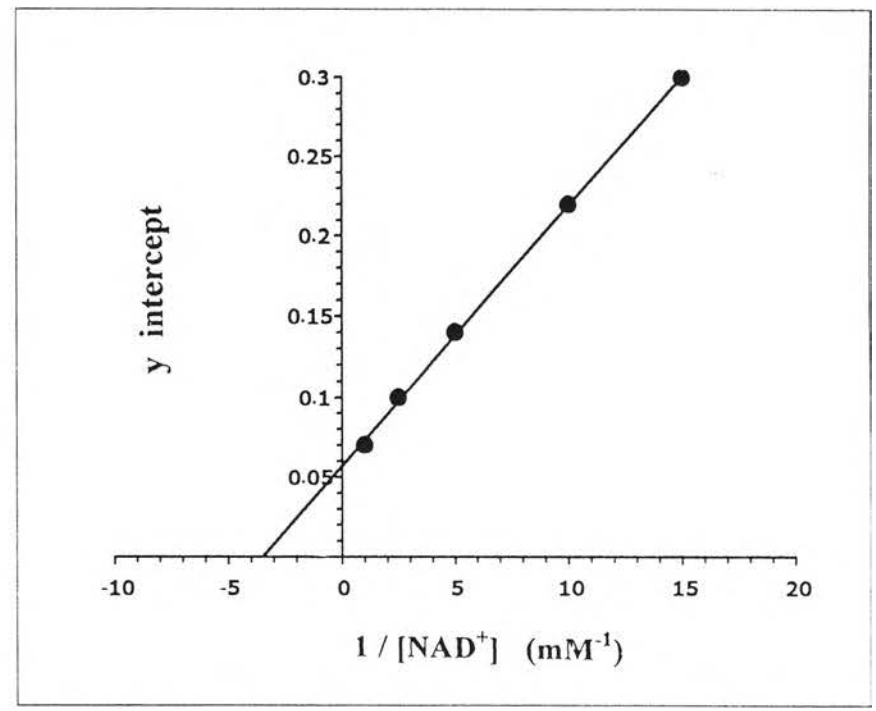


Figure 3.17 Initial velocity patterns for oxidative deamination

A. Double-reciprocal plots of initial velocities versus L-phenylalanine concentrations at a series of fixed concentrations of NAD^+
Concentrations of NAD^+ were: 0.067 mM (1), 0.1 mM (2), 0.2 mM (3), 0.4 mM (4) and 1.0 mM (5).
Concentrations of L-phenylalanine were: 0.1, 0.2, 0.5 and 1.0 mM.

B. Secondary plots of y intercepts versus reciprocal concentrations of NAD^+

3.4.2 Initial velocity studies for reductive amination

A kinetic analysis of reductive amination was performed in order to investigate several possible reaction mechanisms. The observed patterns are shown below.

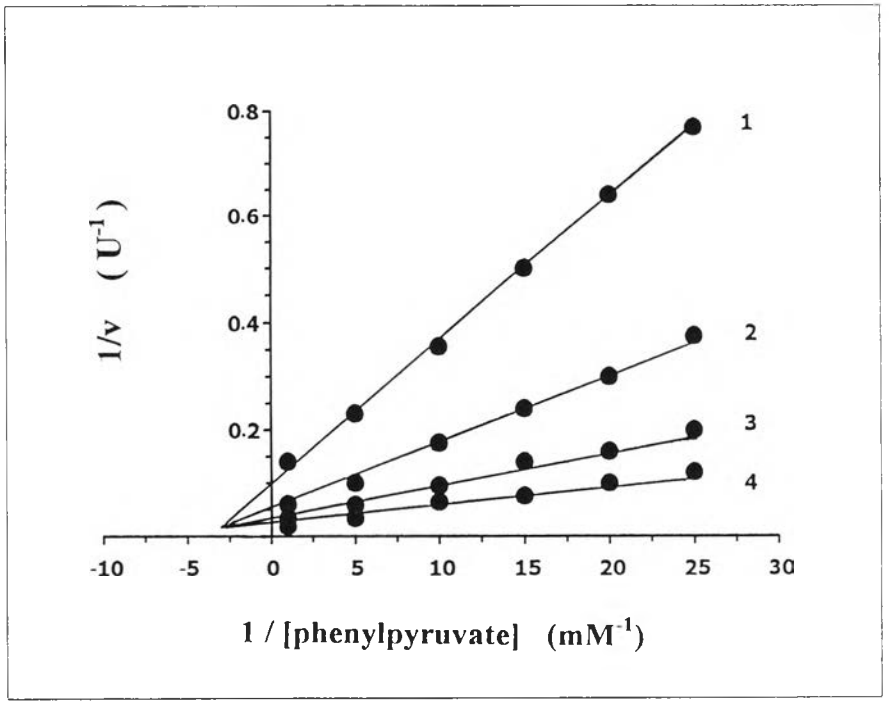
3.4.2.1) At a saturating and constant concentration of NADH (0.2 mM), the double-reciprocal plots of initial velocities versus phenylpyruvate concentrations at several fixed concentrations of NH_4Cl gave straight intersecting lines (Figure 3.18 A) and the apparent K_m value for phenylpyruvate was calculated to be 0.33 mM from this figure. The apparent K_m value for NH_4Cl was calculated to be 200 mM from the secondary plots of the intercepts at the ordinate versus reciprocal concentrations of NH_4Cl (Figure 3.18 B).

