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APPENDICES

APPENDIX A

Preparation for protein determination

Reagent for determination of protein concentration (modified from Lowry *et al.*, 1951)

Solution A (0.5% copper sulfate, 1% potassium tartate, pH 7.0)

Potassium tartate	1	g
Copper sulfate	0.5	g

Adjusted pH to 7.0 and adjust the solution volume to 100 ml.

Solution B (2% sodium carbonate, 1 N sodium hydroxide)

Sodium carbonate	20	g
Sodium hydroxide	4	g

Dissolved in distilled water to 1 litre.

Solution C (phenol reagent)

Folin-Ciocalteu phenol reagent used in this work was reagent grade from Merck, Germany.

APPENDIX B

Preparation for non-denaturing polyacrylamide gel electrophoresis (Native - PAGE)

1. **Stock solutions**

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 % Bromophenol blue (W/V)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. **Working solutions**

Solution A (30 % (W/V) acrylamide, 0.8 % (W/V) bis-acrylamide)

Acrylamide 29.2 g

N,N'-methylene-bis-acrylamide 0.8 g

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl pH 8.8)

2 M Tris-HCl (pH 8.8) 75 ml

Distilled water	25	ml

Solution C (0.5 M Tris-HCl pH 6.8)

1M Tris-HCl (pH 6.8)	50	ml
Distilled water	50	ml

10 % Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5.0	ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	ml

Dissolved in distilled water to 1 litre without pH adjustment

(final pH should be approximately 8.3)

5x Sample buffer

(312.5 mM Tris-HCl pH 6.8 , 50 % glycerol , 1% bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6	ml
Glycerol	5.0	ml
1 % Bromophenol blue	0.5	ml
Distilled water	1.4	ml

3. Native –PAGE

7.7 % Seperating gel

Solution A	2.6	ml
Solution B	2.5	ml
Distilled water	4.9	ml
10 % Ammonium persulfate	50	µl
TEMED	5.0	µl

5.0 % Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % Ammonium persulfate	30	µl
TEMED	5.0	µl

APPENDIX C

Preparation for denaturing polyacrylamide gel electrophoresis

1. **Stock solutions**

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10 % SDS (W/V)

Sodium dodecyl sulfate (SDS) 10 g

Added distilled water to a total volume of 100 ml

50 % Glycerol (W/V)

100 % Glycerol 50 ml

Added 50 ml of distilled water

1 % Bromophenol blue (W/V)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solutions

Solution A (30 % (W/V) acrylamide, 0.8 % (W/V) bis-acrylamide)

Acrylamide	29.2 g
N,N'-methylene-bis-acrylamide	0.8 g

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl pH 8.8 , 0.4 % SDS)

2 M Tris-HCl (pH 8.8)	75 ml
10 % SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl pH 6.8 , 0.4 % SDS)

1M Tris-HCl (pH 6.8)	50 ml
10 % SDS	4 ml
Distilled water	46 ml

10 % Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris , 192 mM glycine , 0.1 % SDS)

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 ml
SDS	1 g

Dissolved in distilled water to 1 litre without pH adjustment

(final pH should be approximately 8.3)

5x Sample buffer

(60 mM Tris-HCl pH 6.8 , 25 % glycerol , 2 % SDS , 0.1% bromophenol blue , 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl (pH 6.8)	0.6 ml
50 % Glycerol	5 ml

10 % SDS	2	ml
1 % Bromophenol blue	1	ml
2-mercaptoethanol	0.5	ml
Distilled water	0.9	ml

3. SDS –PAGE

12.5 % Seperating gel

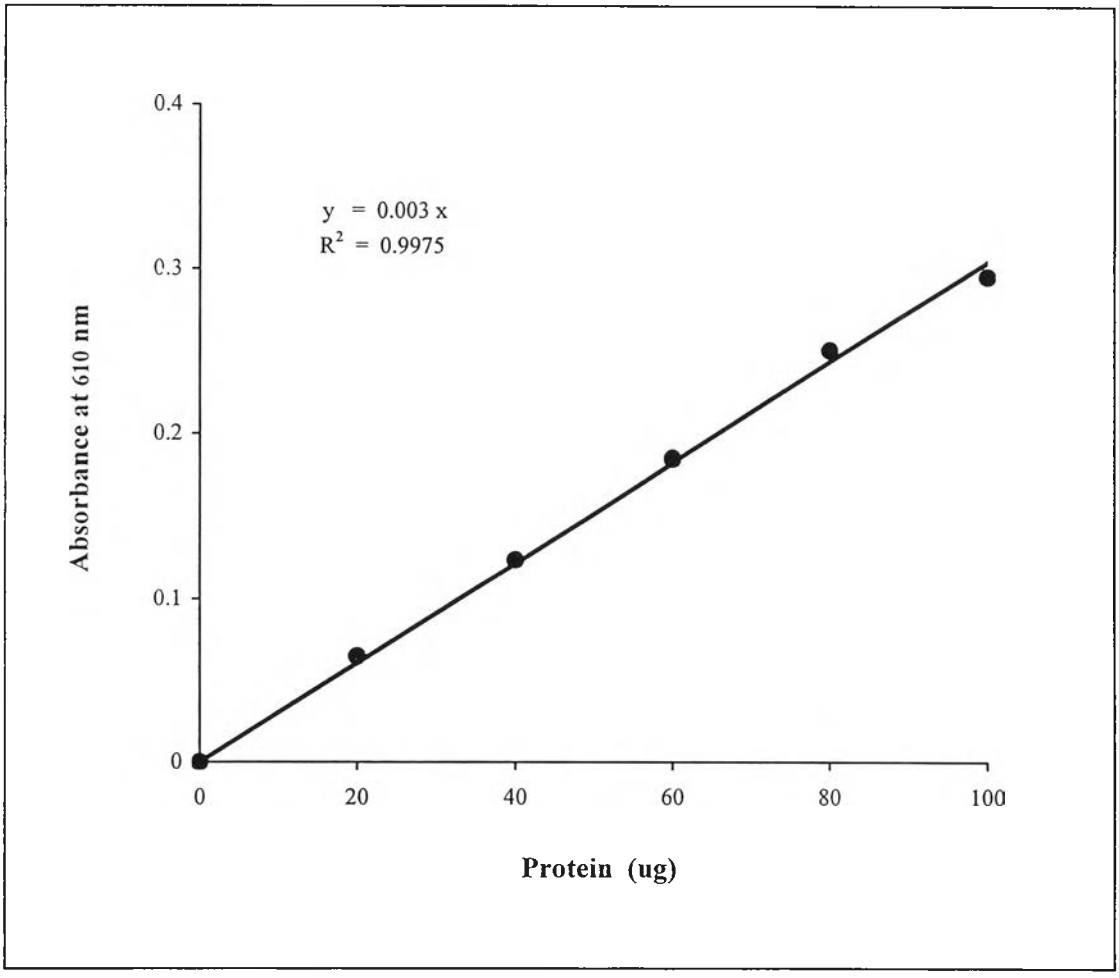
Solution A	4.2	ml
Solution B	2.5	ml
Distilled water	3.3	ml
10 % Ammonium persulfate	50	µl
TEMED	5	µl

