CHAPTER I

INTRODUCTION

Amino acids are biomolecules found in all organisms and can be divided in two groups by the optical activity, L- family and D- family. Amino acids in D- family can be rarely found in nature while L-family plays an important role in all life since it is the building block of enzyme, hormone, antibody and oxygen transported proteins.

Nowadays, amino acid is one of the important substances in food industry, pharmaceutical and cosmetic. For example, phenylalanine is the substrate for synthesizes artificial sweetener, L-aspartyl-L-phenylalanine (Ohshima and Soda, 1990). L-alanine is used as a good substrate to produce L-β- chloroalanine, which is one intermediate in some pesticides, medicines and natural or unnatural amino acid synthesis (Kato, *et al.*, 1993). Not only in drugs but L-alanine is also used as a food additive because of its sweet taste (Suye, *et al.*, 1992). Moreover, some derivative of L-alanine, 3-fluoro-L-alanine, has the potential use to produce antibacterials, antivirus agents and insecticides (Ohshima, 1989).

Many L-amino acids are produced from cheap carbon and nitrogen sources by fermentation with limited kinds of bacterial strains, such as *Bacillus megaterium* ATCC-39118 (Honorat, *et al.*, 1990) *Corynebacterium flaccumfaciens* AHU-1622 linked with *Pseudomonas diminuta* IFO-13182 (Suye, *et al.*, 1992), *B. stearothermophilus* IFO-12550 (Ohshima and Soda, 1989). However, the amino acid is accumulated not only in low yields but also in the form of DL-amino acid because of amino acid racemase in cell (Hashimoto and Katsumata, 1993). Thus, the multienzyme system is introduced to solve the problem, for example L-amino acid dehydrogenase, amino acid racemase, amino acid transaminase and aminotransferase are used to catalyse the formation of amino acid as shown in Figure 1 (Ohshima and Soda, 1990).

The amino acid dehydrogenases are a family of enzymes that are part of the oxidoreductase superfamily, which found in an extensive number of diverse prokaryotic and eucareotic organisms. They catalyze the removal of the amino group in the reversible oxidative of an amino acid to form α-keto acid with the concomitant reduction of $NAD(P)^+$(Brunhuber and Blanchard, 1994). The general reaction is shown in Figure 2.

These enzymes are categorized base on their specificity, which play through their amino acid substrates. Many kinds of amino acid dehydrogenase were discovered as shown in Table 1. The stereospecificity of these enzymes are separated to two groups base on the transference of hydrogen atom to the C-4 position of the nicotinamide ring (Figure 3). The enzymes, which catalize transfering of hydrogen atom to the pro-R direction, are classified as A-enzyme, such as alanine dehydrogenase (Alizade *et al.*, 1975). The later group, which transfers hydrogen atom to pro-S direction, is named B-enzyme such as glutamate dehydrogenase, leucine dehydrogenase, phenylalanine dehydrogenase and valine dehydrogenase. (Levy and Vennesland, 1974 cited in Grimshaw and Cleland, 1981).

The amino acid dehydrogenases are important and essential enzymes in carbon and nitrogen metabolism (Vancura, *et al.*, 1989) in various microorganisms by providing a link between carbohydrate and amino acid metabolism (Vali, *et al.*, 1980). Before the carbon skeleton of amino acid is metabolized for energy through the TCA cycle or glycolytic pathway, the amino group must be firstly removed by three processes, 1. transamination via pyridoxal phosphate-dependent transaminases or 2. deamination such as phenylalanine - ammonialyase or aspartate or 3. removering the amino group as free ammonia by amino acid dehydrogenase (Brunhuber and Blanchard, 1994). This group of enzyme has been used in preparation of many intermediates of some product synthesis in industrial (Kato, *et al.*, 1993) since the equilibrium constant for the reactions ([α keto acid][NH_3][NAD(P)H][H^+] / [amino acid] [$NAD(P)^+$][H_2O]), is around 10^{-15} M (Ohshima and Soda, 1990). They have been used in design of biosensors for monitoring the level of free amino acid in solution and used in

