From *Zymomonas mobilis*
ALCOHOL DEHYDROGENASE (ZM-ADH)
GLUCOKINASE (ZM-GlcK)
GLUCOSE-6-PHOSPHATE DEHYDROGENASE (ZM-G6PDH)

From *Bacillus stearothermophilus*
ACETATE KINASE (AK)
ADENYLATE KINASE (AdK)
ALANINE DEHYDROGENASE (AlaDH)
ALANINE RACEMASE (AlaR)
DIAPHRAGSE I [EC 1.6.99.-] (Di-1)
GLUCOKINASE (GlcK)
α-GLUCOSIDASE (α-Glu)
LEUCINE DEHYDROGENASE (LeuDH)
PHOSPHOFRUCTOKINASE (PFK)
PHOSPHOGLUCOSE ISOMERASE (PGI)
PHOSPHOTRANSACETYLASE (PTA)
POLYNUCLEOTIDE PHOSPHORYLASE (PNPase)
PYRUVATE KINASE (PK)
SUPEROXIDE DISMUTASE (SOD)

From Others
BILIRUBIN OXIDASE (BOD3)
DIAPHRAGSE3 (Di-3)
DIAPHRAGSE22 (Di-22)
GALACTOSE DEHYDROGENASE (GalDH)
GLUCOKINASE2 (GlcK2)
GLUCOSE DEHYDROGENASE (GlcDH2)
D-LACTATE DEHYDROGENASE (D-LDH)
MALATE DEHYDROGENASE (MDH)
MUTAROTASE (MRO)
PHENYLALANINE DEHYDROGENASE (PheDH)
6-PHOSPHOGLUCONATE DEHYDROGENASE (6PGDH)
SORBITOL DEHYDROGENASE (SorDH)

For more information, please contact
NIPRO CORPORATION
3-9-3,Honjo-Nishi,Kita-ku
本社／〒531-8510 大阪市北区本庄西3丁目9番3号
Osaka 531-8510 Japan
Tel (06)6373-3168
Phone +81 6 6373 3168
Fax +81 6 6373 8978
e-mail : nipro-web@nipro.co.jp
http://www.nipro.co.jp/ja/

*Bacillus stearothermophilus* is used as a synonym of *Geobacillus stearothermophilus*.

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The Quality Management System of Enzyme Center, NIPRO Corp. has been certified as to meet the requirements of ISO9001 in the scope of design, development and manufacture of enzymes for analytical reagents and industrial use by JAPAN CHEMICAL QUALITY ASSURANCE LTD.
ALCOHOL DEHYDROGENASE (ZM-ADH)

[EC 1.1.1.1]

from *Zymomonas mobilis*

Alcohol + NAD⁺ ↔ Aldehyde + NADH + H⁺

**SPECIFICATION**

- **State**: Lyophilized
- **Specific activity**: more than 400 U/mg protein
- **Contaminants** (as ZM-ADH activity = 100 %)
  - Glucose-6-phosphate dehydrogenase: < 0.10 %
  - Glucokinase: < 0.02 %
  - Pyruvate kinase: < 0.02 %
  - NADH oxidase: < 0.01 %
  - Lactate dehydrogenase: < 0.01 %

**PROPERTIES**

- **Molecular weight**: ca. 148,000
- **Subunit molecular weight**: ca. 37,000
- **Optimum pH**: 9.5 - 10.0 (Fig. 1)
- **pH stability**: 7.0 - 9.0 (Fig. 2)
- **Thermal stability**: No detectable decrease in activity up to 40 °C. (Fig. 3, 4)
- **Michaelis constants**: (100 mM Glycine-KOH buffer, pH 9.0, at 30 °C)
  - Ethanol: 110 mM
  - Methanol: 350 mM
  - NAD⁺: 0.12 mM
  - Acetaldehyde: 1.66 mM
  - NADH: 0.03 mM
- **Substrate specificity**
  - Ethanol: 100 %
  - Methanol: 0.05 %
  - n-Propanol: 42.3 %
  - n-Butanol: 0.28 %

**STORAGE**

Stable at -20 °C for at least six months

**APPLICATION**

The enzyme is useful for determination of alcohols or aldehydes.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ZM-ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+ 
\]

Unit Definition
One unit of activity is defined as the amount of ZM-ADH that forms 1 μmol of NADH per minute at 30 °C.

Solutions
I  Buffer solution ; 80 mM Glycine-KOH, pH 9.5
II  NAD\(^+\) solution ; 10 mM (0.0663 g NAD\(^+\) free acid/10 mL distilled water)
III  Ethanol solution ; Ethanol (96 %)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM Tris succinate buffer containing 1mg/mL BSA and 0.2 mM CoCl\(_2\), pH 7.0

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  22.90mL
   Solution II  6.00mL
   Solution III  1.10mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs\(_{340}\)) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \[
\frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01}
\] X d.f.

Specific activity (U/mg protein) = \[
\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

d.f. : dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm\(^2\)/μmol)
*Protein concentration ; determined by Bradford's method

REFERENCE
Fig. 1  pH profile

- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 2  pH stability

treated for 24 hr at 4 °C in the following buffer solution (0.1 M), containing 0.5 mM CoCl$_2$:
- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 3  Thermal stability

treated for 15 min in 0.1 M phosphate buffer containing 0.2 mM CoCl$_2$, pH 6.5
- ○ 50 °C,
- □ 55 °C,
- ● 60 °C

Fig. 4  Thermal stability

treated in 0.1 M phosphate buffer containing 0.2 mM CoCl$_2$, pH 6.5
- ○ 50 °C,
- □ 55 °C,
- ● 60 °C
GLUCOKINASE (ZM-GlcK)

[EC 2.7.1.2]

from Zymomonas mobilis

ATP + D-Glucose ↔ ADP + D-Glucose 6-phosphate

SPECIFICATION

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 150 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as ZM-GlcK activity = 100 %)</td>
</tr>
<tr>
<td></td>
<td>Glucose-6-phosphate dehydrogenase &lt; 0.02 %</td>
</tr>
<tr>
<td></td>
<td>Phosphoglucomutase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>6-Phosphogluconate dehydrogenase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Hexose-6-phosphate isomerase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Glutathione reductase &lt; 0.01 %</td>
</tr>
</tbody>
</table>

PROPERTIES

| Molecular weight   | ca. 66,000            |
| Subunit molecular weight | ca. 33,000            |
| Optimum pH         | 7.0 - 8.0 (Fig. 1)    |
| pH stability       | 6.0 - 8.0 (Fig. 2)    |
| Thermal stability  | No detectable decrease in activity up to 40 °C. (Fig. 3, 4) |
| Michaelis constants| (60mM Phosphate buffer, pH 7.0, at 30 °C) |
|                     | Glucose 0.10 mM |
|                     | ATP 0.65 mM       |
| Activator           | Pi                 |

STORAGE

Stable at -20 °C for at least one year

APPLICATION

The enzyme is useful for diagnostic reagent, for example, glucose determination or CK determination, and for the specific determination of glucose. Tris-HCl buffer is not suitable for the practical use of ZM-GlcK.
**ASSAY**

**Principle**

The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{ATP} + \text{Glucose} \xrightarrow{\text{ZM-GlcK}} \text{ADP} + \text{Glucose 6-phosphate}
\]

\[
\text{Glucose 6-phosphate + NAD}^+ \xrightarrow{\text{G6PDH}} \text{Gluconolactone 6-phosphate + NADH + H}^+
\]

**Unit Definition**

One unit of activity is defined as the amount of ZM-GlcK that forms 1 μmol of glucose 6-phosphate per minute at 30 °C.

**Solutions**

- **I** Buffer solution ; 100 mM Triethanolamine - NaOH and 3 mM K₂HPO₄, pH 7.5
- **II** ATP solution ; 100 mM (0.605 g ATP disodium salt·3H₂O/(8.2 mL distilled water + 1.8 mL 1 N-NaOH))
- **III** MgCl₂ solution ; 1 M (20.33 g MgCl₂·6H₂O/100 mL distilled water)
- **IV** NAD⁺ solution ; 100 mM (0.663 g NAD⁺ free acid/10 mL distilled water)
- **V** Glucose solution ; 40mM (0.072 g glucose (anhyd.)/10 mL distilled water)
- **VI** Glucose-6-phosphate dehydrogenase (G6PDH) ; 2000 U/mL (from *Zymomonas mobilis*, Nipro Corp., Dissolve with Buffer solution I )

**Preparation of Enzyme Solution**

Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM potassium phosphate buffer containing 1 mg/mL BSA, pH 7.0.

**Procedure**

1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20.07</td>
</tr>
<tr>
<td>II</td>
<td>1.50</td>
</tr>
<tr>
<td>III</td>
<td>0.30</td>
</tr>
<tr>
<td>IV</td>
<td>0.60</td>
</tr>
<tr>
<td>V</td>
<td>7.50</td>
</tr>
<tr>
<td>VI</td>
<td>0.03</td>
</tr>
</tbody>
</table>

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

**Calculation**

\[
\text{Volume activity (U/mL)} = \frac{(\Delta \text{Abs}_340) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}^*}
\]

\[\text{d.f.} \; ; \text{dilution factor}\]

\[6.22 \; ; \text{millimolar extinction coefficient of NADH (cm}^2/\mu\text{mol)}\]

\[^*\text{Protein concentration} \; ; \text{determined by Bradford's method}\]

**REFERENCE**

treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
acetate, phosphate, Tris-HCl, Gly-KOH

Fig. 2 pH stability
○ MES-KOH, □ TEA-NaOH, ■ Gly-KOH

Fig. 3 Thermal stability
○ 40 °C, □ 50 °C, ● 60 °C

Fig. 4 Thermal stability
● treated in 0.1 M phosphate buffer, pH 7.0
○ 40 °C, □ 50 °C, ● 60 °C
GLUCOSE-6-PHOSPHATE DEHYDROGENASE (ZM-G6PDH)

[EC 1.1.1.49]

from *Zymomonas mobilis*

D-Glucose 6-phosphate + NAD(P)⁺ ↔ D-Gluconolactone 6-phosphate + NAD(P)H + H⁺

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 250 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as ZM-G6PDH activity = 100 %)</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>&lt; 0.02 %</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>&lt; 0.02 %</td>
</tr>
<tr>
<td>Hexose-6-phosphate isomerase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>&lt; 0.01 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

| Molecular weight | ca. 208,000 |
| Subunit molecular weight | ca. 52,000 |
| Optimum pH       | 8.0 (Fig. 1) |
| pH stability     | 5.0 - 10.0 (Fig. 2) |
| Thermal stability| No detectable decrease in activity up to 50 °C. (Fig. 3, 4) |
| Michaelis constants | (30mM Tris-HCl buffer, pH 8.0, at 30 °C) |
| Glucose 6-phosphate | 0.14 mM |
| NADP⁺         | 0.02 mM |
| NAD⁺          | 0.14 mM |
| Substrate specificity | NADP⁺ 70 % |
|                | NAD⁺ 100 % |

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for diagnostic reagent, for example, glucose determination or CK determination, and for the specific determination of glucose.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{Glucose 6-phosphate + NAD}^+ \rightarrow \text{ZM-G6PDH} \rightarrow \text{Gluconolactone 6-phosphate + NADH + H}^+
\]

Unit Definition
One unit of activity is defined as the amount of ZM-G6PDH that forms 1 μmol of NADH per minute at 30 °C.