3.4.2.2) At a saturating and constant concentration of phenylpyruvate (10 mM), the double-reciprocal plots of initial velocities versus NH_4Cl concentrations at several fixed concentrations of NADH gave parallel straight lines as shown in Figure 3.19.

3.4.2.3) At a saturating and constant concentration of NH_4Cl (500 mM), the double-reciprocal plots of initial velocities versus NADH concentrations at several fixed concentrations of phenylpyruvate gave straight intersecting lines (Figure 3.20 A) and the apparent K_m value for NADH was calculated to be 0.07 mM from this figure. The apparent K_m value for phenylpyruvate was calculated to be 0.33 mM from the secondary plots of the intercepts at the ordinate versus reciprocal concentrations of phenylpyruvate (Figure 3.20 B), which was the same value that obtained from Figure 3.18 A.

The apparent K_m values of substrates of the enzyme were summarized in Table 3.8. These observed kinetic patterns from Figure 3.18, 3.19 and 3.20 may rule out the possibility of random addition of substrates and represent a sequential ordered mechanism in which phenylpyruvate binds to the enzyme between NADH and NH_4Cl [85].

A



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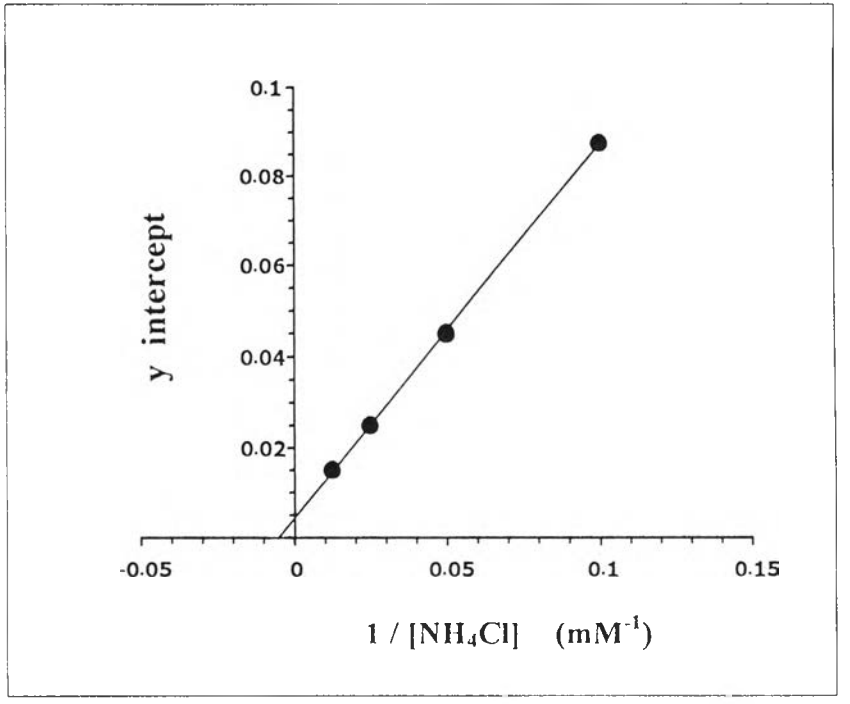