5.0 % Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % Ammonium persulfate	30	µl
TEMED	5	µl

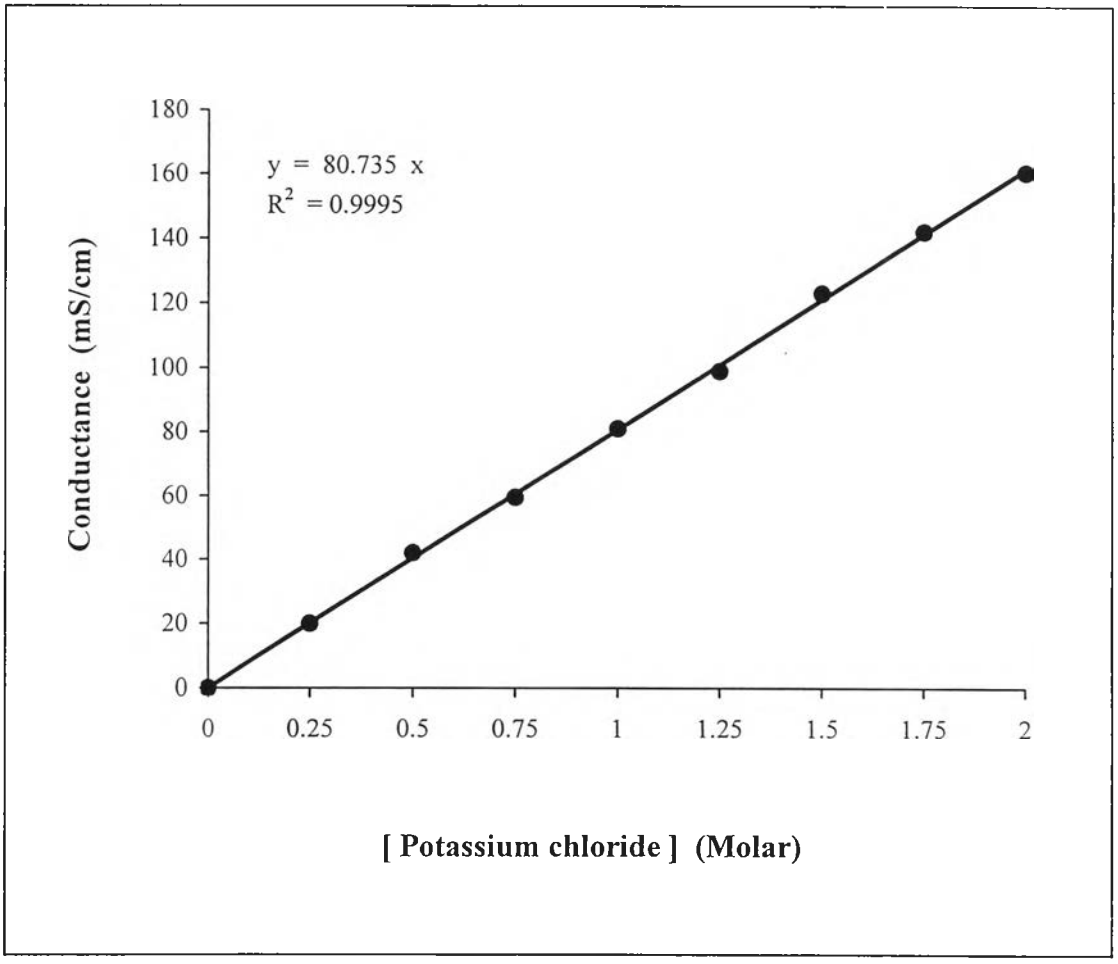
APPENDIX D

Calibration curve for protein determination by Lowry's method



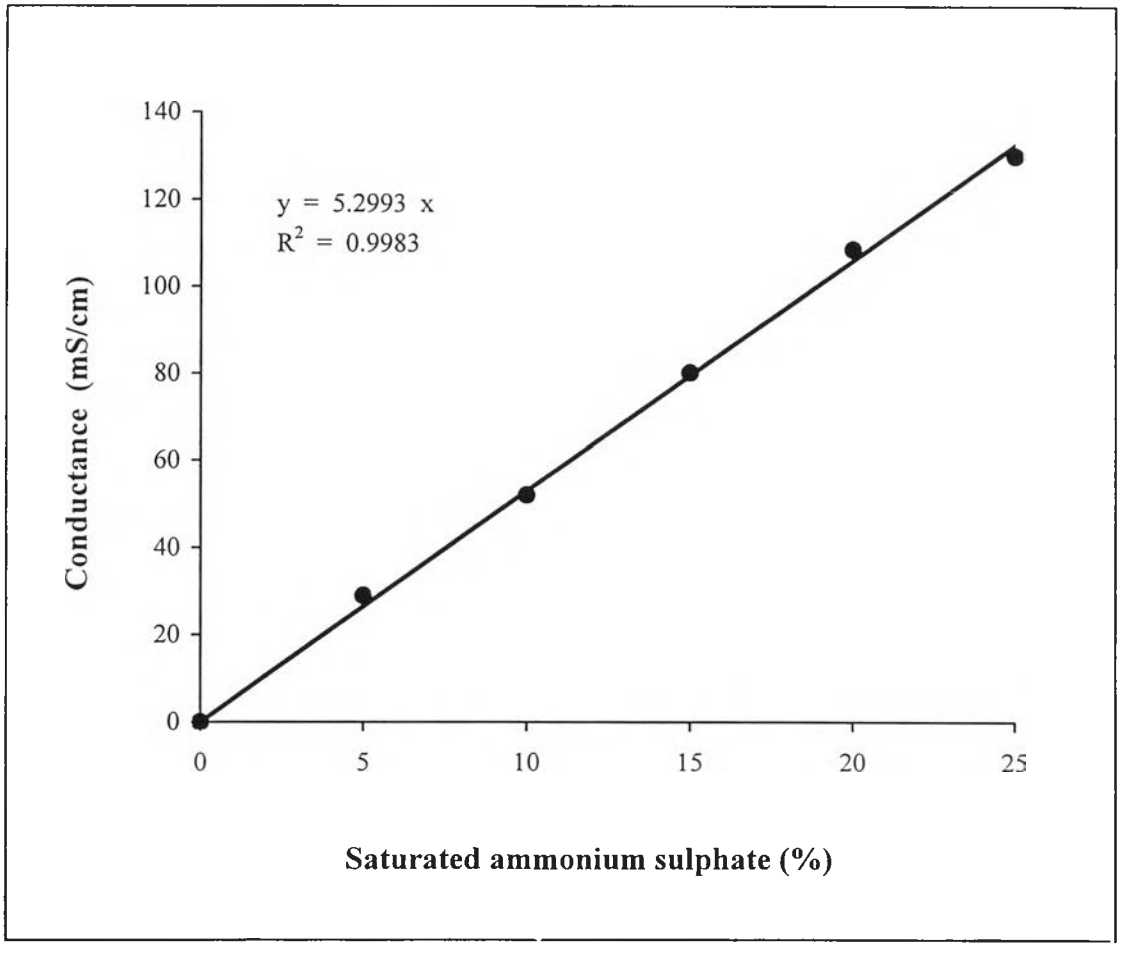
APPENDIX E

Calibration curve for conductivity of potassium chloride



APPENDIX F

Calibration curve for conductivity of ammonium sulfate

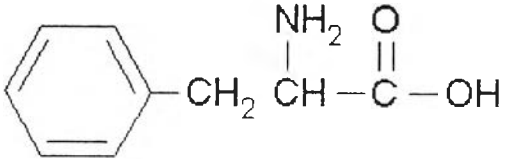


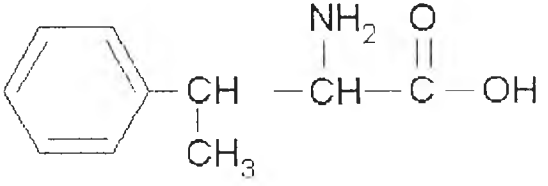
APPENDIX G

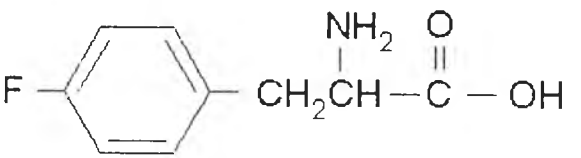
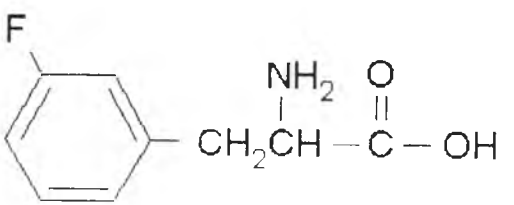
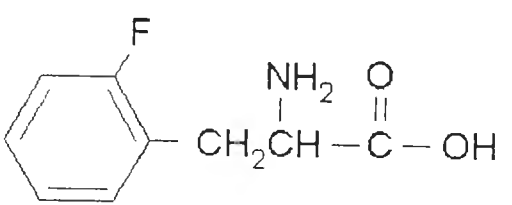
Phenylalanine analogs and their effects as substrates or inhibitors against L-phenylalanine

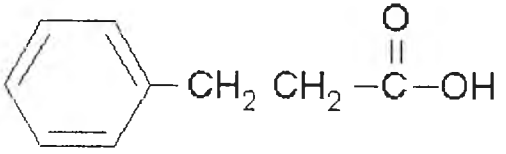
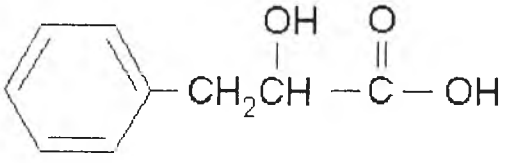
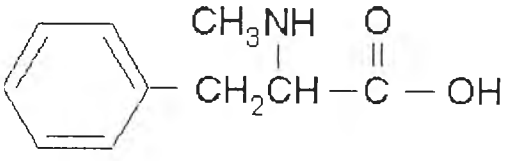
L-phenylalanine

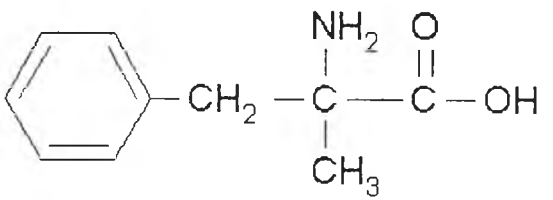
