CHAPTER III

RESULTS

3.1 Purification of alanine dehydrogenase

The crude extract of alanine dehydrogenase was prepared from 30 g wet weight of *Aeromonas hydrophila*. The protein solution was slowly brought to 30-40% saturated ammonium sulfate. The precipitated protein was dissolved with 10 mM KPB, pH 7.4 and dialyzed against the same buffer. The dialyzed enzyme was loaded on DEAE-Toyopearl column. After washing the unbound proteins throughly with 10 mM KPB, pH 7.4, the enzyme was eluted by 0-1 M potassium chloride in the same buffer. It was found that the active fraction between tube number 91-106 was eluted at 0.08-0.2 M of KCl (Figure 8). Fraction with high enzyme activity (fraction 91-103) were pooled and concentrated before dialyzed. The hydroxyapatite column was used to purify the enzyme solution from DEAE-Toyopearl column. The column was washed by 10 mM KPB, pH 7.4 to elute unbound proteins. The bound proteins were eluted by linear gradient of 10-100 mM potassium phosphate buffer, pH 7.4. The active fraction was eluted between fraction 9-20, indicated that alanine dehydrogenase didn't bind with hydroxyapatite gel (Figure 9). These fractions were pooled and concentrated before dialyzed against dialysis buffer. The enzyme solution was then loaded onto Blue Sepharose column and eluted with 0-2 M KCL in 10 mM KPB, pH 7.4. It was found that the active fraction was eluted from tube number 71-89 at 0.2 - 0.4 M of potassium chloride (Figure 10). The active fractions were analyzed for purity by native-PAGE. It was found that the slab gel showed only one band in every fractions. Then, the fractions 73-89 were pooled and concentrated with aquacide before dialyzed against 10 mM KPB, pH 7.4

The purity of alanine dehydrogenase was analyzed by non-denaturing PAGE. The activity stain was performed to compare with protein stain. The result is shown in Figure 11. It was found that protein stain in lane 5 showed of a single band, which corresponded to the activity stain, indicating that the enzyme from Blue Sepharose was pure enzyme.

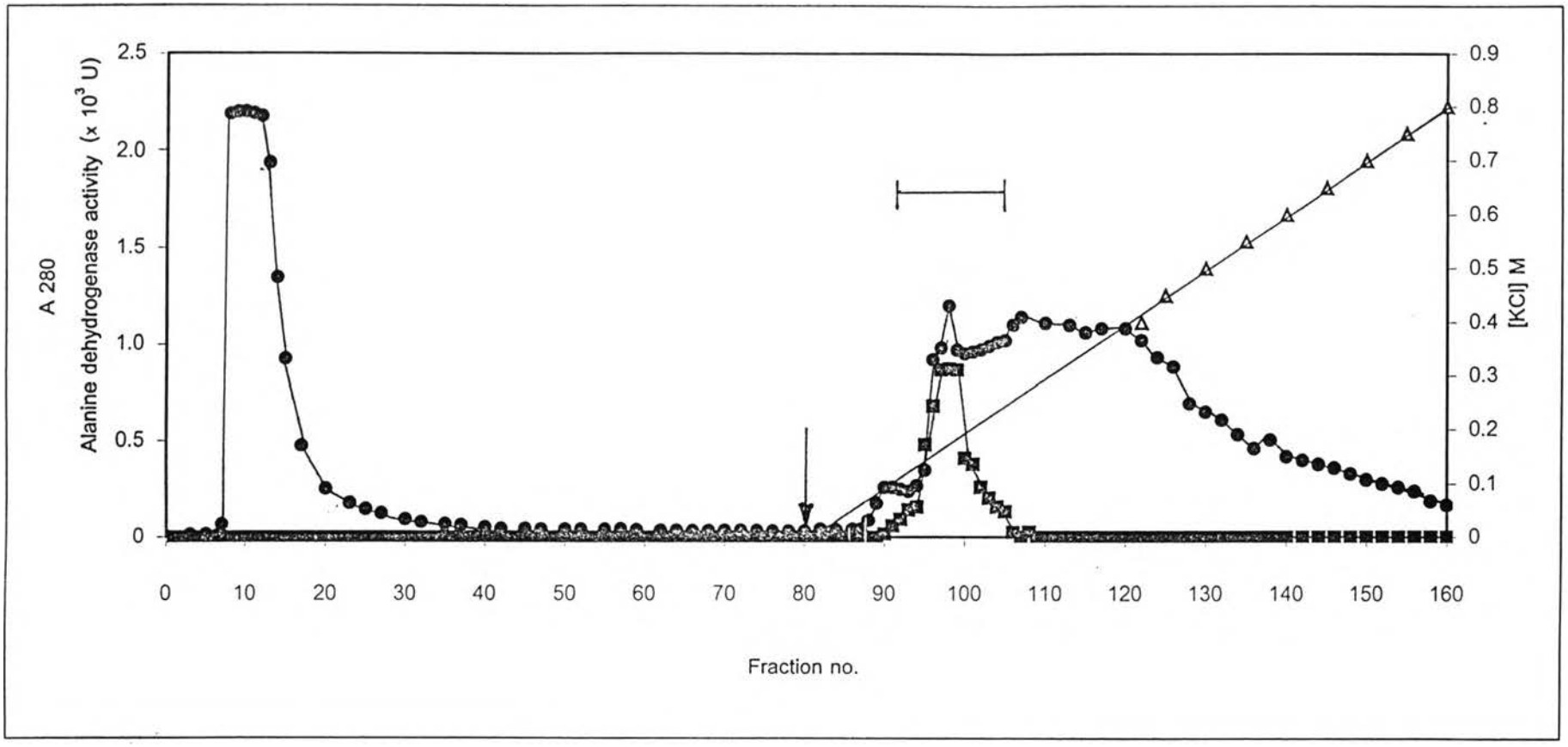

Figure 8 Purification of alanine dehydrogenase from *Aeromonas hydrophila* by DEAE-Toyopearl column

The enzyme solution was applied to DEAE-Toyopearl column and washed with 10 mM KPB, pH 7.4 until A_{280} decreased to background value. Elution of bound proteins was made by 0-1 M KCl in the same buffer at the flow rate of 1 ml/min. The arrow indicates where gradient started. The protein peak from fractions 91 to 103 were pooled.

● A_{280} ■ activity Δ [KCl] ⊢——⊣ the pooled fraction (fraction no.91-103)