Solutions
I. Buffer solution; 50 mM Tris-HCl, pH 8.0
II. NAD\(^+\) solution; 100 mM (0.663 g NAD\(^+\) free acid/10 mL distilled water)
III. Glucose 6-phosphate (G6P) solution; 33 mM (0.112 g G6P disodium salt 2H\(_2\)O/10mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50mM potassium phosphate buffer containing 1 mg/mL BSA, pH 7.0.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  26.40mL
   Solution II 0.90mL
   Solution III 2.70mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340nm per minute (ΔAbs\(_{340}\)) in the linear portion of curve.

Calculation

\[
\text{Volume activity (U/mL)} = \frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)*}}
\]

d.f. : dilution factor
6.22 : millimolar extinction coefficient of NADH (cm\(^2\)/μmol)
*Protein concentration : determined by Bradford's method

REFERENCE
Fig. 1  pH profile

Fig. 2  pH stability

Fig. 3  Thermal stability

Fig. 4  Thermal stability
ACETATE KINASE (AK)

[EC 2.7.2.1]

from *Bacillus stearothermophilus*

ATP + Acetate $\leftrightarrow$ ADP + Acetylphosphate

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 1,100 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as AK activity = 100 %)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>GOT</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>GPT</td>
<td>&lt; 0.01 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

| Molecular weight  | ca. 160,000                        |
| Subunit molecular weight | ca. 40,000               |
| Optimum pH        | 7.2 (Fig. 1)                   |
| pH stability      | 7.0 - 8.0 (Fig. 2)            |
| Isoelectric point | 4.8                              |
| Thermal stability | No detectable decrease in activity up to 65 °C. (Fig. 3, 4) |
| Michaelis constants | (57 mM Imidazole- HCl buffer, pH 7.2, at 30 °C) |
| Acetate          | 120 mM                         |
| Acetylphosphate  | 2.3 mM                         |
| ATP              | 1.2 mM                         |
| ADP              | 0.8 mM                         |
| Substrate specificity | Acetate 100 %         |
| Formate          | 0 %                            |
| Propionate       | 5 %                            |
| Butyrate         | 0 %                            |
| Oxalate          | 0 %                            |
| Citrate          | 0 %                            |
| Malate           | 0 %                            |
| Malate           | 0 %                            |
| Glycine          | 0 %                            |

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for determination of acetate or for ATP regeneration system.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{ATP} + \text{Acetate} \xrightarrow{\text{AK}} \text{ADP} + \text{Acetylphosphate} \\
\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+
\]

Unit Definition
One unit of activity is defined as the amount of AK that forms 1 μmol of ADP per minute at 30 °C.

Solutions
I. Buffer solution ; 100 mM Imidazole-HCl, pH 7.2
II. ATP solution ; 100 mM (0.605 g ATP disodium salt·3H₂O/(8.2 mL distilled water + 1.8 mL 1 N-NaOH))
III. Phosphoenolpyruvate (PEP) solution ; 56 mM (0.150 g PEP MCA salt/10 mL distilled water)
IV. NADH solution ; 13.1 mM (0.100 g NADH disodium/10 mL distilled water)
V. MgCl₂ solution ; 1 M (20.33 g MgCl₂·6H₂O /100 mL distilled water)
VI. KCl solution ; 2.5 M (18.64 g KCl/100 mL distilled water)
VII. Pyruvate kinase (PK) ; (from rabbit muscle, Roche Diagnostics K.K., No. 128 155) crystalline suspension in 3.2 M (NH₄)₂SO₄ solution (10mg/mL) approx. 200 U/mg at 25 °C
VIII. Lactate dehydrogenase (LDH) ; (from pig heart, Oriental Yeast Co. Ltd., Product Code: LDH-02) ammonium sulfate suspension, approx. 5,000 U/mL at 25 °C
IX. Sodium acetate solution ; 2 M (27.22g sodium acetate·3H₂O/100 mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM potassium phosphate buffer, pH 7.5.

Procedure
1. Prepare the following reaction mixture and pipette 2.4 mL of reaction mixture into a cuvette.
   Solution I 16.92mL
   Solution II 3.00mL
   Solution III 1.80mL
   Solution IV 0.60mL
   Solution V 0.60mL
   Solution VI 0.90mL
   Solution VII 0.12mL
   Solution VIII 0.06mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.60 mL of Solution IX and 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

Calculation
\[
\text{Volume activity (U/mL) = } \frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]
\[
\text{Specific activity (U/mg protein) = } \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]
\[\text{d.f. ; dilution factor} \]
\[6.22 ; \text{millimolar extinction coefficient of NADH (cm}^2/\mu\text{mol)} \]
\[*\text{Protein concentration ; determined by Bradford's method} \]

REFERENCE
Fig. 1  pH profile

Fig. 2  pH stability

Fig. 3  Thermal stability

Fig. 4  Thermal stability

- Fig. 1 pH profile
  - Acetate, ○ Phosphate, ● Tris-HCl

- Fig. 2 pH stability
  - Treated for 24 hr at 4 °C in the following buffer solution (0.1 M):
    - ○ Phosphate, ● Tris-HCl, ▲ Carbonate

- Fig. 3 Thermal stability
  - Treated for 15 min in 0.1 M potassium phosphate buffer, pH 7.5

- Fig. 4 Thermal stability
  - Treated in 0.1M potassium phosphate buffer, pH 7.5
    - ○ 60 °C, □ 65 °C, ● 70 °C
ADENYLATED KINASE (AdK)

[EC 2. 7. 4. 3]

from *Bacillus stearothermophilus*

ATP + AMP ↔ 2 ADP

**SPECIFICATION**

State: Lyophilized
Specific activity: more than 200 U/mg protein
Contaminants: (as AdK activity = 100 %)
ATPase < 0.01 %
Phosphoglycerate kinase < 0.10 %

**PROPERTIES**

Molecular weight: ca. 20,000
Optimum pH: 6.5 (Fig. 1)
pH stability: 8.0 - 10.5 (Fig. 2)
Isoelectric point: 5.0
Thermal stability: No detectable decrease in activity up to 65 °C. (Fig. 3, 4)
Michaelis constants: (89 mM Imidazole-HCl buffer, pH 6.5, at 30 °C)
ATP 0.04 mM
ADP 0.05 mM
AMP 0.02 mM

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**
The enzyme is useful for determination of AMP or for system involving ATP regeneration.
**ASSAY**

**Principle**
The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{ATP} + \text{AMP} \xrightarrow{\text{AdK}} 2 \text{ADP}
\]

\[
2 \text{ADP} + 2 \text{PEP} \xrightarrow{\text{PK}} 2 \text{ATP} + 2 \text{Pyruvate}
\]

\[
2 \text{Pyruvate} + 2 \text{NADH} + 2 \text{H}^+ \xrightarrow{\text{LDH}} 2 \text{Lactate} + 2 \text{NAD}^+
\]

**Unit Definition**
One unit of activity is defined as the amount of AdK that forms 2 μmol of ADP per minute at 30 °C.

**Solutions**

1. **Buffer solution**: 100 mM Imidazole-HCl, pH 6.5
2. **AMP solution**: 50 mM (0.250 g AMP disodium salt-6H₂O/10 mL distilled water)
3. **ATP solution**: 100 mM (0.605 g ATP disodium salt-3H₂O/8.2 mL distilled water + 1.8 mL 1 N-NaOH))
4. **NADH solution**: 13.1 mM (0.100 g NADH disodium salt-3H₂O/10 mL distilled water)
5. **Phosphoenolpyruvate (PEP) solution**: 56 mM (0.150 g PEP MCA salt/10 mL distilled water)
6. **MgCl₂ solution**: 1 M (20.33 g MgCl₂-6H₂O/100 mL distilled water)
7. **KCl solution**: 2.5 M (18.64 g KCl/100 mL distilled water)
8. **Pyruvate kinase (PK)**: (from rabbit muscle, Roche Diagnostics K.K., No. 128 155) crystalline suspension in 3.2 M (NH₄)₂SO₄ solution (10 mg/mL) approx. 200 U/mg at 25 °C
9. **Lactate dehydrogenase (LDH)**: (from pig heart, Oriental Yeast Co. Ltd., Product Code: LDH-02) ammonium sulfate suspension, approx. 5,000 U/mL at 25 °C

**Preparation of Enzyme Solution**
Dissolve the lyophilized enzyme with distilled water and dilute to 2.5 to 5 U/mL with 50 mM Tris-HCl buffer, pH 8.5.

**Procedure**
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   - Solution I: 26.70 mL
   - Solution II: 0.24 mL
   - Solution III: 0.30 mL
   - Solution IV: 0.60 mL
   - Solution V: 0.18 mL
   - Solution VI: 0.60 mL
   - Solution VII: 1.20 mL
   - Solution VIII: 0.09 mL
   - Solution IX: 0.09 mL

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

**Calculation**

\[
\text{Volume activity (U/mL)} = \frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{2 \times 6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}} \times
\]

\[
d.f. \quad \text{dilution factor}
\]

\[
2 \quad \text{according to the reaction that forms 2 μmol of ADP, one unit of activity of Adk is defined to form 2 μmol of ADP.}
\]

\[
6.22 \quad \text{millimolar extinction coefficient of NADH (cm}^2/\mu\text{mol)}
\]
*Protein concentration ; determined by Bradford's method

REFERENCE
Fig. 1  pH profile

\[ \text{\text{△ acetate, ○ phosphate, ● Tris-HCl, ▲ carbonate}} \]

Fig. 2  pH stability

\[ \text{treated for 24 hr at 4 °C in the following buffer solution (0.1 M); △ acetate, ○ phosphate, ● Tris-HCl, ▲ carbonate} \]

Fig. 3  Thermal stability

\[ \text{treated for 15 min in 0.1M Tris-HCl buffer, pH 9.0} \]

\[ \text{○ 60 °C, □ 65 °C, ● 70 °C} \]

Fig. 4  Thermal stability

\[ \text{treated in 0.1M Tris-HCl buffer, pH 9.0} \]
\[ \text{○ 60 °C, □ 65 °C, ● 70 °C} \]
ALANINE DEHYDROGENASE (AlaDH)

[EC 1. 4. 1. 1]

from *Bacillus stearothermophilus*

L-Alanine + NAD⁺ + H₂O ↔ Pyruvate + NH₄⁺ + NADH

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 55 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as AlaDH activity = 100 %)</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>&lt; 0.10 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

- Molecular weight: ca. 230,000
- Subunit molecular weight: ca. 38,000
- Optimum pH: 10.4  
  (Fig. 1)
- pH stability: 7.0 - 11.5  
  (Fig. 2)
- Thermal stability: No detectable decrease in activity up to 70 °C.  
  (Fig. 3, 4)
- Michaelis constants:  
  L-Alanine 10.0 mM  
  NAD⁺ 0.26 mM  
  (125 mM Glycine-NaOH buffer, pH 10.5, at 30 °C)
- Substrate specificity:  
  L-Alanine 100 %  
  L-Leucine 0 %  
  L-Isoleucine 0 %

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for determination of L-alanine.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[ \text{L-Alanine} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{AlaDH}} \text{Pyruvate} + \text{NH}_4^+ + \text{NADH} \]

Unit Definition
One unit of activity is defined as the amount of AlaDH that forms 1 μmol of NADH per minute at 30 °C.