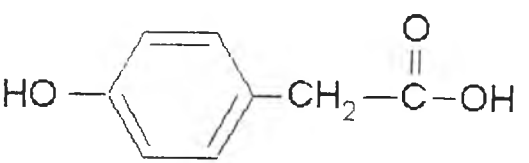
(α-Amino-β-phenylpropionate)



Phenylalanine analog	Structure	Substrate [a]	Inhibitor [b]
α-Amino-β-phenylbutanoate		Y	ND

Phenylalanine analog	Structure	Substrate [a]	Inhibitor [b]
p-Fluoro-DL-phenylalanine		Y	ND
m-Fluoro-DL-phenylalanine		Y	ND
o-Fluoro-DL-phenylalanine		N	Y

Phenylalanine analog	Structure	Substrate [a]	Inhibitor [b]
Hydrocinnamate (3-Phenylpropionate)		**N**	**N**
DL-β-Phenyllactate (DL-2-Hydroxy-3-phenylpropionate, DL-α-Hydroxyhydrocinnamate)		**N**	**N**
***N*-Methyl-L-phenylalanine**		**N**	**N**

Phenylalanine analog	Structure	Substrate [a]	Inhibitor [b]
α-Methyl-DL-phenylalanine (α-Amino-α-methyl-β-phenylpropionate)		N	N
***p*-Hydroxyphenylacetate**		N	N

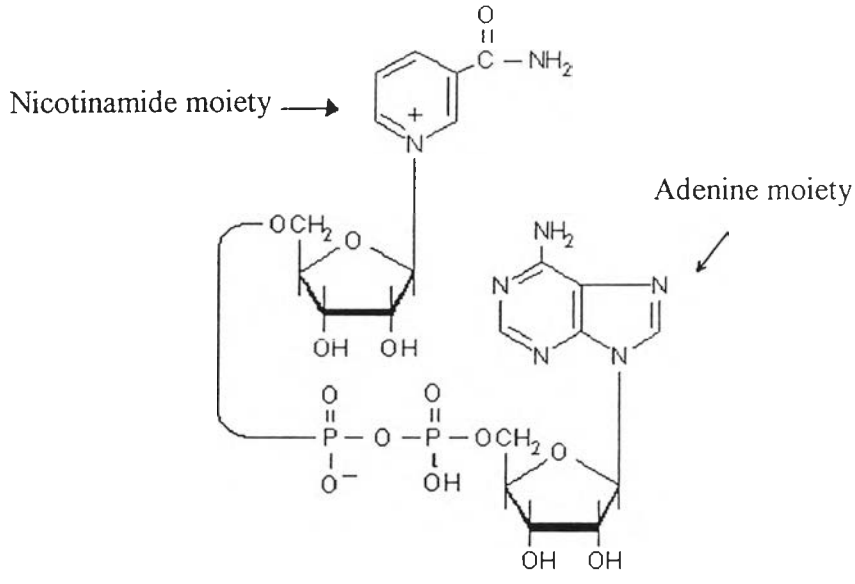
[a] Substrate of the *Bacillus* sp. BC1 PheDH. This result was obtained from substrate specificity on oxidative deamination experiment (section 2.9.2).