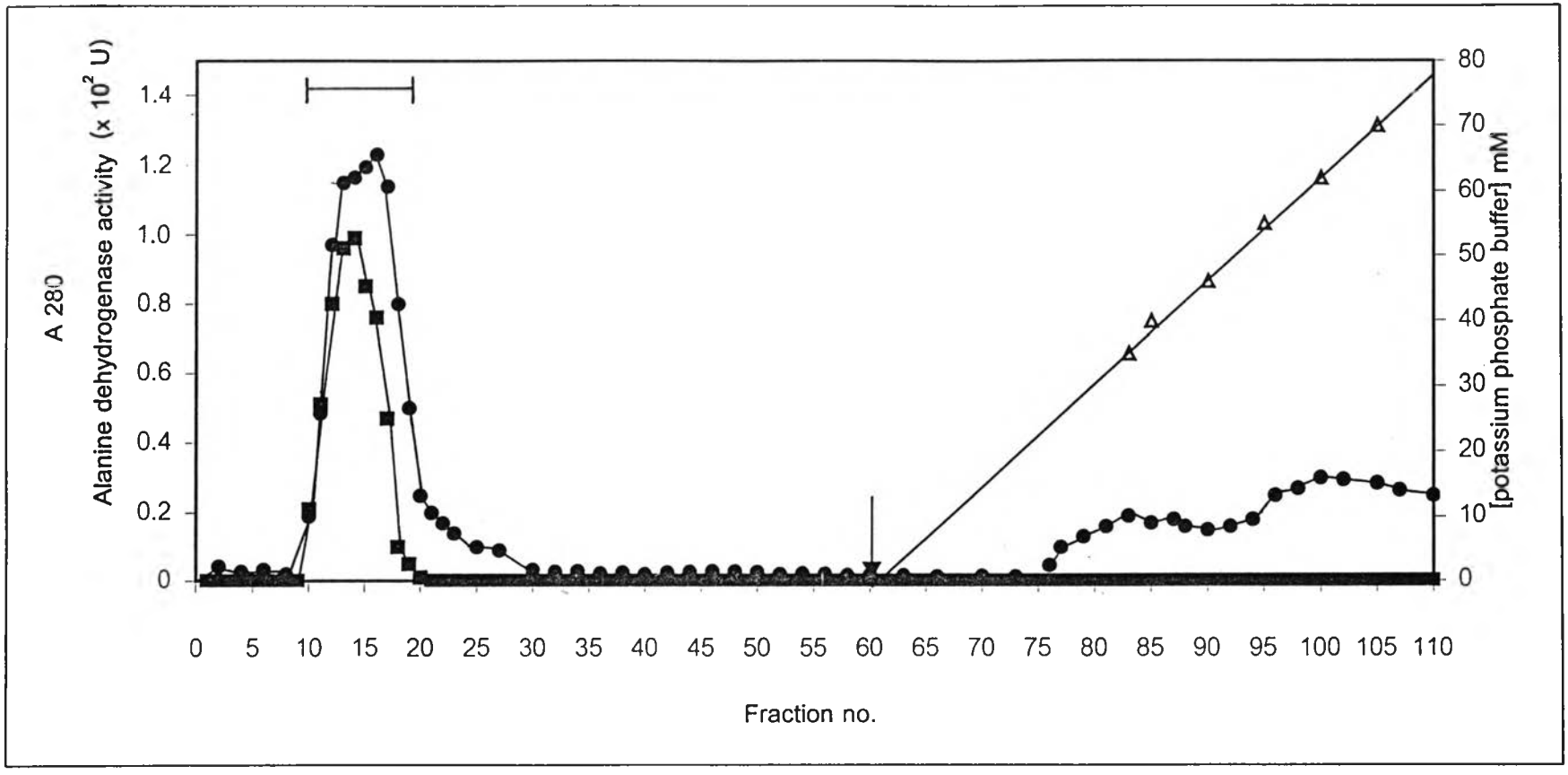

Figure 9 Purification of alanine dehydrogenase from *Aeromonas hydrophila* by hydroxyapatite column

The enzyme solution from DEAE column was applied to hydroxyapatite column and washed with 10 mM KPB, pH 7.4 until A_{280} decreased to background value. Elution of the bound proteins was made by 10 - 100 mM KPB, pH 7.4 at the flow rate 0.25 ml / min. The arrow indicates where gradient started.The protein peak from fractions 9 to 19 were pooled.

● A_{280} ■ activity Δ [potassium phosphate buffer] |——————| the pooled fraction (fraction no. 9 -19)

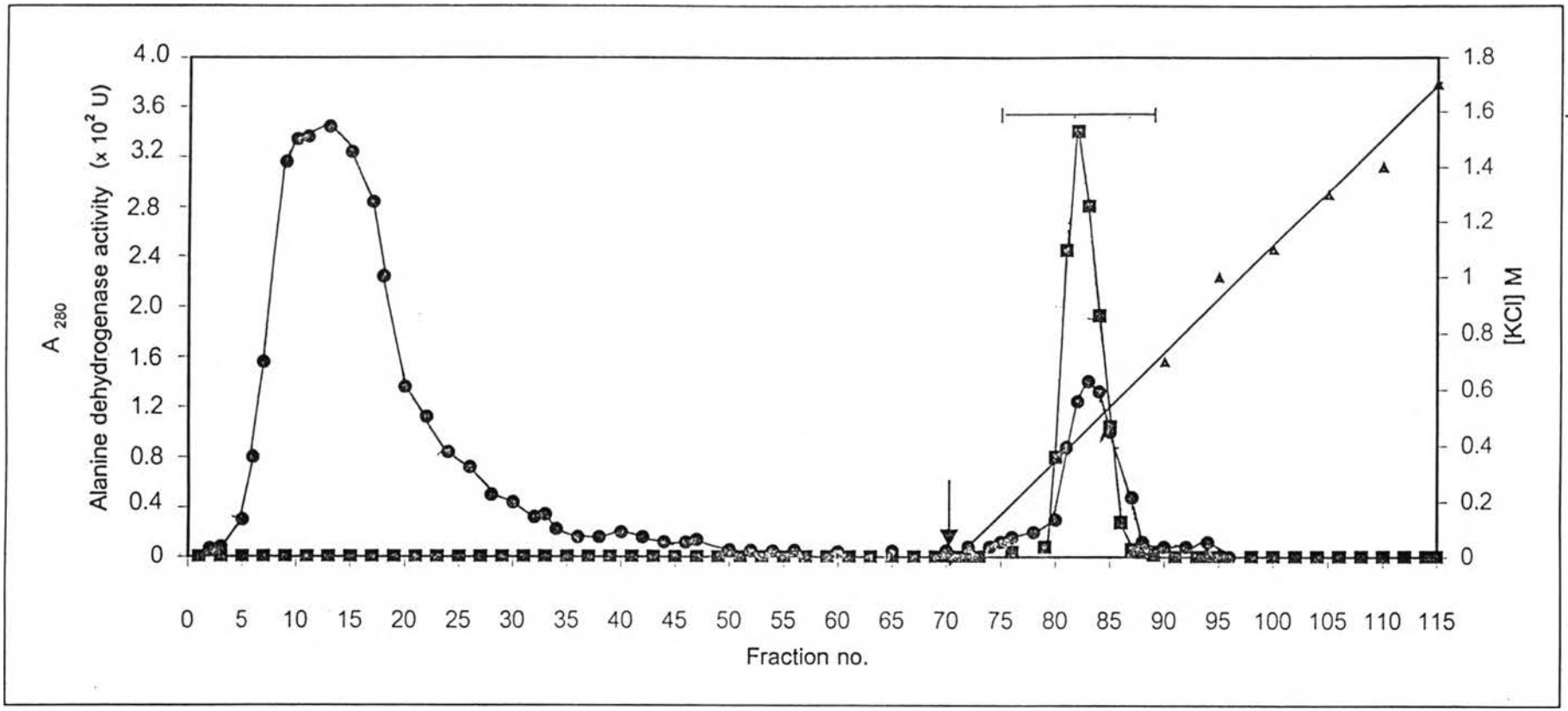

Figure 10 The purification of alanine dehydrogenase from *Aeromonas hydrophila* on Blue Sepharose column

The enzyme from hydroxyapatite was applied to Blue Sepharose column and washed with 10 mM KPB, pH 7.4 until A_{280} decreased to background value. Elution of bound proteins was made by 0 - 2 M KCl in the same buffer at flow rate 1 ml /min. The arrow indicates where gradient started.The protein peak from fractions 73 to 89 were pooled.

● A_{280} ■ activity △ [KCl] ⊢——⊣ the pooled fraction (fraction no. 73 - 89)

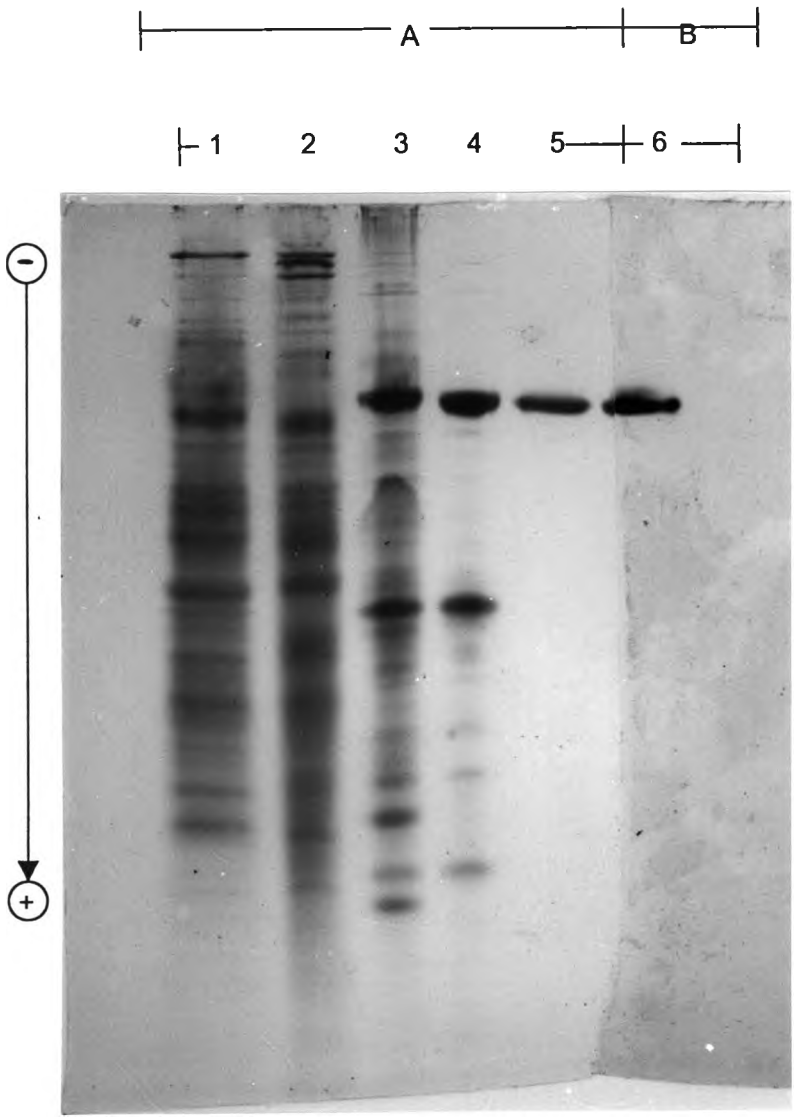

Figure 11 Non-denaturing PAGE of alanine dehydrogenase from each step of purification

A: Protein staining

Lane 1.	crude enzyme	102.2 μg
2.	30-40% ammonium sulfate precipitation	123.0 μg
3.	DEAE-Toyopearl column	27.5 μg
4.	Hydroxyapatite column	9.0 μg
5.	Blue Sepharose column (purified enzyme)	9.0 μg

B: Activity staining

Lane 6.	Blue Sepharose column	9.0 μg

The recovery and yield of alanine dehydrogenase obtained from each purification step was shown in Table 3. The specific activity of alanine dehydrogenase increased through each step. After the final step of purification, an activity yield of 24.8% was obtained with 150.8 folds of purity.