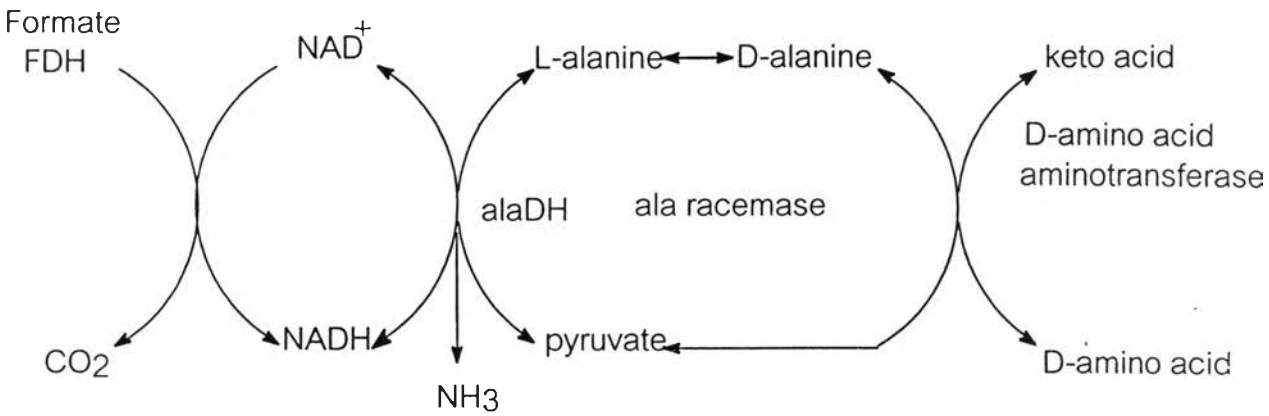


Figure 1 The multi-enzyme systems for the synthesis of amino acids (Ohshima and Soda,1990)

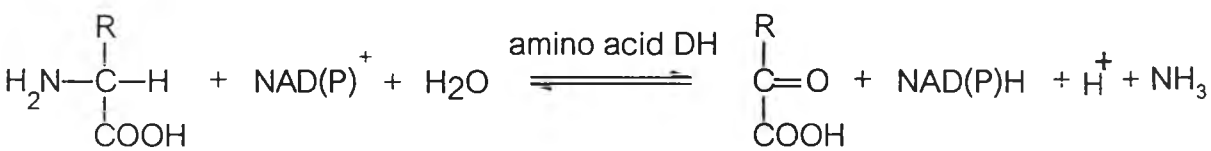


Figure 2 The general reaction of amino acid dehydrogenase (Brunhuber and Blanchard, 1994)

Table 1 NAD(P)-dependent amino acid dehydrogenases (Ohshima and Soda, 1990)

EC number	Enzyme	Coenzyme*	Organisms[τ]
1.4.1.1	Ala DH	NAD	B (*Bacillus, Steptomyces, Thermus, Halobacterium, Pseudomonas, Aeromonas hydrophila*, etc.)
1.4.1.2	Glu DH	NAD	B,F,Y,P
1.4.1.3	Glu DH	NAD(P)	A,F,*Tetrahymena*
1.4.1.4	Glu DH	NAD	B,F,Y,*Chlorella*
1.4.1.7	Ser DH	NAD	P
1.4.1.8	Val DH	NAD, NADP	B(*Alcaligenes,Steptomyces*),P
1.4.1.9	Leu DH	NAD	B(*Bacillus,Clostidium*)
1.4.1.10	Gly DH	NAD	B(*Myobacterium*)
1.4.1.11	3,5-Diaminohexanoate DH	NAD	B(*Clostridium,Brevibacterium*)
1.4.1.12	2,4- Diaminopentanoate DH	NAD(P)	B(*Clostridium*)
1.4.1.15	Lys DH	NAD	Human,D(*Agrobacterium*)
1.4.1.16	Diaminopimelate DH	NADP	B(*Bacillus,Corynebacterium*)
1.4.1.-	Phe DH	NAD	B(*Brevibacterium,Sporosarucina, Bacillus,Rhodococcus, Thermoactinomyces*)
1.4.1.-	Try DH	NAD(P)	P

* NAD(P) indicates the enzyme may use equally NAD or NADP; NAD, NADP indicates two different enzymes, each specific to one coenzyme.