[b] Inhibitor of the *Bacillus* sp. BC1 PheDH against L-phenylalanine. This result was obtained from the inhibitory effects of nonsubstrate phenylalanine analogs on oxidative deamination experiment (section 2.9.9). Only nonsubstrate phenylanine analogs were test on this experiment.

Y = Yes, **N** = No, **ND** = Not detected

APPENDIX H

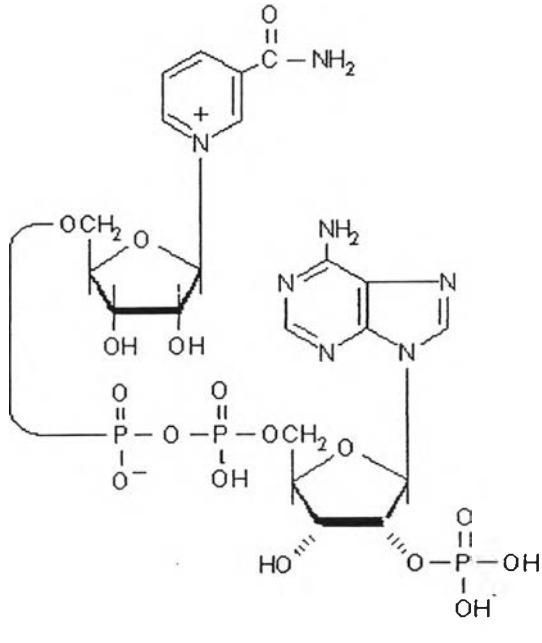
NAD^+ analogs



Nicotinamide adenine dinucleotide (NAD^+)

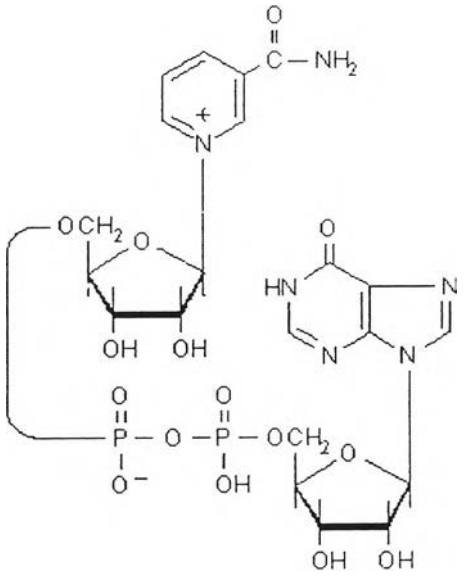
The NAD^+ analogs used in this work can be divided into 3 groups based on their modified structure.

1. **<u>Coenzyme analog modified at C-2 position of the adenosyl ribose</u>**

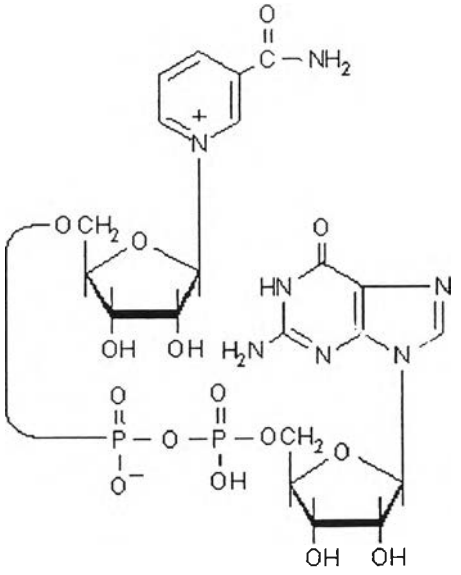


Nicotinamide adenine-dinucleotide phosphate ($NADP^+$)

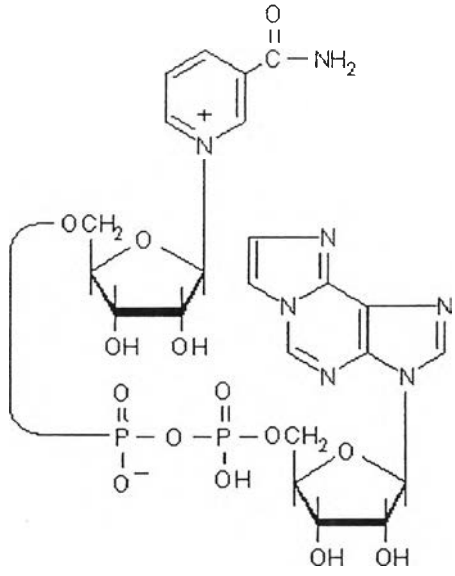
2. Coenzyme analog modified at the amino group in the adenine moiety



Nicotinamide hypoxanthine dinucleotide (Deamino-NAD$^+$)

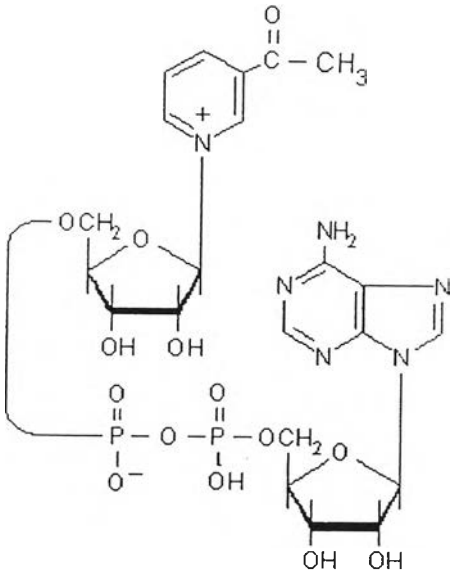


Nicotinamide guanine dinucleotide

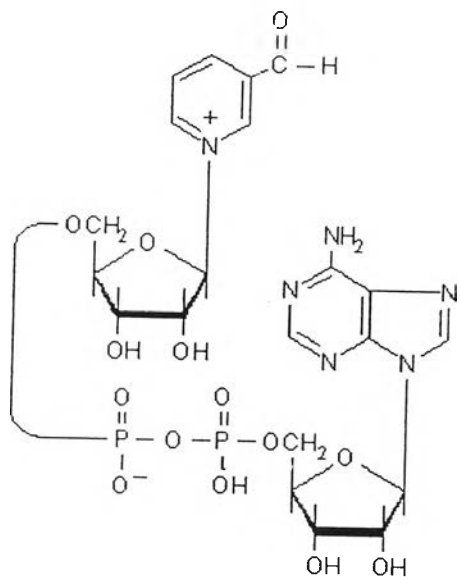


Nicotinamide 1, N^6-ethenoadenine dinucleotide

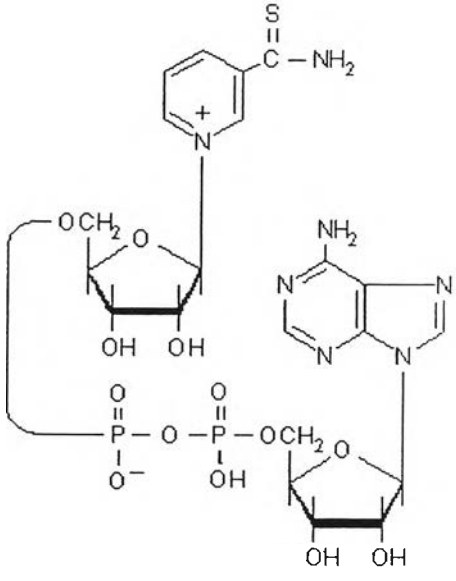
3. Coenzyme analog modified at the nicotinamide moiety