3.2 Chemical modification of alanine dehydrogenase

To determine the effect of group-specific reagents on the enzyme activity, purified enzyme was incubated with 10 mM of each modifying reagent at 30 °C for 20 minutes under the selective condition for each reagent as described in section 2.8. The residual activity for oxidative deamination and reductive amination were then determined and compared with those of untreated enzyme as shown in Table 4. Alanine dehydrogenase activity was completely inhibited by 10 mM CT, DEPC, PG, and TNBS whereas NAI slightly inhibited the enzyme activity with 88.9 % and 88.7% residual activity for oxidative deamination and reductive amination, respectively. In contrast, modification by DTT did not affect the enzyme activity. Hence methionine, histidine, arginine and lysine residues may play an important role in the active site of the enzyme. To prove whether or not the modification occured at or near the active site, substrate protection technique was performed.

At first the suitable concentration of each modifying reagent was determined. The enzyme was incubated with various concentrations of the reagent at 30 °C for 20 minutes before monitoring the residual activity as described in section 2.9. The suitable concentration is the minimum concentration of reagent that leads to the maximum inactivation of the enzyme. Then, suitable incubation time was carried out by incubation the enzyme with suitable concentration of modifier at various times. The residual acivity was determined and compared with untreated enzyme as described in section 2.10. The suitable incubation time is the time at which about 50% of enzyme activity was left. The suitable concentration and incubation time were then used to identify the amino acids involved in the catalytic site using substrate protection technique as described in section 2.11.

Table 3 Purification of alanine dehydrogenase from *Aeromonas hydrophila*

Purification steps	Total activity (unit)	Total protein (mg)	Specific activity (unit/ mg protein)	% Yield	Purification fold
Crude enzyme	387.0	3289.5	0.1	100.0	1
30-40 % ammonium sulfate precipitation	265.6	1029.2	0.3	68.6	2.2
DEAE-Toyopearl	186.0	71.0	2.6	48.1	21.8
Hydroxyapatite	123.0	23.2	5.3	31.8	44.2
Blue Sepharose	95.9	5.3	18.1	24.8	150.7

Table 4 Effect of various group-specific reagents on alanine dehydrogenase activity

Reagent (10mM)	Amino acid	Residual activity(%)	
		Oxidative deamination	Reductive amination
None	-	100.0	100.0
N-acetylimidazole(NAI)	tyrosine	88.9	88.7
chloramine T(CT)	methionine	0	0
diethylpyrocarbonate (DEPC)	histidine	0	0
dithiothreitol (DTT)	cysteine	100.0	100.0
phenylglyoxal (PG)	arginine	0	0
2,4,6-trinitrobenzenesulfonic acid (TNBS)	lysine	0	0

The enzyme was treated at 30°C for 20 minutes under the condition of pH specified in the respective modifiers. The residual activity was assayed.

3.2.1 Modification of methionine residues

Methionine residues of alanine dehydrogenase were modified by CT as shown in Figure 12. The enzyme activity was completely lost by 2.4 mM of CT. Thus the suitable concentration of CT was 2.4 mM. Figure 13 shows that at 2 minutes of incubation, alanine dehydrogenase activity decreased to 57.0 % whereas complete loss was observed at 15 minutes. Therefore, the suitable incubation time of the enzyme with CT was 2 minutes.

To determine if methionine residue was involved at the active site of alanine dehydrogenase, modifications by CT in the presence or absence of substrates were compared in eight different conditions: 1.) alanine dehydrogenase alone, 2.) alanine dehydrogenase incubated with pyruvate and NADH, 3.) alanine dehydrogenase incubated with pyruvate, 4.) alanine dehydrogenase incubated with NADH, 5.) alanine dehydrogenase incubated with both substrates, then modified by CT, 6.) alanine dehydrogenase incubated with pyruvate, then modified by CT, 7.) alanine dehydrogenase incubated with NADH, then modified by CT, and 8.) alanine dehydrogenase modified by CT. After the reaction, residual activity of alanine dehydrogenase was then determined. The result in Table 5 shows that the modification by 2.4 mM CT led to 48.5% loss of alanine dehydrogenase activity. When it was preincubated with both substrates, the activity regained was about 8.0%. However, less activity of regain about 6.2% and 2.5% could be observed in the presence of pyruvate or NADH, respectively.

3.2.2 Modification of histidine residues

Histidine residues of alanine dehydrogenase were modified by DEPC. The result in Figure 14 shows that enzyme activity was completely lost by 0.08 mM of DEPC. Thus, the suitable concentration of DEPC was 0.08 mM. Figure 15 shows that after 5 minutes of incubation, alanine dehydrogenase activity decreased to 49.2 % whereas total activity loss was observed at 15 minutes. Thus, the suitable incubation time with DEPC was 5 minutes.

To determine if histidine residue was involved at the active site of alanine dehydrogenase, modification by DEPC in the presence or absence of substrate were compared. The procedure was performed in similar with modification by CT. The result in

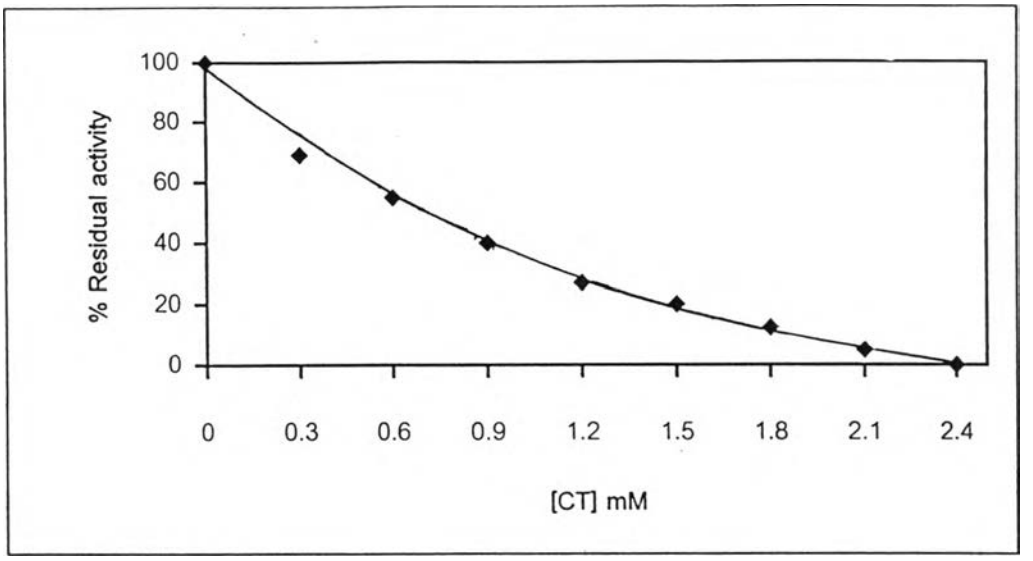

Figure 12 Effect of CT on alanine dehydrogenase activity

Alanine dehydrogenase was incubated with varying concentrations of CT at 30° C for 20 minutes according to the method in 2.9. The residual activity of the enzyme was determined and compared with untreated enzyme.