τ Major organisms containing amino acid dehydrogenase. Abbreviations used: B, bacterium; F, fungi; Y, yeast; A, animal; P, plant and DH, dehydrogenase.

the industrial synthesis of amino acids and many intermediate of some product (Brunhuber and Blanchard, 1994 and Kato *et al.*, 1993)

The first report on purification and characterization of alanine dehydrogenase was performed in *Bacillus subtilis* (Yoshida and Freese, 1964). The enzyme catalyzes the reverseible deamination of L-alanine to pyruvate (Figure 4) (McCowen and Phibbs, 1974). The specific activity of the enzymes in crude extract from *Bacillus* such as *B. sphericus* (Ohshima and Soda, 1979), *B. cereus* (Porumb, *et al.*, 1987), *B. aneurinolyticus* and *B. circulans* (referred in Hummel and Kula, 1989) ranges between 0.15-0.41 units /mg protein. The enzymes have molecular mass of 230 Kda and consist of 6 identical subunits. This group of enzymes oxidizes quite specifically L-alanine. The pH optimum for the reductive amination is around 9.0 and between 10.0-10.5 for the oxidative deamination (Hummel and Kula, 1989).

In addition, alanine dehydrogenase from thermophilic *Halobacterium salinarium* is monomer with molecular mass of 60,000 Da and has high specificity for L-alanine (Keradjopulos and Holldrof, 1979). This enzyme requires 1.0-5.0 M sodium chloride for optimum activity and 3.0-5.0 M sodium chloride for maximum stability. The enzyme is thermophilic with an optimum temperature of 70^{o}C in the presence of 3.4 M potassium chloride and 60 ^{o}C in presence of 3.4 M sodium chloride. Alanine dehydrogenase from phototrophic bacterium *Rhodobacterium capsulatus* was purified by Carballero, *et al.* (1989). The molecular mass of the enzyme and subunit are similar to those of *Bacillus*. Surprisingly, the aminating activity is absolutely specific for NADPH whereas deaminating activity is strictly NAD dependent. Hence, the enzyme properties are different in various organisms (Table 2).

Kinetic mechanism of alanine dehydrogenases show different patterns depended on source of enzyme. The enzyme for *B. sphaericus*, *B. subtilis* and

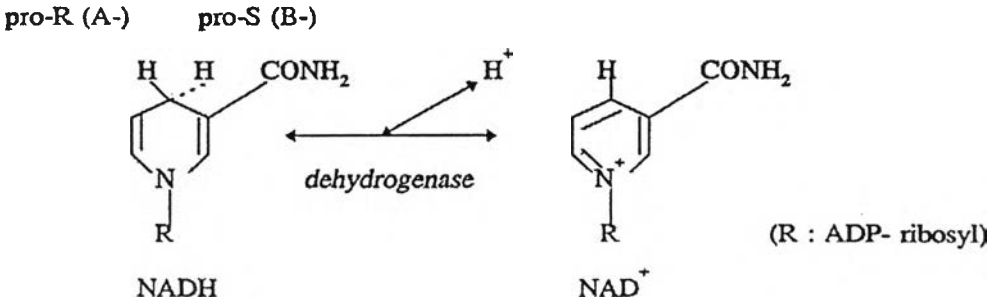


Figure 3 Stereospecific of hydrogen transfer to C-4 position of NADH by dehydrogenase family (Ohshima and Soda, 1990)

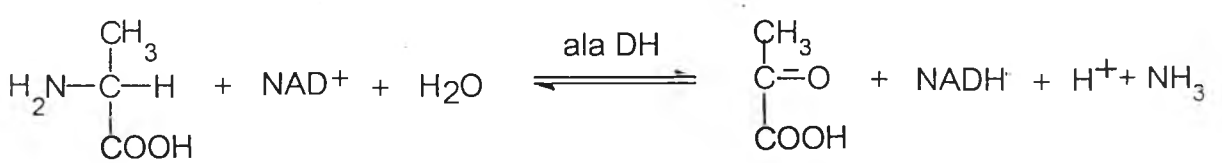


Figure 4 General reaction of alanine dehydrogenase (modified from Oshima and Soda, 1990)