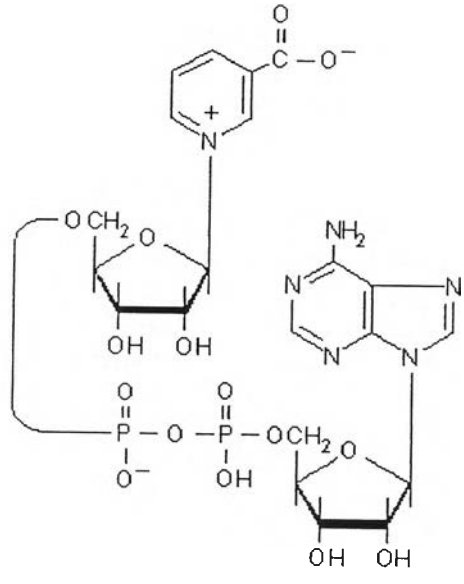
3-Acetylpyridine adenine dinucleotide



3-Pyridinealdehyde adenine dinucleotide



Thionicotinamide adenine dinucleotide



Nicotinic acid adenine dinucleotide (Deamido-NAD$^+$)

APPENDIX I

Effect of amino acids as substrates or inhibitors against L-phenylalanine on phenylalanine dehydrogenase from *B. badius* BC1

1. Amino acids with non-polar side chains

Amino acid	Substrate [a]	Inhibitor [b]
D-Gly	N	N
D-Ala	N	N
D-Val	N	Y
D-Leu	N	Y
D-Met	N	Y
D-Trp	N	Y
D-Phe	N	Y
L-Gly	N	N
L-Ala	N	N
L-Val	Y	ND
L-Leu	N	Y
L-Ile	N	Y
L-Met	Y	ND
L-Trp	Y	ND
L-Tyr	N	Y

2. Amino acids with polar uncharged side chains

Amino acid	Substrate [a]	Inhibitor [b]
D-Ser	N	N
D-Thr	N	N
L-Ser	N	N
L-Thr	N	N
L-Asn	N	N
L-Cys	N	N

3. Amino acids with positive charged side chains (basic side chains)

Amino acid	Substrate [a]	Inhibitor [b]
D-Arg	N	N
D-His	N	N
D-Lys	N	N
L-Arg	N	N
L-His	N	N
L-Lys	N	N

4. Amino acids with negative charged side chains (acidic side chains)

Amino acid	Substrate [a]	Inhibitor [b]
D-Asp	N	Y
D-Glu	N	Y
L-Asp	N	Y
L-Glu	N	Y

[a] Substrate of the *B. badius* BC1 PheDH. This result was obtained from substrate specificity on oxidative deamination experiment (section 2.9.2).

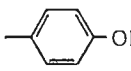
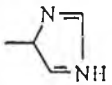
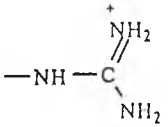
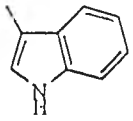
[b] Inhibitor of the *B. badius* BC1 PheDH against L-phenylalanine. This result was obtained from the inhibitory effects of nonsubstrate D- and L-amino acids on oxidative deamination experiment (section 2.9.9). Only nonsubstrate phenylalanine analogs were tested on this experiment.

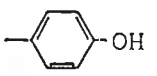
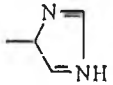
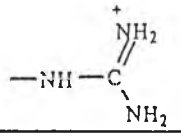
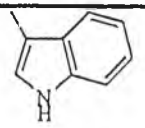
Y = Yes, **N** = No, **ND** = Not detected

APPENDIX J

Reactivities of amino acid side chains [79]

Reagent	—NH_2	—SH	—OH	—NH—	—NH—C(=NH_2^+)—NH_2	—COOH	—S—S—	—S—CH_3
Acetic anhydride	+++	+++[b]	+++[c]	+++[b]	-	-	-	-
N-acetylimidazole	±	+++[b]	+++[c]	+++[b]	-	-	-	-
acrylonitrile	±	+++	-	-	-	-	-	-
Aldehyde/ $NaBH_4$	+++	-	-	-	-	-	-	-
N-bromosuccinimide	-	+++	++	+	-	-	+++	-
N-carboxyanhydrides	+++	-	-	-	-	-	-	-
Cyanate	+++	+++[b]	++[b]	+[b]	-	+[b]	-	-
Cyanogen bromide	-	+	-	-	-	-	-	+++
1,2-cyclohexanedione	±	-	-	-	+++	-	-	-
Diacetyl trimer	+	-	-	-	+++	-	-	-
Diazoacetates	-	++	-	-	-	+++	-	-
Diazonium salts	+++	+	+++	+++	+	-	+	-
Diethylpyrocarbonate	+++	-	-	+++[c]	-	-	-	-
Diketone	+++[c]	-	+	-	-	-	-	-
Dinitrofluorobenzene	+++	+++	++	++	-	-	-	-
5,5'-dithiobis (2-nitrobenzoic acid)	-	+++[c]	-	-	-	-	-	-
Ethyleneimine	-	+++	-	-	-	-	-	+

Reagent	—NH_2	—SH				—COOH		—S—S—	—S—CH_3
N-ethylmaleimide	±	+++	-	-	-	-	-	-	
Ethyl thiotrifluoacetate	+++[b]	-	-	-	-	-	-	-	-
Formaldehyde	+++	+++	+++	+++	+	-	+	-	-
glyoxal	++	-	-	-	+++	-	-	-	-
Haloacetates	+	+++	-	+	-	-	-	-	+
Hydrogen peroxide	-	+++	-	-	-	-	+	+	+++
2-hydroxy-5-nitrobenzyl bromide	-	++	-	-	-	-	+++	-	-
Iodine	-	+++	+++	+++	-	-	-	-	-
0-iodosobenzoate	-	+++	-	-	-	-	-	-	-
Maleic anhydride	+++[c]	++[c]	++[b]	++[b]	-	-	-	-	-
p-mercuribenzoate	-	+++	-	-	-	-	-	-	-
Methanol/ HCl	-	-	-	-	-	+++	-	-	-
2-methoxy-5-nitrotropone	+++[c]	-	-	-	-	-	-	-	-
Methyl acetimidate	+++	-	-	-	-	-	-	-	-
O-methylisourea	+++	-	-	-	-	-	-	-	-
Nitrous acid	+++	+++	±	-	-	-	-	+	-
Performic acid	-	+++	-	-	-	-	++	+++	+++