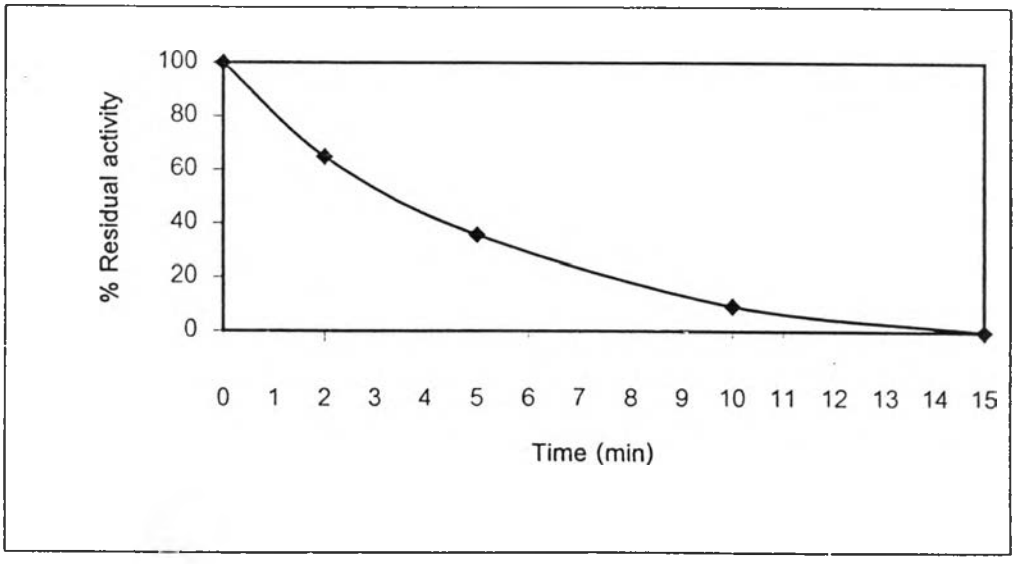

Figure 13 Inactivation of alanine dehydrogenase activity by 2.4 mM CT at various incubation times

Alanine dehydrogenase was incubated with 2.4 mM CT at 30° C, at various time. The residual activity of enzyme was determined and compared with untreated enzyme.

Table 5 Residual alanine dehydrogenase activity of CT modified enzyme in the presence and the absence of protective substance(s)

Compound added	Relative residual activity (%)
1. None	100.0
2. 6.65 mM pyruvate + 2.5 mM NADH	100.0
3. 6.65 mM pyruvate	100.0
4. 2.5 mM NADH	99.4
5. 6.65 mM pyruvate + 2.5 mM NADH then 2.4 mM CT	59.5
6. 6.65 mM pyruvate then 2.4 mM CT	57.7
7. 2.5 mM NADH then 2.4 mM CT	54.0
8. 2.4 mM CT	51.5

The relative residual activity values are average of two separate experiments.

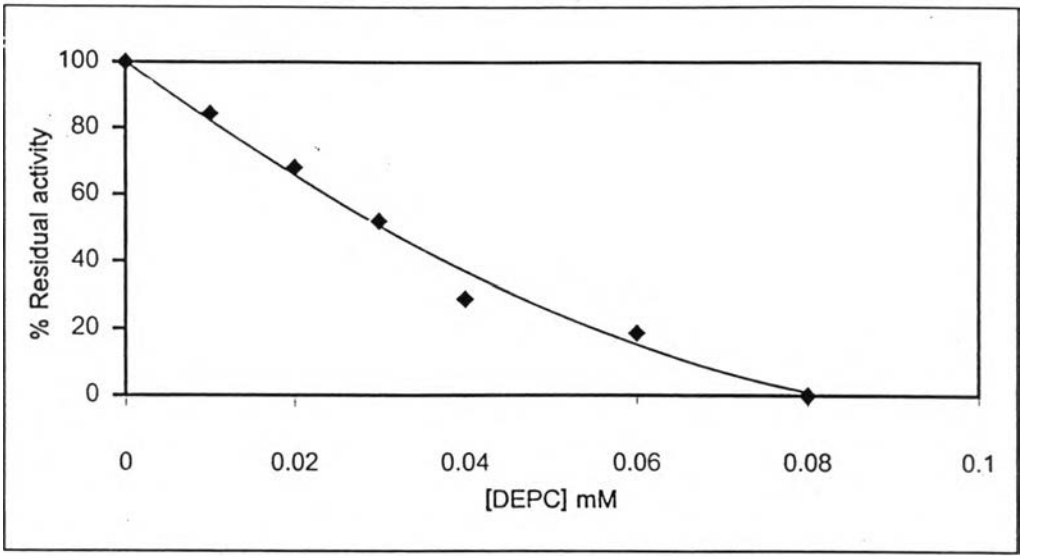

Figure 14 Effect of DEPC on alanine dehydrogenase activity

Alanine dehydrogenase was incubated with varying concentrations of DEPC at 30° C for 20 minutes according to the method in 2.9. The residual activity of the enzyme was determined and compared with untreated enzyme.

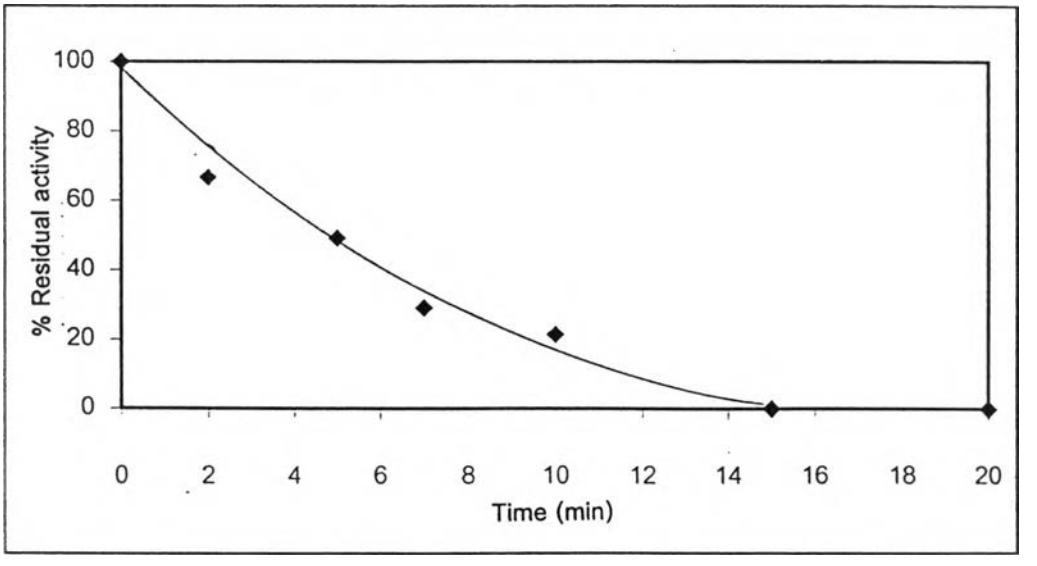

Figure 15 Inactivation of alanine dehydrogenase activity by 0.08 mM DEPC at various incubation times

Alanine dehydrogenase was incubated with 0.08 mM DEPC at 30° C, at various time. The residual activity of enzyme was determined and compared with untreated enzyme.

Table 6 shows that modification by 0.08 mM DEPC led to 50.8 % loss of alanine dehydrogenase activity. When pyruvate and/or NADH was included, the activity of enzyme increased significantly. In the presence of both pyruvate and NADH, only 10.0% of enzyme activity was lost when compared to control. About 15.0 and 17.6% activity loss could be observed in the presence of pyruvate or NADH, respectively.

3.2.3 Modification of arginine residues

Arginine residues of alanine dehydrogenase were modified by phenylglyoxal (PG) as described in section 2.9. At 10 mM PG, the enzyme activity was completely lost while at 9 mM, little activity was still observed (Figure 16). Thus, the suitable concentration was 10 mM. The suitable incubation time was determined as shown in Figure 17. After incubation for 5 minutes, alanine dehydrogenase activity decreased to 50.0%, while at 20 minutes, total activity loss was observed. Thus, the suitable incubation time of enzyme with PG was 5 minutes.

Modifications by PG in the presence or absence of substrate were compared to determine if arginine residue was involved at the active site of alanine dehydrogenase. The result in Table 7 shows that modification by 10 mM PG led to 47.5% loss of alanine dehydrogenase activity. In the presence of pyruvate or NADH, the activity loss due to the modification of arginine was reduced to 15 % and 18%, respectively. The highest residual activity (about 90%) was found when the modification was performed in the presence of both pyruvate and NADH.

3.2.4 Modification of lysine residues

Lysine residues of alanine dehydrogenase were modified by 2,4,6-trinitrobenzenesulfonic acid (TNBS) as described in section 2.9. After the enzyme was inactivated at suitable incubation time, excess chemicals were removed by ultrafilter Suprec $^{Tm-01}$. Then, the residual activity of enzyme was determined. It was found that at 5 mM of TNBS, enzyme activity was completely lost (Figure 18). Thus, the suitable concentration is 5 mM. To determine the suitable incubation time , the enzyme was

Table 6 Residual alanine dehydrogenase activity of DEPC modified enzyme in the presence and the absence of protective substance(s)

Compound added	Relative residual activity (%)
1. None	100.0
2. 6.65 mM pyruvate + 2.5 mM NADH	100.0
3. 6.65 mM pyruvate	100.0
4. 2.5 mM NADH	98.9
5. 6.65 mM pyruvate + 2.5 mM NADH then 0.08 mM DEPC	90.0
6. 6.65 mM pyruvate then 0.08 mMDEPC	85.0
7. 2.5 mM NADH then 0.08 mM DEPC	82.4
8. 0.08 mM DEPC	49.2

The relative residual activity values are average of two separate experiments.