Solutions
I. Buffer solution ; 250 mM Glycine-NaOH, pH 10.5
II. L-Alanine solution ; 150 mM (1.336 g L-alanine/80 mL distilled water, adjusted to pH 10.5 with 1 N-NaOH and filled up to 100 mL with distilled water)
III. NAD⁺ solution ; 100 mM (0.663 g NAD⁺/ 10 mL with distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 100 mM glycine - NaOH buffer, pH 9.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I 15.00 mL  Solution III 1.50 mL
   Solution II 10.00 mL  H₂O 3.50 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

Calculation
\[
\text{Volume activity (U/mL)} = \frac{(\Delta\text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

 Reference
Fig. 1  pH profile

- Tris-HCl,
- Gly-KOH,
- phosphate

Fig. 2  pH stability

treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
- acetate,
- phosphate,
- Tris-HCl,
- Gly-KOH

Fig. 3  Thermal stability

treated for 15 min in 0.1 M Gly-KOH buffer, pH 9.0

Fig. 4  Thermal stability

treated in 0.1 M Gly-KOH buffer, pH 9.0
- 70 °C,
- 80 °C,
- 90 °C
ALANINE RACEMASE (AlaR)

[EC 5.1.1.1]

from *Bacillus stearothermophilus*

D-Alanine ↔ L-Alanine

SPECIFICATION

State: Liquid
Specific activity: more than 950 U/mg protein
Contaminants: (as AlaR activity = 100 %)
  - Lactate dehydrogenase < 0.01 %
  - NADH oxidase < 0.01 %
  - Alanine dehydrogenase < 0.01 %

PROPERTIES

Molecular weight: ca. 78,000
Subunit molecular weight: ca. 39,000
Optimum pH: 10.5 - 12.0 (Fig. 1)
ph stability: 5.5 - 11.0 (Fig. 2)
Thermal stability: No detectable decrease in activity up to 70 °C, (Fig. 3, 4)
Michaelis constants: (100 mM Carbonate buffer, pH 10.5, at 30 °C)
  - D-Alanine: 31 mM

STORAGE

Stable at least one year at -25 °C.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reactions.

D-Alanine $\xrightarrow{\text{AlaR}}$ L-Alanine

L-Alanine + NAD$^+$ + H$_2$O $\xrightarrow{\text{AlaDH}}$ Pyruvate + NH$_4^+$ + NADH

Unit Definition
One unit of activity is defined as the amount of AlaR that forms 1 μmol of L-alanine per minute at 30 °C.

Solutions
I  Buffer solution ; 200 mM Sodium hydrogencarbonate, pH 10.5
II  D-Alanine solution ; 1 M (0.891 g D-alanine/10 mL distilled water)
III  NAD$^+$ solution ; 100 mM (0.663 g NAD$^+$/10 mL distilled water)
IV  L-Alanine dehydrogenase (AlaDH) ; 1000 U/mL (from Bacillus stearothermophilus, Nipro Corp., Dissolve with distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM potassium phosphate buffer, pH 7.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  16.50mL     Solution IV  1.50mL
   Solution II  3.00mL     H$_2$O  8.25mL
   Solution III  0.75mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute ($\Delta$Abs$_{340}$) in the linear portion of curve.

Calculation
Volume activity (U/mL) = $\frac{(\Delta$Abs$_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01}$ \times \text{d.f.}

Specific activity (U/mg protein) = $\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}$

\text{d.f.} ; \text{dilution factor}
6.22 ; \text{millimolar extinction coefficient of NADH (cm}^2/\mu\text{mol)}
*\text{Protein concentration} ; \text{determined by Bradford's method}

REFERENCE
Fig. 1 pH profile
- ○ phosphate,
- ■ Gly-KOH,
- ▲ acetate,
- ■ Tris-HCl,
- □ NaHCO₃-NaOH

Fig. 2 pH stability
- ▲ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 3 Thermal stability
- treated for 15 min in 50 mM Tris-HCl buffer, pH 9.0

Fig. 4 Thermal stability
- treated in 50 mM Tris-HCl buffer, pH 9.0
- ○ 70 °C, □ 80 °C, ● 85 °C
Fig. 5  Stability (Liquid form) at -25 °C
DIAPHORASE I (Di-1)

[EC 1. 6. 99. - ]

from Bacillus stearothermophilus

NAD(P)H + Acceptor(ox.) + H⁺ ↔ NAD(P)⁺ + Acceptor(red.)

SPECIFICATION

State: Lyophilized
Specific activity: more than 1,000 U/mg protein
Contaminants: (as Diaphorase activity = 100 %)
  Adenylate kinase: < 0.01 %
  NADH oxidase: < 0.01 %

PROPERTIES

Molecular weight: ca. 30,000
Optimum pH: 8.0 (Fig. 1)
pH stability: 7.5 - 9.5 (Fig. 2)
Isoelectric point: 4.7
Optimum temperature: 70 °C
Thermal stability: No detectable decrease in activity up to 50 °C. (Fig. 3, 4)
Michaelis constants: See Table 1
Substrate specificity: See Table 1
Effectors: cations and anions (Fig. 5, 6)

STORAGE

Stable at -20 to 5 °C for at least one year

APPLICATION

The enzyme is useful for the measurement of various dehydrogenase reactions in visible spectral range.
ASSAY

Principle
The change in absorbance is measured at 600 nm according to the following reaction.

\[
\text{NAD(P)H + DCIP(ox.) + H}^+ \rightarrow \text{Di-1} \rightarrow \text{NAD(P)^+ + DCIP(red.)}
\]

Unit Definition
One unit of activity is defined as the amount of Di-1 that reduces 1 μmol of DCIP per minute at 30 °C.

Solutions
I Buffer solution; 500 mM Tris-HCl, pH 8.5
II NADH solution; 13.1 mM (0.100 g NADH disodium salt·3H₂O/10 mL distilled water)
III 2,6-Dichlorophenolindophenol (DCIP) solution; 1.2 mM (2.0 mg DCIP sodium salt·2H₂O/5 mL distilled water) (prepare freshly)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 1.0 to 2.0 U/mL with 50 mM potassium phosphate buffer, pH 7.5.

Procedure
1. Prepare the following reaction mixture and pipette 2.85 mL of reaction mixture into a cuvette.
   Solution I 3.00 mL
   Solution II 2.28 mL
   H₂O 23.22 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.15 mL of Solution III and 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 600 nm per minute (ΔAbs(test)) in linear portion of curve. Repeat the Procedure 3 using distilled water in place of enzyme solution, and ΔAbs(blank) is obtained.

Calculation
Volume activity (U/mL) = \( \frac{(\Delta\text{Abs (test)} - \Delta\text{Abs (blank)}) \times (3.00 + 0.01)}{19 \times 0.01} \times \text{d.f.} \)

Specific activity (U/mg protein) = \( \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}} \)

\( \text{d.f.} \); dilution factor
\( 19 \); millimolar extinction coefficient of DCIP (cm²/μmol)
*Protein concentration; determined by Bradford's method

REFERENCE
Fig. 1 pH profile

- ○ phosphate,
- ■ Gly-KCl-KOH
- ● Tris-HCl

Fig. 2 pH stability

- ○ phosphate,
- ● Tris-HCl

-treated for 24 hr at 4 °C in the following buffer solution (0.1 M):

- △ acetate,
- □ Gly-KCl-KOH

Fig. 3 Thermal stability

-treated for 15 min in 0.1 M potassium phosphate buffer, pH 7.5

Fig. 4 Thermal stability

-treated in 0.1 M potassium phosphate buffer, pH 7.5

- ○ 50 °C,
- □ 60 °C,
- ● 70 °C
Fig. 5  Effect of various cations on the activity of DIAHORASE

Measurement: 0.30 mL of each cation solution and 3.00 mL of assay mixture were mixed, and incubated at 30°C for about 3 minutes. After incubation, 0.01 mL of enzyme solution was added to the reaction mixture and the activity of DIAHORASE was measured.

○ NaCl, △ KCl, □ MgCl₂, ● CaCl₂

Fig. 6  Effect of various anions on the activity of

Measurement: 0.30 mL of each anion solution and 3.00 mL of assay mixture were mixed, and incubated at 30°C for about 3 minutes. After incubation, 0.01 mL of enzyme solution was added to the reaction mixture and the activity of DIAHORASE was measured.

○ NaCl, △ CH₃COONa, □ Na₂SO₄, ● NaHCO₃
# Table 1. SUBSTRATE SPECIFICITY OF DIAPHORASE

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>DCIP&lt;sup&gt;1&lt;/sup&gt; (mM)</th>
<th>NBT&lt;sup&gt;2&lt;/sup&gt; (mM)</th>
<th>INT&lt;sup&gt;3&lt;/sup&gt; (mM)</th>
<th>FMN&lt;sup&gt;4&lt;/sup&gt; (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km&lt;sub&gt;Acceptors&lt;/sub&gt; (mM)</td>
<td>0.015</td>
<td>0.15</td>
<td>0.40</td>
<td>-</td>
</tr>
<tr>
<td>Km&lt;sub&gt;NADH&lt;/sub&gt; (mM)</td>
<td>0.50</td>
<td>0.02</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>Km&lt;sub&gt;NADPH&lt;/sub&gt; (mM)</td>
<td>0.52</td>
<td>0.19</td>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>8.0</td>
<td>&gt; 10</td>
<td>7.5</td>
<td>&lt; 6.5</td>
</tr>
<tr>
<td>Activity&lt;sub&gt;NADH&lt;/sub&gt; (U/mg)</td>
<td>1,200</td>
<td>225</td>
<td>290</td>
<td>18</td>
</tr>
<tr>
<td>Activity&lt;sub&gt;NADPH&lt;/sub&gt; (U/mg)</td>
<td>4</td>
<td>150</td>
<td>120</td>
<td>-</td>
</tr>
</tbody>
</table>

---

**Assay Mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 8.5)</td>
<td>50 mM NAD(P)H 1 mM DCIP 0.06 mM</td>
</tr>
<tr>
<td>TEA (pH 7)</td>
<td>50 mM NAD(P)H 1 mM NBT 0.5 mM Triton X-100 0.1 %</td>
</tr>
<tr>
<td>Phosphate (pH 7.5)</td>
<td>96 mM NAD(P)H 1 mM INT 3 mM DMSO&lt;sup&gt;6&lt;/sup&gt; 2 % BSA&lt;sup&gt;5&lt;/sup&gt; 1 mg/mL</td>
</tr>
<tr>
<td>Phosphate (pH 7)</td>
<td>88 mM NADH 0.2 mM FMN 0.13 mM</td>
</tr>
</tbody>
</table>

**Wavelength for measurement (nm)**

| Extinction coefficient (cm<sup>2</sup>/μmol) | 600 | 550 | 492 | 340 |

*1 2,6-Dichlorophenolindophenol
*2 Nitro blue tetrazolium
*3 p-Iodonitrotetrazolium violet
*4 Flavin mononucleotide
*5 Bovine serum albumin
*6 Added 1/40 volume of 120mM INT (0.607g/10mL 80% DMSO) into the Assay Mixture

---

**Effect of BSA on the activity of DIAPHORASE:**

BSA stimulates the activity with INT as electron acceptor and the activation can be increased 30 fold with concentrations above 1 mg/mL BSA (Fig. 10). The extent of activation for DCIP is about 35 %, whereas the activities with NBT and FMN are not affected by BSA.