Figure 3.18 Initial velocity patterns for reductive amination (phenylpyruvate vs NH_4Cl)

A. Double-reciprocal plots of initial velocities versus phenylpyruvate concentrations at several fixed concentrations of NH_4Cl in the presence of a saturating and constant concentration (0.2 mM) of NADH. Concentrations of NH_4Cl were: 10 mM (1), 20 mM (2), 40 mM (3) and 80 mM (4).

B. Secondary plots of y intercepts versus reciprocal concentrations of NH_4Cl

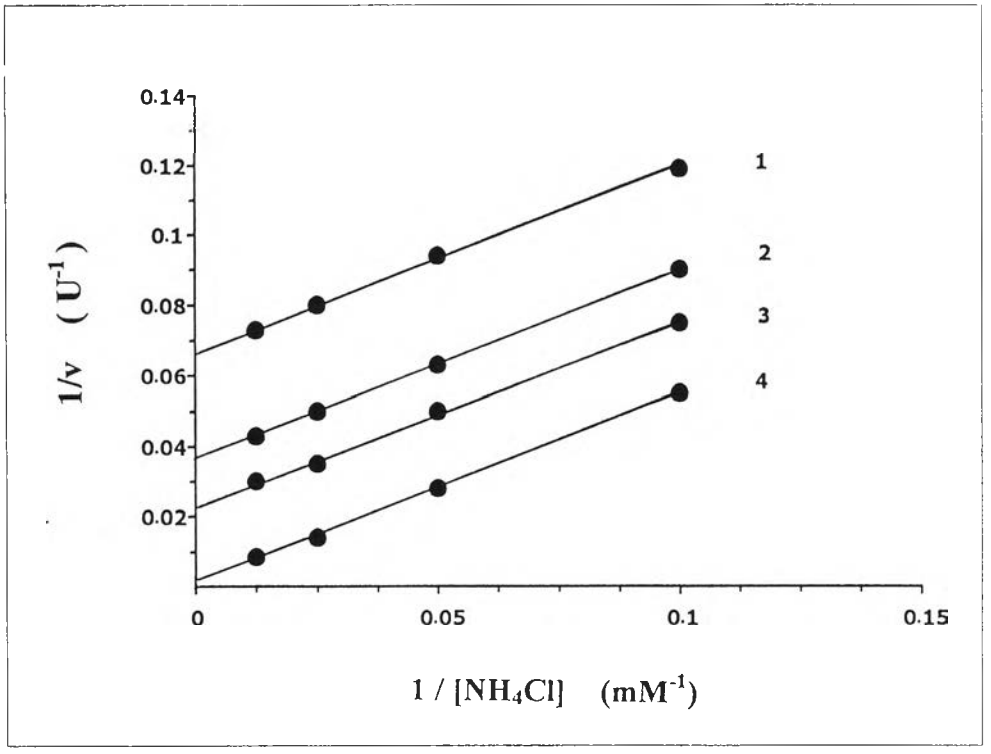

Figure 3.19 Initial velocity pattern for reductive amination (NH_4Cl vs NADH)

Double-reciprocal plots of initial velocities versus NH_4Cl concentration at several fixed concentration of NADH in the presence of a saturating and constant concentration (10 mM) of phenylpyruvate. Concentrations of NADH were: 0.0125 mM (1), 0.025 mM (2), 0.050 mM (3) and 0.1 mM (4).