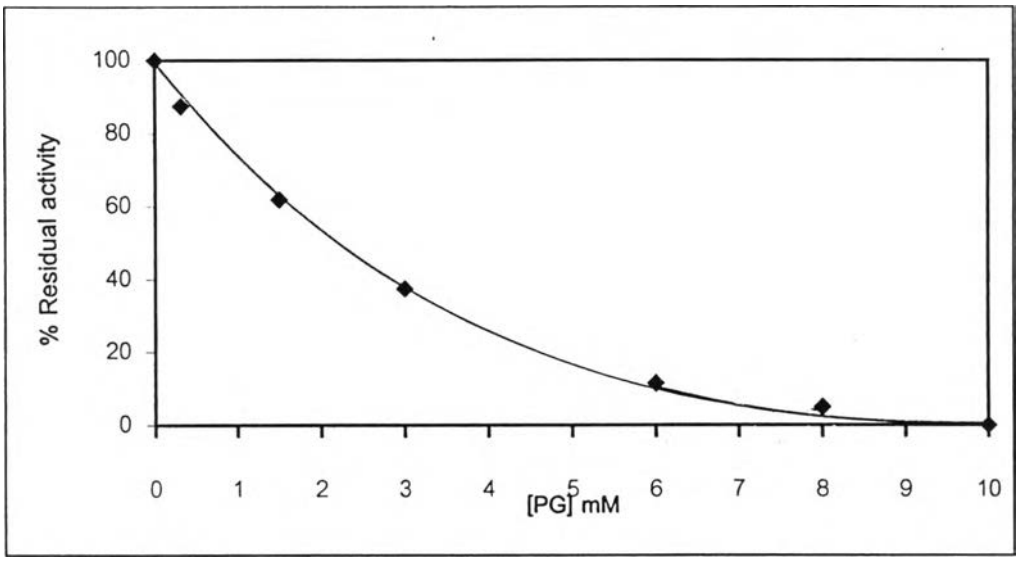

Figure 16 Effect of PG on alanine dehydrogenase activity

Alanine dehydrogenase was incubated with varying concentrations of PG at 30° C for 20 minutes according to the method in 2.9. The residual activity of the enzyme was determined and compared with untreated enzyme.

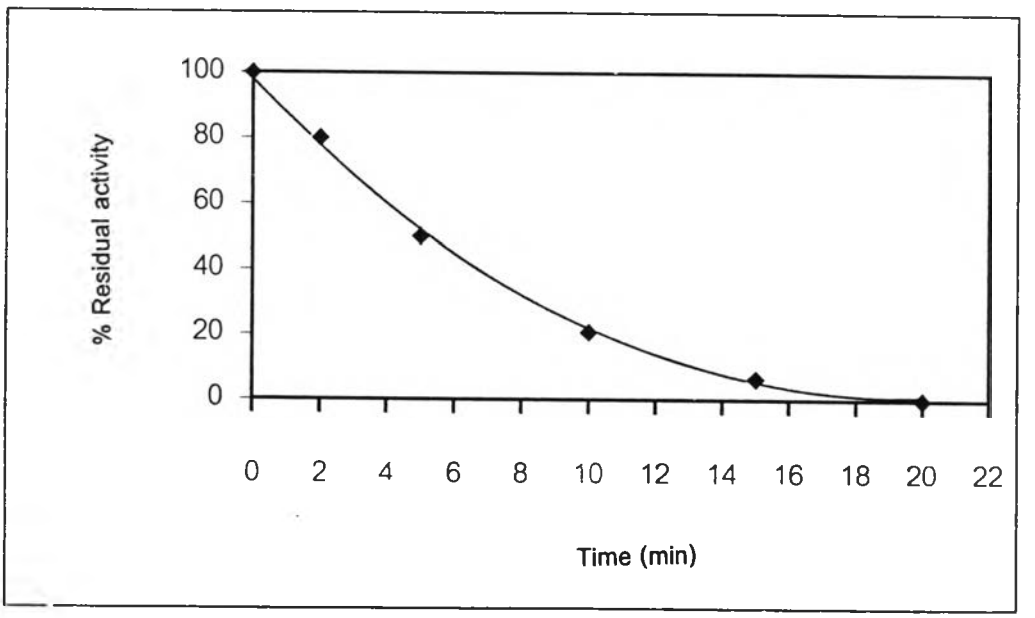

Figure 17 Inactivation of alanine dehydrogenase activity by 10 mM PG at various incubation times

Alanine dehydrogenase was incubated with 10 mM PG at 30° C, at various time. The residual activity of enzyme was determined and compared with untreated enzyme.

Table 7 Residual alanine dehydrogenase activity of PG modified enzyme in the presence and the absence of protective substance(s)

Compound added	Relative residual activity (%)
1. None	100.0
2. 6.65 mM pyruvate + 2.5 mM NADH	100.0
3. 6.65 mM pyruvate	100.0
4. 2.5 mM NADH	100.0
5. 6.65 mM pyruvate + 2.5 mM NADH then 10 mM PG	90.0
6. 6.65 mM pyruvate then 10 mM PG	85.0
7. 2.5 mM NADH + then 10 mM PG	82.0
8. 10 mM PG	52.5

The relative residual activity values are average of two separate experiments.

incubated with the suitable concentration at 5 mM at various times. After the reaction, the enzyme activity was then determined and compared with untreated enzyme (Figure 19). After incubated for 5 minutes, alanine dehydrogenase activity decreased to 50.6% and was completely lost at 25 minutes of incubation time. Thus, the suitable incubation time of TNBS was 5 minutes.

To determine if lysine residue was involved at the active site of alanine dehydrogenase, modifications by TNBS in the presence or absence of substrate were compared in eight different conditions. The result in Table 8 shows that modification by 5 mM TNBS led to 50.6% loss of alanine dehydrogenase activity. When pyruvate and/or NADH was included, the residual activity of enzyme were increased. In the presence of pyruvate and NADH, an estimated 13.0% of enzyme activity was lost. While, in the presence of pyruvate or NADH, about 23.4 % and 18.8 % loss of alanine dehydrogenase activity could be observed, respectively.

From the above result, 30-35% of the activity could be regained when the enzyme was modified with DEPC, PG and TNBS in the presence of pyruvate or NADH. Moreover, up to 90 % of the residual activity was found when the enzyme was pretected by both pyruvate and NADH. No significant protection (about 10%) was obtained when CT was used as the modifying reagents. Thus, histidine, arginine and lysine were supposed to be essential residues, which play the important role in an active site where as methionine should far from the active site.

Since lysine in the active site of alanine dehydrogenase was already identified (Delforge *et al.*, 1997), only arginine and histidine were further studied for inactivation and protection patterns.

3.3 Inactivation pattern

3.3.1 Inactivation pattern of histidine residues by DEPC

Modification of histidine residues by DEPC was performed by incubating alanine dehydrogenase with various concentrations of DEPC at various times as described in section 2.12.1. The linear plots of the logarithm of residual enzyme activity against the

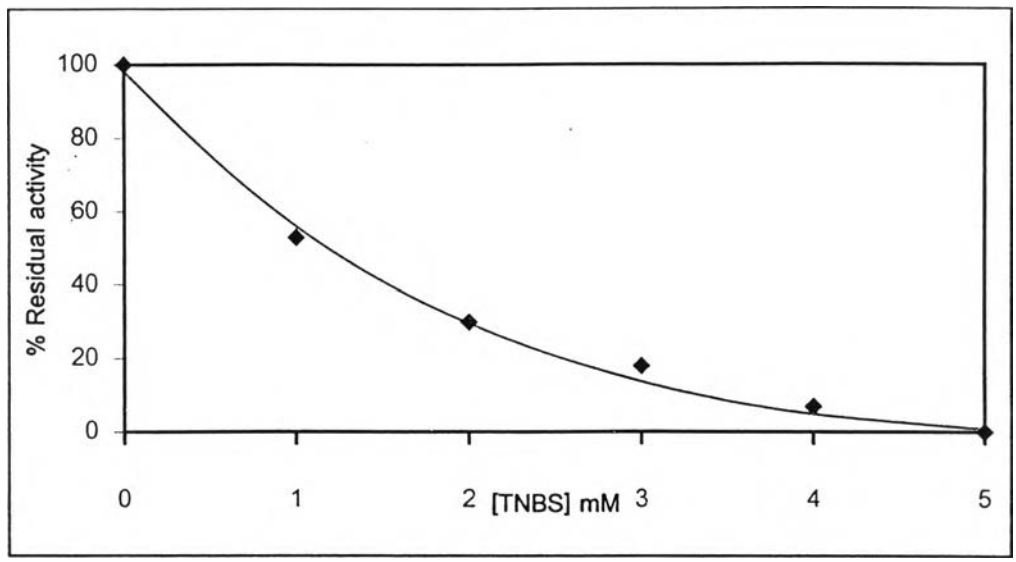

Figure 18 Effect of TNBS on alanine dehydrogenase activity

Alanine dehydrogenase was incubated with varying concentrations of TNBS at 30° C for 20 minutes according to the method in 2.9. The residual activity of the enzyme was determined and compared with untreated enzyme.