---

**Effect of Triton X-100 on the activity of DIAPHORASE:**

The activity with NBT is little in the absence of Triton X-100, but is greatly increased by the addition of Triton X-100 (Fig. 8). On the other hand, Triton X-100 has no effect on the activities with DCIP, INT and FMN.
NBT (Nitro blue tetrazolium)

Fig. 7  pH profile

INT (p-iodonitrotetrazolium violet)

Fig. 9  pH profile

Fig. 8  Effect of Trion- X-100 on the activity of DIAPHORASE

Fig. 10  Effect of BSA on the activity of DIAPHORASE
GLUCOKINASE (GlcK)

[EC 2. 7. 1. 2]

from *Bacillus stearothermophilus*

ATP + D-Glucose ↔ ADP + D-Glucose 6-phosphate

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 350 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as GlcK activity = 100 %)</td>
</tr>
<tr>
<td></td>
<td>Glucose-6-phosphate dehydrogenase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Phosphoglucomutase    &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>6-Phosphogluconate dehydrogenase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Hexose-6-phosphate isomerase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Glutathione reductase  &lt; 0.01 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>ca. 68,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subunit molecular weight</td>
<td>ca. 32,000</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>8.5</td>
</tr>
<tr>
<td>pH stability</td>
<td>8.0 - 11.0</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>5</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>65 °C</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>No detectable decrease in activity up to 60 °C. (Fig. 3, 4)</td>
</tr>
<tr>
<td>Michaelis constants</td>
<td>(60mM Tris-HCl buffer, pH 8.5, at 30 °C)</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>D-Glucose</td>
</tr>
<tr>
<td></td>
<td>D-Mannose</td>
</tr>
<tr>
<td></td>
<td>D-Fructose</td>
</tr>
</tbody>
</table>

**STORAGE**

Stable at -20 to 5 °C for at least one year

**APPLICATION**

The enzyme is useful for diagnostic reagent, for example, glucose determination or CK determination, and for the specific determination of glucose.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{ATP + Glucose} \xrightarrow{\text{GlcK}} \text{ADP + Glucose 6-phosphate}
\]

\[
\text{Glucose 6-phosphate + NADP}^+ \xrightarrow{\text{G6PDH}} \text{Gluconolactone 6-phosphate + NADPH + H}^+
\]

Unit Definition
One unit of activity is defined as the amount of GlcK that forms 1 μmol of glucose 6-phosphate per minute at 30 °C.

Solutions
I. Buffer solution; 100 mM Tris-HCl, pH 9.0
II. ATP solution; 100 mM (0.605 g ATP disodium salt·3H₂O/(8.2 mL distilled water + 1.8 mL 1 N-NaOH))
III. MgCl₂ solution; 1 M (20.33 g MgCl₂·6H₂O/100 mL distilled water)
IV. NADP⁺ solution; 22.5 mM [(0.172 g NADP⁺ monosodium salt or 0.177 g NADP⁺ disodium salt)/10 mL distilled water]
V. Glucose solution; 40 mM (0.072 g glucose (anhyd.))/10 mL distilled water
VI. Glucose-6-phosphate dehydrogenase (G6PDH) ; (from yeast. Roche Diagnostics K.K., No. 127 671) suspension in 3.2 M (NH₄)₂SO₄ solution (10 mg/2 mL) approx. 140 U/mg at 25 °C

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM Tris-HCl buffer, pH 8.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  17.97 mL
   Solution II  1.20 mL
   Solution III  0.60 mL
   Solution IV  1.20 mL
   Solution V  9.00 mL
   Solution VI  0.03 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \( \frac{(\Delta \text{Abs}₃₄₀) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.} \)

Specific activity (U/mg protein) = \( \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)*}} \)

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADPH (cm²/μmol)
*Protein concentration ; determined by Bradford's method

REFERENCE
3. Tomita, K., Kamei, S., Nagata, K., Okuno, H., Shiraishi, T., Motoyama, A., Ohkubo, A., and
Yamanaka, M.; *ibid.*, 3, 11 (1987)
Fig. 1  pH profile
○ phosphate,  ● Tris-HCl,  ▲ carbonate

Fig. 2  pH stability
treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
△ acetate,  ○ phosphate,  ● Tris-HCl,  ▲ carbonate

Fig. 3  Thermal stability
treated for 15 min in 0.1 M Tris-HCl buffer, pH 8.9
○ 60 °C,  □ 70 °C,  ● 80 °C

Fig. 4  Thermal stability
treated in 0.1 M Tris-HCl buffer, pH 8.9
○ 60 °C,  □ 70 °C,  ● 80 °C
α-GLUCOSIDASE (α-Glu)

[EC 3.2.1.20]

from Bacillus stearothermophilus

α-D-Glucoside + H₂O ↔ D-Glucose + Alcohol

SPECIFICATION

State : Lyophilized
Specific activity : more than 40 U/mg protein
Contaminants : (as α-Glu activity = 100 %)
  Phosphoglucomutase < 0.01 %
  NADH oxidase < 0.01 %
  Alcohol dehydrogenase < 0.01 %

PROPERTIES

Molecular weight : ca. 50,000
Optimum pH : 6.0 - 7.0 (Fig. 1)
pH stability : 5.0 - 11.0 (Fig. 2)
Isoelectric point :
Thermal stability : No detectable decrease in activity up to 60 °C. (Fig. 3, 4)
Michaelis constants : (50 mM Potassium phosphate buffer, pH 6.3, at 30 °C)
  p-Nitrophenyl-α-glucopyranoside (PNPG) 0.73 mM
  Maltose 1.3 mM
  Phenyl-α-glucopyranoside 2.4 mM
Substrate specificity : PNPG 100 %
  Maltose 177 %
  Phenyl-α-glucopyranoside 59 %

STORAGE

Stable at -20 °C for at least one year

APPLICATION

The enzyme is useful for diagnostic reagent, for example, α-amylase determination.
ASSAY

Principle
The change in absorbance is measured at 400 nm according to the following reaction.

\[
\text{p-Nitrophenyl-} \alpha - \text{glucopyranoside (PNPG)} \xrightarrow{\alpha - \text{Glu}} \text{p-Nitrophenol (PNP)} + \text{Glucose}
\]

Unit Definition
One unit of activity is defined as the amount of \( \alpha - \text{Glu} \) that forms 1 \( \mu \text{mol} \) of PNP per minute at 30 °C.

Solutions

I  Buffer solution ; 100 mM Potassium phosphate buffer, pH 6.3
II PNPG solution ; 20 mM (0.603 g PNPG/100 mL distilled water) (Stable for two weeks if stored at 0 - 5 °C)
III \( \text{Na}_2\text{CO}_3 \) solution ; 0.2 M (2.12 g \( \text{Na}_2\text{CO}_3 \)/100 mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 0.006 to 0.022 U/mL with 10 mM Potassium phosphate buffer containing 1 mg/mL BSA, pH 7.5.

Procedure
1. Prepare the following reaction mixture and pipette 1.5 mL of reaction mixture into a test tube.
   Solution I 10.0mL
   Solution II 5.0mL
2. Incubate at 30 °C for 5 minutes.
3. Add 0.5 mL of the enzyme solution and mix.
4. Incubate at 30 °C for exactly 15 minutes.
5. After incubation, add 2.0 mL of Solution III and mix.
6. Read absorbance at 400 nm (Abs•test).
   At the same time, prepare the blank with 1.5 mL of the reaction mixture, and add 2.0 mL of Solution III after incubation at 30 °C for 15 minutes, followed by addition of the enzyme solution (Abs•blank).

Calculation

Volume activity (U/mL) = ((Abs•test) - (Abs•blank)) \times \frac{4.0}{18.1 \times 15 \times 0.5} \times \text{d.f.}

Specific activity (U/mg protein) = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}

d.f. ; dilution factor
18.1 ; millimolar extinction coefficient of PNP (cm\(^2/\mu\text{mol})
*Protein concentration ; determined by Bradford's method
NIPRO ENZYMES

Fig. 1  pH profile

△ acetate, ○ phosphate, □ Gly-NaOH

Fig. 2  pH stability

treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
△ acetate, ○ phosphate, □ TEA-NaOH, ■ Gly-NaOH

Fig. 3  Thermal stability

treated for 15 min in 0.1M potassium phosphate buffer, pH 8.0

Fig. 4  Thermal stability

treated in 0.1M potassium phosphate buffer, pH 8.0
○ 60 °C, ● 65°C
LEUCINE DEHYDROGENASE (LeuDH)

[EC 1. 4. 1. 9]

from *Bacillus stearothermophilus*

\[
\text{L-Leucine} + \text{NAD}^+ + \text{H}_2\text{O} \leftrightarrow \alpha\text{-Ketoisocaprate} + \text{NH}_4^+ + \text{NADH}
\]

**SPECIFICATION**

- **State**: Lyophilized
- **Specific activity**: more than 40 U/mg protein
- **Contaminants**: (as LeuDH activity = 100 %)
  - NADH oxidase < 0.01 %
  - Lactate dehydrogenase < 0.01 %

**PROPERTIES**

- **Molecular weight**: ca. 300,000
- **Subunit molecular weight**: ca. 49,000
- **Optimum pH**: 10.6
- **pH stability**: 6.0 - 11.5
- **Thermal stability**: No detectable decrease in activity up to 60 °C.
- **Michaelis constants**: (125mM Sodium phosphate buffer, pH 10.5, at 30 °C)
  - L-Leucine 3.4 mM
  - NAD' 0.3 mM
- **Substrate specificity**: L-Leucine 100 %
  - L-Valine 86 %
  - L-Isoleucine 73 %

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for determination of L-leucine, L-valine or L-isoleucine.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{L-Leucine} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{LeuDH}} \text{α-Ketoisocaprate} + \text{NH}_4^+ + \text{NADH}
\]

Unit Definition
One unit of activity is defined as the amount of LeuDH that forms 1 μmol of NADH per minute at 30 °C.