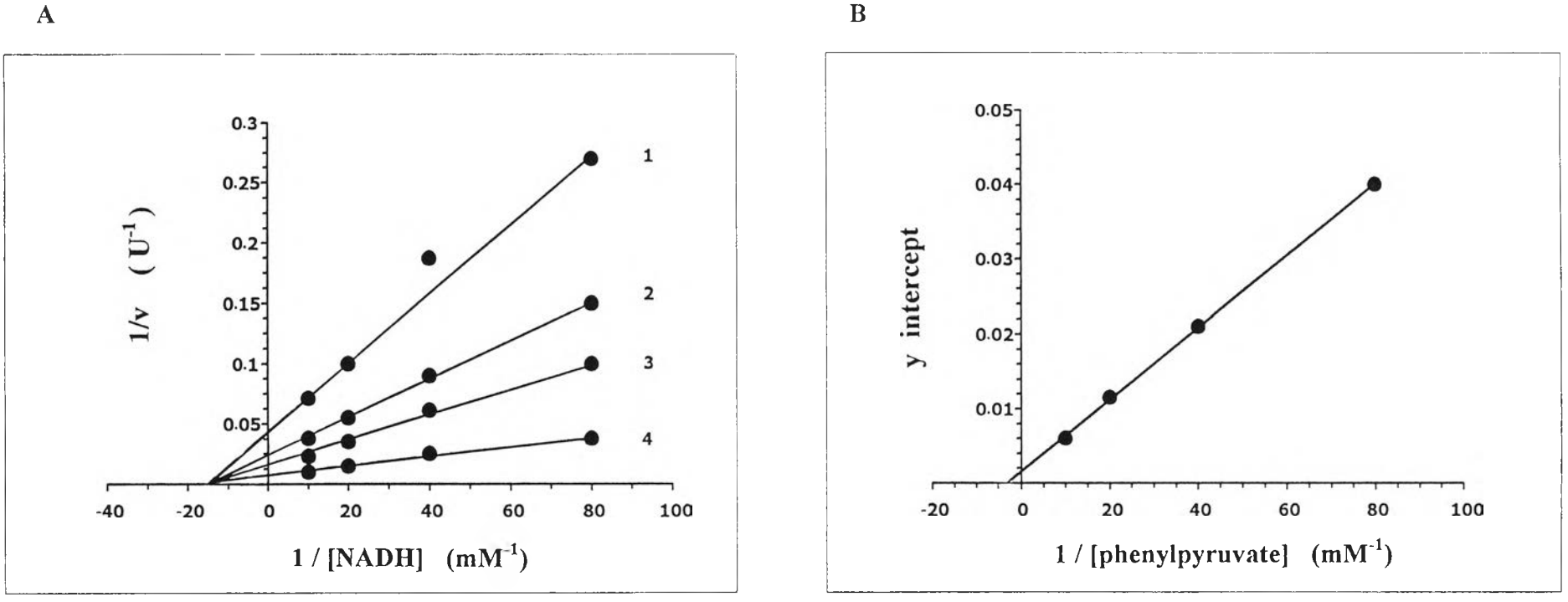

Figure 3.20 Initial velocity patterns for reductive amination (NADH vs phenylpyruvate)

A. Double-reciprocal plots of initial velocities versus NADH concentration at several fixed concentrations of phenylpyruvate in the presence of a saturating and constant concentration (500 mM) of NH_4Cl. Concentrations of phenylpyruvate were: 0.0125 mM (1), 0.025 mM (2), 0.050 mM, (3) and 0.1 mM (4).

B. Secondary plots of y intercepts versus reciprocal concentrations of phenylpyruvate

Table 3.8 The apparent K_m values of substrates of phenylalanine dehydrogenase from *B. badius* BC1

Substrate	K_m (mM)
L-phenylalanine	0.59
NAD^+	0.28
phenylpyruvate	0.33
NH_4Cl	200
NADH	0.07

3.4.3 Product inhibition studies

Product inhibition studies of the oxidative deamination by NADH, phenylpyruvate and NH_4Cl to determine the order of substrate addition and product release were performed according to the method of Cleland [85]. With NADH as an inhibitor, the double reciprocal plots of velocities versus NAD^+ concentrations at a constant concentration of L-phenylalanine showed a typical competitive inhibition pattern (Figure 3.21 A). This finding indicates that NAD^+ and NADH can bind to the free form of the enzyme. Uncompetitive inhibition by NH_4Cl with respect to L-phenylalanine (Figure 3.23 B) rules out the Theorell-Chance mechanism [85]. The other product inhibition patterns observed with phenylpyruvate (Figure 3.22) and NH_4Cl (Figure 3.23) as inhibitors were identical to the predicted patterns for the sequential ordered binary-ternary mechanism except for the noncompetitive inhibition by phenylpyruvate with respect to L-phenylalanine as shown in Figure 3.22 B. Observed product inhibition patterns of oxidative deamination were summarized in Table 3.9.