Reagent	—NH_2	—SH				—COOH		—S—S—	—S—CH_3
Phenylglyoxal	++	-	-	-	+++	-	-	-	-
Photooxidation	-	+++	±±	+++	-	-	+++	±	+++
Sodium borohydride	-	+++[b]	++[b]	++[b]	-	-	-	-	-
Succinic anhydride	+++	+++	-	-	-	-	+++	-	-
Sulfite	-	+++	+++	+++	-	-	-	-	-
Sulfonyl halides	+++	+++	+++	-	-	-	+	-	+
Tetranitromethane	-	+++	+++	-	-	-	+	-	+
Tetrathionate	-	+++	-	-	-	-	-	-	-
Thiols	-	-	-	-	-	-	-	+++	-
Trinitrobenzenesulfonic acid	+++	++[b]	-	-	-	-	-	-	-
Water-soluble carbodiimide and nucleophile	±	±	±	-	-	+++	-	-	-

[a] - ,+ ,++ , and +++ indicate relative reactivities; ±, ±±, and ±±± likewise indicate reactivities which may or may not be attained depending on the condition used.

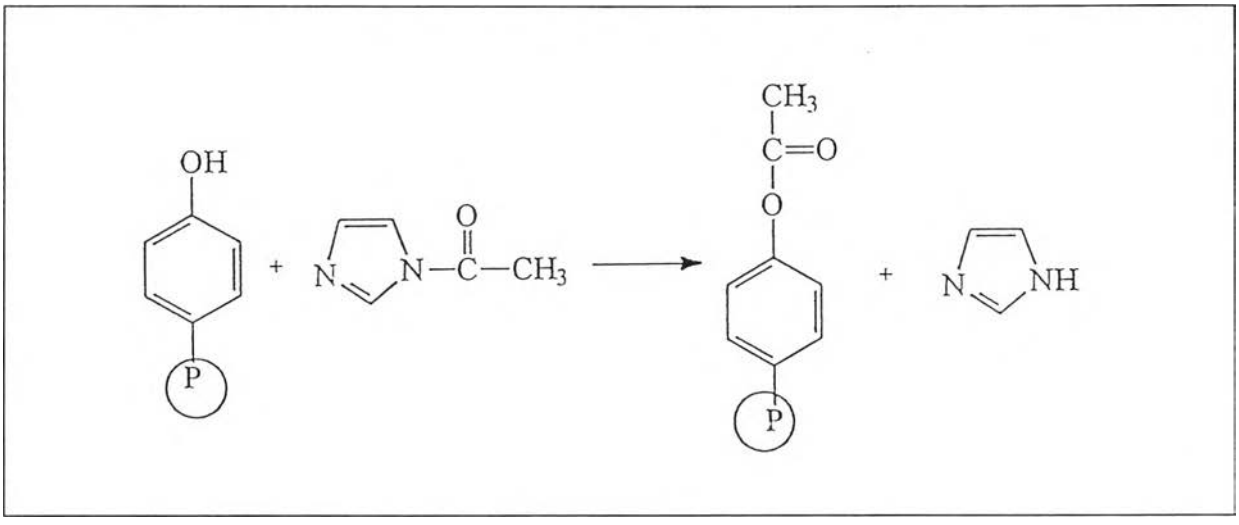
[b] Spontaneously reversible under the reaction conditions or upon dilution, regenerating original group.

[c] Easily reversible, regenerating original group.

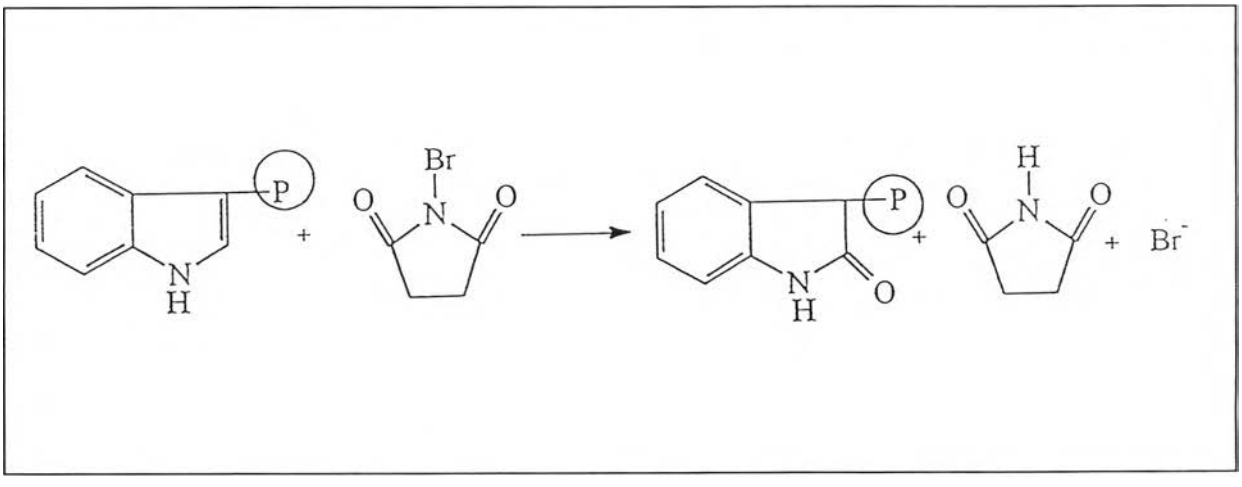
APPENDIX K

Modification reactions of group-specific reagents [79-84]

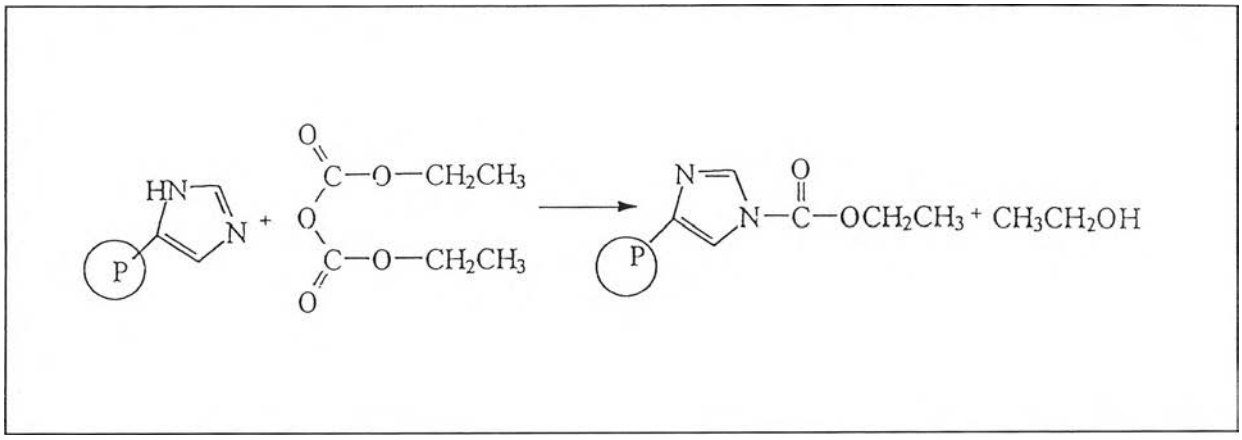
Modification reaction of *N*-acetylimidazole (NAI) with tyrosine residue in protein (P)