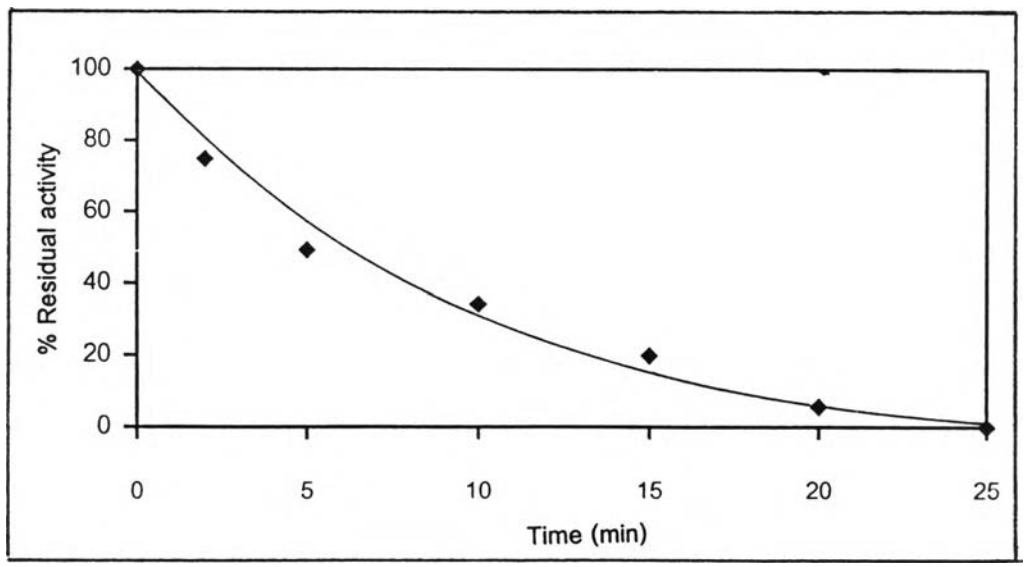

Figure 19 Inactivation of alanine dehydrogenase activity by 5 mM TNBS at various incubation times

Alanine dehydrogenase was incubated with 5 mM TNBS at 30° C, at various time. The residual activity of enzyme was determined and compared with untreated enzyme.

Table 8 Residual alanine dehydrogenase activity of TNBS modified enzyme in the presence and absence of protective substance(s)

Compound added	Relative residual activity (%)
1. None	100.0
2. 6.65 mM pyruvate + 2.5 mM NADH	99.8
3. 6.65 mM pyruvate	99.5
4. 2.5 mM NADH	100.0
5. 6.65 mM pyruvate + 2.5 mM NADH then 5.0 mM TNBS	87.0
6. 6.65 mM pyruvate then 5.0 mM TNBS	76.6
7. 2.5 mM NADH then 5.0 mM TNBS	81.2
8. 5.0 mM TNBS	49.4

The relative residual activity values are average of two separate experiments.

reaction time indicated that the time-dependent decrease in activity displayed pseudo-first order kinetics (Figure 20a). The first-order reaction can be described by the following equation, where (v/v_0) is the residual enzymic activity, k is the observed first-order rate constant of inactivation and t is the reaction time with DEPC; $\log(v/v_0) = -(k_{inact} / 2.303)t$ (Foster *et al.*, 1981). The double logarithmic plot of pseudo-first order rate constant versus DEPC concentrations yielded a straight line with slope value of 1 $mM^{-1}min^{-1}$ (Figure 20b), indicating that the modification was the result of a simple bimolecular reaction. The relationship between the observed first-order rate constants (k_{inact}) and the concentration of DEPC may be described by the equation; $k_{inact} = k\,[DEPC]^n$, where n is the reaction order with respect to DEPC concentration, k_{inact} is the pseudo-first order rate constant, and k is the second-order rate constant with respect to DEPC concentration (Levy *et al.*, 1963 cited in Drabikowsva and Woz'niak, 1990). Thus, plotting log k_{inact} versus log of reagent concentration yielded an apparent reaction order of 1.0 (Figure 20c), indicating that the inactivation resulted from the reaction of approximately 1mole of DEPC with 1 mole of enzyme subunit.

3.3.2 Inactivation pattern of arginine residues by PG

Inactivation pattern of modification of arginine residues by PG was determined as described in section 2.12.2. Incubation of alanine dehydrogenase with varying concentrations of PG resulted in a time-dependent loss of enzyme activity as shown in Figure 21a. The logarithmic plot of the residual activity versus incubation time at different reagent concentrations indicated pseudo-first order kinetics. A straight line with slope value of 1.2 mM^{-1} min^{-1} was observed from plotting of pseudo-first order rate (k_{inact}) versus reagent concentration, indicating that the modification is the result of a simple bimolecular reaction (Figure 21b). Plotting log k_{inact} versus log of reagent concentration yielded an apparent reaction order of 1.1 as shown in Figure 21c indicating that the inactivation resulted from the reaction of approximately 1 mole of PG with 1 mole of enzyme subunit.

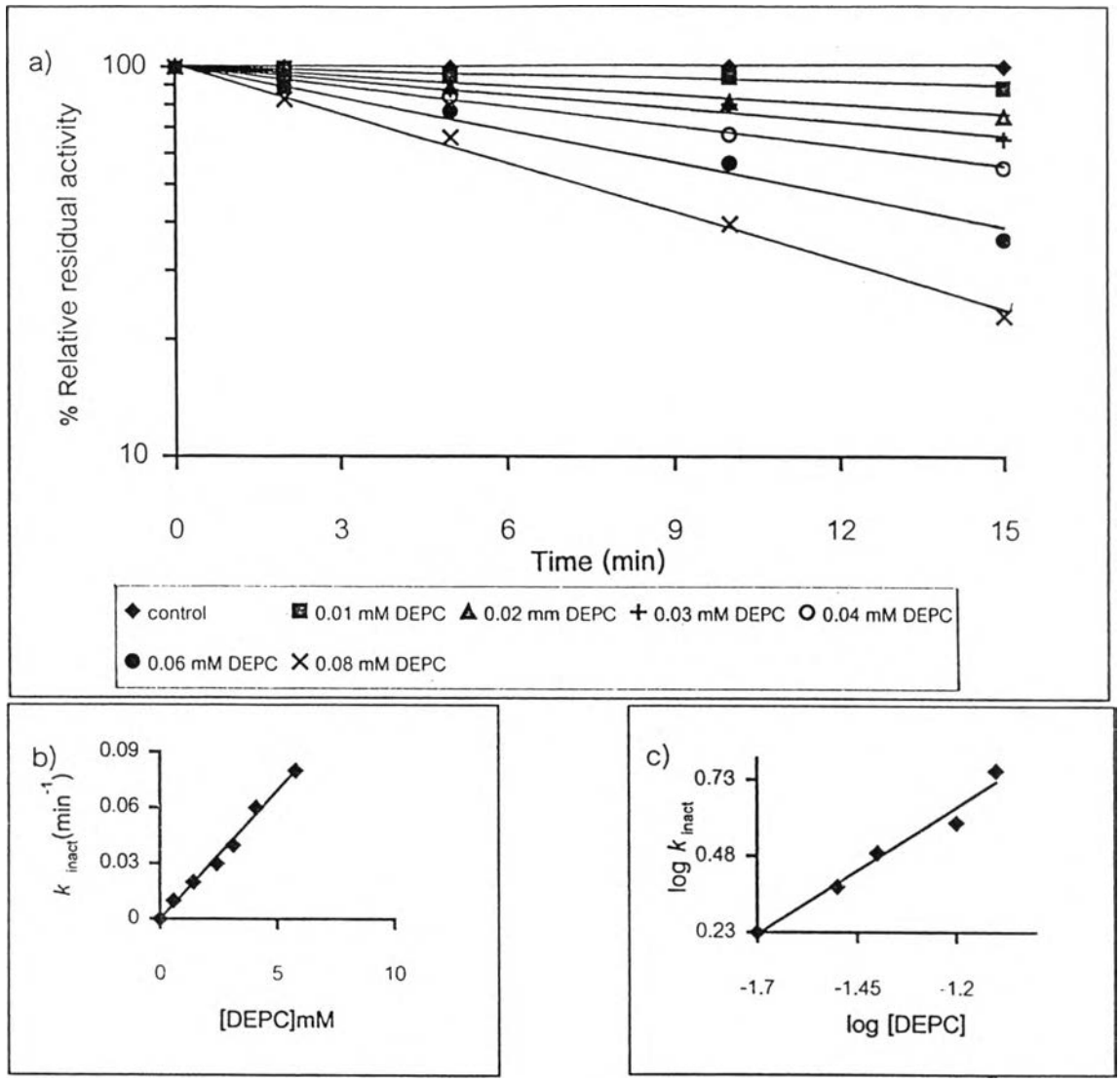

Figure 20 Inactivation of the alanine dehydrogenase with DEPC

a) Alanine dehydrogenase was incubated with various concentrations of DEPC. The experiment conditions were described in Section 2.12. The residual activity was determined. Pseudo first-order rate constants of inactivation k_{inact} are obtained from the slopes of straight lines fitted to data points by least square linear regression. b) determination of second order rate constant of inactivation. c) determination of apparent order of reaction with respect to reagent concentration.