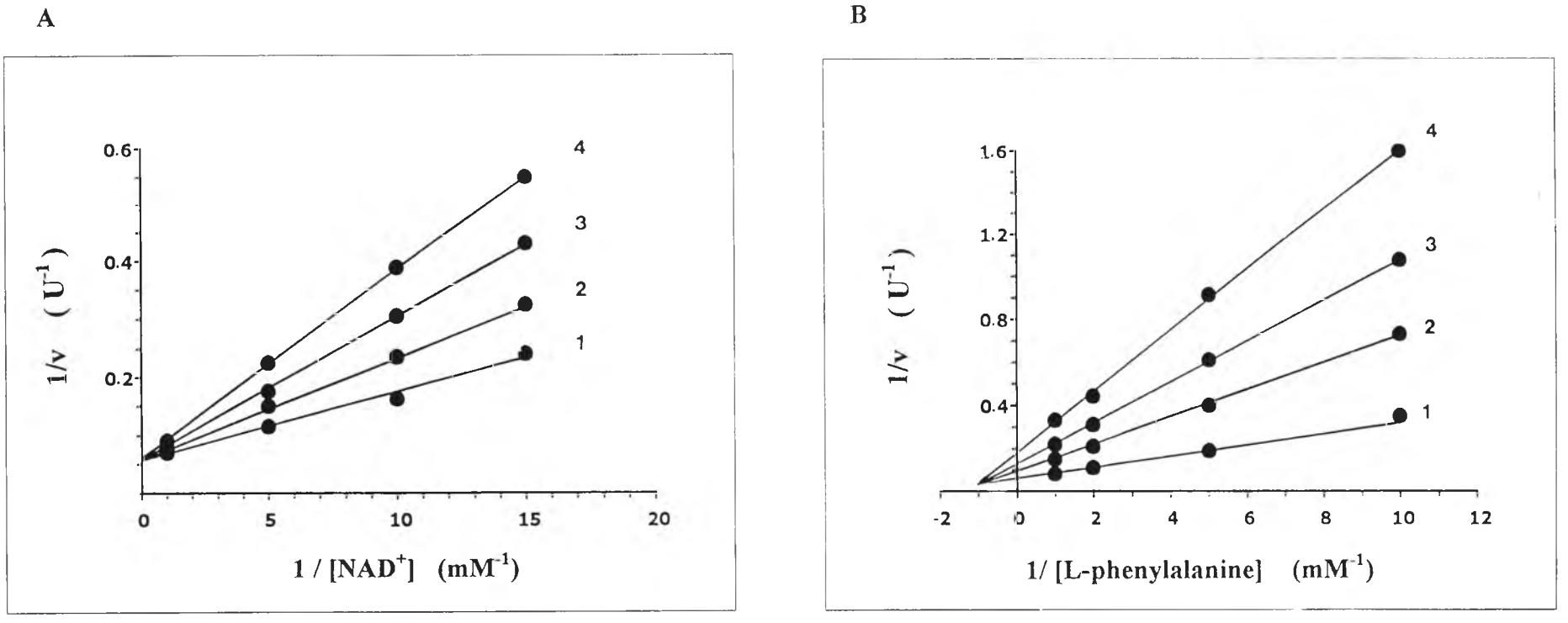

Figure 3.21 Product inhibition patterns of oxidative deamination by NADH

A. Product inhibition pattern by NADH with respect to NAD^+ as the varied substrate. L-phenylalanine (20 mM) was held at saturating and constant concentration. Concentrations of NADH were 0 mM (1), 0.025 mM (2), 0.05 mM (3) and 0.1 mM (4).

B. Product inhibition pattern by NADH with respect to L-phenylalanine as the varied substrate. NAD^+ (2 mM) was held at saturating and constant concentration. Concentrations of NADH were 0 mM (1), 0.025 mM (2), 0.05 mM (3) and 0.1 mM (4).