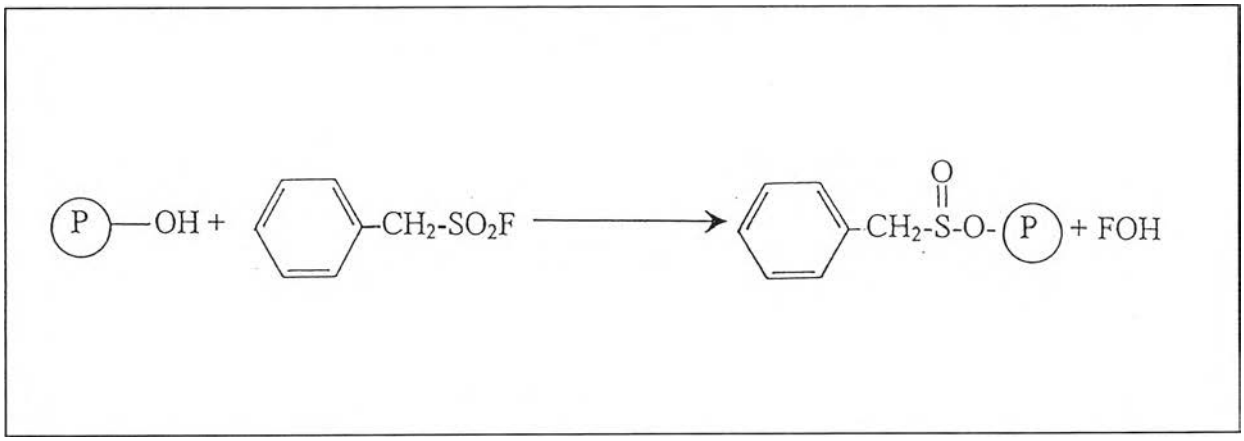
Modification reaction of *N*-bromosuccinimide (NBS) with tryptophan residue in protein (P)



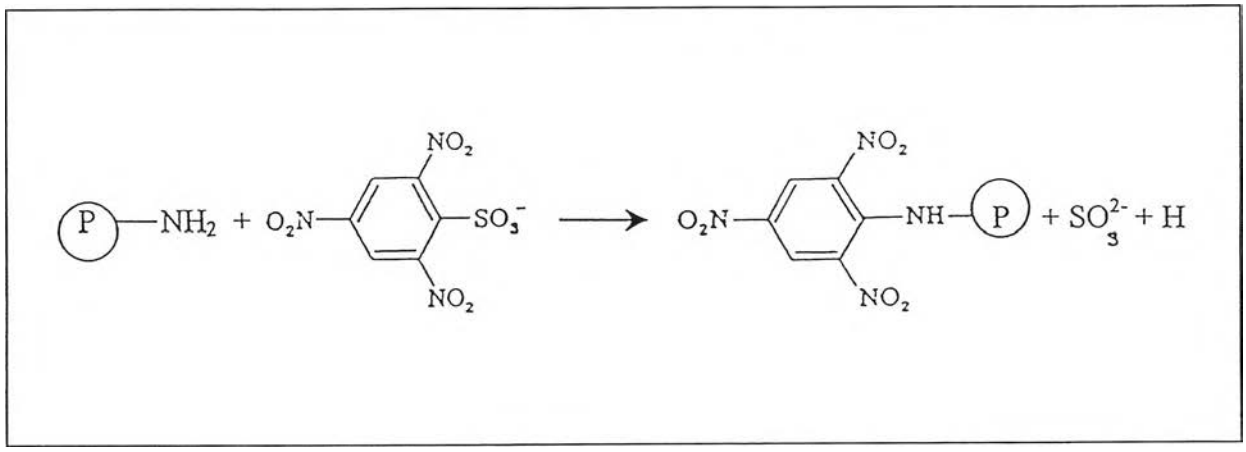
Modification reaction of diethylpyrocarbonate (DEPC) with histidine residue in protein (P)



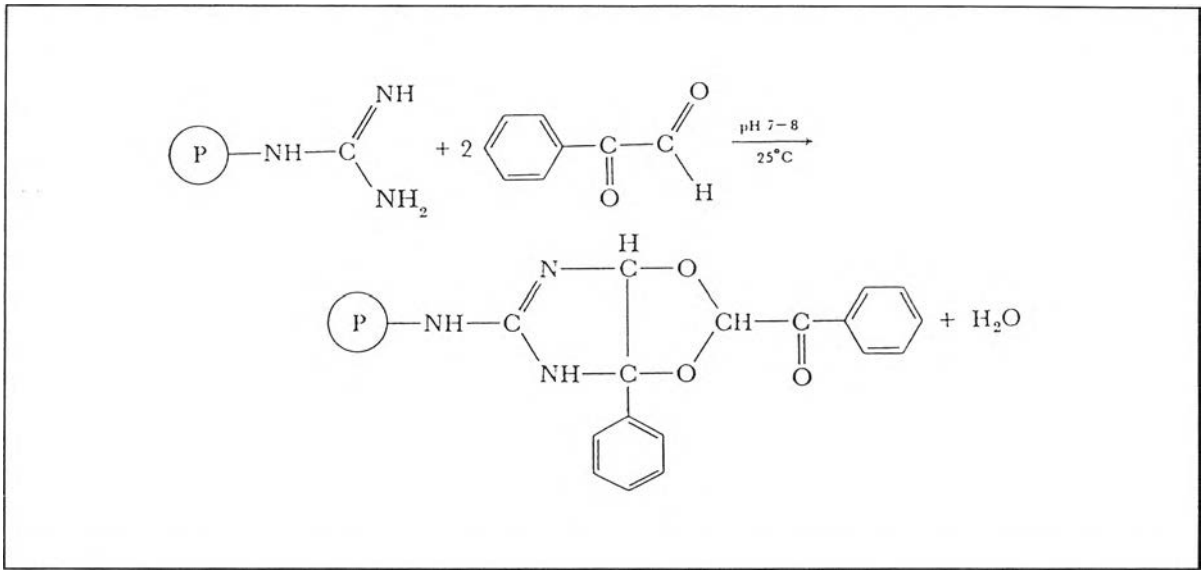
Modification reaction of phenylmethylsulfonyl fluoride (PMSF) with serine residue in protein (P)