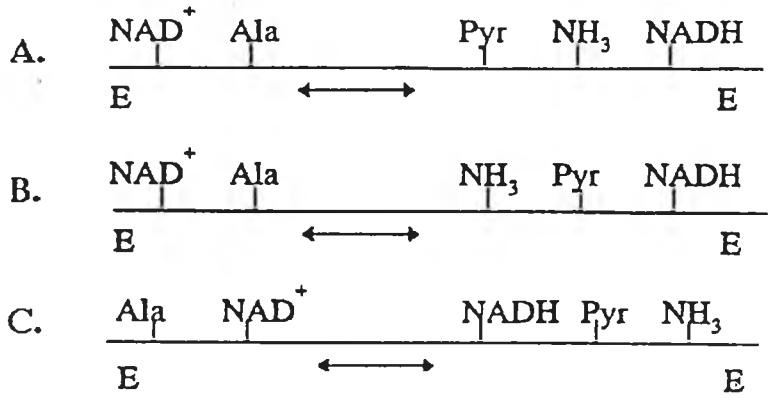
Table 2 Some properties of alanine dehydrogenase from various microorganisms (modified from Ohshima and Soda, 1990)

Source	Mr ($X10^3$) (subunit structure)	Degree of purification	k_m values (mM)				
			L-ala	NAD^+	pyr	NH_3	NADH
Bacillus subtilis	228(6x38,000)	X356	1.73	0.18	0.54	38	0.023
Bacillus sphaericus	230(6x38,000)	X340	18.9	0.23	1.7	28	0.010
Bacillus cereus	255(6x42,000)	-	-	-	-	-	-
Bacillus stearothermophilus (*Escherichia coli* clone cell)	235(6x39,465)	X30	-	-	-	-	-
Thermus thermophilus	290(6x48,000)	X85	4.2	0.12	0.75	59	0.035
Streptomyces clavuligerus	92(monomer)	X38	9.1	0.5	1.1	20	0.14
Steptomyces phaeochromogenes	240(6x39,000)	X20	7.1	0.036	0.29	61	0.047
Steptomyces aureofaciens	395(8x48,000)	X714	5.0	0.11	0.56	6.7	0.029
Steptomyces fradiae	205-210(4x51,000)	X1,180	10.0	0.18	0.23	12	0.050
Pseudomonas sp. (methylotrophic)	214(4x53,000)	X400	-	-	-	-	-
Desufvivrio desuficans	-	X56	-	-	-	-	-
Halobacterium cutirubrum	72.5(monomer)	X100	k_m values is salt dependent				
Halobacterium salinarium	60(monomer)	X500	k_m values is salt dependent				
Anabeana cylindrica	270(6x43,000)	X700	0.4	0.014	0.11	8-133	-
Rhizobium lupini bacteroids	180(4x41,000)	-	-	-	-	-	-
Rhodobacter capsulatus E1F1 (phototrophic)	246(6x42,000)	X50	1.25	0.15	0.13	16	0.25 (NADPH)
Phormidium lapideum	240(6x41,000)	-	5.0	0.04	0.33	60.6	0.02
Aeromonas hydrophila	230(6x40,000)	X100	20.0	0.17	1.33	77	0.25

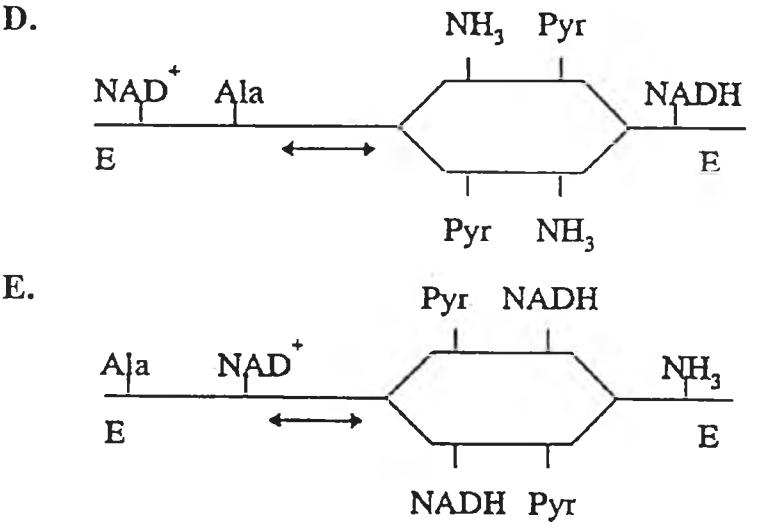
Mycobacterium catalyse the reaction through sequential ordered binary-ternary mechanism whereas those of *B. sphaericus* (Ohshima and Soda, 1990) and *Propionibacterium* (Crow, 1987) show sequential random binary-ternary mechanism (Figure 5).