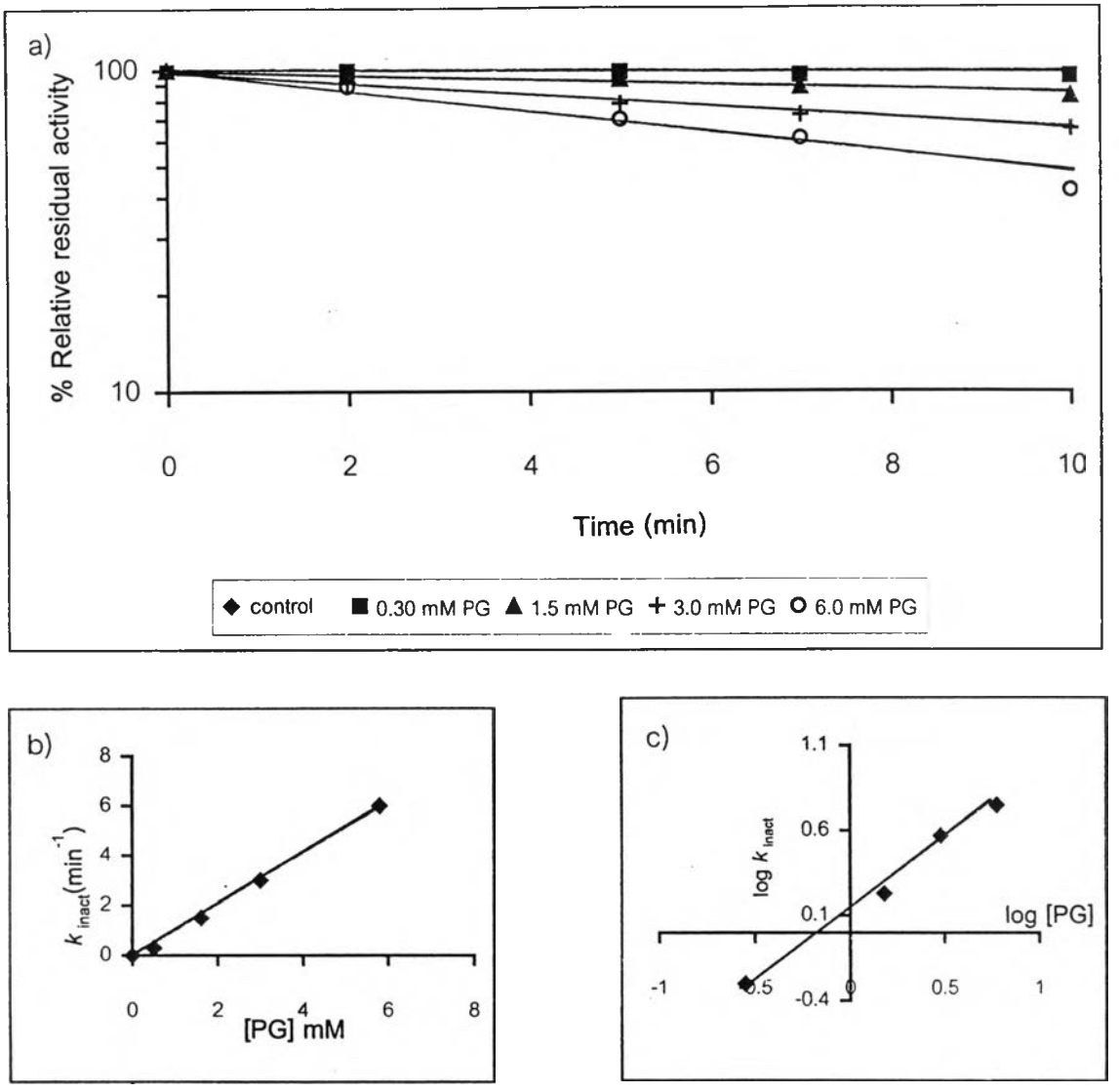

Figure 21 Inactivation of the alanine dehydrogenase with PG

a) Alanine dehydrogenase was incubated with various concentrations of PG. The experiment conditions were described in Section 2.12. The residual activity was determined. Pseudo first-order rate constants of inactivation k_{inact} are obtained from slopes of straight lines fitted to data points by least square linear regression. b) determination of second order rate constant of inactivation. c) determination of apparent order of reaction with respect to reagent concentration.

3.4 Protection pattern

To obtain more information on substrate protection, alanine dehydrogenase was incubated with suitable concentration of substrates at 30 °C for 5 minutes. After that suitable concentration of each modifying reagent was added and incubated at varying times. The residual activity was determined and compared with unmodified enzyme.

3.4.1 Substrate protection of alanine dehydrogenase against inactivation by DEPC

The result in Figure 22 shows that when either the substrate pyruvate or NADH was used, protection effect could not reach maxima. However, the k_{inact} was reduced when NADH and pyruvate were used together. Thus, the histidine residues reacting with the DEPC should be locate in an active site and can be successfully protected in the presence of the ternary complex E· NADH · pyruvate.

3.4.2 Substrate pretection of alanine dehydrogenase against inactivation by PG

Similar result as 3.4.1 was obtained for substrate protection against the inactivation by PG (Figure 23). Therefore, essential arginine residue should locate at or near enzyme active site.

3.5 Identification of modified histidine residues

3.5.1 Separation of peptides by HPLC

Alanine dehydrogenase was inactivated by 1.5 mM DEPC as described in section 2.8 and then passed through Hitrap desalting column, reconcentrated and digested with lysyl endopeptidase. The digested peptides were separated by HPLC.

The HPLC profiles of native enzyme and inactivated enzyme were presented in Figure 24. The comparison of the profiles showed that the peaks I, II and III significantly decreased when the enzyme was modified. After repeating the experiment for several times, the height of peak II of the native and modified enzyme did not differ. This suggests that, incomplete digestion by lysylendopeptidase might be occurred. The peptide