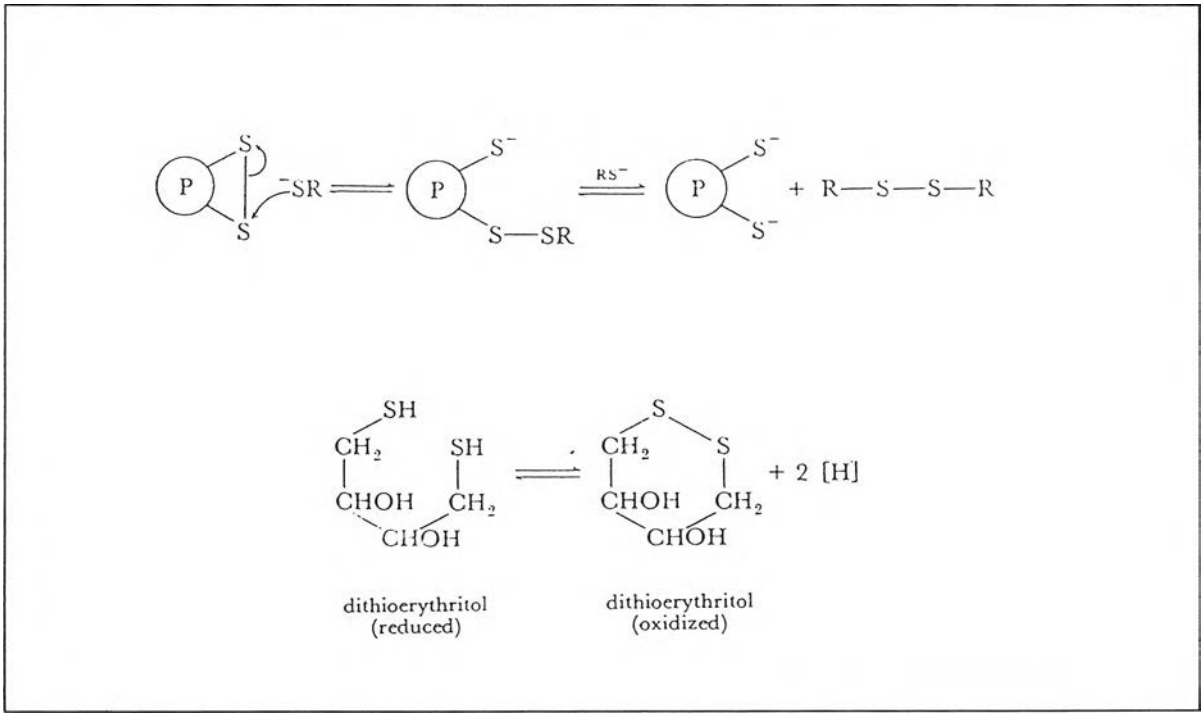
Modification reaction of 2,4,6-trinitrobenzene sulfonic acid (TNBS) with lysine residue in protein (P)



Modification reaction of phenylglyoxal (PG) with arginine residue in protein (P)



Modification reaction of dithiothreitol (DTT) with cysteine residue in protein (P)



APPENDIX L

Comparison of product inhibition patterns of phenylalanine dehydrogenases from various bacteria

Substrate	Product inhibition patterns											
	NADH				Phenylpyruvate				Ammonia			
	B. badius BC1	*R. maris*	*Rhodococcus* sp. M4	*T. inter-medius*	*B. badius* BC1	*R. maris*	*Rhodococcus* sp. M4	*T. inter-medius*	*B. badius* BC1	*R. maris*	*Rhodococcus* sp. M4	*T. inter-medius*
NAD^+	C	C	C	C	UC	UC	NC	UC	UC	UC	NC	UC
L-phenyl-alanine	NC	UC	NC	NC	NC	NC	NC	NC	UC	NC	-	UC

C; Competitive inhibition pattern

NC; Noncompetitive inhibition pattern

UC; Uncompetitive inhibition pattern

- ; No data

APPENDIX M

Comparison of properties of PheDHs from *Bacillus badius* BC1 and *Bacillus badius* IAM 11059

Properties	*B. badius* IAM 11059 [a]	*B. badius* BC1
Native molecular weight	335,000	358,000
Subunit molecular weight	41,350	44,500
Structure	octamer	octamer
Isoelectric point (pI)	3.5	ND
pH optimun		
Oxidative deamination	10.4	10.7
Reductive amination	9.4	8.3
Inhibitors	$AgNO_3$, $HgCl_2$, *p*-chloromercuribenzoate	$AgNO_3$, $HgCl_2$, $FeCl_3$
Substrate specificity (% relative activity)		
Oxidative deamination		
L-phenylalanine	100	100
L-tyrosine	9	0
L-tryptophan	4	3
L-methionine	8	4
L-valine	4	2
L-leucine	3	0
L-isoleucine	0.2	0

Properties	*B. badius* IAM 11059 [a]	*B. badius* BC1
L-norvaline	5	ND
L-norleucine	19	ND
L-phenylalaninamide	9	ND
L-phenylalaninol	9.4	ND
L-phenylalanine methyl ester	38	ND
p-fluoro-DL-phenylalanine	34	11
m-fluoro-DL-phenylalanine	11	5
o-fluoro-DL-phenylalanine	2	0
α-amino-β-phenylbutanoate	ND	8
D-amino acids	ND	0
Reductive amination		
phenylpyruvate	100	100
p-hydroxyphenylpyruvate	53	0
α-ketovalerate	12	3
α-ketocaproate	ND	12
α-ketoisovalerate	ND	5
α-ketoisocaproate	ND	4
α-ketobutyrate	3	0
α-ketohexanoate	31	ND
α-keto-γ-methylthiobutyrate	16	0
α-keto-γ-methylvalerate	4	0
α-keto-γ-methylpentanoate	13	ND
Apparent K_m (mM)		
L-phenylalanine	0.088	0.59
NAD^+	0.15	0.28
NADH	0.21	0.067
phenylpyruvate	0.106	0.33
ammonia	127	200

[a] *Bacillus badius* IAM 11059

Asano, Y., Nakazawa, A., Endo, K., Hibino, Y., Ohmori, M., Numao, N., and Kondo, K. 1987. Phenylalanine dehydrogenase of *Bacillus badius*: Purification, characterization and gene cloning. *Eur. J. Biochem.* 168: 153-159.

ND = Not determined

APPENDIX N

Abbrevation for amino acid residues [98]

Amino acid	3 Letter-Abbreviation	1-Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown	-	X

BIOGRAPHY

Miss Arunee Leksakorn was born on October 15, 1978 in Chonburi province. She finished High school at Navamintrachutid Bangkok school, Bangkok and enrolled in the Faculty of Science, Chiangmai University. She graduated with the B.Sc. in Biochemistry and Biochemical technology in 1999 and continue studying for M.Sc. in Biochemistry Program, Faculty of Science, Chulalongkorn University in that year.