Solutions
- **I** Buffer solution ; 250 mM Sodium phosphate, pH 10.5
- **II** L-Leucine solution ; 60 mM (0.787 g L-leucine/80 mL distilled water, adjusted to pH 10.5 with 1 N-NaOH and filled up to 100 mL with distilled water)
- **III** NAD\(^+\) solution ; 100mM (0.663 g NAD\(^+\)/ 10mL with distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 100 mM sodium phosphate buffer, pH 9.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette,
   - Solution I 15.00mL
   - Solution II 10.00mL
   - Solution III 0.93mL
   - H\(_2\)O 4.07mL
2. Incubate at 30°C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs\(_{340}\)) in the linear portion of curve.

Calculation

\[
\text{Volume activity (U/mL)} = \frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

\[
\text{d.f.} \text{ ; dilution factor}
\]

\[
6.22 \text{ ; millimolar extinction coefficient of NADH (cm}^2/\mu\text{mol)}
\]

\[
^*\text{Protein concentration ; determined by Bradford's method}
\]

REFERENCE
Fig. 1  pH profile

- ▲ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 2  pH stability

treated for 24 hr at 4 °C in the following buffer solution (0.1 M):
- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 3  Thermal stability

treated for 15 min in 0.1M Gly-KOH buffer, pH 9.0

Fig. 4  Thermal stability

treated in 0.1M Gly-KOH buffer, pH 9.0
- ○ 60°C, □ 70°C, ● 80°C
PHOSPHOFRACTOKINASE (PFK)

[EC 2.7.1.11]

from Bacillus stearothermophilus

ATP + D-Fructose 6-phosphate ↔ ADP + D-Fructose 1, 6-bisphosphate

SPECIFICATION

State: Lyophilized
Specific activity: more than 100 U/mg protein
Contaminants: (as PFK activity = 100 %)
  Adenylate kinase < 0.01 %
  ATPase < 0.01 %
  6-Phosphogluconate dehydrogenase < 0.01 %
  Glutathione reductase < 0.01 %
  Phosphoglucomutase < 0.01 %
  Glucose phosphate isomerase < 0.01 %

PROPERTIES

Molecular weight: ca. 74,000
Subunit molecular weight: ca. 34,000
Optimum pH: 9.0 (Fig. 1)
pH stability: 6.5 - 10.0 (Fig. 2)
Isoelectric point: 6.0 - 6.2
Thermal stability: No detectable decrease in activity up to 50 °C. (Fig. 3, 4)
Michaelis constants: (91 mM Tris-HCl buffer, pH 9.0, at 30 °C)
  Fructose 6-phosphate 1.6 mM
  ATP 0.035 mM

Activators: K⁺, (NH₄)₂SO₄
Inhibitors: PEP, Citrate

STORAGE

Stable at -20 °C for at least one year
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{Fructose 6-phosphate + ATP} \xrightarrow{\text{PFK}} \text{Fructose 1, 6-bisphosphate + ADP}
\]
\[
\text{ADP + PEP} \xrightarrow{\text{PK}} \text{ATP + Pyruvate}
\]
\[
\text{Pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{Lactate + NAD}^+
\]

Unit Definition
One unit of activity is defined as the amount of PFK that forms 1 μmol of fructose 1, 6-bisphosphate per minute at 30 °C.

Solutions
I. Buffer solution ; 100 mM Tris-HCl, pH 9.0
II. ATP solution ; 100 mM (0.605 g ATP disodium salt·3H2O/(8.2 mL distilled water + 1.8 mL 1 N-NaOH))
III. Phosphoenolpyruvate (PEP) solution ; 56 mM (0.150 g PEP MCA salt/10 mL distilled water)
IV. NADH solution ; 13.1 mM (0.100 g NADH disodium salt·3H2O/10 mL distilled water)
V. Fructose 6-phosphate (F6P) solution ; 500 mM (1.55 g F6P disodium salt/10 mL distilled water)
VI. KCl solution ; 2.5 M (16.64g KCl/100 mL distilled water)
VII. MgSO4 solution ; 100 mM (2.47 g MgSO4·7H2O/100 mL distilled water)
VIII. Pyruvate kinase (PK) ; (from rabbit muscle, Roche Diagnostics K.K., No. 128 155) crystalline suspension in 3.2 M (NH4)2SO4 solution (10 mg/mL) approx. 200 U/mg at 25 °C
IX. Lactate dehydrogenase (LDH) ; (from pig heart, Oriental Yeast Co. Ltd., Product Code: LDH-02) ammonium sulfate suspension, approx. 5,000 U/mL at 25 °C

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50mM potassium phosphate buffer, pH 8.0.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL reaction mixture into a cuvette.
   Solution I  27.33mL
   Solution II 0.30mL
   Solution III 0.39mL
   Solution IV 0.60mL
   Solution V  0.60mL
   Solution VI 0.06mL
   Solution VII 0.60mL
   Solution VIII 0.06mL
   Solution IX  0.06mL

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs340) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \( \frac{(\Delta \text{Abs}340) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.} \)

Specific activity (U/mg protein) = \( \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}} \)

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm²/μmol)
*Protein concentration; determined by Bradford's method

**REFERENCE**

Fig. 1 pH profile

- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 2 pH stability

- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 3 Thermal stability

- treated for 15 min in 50 mM Tris-HCl buffer, pH 8.5, or potassium phosphate buffer, pH 7.5
- ○ phosphate,
- ● Tris-HCl

Fig. 4 Thermal stability

- treated in 50 mM potassium phosphate buffer, pH 7.5
- ○ 50 °C,
- □ 60 °C,
- ● 70 °C
PHOSPHOGLUCOSE ISOMERASE (PGI)

[EC 5. 3. 1. 9]

from Bacillus stearothermophilus

D-Glucose 6-phosphate ↔ D-Fructose 6-phosphate

SPECIFICATION

State : Lyophilized
Specific activity : more than 400 U/mg protein
Contaminants : (as PGI activity = 100 %)
  Phosphofructokinase < 0.01 %
  6-Phosphogluconate dehydrogenase < 0.01 %
  Phosphoglucomutase < 0.01 %
  NA DPH oxidase < 0.01 %
  Glutathione reductase < 0.01 %

PROPERTIES

Molecular weight : ca. 200,000
Subunit molecular weight : ca. 54,000
Optimum pH : 9.0 - 10.0 (Fig. 1)
pH stability : 6.0 - 10.5 (Fig. 2)
Isoelectric point : 4.2
Thermal stability : No detectable decrease in activity up to 60 ºC. (Fig. 3, 4)
Michaelis constants : (95mM Tris-HCl buffer, pH 9.0, at 30 ºC)
  Fructose 6-phosphate 0.27 mM

STORAGE

Stable at -20 ºC for at least one year
ASSAY

Principle
The change in absorbance is measured at 340nm according to the following reactions.

\[
\text{Fructose 6-phosphate} \xrightarrow{\text{PGI}} \text{Glucose 6-phosphate}
\]

\[
\text{Glucose 6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{Gluconolactone 6-phosphate} + \text{NADPH} + \text{H}^+
\]

Unit Definition
One unit of activity is defined as the amount of PGI that forms 1 μmol of glucose 6-phosphate per minute at 30 °C.

Solutions
I Buffer solution ; 100 mM Tris-HCl, pH 9.0
II Fructose 6-phosphate (F6P) solution ; 100 mM (0.310 g F6P disodium salt/10 mL distilled water)
III NADP⁺ solution ; 22.5 mM (0.188 g NADP⁺ sodium salt·4H₂O/10 mL distilled water)
IV Glucose-6-phosphate dehydrogenase (G6PDH) ; (from yeast, Roche Diagnostics K.K., No. 127 671)
suspension in 3.2 M (NH₄)₂SO₄ solution (10 mg/2 mL) approx. 140 U/mg at 25 °C

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50mM Tris-HCl buffer, pH 8.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  28.44 mL
   Solution II 0.90 mL
   Solution III 0.60 mL
   Solution IV 0.06 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of the curve.

Calculation
\[
\text{Volume activity (U/mL)} = \frac{\left(\Delta\text{Abs}_340\right) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

\text{d.f.} ; \text{dilution factor}
\text{6.22} ; \text{millimolar extinction coefficient of NADPH (cm}^2/\mu\text{mol)}
*\text{Protein concentration} ; \text{determined by Bradford’s method}

REFERENCE
Fig. 1  pH profile

- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 2  pH stability

- treated for 24 hr at 4 °C in the following buffer solution (0.1 M):
  - △ acetate,
  - ○ phosphate,
  - ● Tris-HCl,
  - ■ Gly-KOH

Fig. 3  Thermal stability

- treated for 15 min in 50 mM Tris-HCl buffer, pH 8.5

Fig. 4  Thermal stability

- treated in 50 mM Tris-HCl buffer, pH 8.5
  - ○ 60 °C,
  - □ 65 °C,
  - ● 70 °C
PHOSPHOTRANSACETYLASE (PTA)

[EC 2. 3. 1. 8]

from *Bacillus stearothermophilus*

Acetyl-CoA + Pi ↔ Acetylphosphate + CoA

**SPECIFICATION**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>State</td>
<td>Lyophilized</td>
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<tr>
<td>Specific activity</td>
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</tr>
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<td>Contaminants</td>
<td>(as PTA activity = 100 %)</td>
</tr>
<tr>
<td></td>
<td>Acetate kinase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Adenylate kinase &lt; 0.01 %</td>
</tr>
<tr>
<td></td>
<td>Lactate dehydrogenase &lt; 0.01 %</td>
</tr>
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</table>

**PROPERTIES**

<table>
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<th>Value</th>
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<td>Molecular weight</td>
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<tr>
<td>Subunit molecular weight</td>
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<td>Optimum pH</td>
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<tr>
<td>pH stability</td>
<td>7.0 - 11.0 (Fig. 2)</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.5 (Fig. 1)</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>No detectable decrease in activity up to 50 °C. (Fig. 3, 4)</td>
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<tr>
<td>Michaelis constants</td>
<td>(87mM Tris-HCl buffer, pH 7.5, at 30 °C)</td>
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<tr>
<td>Coenzyme A</td>
<td>0.4 mM</td>
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<tr>
<td>Acetyl Phosphate</td>
<td>1.1 mM</td>
</tr>
</tbody>
</table>

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for determination of CoA or acetate.
ASSAY

Principle

The change in absorbance is measured at 233 nm according to the following reaction.

\[
\text{Acetylphosphate} + \text{CoA} \xrightarrow{\text{PTA}} \text{Acetyl-CoA} + \text{Pi}
\]

Unit Definition

One unit of activity is defined as the amount of PTA that forms 1 μmol of acetyl-CoA per minute at 30 °C.

Solutions

I. Buffer solution; 100 mM Tris-HCl, pH 7.5
II. CoA solution; 6.4 mM (50 mg CoA trilithium salt/10 mL distilled water)
III. Acetylphosphate solution; 217 mM (0.400 g acetylphosphate potassium lithium salt/10 mL distilled water)
IV. Ammonium sulfate (AmS) solution; 1 M (13.2 g AmS/100 mL distilled water)

Preparation of Enzyme Solution

Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 20 U/mL with 50 mM Tris-HCl buffer, pH 8.0.