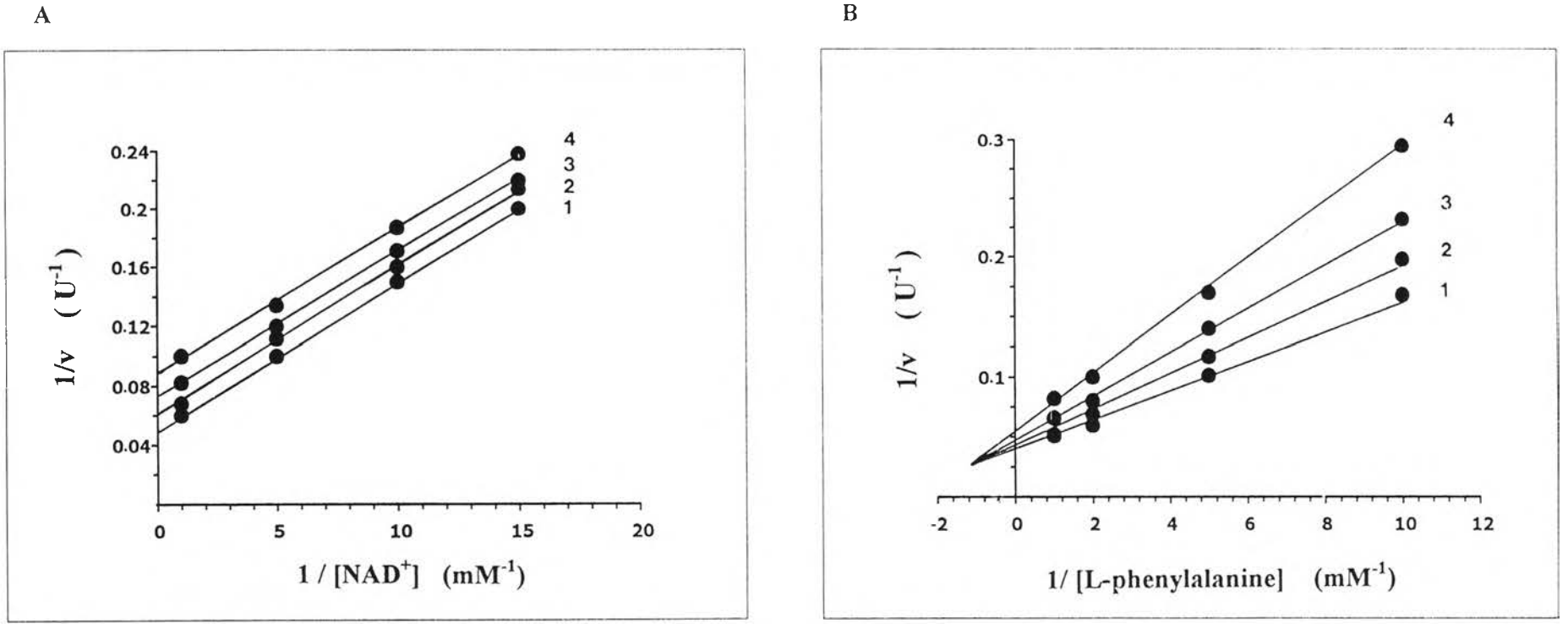

Figure 3.22 Product inhibition patterns of oxidative deamination by phenylpyruvate

A. Product inhibition pattern by phenylpyruvate with respect to NAD^+ as the varied substrate. L-phenylalanine (20 mM) was held at saturating and constant concentration. Concentrations of phenylpyruvate were 0 mM (1), 0.2 mM (2), 0.4 mM (3) and 1.0 mM (4).

B. Product inhibition pattern by phenylpyruvate with respect to L-phenylalanine as the varied substrate. NAD^+ (2 mM) was held at saturating and constant concentration. Concentrations of phenylpyruvate were 0 mM (1), 0.2 mM (2), 0.4 mM (3) and 1.0 mM (4).