Gene encoding alanine dehydrogenase from various kinds of microorganism were cloned and their completed DNA sequences were determined. Comparison for their deduced amino acid sequence indicates the high similarity among this group of enzyme (Figure 6). The amino acid sequence of alanine dehydrogenase from mesophile *B. sphaericus* (Kuroda, *et al.*, 1991) is very high (> 70%) similarity to that from modorate mesophile *B. stearothermophilus* (Kuroda, *et al.*, 1990) whereas the enzyme sequence from *Aeromonas hydrophila* (Poomipark, 2000) has 54, 54,53,53 and 51% similarity to those of different genus (*B. stearothermophilus, Enterobactor aerogenes, B. subtilis, Phormidium lapideum* and *B. sphaericus*). The sequence comparison of alanine dehydrogenase and the other proteins available in data bank shows high similarities between alanine dehydrogenase and the N-terminal part of pyridine nucleotide transhydrogenase, for which a three-dimensional model of the NAD^+ binding site and several catalytic residues in the primary structure have been purposed (Delforge, *et al.*, 1997). Furthermore the characteristic active-site motif $K(X_8)$ GGXK identified in glutamate, leucine, phenylalanine and valine dehydrogenase (Delforge, *et al.*, 1997) is not found in alanine dehydrogenase, suggesting a separate evolution of these two groups of amino acid dehydrogenase. Since similarity of sequence is found in one part of pyridine nucleotide transhydrogenase, it suggests that alanine dehydrogenase, contrary to the B- stereospecific amino acid dehydrogenase, may have revolved along with pyridine nucleotide transhydrogenase rather than with the other dehydrogenases of the amino acid dehydrogenase super family (Delforge, *et al.*, 1997).

Chemical modification of the *P. lapideum* enzyme with pyridoxal 5'-phosphate and site-directed mutagenesis indicated that Lys-74 is a catalytically important residue



Sequential ordered binary-ternary mechanism



Sequential random binary- ternary mechanism

Figure 5 The kinetic mechanism of L-alanine dehydrogenase from various sources A) mesophilic *Bacillus sphearicus* and *Aeromonas hydrophila* B) *Bacillus subtilis* C) *Mycobacterium* D) thermophilic *Bacillus sphaericus* E) *Propionibacterium* (modified from Phungsangthum,1997)

(continued)

Figure 6 Linear alignment of the amino acid sequences of alanine dehydrogenase of *Enterobacter aerogenase* (EAE), *Bacillus subtilis* (BSU), *Bacillus sphearicus* (BSP), *Bacillus stearothermophilus* (BST), *Phormidium lapideum* (PLA), *Mycobacterium tuberculosis* (MTU) and *Aeromonas hydrophila* (AHY) (Poomipark, 2000). Conserved amino acids are blocked.