Procedure

1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   
<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>26.0mL</td>
</tr>
<tr>
<td>II</td>
<td>2.0mL</td>
</tr>
<tr>
<td>III</td>
<td>1.0mL</td>
</tr>
<tr>
<td>IV</td>
<td>1.0mL</td>
</tr>
</tbody>
</table>

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 233 nm per minute (ΔAbs\text{233}) in the linear portion of curve.

Calculation

Volume activity (U/mL) = \(\frac{(\Delta\text{Abs}_{233}) \times (3.00 + 0.01)}{4.44 \times 0.01}\) X d.f.

Specific activity (U/mg protein) = \(\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}\)

\(\text{d.f.} \); dilution factor
4.44; differential millimolar extinction coefficient between acetyl-CoA and CoA (cm\(^2\)/μmol)
*Protein concentration; determined by Bradford's method
Fig. 1  pH profile  
\[ \bullet \text{Tris-HCl} \]  

Fig. 2  pH stability  
\[ \text{treated for 24 hr at 4 \degree C in the following buffer solution (0.1 M); } \]
\[ \triangle \text{acetate, } \circ \text{phosphate,} \]
\[ \bullet \text{Tris-HCl, } \blacksquare \text{Gly-KOH} \]  

Fig. 3  Thermal stability  
\[ \text{treated for 15 min in 50 mM Tris-HCl buffer, pH 8.0} \]  

Fig. 4  Thermal stability  
\[ \text{treated in 50 mM Tris-HCl buffer, pH 8.0} \]
\[ \circ 50 \degree C, \square 60 \degree C, \bullet 65 \degree C \]
POLYNUCLEOTIDE PHOSPHORYLASE (PNPase)

[EC 2.7.7.8]

from *Bacillus stearothermophilus*

\[ RNA_{n+1} + Pi \leftrightarrow RNA_n + \text{Nucleoside diphosphate} \]

**FOR DEPOLYMERIZATION REACTION**

**SPECIFICATION**

State : Lyophilized
Specific activity : more than 2,000 U/mg protein

**PROPERTIES**

Molecular weight : 300,000 - 340,000
Subunit molecular weight : ca. 85,000
Optimum pH : 9.0 - 9.5 (Fig. 1)
pH stability : 9.0 - 11.0 (Fig. 2)
Isoelectric point : 4.0
Thermal stability : No detectable decrease in activity up to 55 °C (Fig. 3, 4)
Michaelis constants : (38 mM Tris-HCl buffer, pH 9.5, at 60 °C)
  Poly A : 0.27 mM**
  KH₂PO₄ : 3.0 mM

**concentration of poly A was calculated as AMP concentration**

Effectors : cations and anions (Fig. 5, 6)

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for the preparation of polyribonucleotide.
**ASSAY**

**Principle**

The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{Poly A}_n + Pi \xrightarrow{\text{PNPase}} \text{Poly A}_{n-1} + \text{ADP} \quad (I)
\]

\[
\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}
\]

\[
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+ \quad (II)
\]

**Unit Definition**

One unit of activity is defined as the amount of PNPase that forms 1 μmol of ADP per hour at 60 °C by depolymerizing of Poly A.

**Solutions**

(Reaction I)

I. Buffer solution ; 100 mM Tris-HCl, pH 9.5 ((1.212 g Tris + 0.074 g EDTA + 0.014 mL 2-mercaptoethanol + 0.610 g MgCl₂·6H₂O + 0.746 g KCl)/80 mL distilled water, adjusted to pH 9.5 with 1 N-HCl and filled up to 100 mL with distilled water)

II. KH₂PO₄ solution ; 65 mM (0.088 g KH₂PO₄/10 mL distilled water)

III. polyadenylate (Poly A) solution ; (25 mg Poly A potassium salt/1 mL distilled water; ca. 35 mM based on AMP concentration)

(Reaction II)

IV. Buffer solution ; 100 mM Triethanolamine buffer, pH 7.6 ((9.300 g triethanolamine-HCl + 0.407 g MgCl₂·6H₂O + 0.373 g KCl)/400 mL distilled water, adjusted to pH 7.6 with 1 N-NaOH and filled up to 500 mL with distilled water)

V. NADH solution ; 13.1 mM (0.100 g NADH disodium salt·3H₂O/10 mL distilled water)

VI. Phosphoenolpyruvate (PEP) solution ; 56mM (0.150 g PEP MCA salt/10 mL distilled water)

VII. Pyruvate kinase (PK) ; (from rabbit muscle, Roche Diagnostics K.K., No. 128 155) crystalline suspension in 3.2 M (NH₄)₂SO₄ solution (10 mg/mL) approx. 200 U/mg at 25 °C

VIII. Lactate dehydrogenase (LDH) ; (from pig heart, Oriental Yeast Co. Ltd., Product Code: LDH-02) ammonium sulfate suspension, approx. 5,000 U/mL at 25 °C

**Preparation of Enzyme Solution**

Dissolve the lyophilized enzyme with distilled water and dilute to 1 to 5 U/mL with 50 mM Tris-HCl buffer, pH 8.5.

**Procedure**

(Reaction I)

1. Prepare the following reaction mixture and pipette 0.55 mL of reaction mixture into a test tube.

   | Solution I | Solution II | Solution III | Solution IV |
   | 2.50mL | 1.00mL | 1.00mL | H₂O 1.00mL |

2. Add 0.10 mL of enzyme solution and mix.

3. Incubate at 60 °C for exactly 10 minutes.

4. After incubation, add 0.01 mL conc. HCl and mix.

5. Centrifuge at 10,000 rpm for 30 seconds.

   At the same time, repeat the Procedure 1 to 5 using distilled water in place of enzyme solution in Procedure 2 (as blank).

(Reaction II)

6. Prepare the following reaction mixture and pipette 2.50 mL of the reaction mixture into a cuvette.

   | Solution IV | Solution VII |
   | 24.18mL | 0.12mL |
Solution V  0.40 mL  
Solution VI  0.25 mL  
Solution VII  0.05 mL

7. Incubate at 30 °C for about 3 minutes.
8. Add 0.10 mL of supernatant of Procedure 5 and mix.
9. Read absorbance at 340 nm (Abs•test).
   Repeat the Procedure using blank (Abs•blank).

**Calculation**

\[
\text{Volume activity (U/mL)} = \left( \frac{(\text{Abs}•\text{blank}) - (\text{Abs}•\text{test})}{60} \right) \times \left( \frac{2.60 \times 0.65}{6.22 \times 0.10 \times 0.10} \right) \times 10 \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

- d.f. = dilution factor
- 6.22 = millimolar extinction coefficient of NADH (cm\(^2\)/μmol)
- *Protein concentration = determined by the absorbance at 280nm (Abs280), where 1 Abs280 = 1 mg/mL

**REFERENCES**
Fig. 1  pH profile

- ■ Tris-HCl

Fig. 2  pH stability

- ■ Tris-HCl
- ● Gly-KCl-KOH

Tris-HCl treated for 24 hr at 4 °C in the following buffer solution (0.1 M):
- ● Tris-HCl
- ■ Gly-KCl-KOH

Fig. 3  Thermal stability

- ○ 55 °C
- □ 60 °C
- ● 65 °C

Treated for 15 min in 0.1 M Tris-HCl buffer, pH 8.5

Fig. 4  Thermal stability

Treated in 0.1 M Tris-HCl buffer, pH 8.5
- ○ 55 °C
- □ 60 °C
- ● 65 °C
Fig. 5  Effect of various cations on the activity of Polynucleotide phosphorylase in the following Assay Method

Measurement: 0.015 mL of each cation solution, 0.010 mL of enzyme solution and 0.055 mL of reaction mixture were mixed, and reacted at 60 °C. After 10 minutes, the quantity of ADP was determined.

○ NaCl, △ KCl, □ MgCl₂, ● CaCl₂, ▲ ZnCl₂

Fig. 6  Effect of various anions on the activity of Polynucleotide phosphorylase in the following Assay Method

Measurement: 0.015 mL of each anion solution, 0.010 mL of enzyme solution and 0.055 mL of reaction mixture were mixed, and reacted at 60 °C. After 10 minutes, the quantity of ADP was determined.

○ NaCl, △ CH₃COONa, □ Na₂SO₄, ● NaHCO₃, ▲ NaH₂PO₄
**PYRUVATE KINASE (PK)**

[EC 2.7.1.40]

from *Bacillus stearothermophilus*

\[
\text{ATP + Pyruvate} \leftrightarrow \text{ADP + Phosphoenolpyruvate}
\]

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 230 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as PK activity = 100 %)</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>&lt; 0.01 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

| Molecular weight | ca. 260,000 |
| Subunit molecular weight | ca. 68,000 |
| Optimum pH | 7.0 (Fig. 1) |
| pH stability | 8.0 - 10.0 (Fig. 2) |
| Isoelectric point | 5.2 |
| Thermal stability | No detectable decrease in activity up to 55 °C. (Fig. 3, 4) |
| Michaelis constants | (76 mM Imidazole-HCl buffer, pH 7.2, at 30 °C) |
| Phosphoenolpyruvate | 0.6 mM |
| ADP | 0.9 mM |

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for diagnostic reagent, for example, ADP determination.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\begin{align*}
\text{ADP} + \text{PEP} & \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate} \\
\text{Pyruvate} + \text{NADH} + \text{H}^+ & \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+
\end{align*}
\]

Unit Definition
One unit of activity is defined as the amount of PK that forms 1 μmol of pyruvate per minute at 30 °C.

Solutions
I. Buffer solution; 100 mM Imidazole-HCl, pH 7.2
II. ADP solution; 100 mM (0.507 g ADP disodium salt-2H₂O/(9.0 mL distilled water + 1.0 mL 1 N NaOH))
III. NADH solution; 13.1 mM (0.100 g NADH disodium salt-3H₂O/10 mL distilled water)
IV. Phosphoenolpyruvate (PEP) solution; 56 mM (0.150 g PEP MCA salt/10 mL distilled water)
V. MgCl₂ solution; 1.0 M (20.33 g MgCl₂-6H₂O/100 mL distilled water)
VI. KCl solution; 2.5 M (18.64 g KCl/100 mL distilled water)
VII. Lactate dehydrogenase (LDH); (from pig heart, Oriental Yeast Co. Ltd., Product Code: LDH-02) ammonium sulfate suspension, approx. 5,000 U/mL at 25 °C

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM Tris-HCl buffer, pH 8.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I 22.71 mL  
   Solution II 2.40 mL  
   Solution III 0.45 mL  
   Solution IV 3.00 mL  
   Solution V 0.48 mL  
   Solution VI 0.90 mL  
   Solution VII 0.06 mL

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

Calculation

\[
\text{Volume activity (U/mL)} = \frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

d.f.; dilution factor
6.22; millimolar extinction coefficient of NADH (cm²/μmol)
*Protein concentration; determined by Bradford’s method

REFERENCE
treated for 24 hr at 4 °C in the following buffer solution (0.1 M); △ acetate, □ imidazole-HCl, ● Tris-HCl, ▲ carbonate

Fig. 1  pH profile
△ acetate, □ imidazole-HCl, ● Tris-HCl

Fig. 2  pH stability
treated for 15 min in 0.1 M Tris-HCl buffer, pH 8.5
○ 55 °C, □ 60 °C, ● 65 °C
△ acetate, □ imidazole-HCl, ● Tris-HCl

Fig. 3  Thermal stability
treated for 15 min in 0.1 M Tris-HCl buffer, pH 8.5
○ 55 °C, □ 60 °C, ● 65 °C

Fig. 4  Thermal stability
treated in 0.1 M Tris-HCl buffer, pH 8.5
○ 55 °C, □ 60 °C, ● 65 °C
SUPEROXIDE DISMUTASE (SOD)

[EC 1.15.1.1]

from *Bacillus stearothermophilus*

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \leftrightarrow \text{O}_2 + \text{H}_2\text{O}_2 \]

**SPECIFICATION**

- State: Lyophilized
- Specific activity: more than 9,000 U/mg protein
- Contaminants: (as SOD activity = 100 %)
  - Catalase: < 0.01 %

**PROPERTIES**

- Molecular weight: ca. 50,000
- Subunit molecular weight: ca. 25,000
- Metal content: 1.5 g atoms of Mn per mole of enzyme
- Optimum pH: 9.5 (Fig. 1)
- pH stability: 6.0 - 9.0 (Fig. 2)
- Isoelectric point: 4.5
- Thermal stability: No detectable decrease in activity up to 60 °C. (Fig. 3, 4)

**STORAGE**

Stable at -20 °C for at least one year

**APPLICATION**

The enzyme is useful for medicine, cosmetic material and nutrition or antioxidant.
ASSAY

Principle
To determine the enzyme activity of cytochrome c reduction is measured by the following reactions.