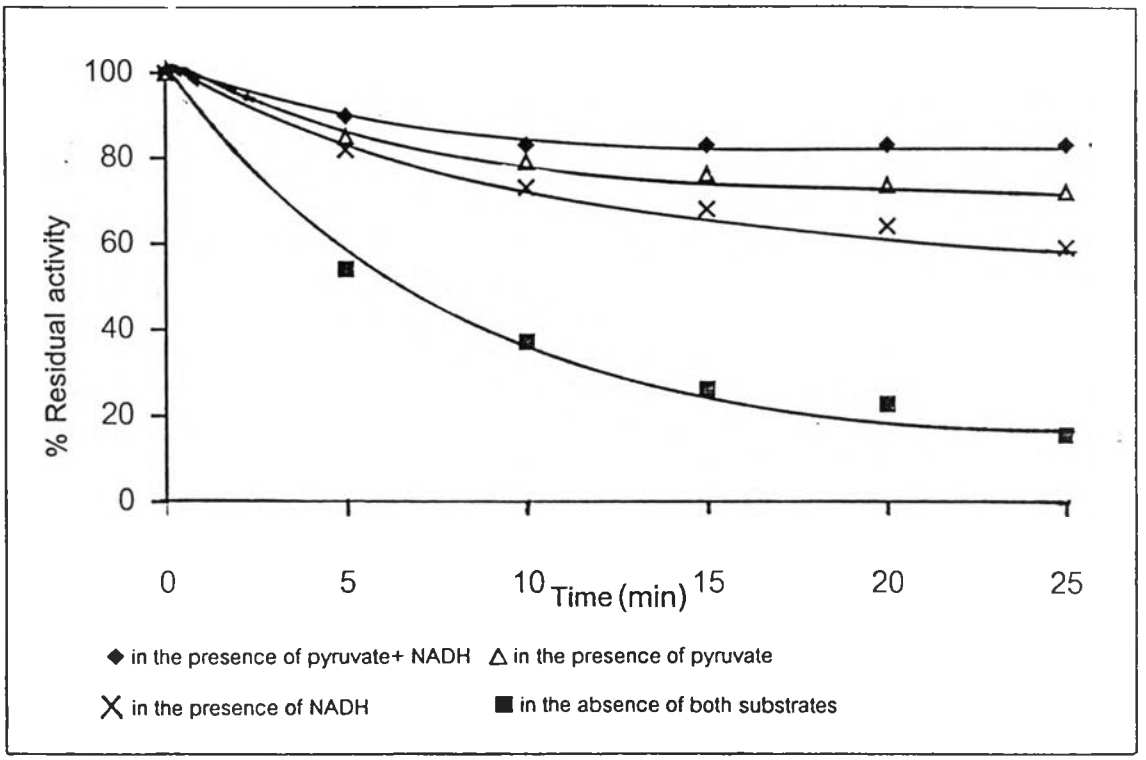

Figure 22 Protection of alanine dehydrogenase from inactivation by DEPC

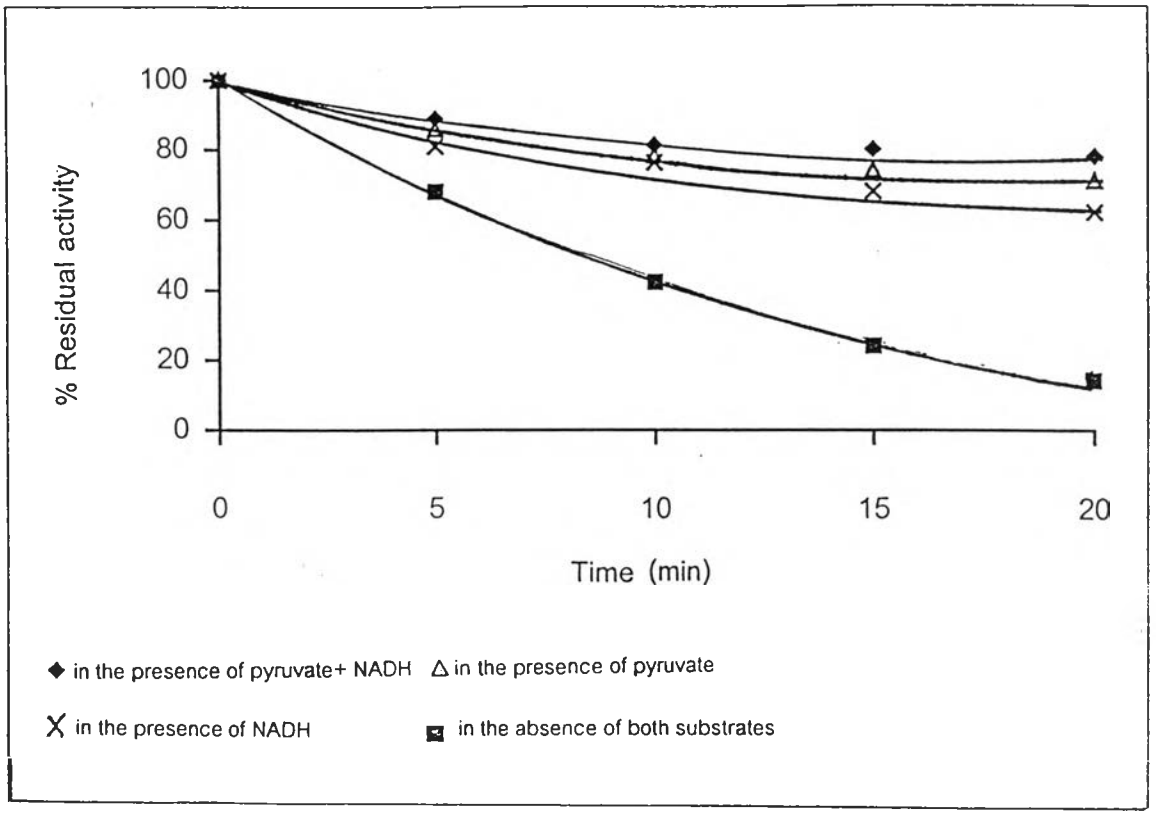

Figure 23 Protection of alanine dehydrogenase from inactivation by PG

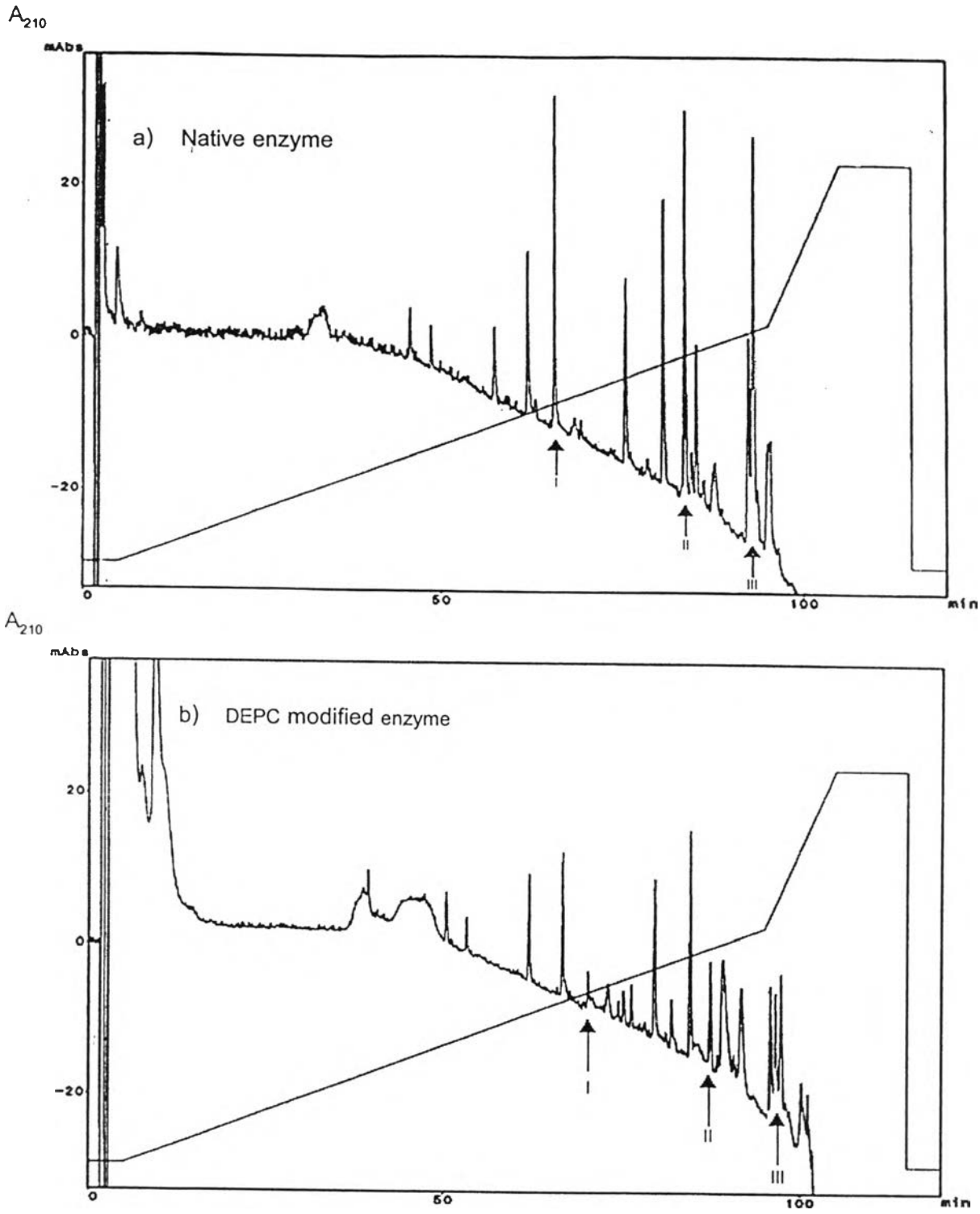

Figure 24 The reverse-phase HPLC profiles of lysyl endopeptidase digested peptides
a) native alanine dehydrogenase b) DEPC-modified alanine dehydrogenase

fragments from peak I and III were further investigated for their N-terminal amino acid sequences.

3.5.2 Identification of modified histidine residues

The amino acid sequence determination of the fractionated peptide fragments were investigated by automate Edman degradation amino acid sequencer. For peak I, the N-terminal sequence AMLRPGQTLF could be identified whereas N- terminal amino acid sequence of peptide from peak III was NHEYRVGMVP. Comparison of the obtained sequence with deduced amino acid sequence of *Aeromonas hydrophila* alanine dehydrogenase (Poomipark, personal communication) indicated that the peptide from peak I contained His-95 while peak III had two histidine residues, His –12 and His-31.