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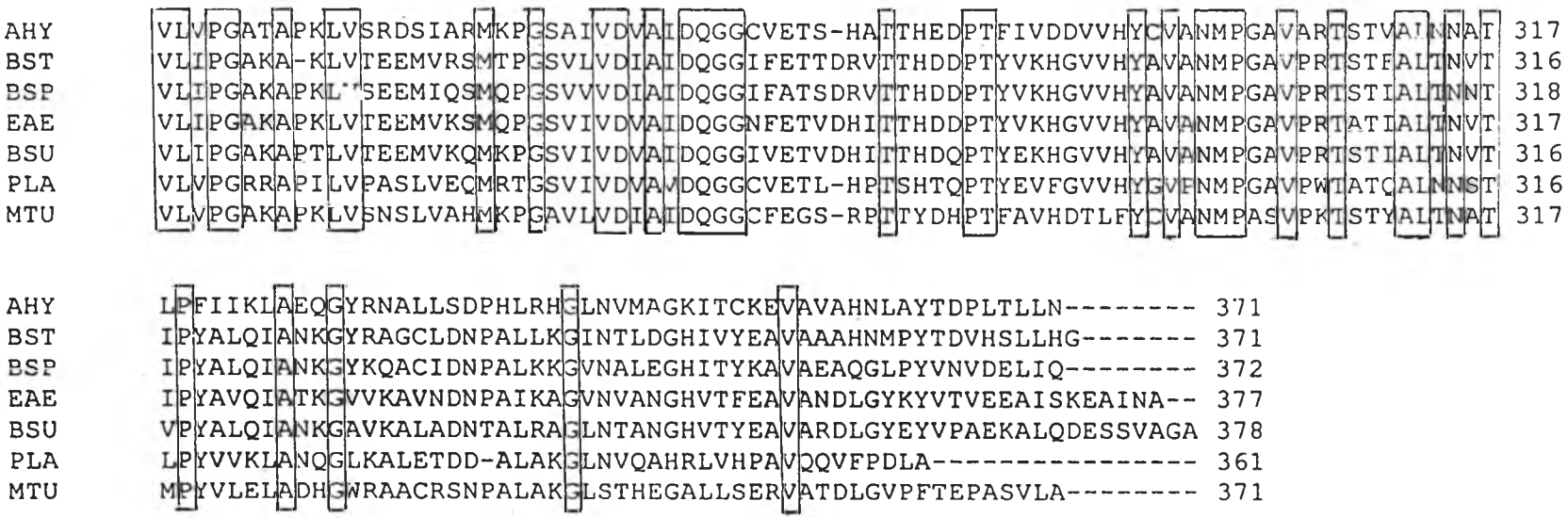

Figure 6 Linear alignment of the amino acid sequences of alanine dehydrogenase of *Enterobacter aerogenase* (EAE), *Bacillus subtilis* (BSU), *Bacillus sphearicus* (BSP), *Bacillus stearothermophilus* (BST), *Phormidium lapideum* (PLA), *Mycobacterium tuberculosis* (MTU) and *Aeromonas hydrophila* (AHY) (continued). Conserved amino acids are blocked.

(Sawa, *et al.*, 1994 cited in Chowdhury, *et al.*, 1998). Delforge, *et al.*, (1997) also reported that Lys-74 was necessary for the binding of pyruvate in the active site of the *B. subtilis* enzyme. Lys-74 is conserved among all the alanine dehydrogenase to date, and located in an important stretch of five conserved residued KVKEP from Lys-72 to Pro-76.

The information of the A-stereospecific amino acid dehydrogenase such as alanine dehydrogenase is still scantly. Only Lys-74 has been known as the essential residue that involves in substrate binding. Thus this research is aimed to study the other essential amino acid residues in pyruvate binding site of alanine dehydrogenase from *Aeromonas hydrophila*.

Alanine dehydrogenase from *Aeromonas hydrophila* was screened from soil in Thailand by Phungsangthum (1997). The enzyme has molecular mass of 230 KDa and consists of 6-identical subunits. It is highly specific for L-alanine and NAD^+. Sulfhydyl group of the enzyme plays an important role in the catalysis. Optimum temperature for reductive amination and oxidative deamination were 45 and 55 ^{o}C, respectively. Enzyme activity remained high when incubated at 55 ^{o}C for 16 hours. The optimum pH for reductive amination was 8.0 while the reverse reaction rate was highest at pH 10.5. The steady state kinetic studies including product inhibition on the enzyme reaction indicated that the oxidative deamination proceeds through a sequential ordered binary-ternary mechanism in which NAD^+ binds first to the enzyme followed by L-alanine and products are released in the order of pyruvate, ammonia and NADH, respectively. The k_m value for NAD^+, L-alanine, pyruvate, ammonia and NADH were 0.17, 20, 1.33, 77 and 0.25 mM, respectively. The N-terminal and some internal amino acid sequences of this enzyme were investigated in order to design primer for sequencing and cloning of alanine dehydrogenase gene. The gene consists of 1,113 bp open reading frame which encodes for 371 amino acid residues corresponding to the subunit (M_r = 40 KDa) of the hexameric enzyme (Poomipark, 2000).

The objective of this research

1. To determine the effect of various group-specific reagents on the alanine dehydrogenase acitivity of *Aeromonas hydrophila*
2. To determine essential amino acids in the substrate binding site
3. To identify the modified amino acid residues at the substrate binding site