Xanthine + O₂ \xrightarrow{\text{Xanthine oxidase}} \text{Urate} + O₂⁻ + H₂O₂

O₂⁻ \xrightarrow{\text{SOD}} \text{O}_2 + H₂O₂

Unit Definition
One unit of activity is defined as the amount of SOD required to inhibit the rate of reduction of cytochrome C by 50 % at 30 °C.

Solutions
I. Buffer solution ; 75 mM Potassium phosphate buffer, pH 7.8
II. Xanthine solution ; 0.75 mM (0.010 g xanthine/50 mL N/250 NaOH)
III. Cytochrome c solution ; 0.15 mM (0.019 g cytochrome c/10 mL distilled water, Sigma-Aldrich Co., No. C-2506, from horse heart)
IV. EDTA solution ; 1.5 mM (0.028 g EDTA disodium salt·2H₂O/50 mL distilled water)
V. Xanthine oxidase (XOD) ; (from buttermilk, Sigma-Aldrich Co., No. X-1875) suspension in 2.3 M (NH₄)₂SO₄ solution is diluted to 0.04 U/mL with distilled water. (prepare freshly)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to approx. 600 U/mL with 50 mM potassium phosphate buffer, pH 7.5.

Procedure
1. Prepare the following reaction mixture and pipette 2.80 mL of reaction mixture and 0.005 mL of enzyme solution into a cuvette.
   Solution I  22.00mL  Solution III  2.00mL
   Solution II 2.00mL  Solution IV  2.00mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.20 mL of Solution V into the cuvette and mix.
4. Read absorbance change at 550 nm per minute for the linear portion of curve (ΔAbs•test)*.
5. Add 0.005 mL of Solution I in place of enzyme solution and measure the same above 4 (ΔAbs•blank).
   *Dilute enzyme solution with 50 mM potassium phosphate buffer, pH 7.5, because the decrease in the initial rate should not fall outside the range of 40 to 60 % for the results to be valid.

Calculation
Volume activity (U/mL) = \frac{(ΔAbs•blank)}{(ΔAbs•test)} - 1 \times \frac{601}{1} \times \text{d.f.}

Specific activity (U/mg protein) = \frac{\text{Volume activity (U/mL)}}{\text{protein concentration (mg/mL)}^*}

\text{d.f.} ; \text{dilution factor}
*Protein concentration ; determined by Bradford's method

REFERENCE
Fig. 1  pH profile

Fig. 2  pH stability

Fig. 3  Thermal stability

Fig. 4  Thermal stability

△ acetate, ○ phosphate,

treated for 24 hr at 4 °C in the

treated for 15 min in 0.1 M potassium phosphate buffer, pH 7.5

△ acetate, ○ phosphate,

treated for 24 hr at 4 °C in the

treated in 0.1 M potassium phosphate buffer, pH 7.5

△ acetate, ○ phosphate,

treated for 24 hr at 4 °C in the

treated in 0.1 M potassium phosphate buffer, pH 7.5

△ acetate, ○ phosphate,
BILIRUBIN OXIDASE (BOD3)

[EC 1.3.3.5]

from *Trachyderma tsunodae*

2 Bilirubin + O$_2$ → 2 Biliverdin + 2 H$_2$O

**SPECIFICATION**

State: Lyophilized
Specific activity: more than 100 U/mg protein

**PROPERTIES**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>ca. 60,000</td>
<td>(SDS-electrophoresis)</td>
<td>(Fig. 1)</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>5.0</td>
<td>(Gel filtration)</td>
<td></td>
</tr>
<tr>
<td>pH stability</td>
<td>4.0 – 11.0</td>
<td>(4 °C, 24 hr)</td>
<td>(Fig. 2)</td>
</tr>
<tr>
<td>Isoelectric point (calculation)</td>
<td>3.8</td>
<td>(Fig. 3)</td>
<td></td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>65 – 80 °C</td>
<td>(Fig. 3)</td>
<td></td>
</tr>
<tr>
<td>Thermal stability</td>
<td>No detectable decrease in activity up to 50 °C. (pH 7.0)</td>
<td>(Fig. 4, 5)</td>
<td></td>
</tr>
<tr>
<td>Michaelis constants</td>
<td>See table 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>See table 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**STORAGE**

Stable at -20 °C for one year

**APPLICATION**

The enzyme is useful for enzymatic determination of bilirubin. It could be used as a cathode catalyst in biofuel cells.
ASSAY

Principle
The change in absorbance is measured at 500 nm according to the following reaction.

\[
\text{Phenol} + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Quinone and/or Phenoxy radical} + \text{H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \rightarrow \text{Quinoneimine} + 4 \text{H}_2\text{O}
\]

Unit Definition
One unit of activity is defined according to the calculation formula below.

Solutions
I  Buffer solution; 300 mM Potassium phosphate buffer, pH 7.0
II 4-Aminoantipyrine (4-AA) solution; 24.6 mM (0.25 g 4-AA / 50 mL distilled water)
III Phenol solution; 420 mM (1.98 g phenol/50 mL distilled water)
IV Peroxidase \(^1\) (POD) solution; 240 U/mL (2,400 U/10 mL distilled water)

\(^1\)POD: TOYOBO Co., LTD. Grade III #PEO-302

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 15 to 60 U/mL with 10 mM potassium phosphate buffer, pH 7.0 containing 0.1 % BSA.

Procedure
1. Prepare the following reaction mixture and pipette 0.90 mL of reaction mixture into a cuvette.
   Solution I  4.00 mL
   Solution II 0.40 mL
   Solution III 0.40 mL
   Solution IV 0.40 mL
   H\(_2\)O 6.40 mL
2. Incubate at 37 °C for about 3 minutes.
3. Add 0.005 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 500 nm per minute (\(\Delta\text{Abs (test)}\)) in linear portion of curve. Repeat the procedure 3 using distilled water in place of enzyme solution, and \(\Delta\text{Abs (blank)}\) is obtained.

Calculation
Volume activity (U/mL) = \(\frac{(\Delta\text{Abs (test)} - \Delta\text{Abs (blank)}) X (0.90 + 0.005)}{11.11 X 0.005 X 1/20}\) X d.f.

Specific activity (U/mg protein) = \(\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}\) \(^2\)

d.f. ; dilution factor
11.11 ; millimolar extinction coefficient of quinoneimine dye at 500 nm (cm\(^2\)/μmol)
1/20 ; coefficient of transformation for internal unit definition
\(^2\)Protein concentration ; determined by Bradford’s method
Fig. 1 pH profile

- △ acetate, ○ phosphate
- ● Tris-HCl, ■ Glycine-KOH

Fig. 2 pH stability

treated for 24 hr at 4 °C in the following buffer solution (50 mM):
- △ acetate, ○ phosphate
- ● Tris-HCl, ■ Glycine-KOH

Fig. 3 Thermal activity

Fig. 4 Thermal stability

treated for 15 min in 20 mM potassium phosphate buffer, pH 7.0
**Fig. 5** Thermal stability
treated in 20 mM potassium phosphate buffer, pH 7.0
○ 50 °C, ● 55 °C, □ 60 °C, ■ 65 °C

**Fig. 6** pH profile (ABTS*)
**Fig. 7** pH profile (Bilirubin C*)
**Fig. 8** pH profile (Bilirubin F*)

Measured in 20 mM buffer.
△ Glycine-HCl, △ acetate, ○ phosphate, ● Tris-HCl, ■ Glycine-KOH

*3 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic Acid) Diammonium Salt
*4 Bilirubin C (conjugated type) and Bilirubin F (free type) are from [Interference Check, A Plus] (Sysmex, Kobe, Japan).
Table 1. Substrate specificity of BOD3

<table>
<thead>
<tr>
<th></th>
<th>Phenol</th>
<th>ABTS</th>
<th>Bilirubin C</th>
<th>Bilirubin F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>5.0</td>
<td>4.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Michaelis constants (μM)</td>
<td>41</td>
<td>39</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Relative activity (%)</td>
<td>100</td>
<td>427</td>
<td>36</td>
<td>8</td>
</tr>
<tr>
<td>Wavelength for</td>
<td>500</td>
<td>405</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>Measurement (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extinction Coefficient</td>
<td>11.11</td>
<td>29</td>
<td>74</td>
<td>32</td>
</tr>
<tr>
<td>(cm²/μmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Michaelis constants and activity of phenol were defined at pH 7.0. They were defined at each optimum pH when the substrate was ABTS, Bilirubin C, or Bilirubin F.
DIAPHORASE 3 (DI-3)

[EC 1. 6. 99. - ]

from recombinant *E. coli*

NAD(P)H + Acceptor(ox.) + H⁺ ↔ NAD(P)⁺ + Acceptor(red.)

**SPECIFICATION**

State: Lyophilized
Specific activity: more than 1,000 U/mg protein
Contaminants: (as Diaphorase activity = 100 %)
  - Adenylate kinase < 0.01 %
  - NADH oxidase < 0.01 %

**PROPERTIES**

- Subunit molecular weight: ca. 20,000 (SDS-electrophoresis)
- Optimum pH: 8.0 (Fig. 1)
- pH stability: 7.5 - 9.5 (Fig. 2)
- Isoelectric point: 4.7
- Thermal stability: No detectable decrease in activity up to 60 °C. (Fig. 3, 4)
- Michaelis constants: See Table 1

**STORAGE**

Stable at -20 to 5 °C for one year

**APPLICATION**

The enzyme is useful for the measurement of various dehydrogenase reactions in visible spectral range.
ASSAY

Principle
The change in absorbance is measured at 600 nm according to the following reaction.

\[
\text{NAD(P)} + \text{DCIP(ox.)} + H^+ \xrightarrow{\text{DI-3}} \text{NAD(P)^+} + \text{DCIP(red.)}
\]

Unit Definition
One unit of activity is defined as the amount of DI-3 that reduces 1 μmol of DCIP per minute at 30 °C.