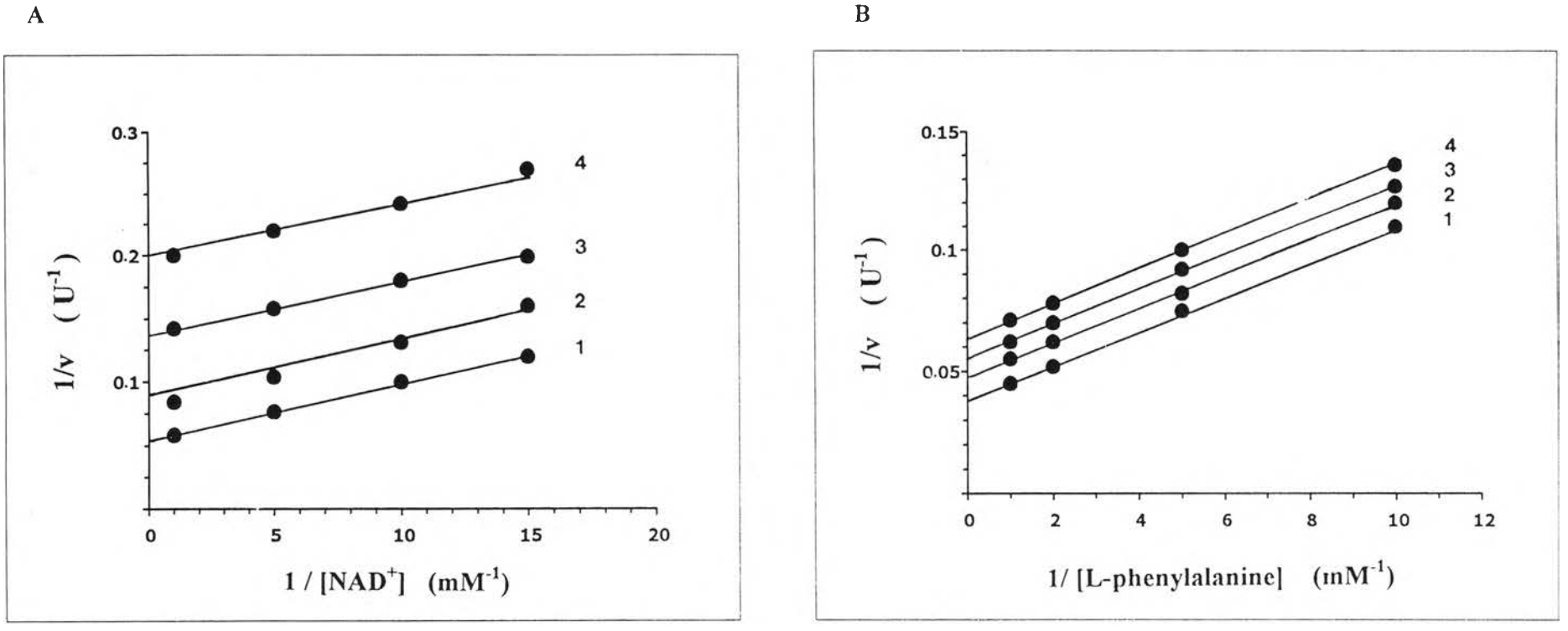

Figure 3.23 Product inhibition patterns of oxidative deamination by NH_4Cl

A. Product inhibition pattern by NH_4Cl with respect to NAD^+ as the varied substrate. L-phenylalanine (20 mM) was held at saturating and constant concentration. Concentrations of NH_4Cl were 0 mM (1), 20 mM (2), 40 mM (3) and 80 mM (4).

B. Product inhibition pattern by NH_4Cl with respect to L-phenylalanine as the varied substrate. NAD^+ (2 mM) was held at saturating and constant concentration. Concentrations of NH_4Cl were 0 mM (1), 20 mM (2), 40 mM (3) and 80 mM (4).

Table 3.9 Product inhibition patterns of oxidative deamination of phenylalanine dehydrogenase from *B. badius* BC1

Substrate	Product inhibition pattern by		
	NADH	**phenylpyruvate**	**NH_4Cl**
NAD^+	competitive	uncompetitive	uncompetitive
L-phenylalanine	noncompetitive	noncompetitive	uncompetitive