Solutions
I. Buffer solution; 500 mM Tris-HCl, pH 8.5
II. NADH solution; 13.1 mM (0.100 g NADH disodium salt·3H₂O/10 mL distilled water)
III. 2,6-Dichlorophenolindophenol (DCIP) solution; 1.2 mM (2.0 mg DCIP sodium salt·2H₂O/5 mL distilled water) (prepare freshly)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 1.0 to 2.0 U/mL with 50 mM potassium phosphate buffer, pH 7.5.

Procedure
1. Prepare the following reaction mixture and pipette 2.28 mL of reaction mixture and 0.12 mL of Solution III into a cuvette.
   Solution I 3.00 mL
   Solution II 2.28 mL
   H₂O 23.22 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.008 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 600 nm per minute (\(\Delta\text{Abs(test)}\)) in linear portion of curve. Repeat the Procedure 3 using distilled water in place of enzyme solution, and \(\Delta\text{Abs(blank)}\) is obtained.

Calculation
Volume activity (U/mL) = \[
\frac{(\Delta\text{Abs(test)} - \Delta\text{Abs(blank)}) \times (2.40 + 0.008)}{19 \times 0.008}
\] X d.f.

Specific activity (U/mg protein) = \[
\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]
d.f. ; dilution factor
19 ; millimolar extinction coefficient of DCIP (cm²/μmol)
*Protein concentration ; determined by Bradford's method

REFERENCE
Fig. 1  pH profile
- ○ phosphate,
- ■ Gly-KCl-KOH,
- ● Tris-HCl

Fig. 2  pH stability
- ○ phosphate,
- ● Tris-HCl

Fig. 3  Thermal stability
- treated for 15 min in 0.1 M potassium phosphate buffer, pH 7.5
- △ acetate,
- □ 50 °C,
- ● 60 °C,
- ● 70 °C

Fig. 4  Thermal stability
- treated in 0.1 M potassium phosphate buffer, pH 7.5
- ○ 50 °C,
- □ 60 °C,
- ● 70 °C
### Table 1. SUBSTRATE SPECIFICITY OF DIAPHORASE

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>DCIP $^1$</th>
<th>NTB $^2$</th>
<th>MTT $^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km $^{\text{Acceptor}}$ (mM)</td>
<td>0.02</td>
<td>0.15</td>
<td>0.9</td>
</tr>
<tr>
<td>Km $^{\text{NADH}}$ (mM)</td>
<td>0.37</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Km $^{\text{NADPH}}$ (mM)</td>
<td>32.7</td>
<td>0.31</td>
<td>2.0</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>8.0</td>
<td>10</td>
<td>8.0</td>
</tr>
</tbody>
</table>

| Assay Mixture | Tris-HCl (pH 8.5) 50 mM NAD(P)H 1 mM DCIP 0.06 mM | TEA (pH 7.0) 50 mM NAD(P)H 1 mM NBT 0.5 mM Triton X-100 0.1% | TEA (pH 7.0) 50 mM NAD(P)H 1 mM MTT 0.5 mM Triton X-100 0.5% |

| Wavelength for Measurement (nm) | 600 | 550 | 565 |
| Extinction Coefficient (cm$^2$/μmol) | 19 | 12.4 | 20 |

$^1$ 2,6-Dichlorophenolindophenol  
$^2$ Nitrotetrazolium Blue  
$^3$ Thiazolyl Blue Tetrazolium Bromide

**pH profiles of DI-3 (Acceptor; NTB or MTT)**

![pH profile (NTB)](image1.png)  
![pH profile (MTT)](image2.png)
DIAPHORASE 22 (Di-22)  
[EC 1. 8. 1. 4]  

from recombinant *E.coli*  

NADH + Acceptor(ox.) + H⁺ ↔ NAD⁺ + Acceptor(red.)

**SPECIFICATION**

- **State**: Lyophilized  
- **Specific activity**: more than 150 U/mg protein  
- **Contaminants**: (as Diaphorase activity = 100 %)  
  - Adenylate kinase: < 0.01 %  
  - NADH oxidase: < 0.20 %

**PROPERTIES**

- **Molecular weight**: ca. 110,000  
- **Subunit molecular weight**: ca. 50,000  
- **Optimum pH**: 8.0 
- **pH stability**: 6.0 – 9.0 (Fig.1)  
- **Thermal stability**: No detectable decrease in activity up to 70 °C. (Fig. 3, 4)  
- **Michaelis constants**
  - (50 mM HEPES buffer, pH 7.0, at 30 °C)
  - 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) 0.345 mM  
  - NADH 0.033 mM (Table 1)  
- **Substrate specificity**:  
  - NADH 100 %  
  - NADPH 1 %  
  - MTT 100 %  
  - Lipoate 103 % (Table 1)

**STORAGE**

Store at -20°C

**APPLICATION**

The enzyme is useful for measurement of various dehydrogenase reactions in the visible spectral range.
ASSAY

Principle
The change in absorbance is measured at 565 nm according to the following reaction.

NADH + MTT(ox.) + H+ \rightarrow NAD+ + MTT(red.)

Unit Definition
One unit of activity is defined as the amount of Diaphorase that forms 1 μmol of NAD⁺ per minute at 30 °C.

Solutions
I. Buffer solution; 100 mM HEPES, pH 7.0
II. 3-(4,5-Dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution; 10 mM (20 mg MTT disodium salt·2H₂O/5 mL distilled water)
III. NADH solution; 13.1 mM (0.100g NADH disodium salt·3H₂O /10 mL distilled water)
IV. Triton solution; 10 % (1 mL TritonX-100 dilute with distilled water up to10 mL)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 1 to 5 U/mL with 50 mM potassium phosphate buffer, pH 7.5, 1mg/mL BSA.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I 15.00 mL
   Solution IV 1.50mL
   Solution II 1.50mL
   Solution III 1.20mL
   H₂O 10.80mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 565 nm per minute (∆Abs₅₆₅) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \frac{(\Delta Abs₅₆₅) \times (3.00 + 0.01)}{20.0 \times 0.01} \times \text{d.f.}

Specific activity (U/mg protein) = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}

\text{d.f.} ; \text{dilution factor}
20.0 ; \text{millimolar extinction coefficient of MTT (cm}^2/\text{μmol)}
*Protein concentration ; determined by Bradford's method

REFERENCE
<table>
<thead>
<tr>
<th>Acceptor</th>
<th>MTT</th>
<th>Lipoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Km}^{\text{Acceptor}} ) (mM)</td>
<td>0.345</td>
<td>2.0</td>
</tr>
<tr>
<td>( \text{Km}^{\text{NADH}} ) (mM)</td>
<td>0.033</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Relative Activity

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th>MTT</th>
<th>Lipoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (pH 7.0) 50 mM</td>
<td>Potassium Phosphate (pH 6.5) 70.5 mM</td>
<td></td>
</tr>
<tr>
<td>NADH 0.5 mM</td>
<td>NADH 0.2 mM</td>
<td></td>
</tr>
<tr>
<td>MTT 0.5 mM</td>
<td>NAD 0.3 mM</td>
<td></td>
</tr>
<tr>
<td>Triton X-100 0.5 %</td>
<td>Lipoate 10.2 mM</td>
<td></td>
</tr>
<tr>
<td>BSA 0.7mg/mL</td>
<td>EDTA 0.81 mM</td>
<td></td>
</tr>
</tbody>
</table>

| Wavelength for Measurement (nm) | 565 | 340 |
| Extinction coefficient (cm\(^2\)/μmol) | 20 | 6.22 |
**Fig. 1** pH profile

Fig. 1 shows the pH profile of the enzyme. The x-axis represents pH values ranging from 4 to 12, and the y-axis represents relative activity (%) ranging from 0 to 200. The graph indicates the enzyme's activity across different pH values.

**Fig. 2** pH stability

Fig. 2 illustrates the pH stability of the enzyme. The x-axis represents pH values ranging from 4 to 12, and the y-axis represents remaining activity (%) ranging from 0 to 100. The graph shows the percentage of remaining activity at different pH levels.

**Fig. 3** Thermal stability

Fig. 3 displays the thermal stability of the enzyme. The x-axis represents temperature (°C) ranging from 20 to 90, and the y-axis represents relative activity (%) ranging from 0 to 100. The graph demonstrates the enzyme's activity at various temperatures.

**Fig. 4** Thermal stability

Fig. 4 presents the thermal stability of the enzyme. The x-axis represents time (min) ranging from 0 to 60, and the y-axis represents remaining activity (%) ranging from 0 to 100. The graph shows the percentage of remaining activity over time.

- **treated for 24 hr at 4°C in the following buffer solution (0.1 M),**:
  - △ Gly-KOH,
  - ○ Bicine,
  - ● phosphate,
  - ▲ HEPES,
  - □ MES,
  - ■ Citrate

- **treated for 15 min in 0.1M potassium phosphate buffer, pH 7.5**
GALACTOSE DEHYDROGENASE (GalDH)

[EC 1.1.1.48]

from recombinant E. coli

D-Galactose + NAD(P)^+ ↔ D-Galactono-δ-lactone + NAD(P)H + H^+

SPECIFICATION

State : Ammonium sulphate suspension
Specific activity : more than 80 U/mg protein
Contaminants : (as GalDH activity = 100 %)
  NADH oxidase < 0.10 %
  LDH < 0.10 %
  ADH < 0.01 %

PROPERTIES

Subunit molecular weight : ca. 33,800
Optimum pH : 10.5 (Fig. 1)
pH stability : 5.0 - 10.0 (Fig. 2)
Thermal stability : No significant decrease in activity up to 50 °C with Ammonium sulphate and 40 °C without Ammonium sulphate. (Fig. 3, 4)
Michaelis constants : D-Galactose 0.25 mM
  NAD^+ 0.15 mM
Substrate specificity (100mM) : D-Galactose 100 %
  D-Glucose 0.2 %
  D-Xylose 8.7 %
  D-Maltose 0.1 %
  D-Sucrose 0.1 %

STORAGE

Store at 4 to 10 °C (Do not freeze)
Stable at 4 °C for at least one year

APPLICATION

This enzyme is useful for determination of galactose.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{D-Galactose + NAD}^+ \xrightarrow{\text{GalDH}} \text{D-Galactono-\(\delta\)-lactone + NADH + H}^+
\]

Unit Definition
One unit of activity is defined as the amount of GalDH that forms 1 \(\mu\)mol of NADH per minute at 30 \(^\circ\)C.

Solutions
I. Buffer solution ; 100 mM Tris-HCl, pH9.1 (at 30\(^\circ\)C)
II. NAD\(^+\) solution ; 100 mM
III. D-Galactose solution ; 1 M
IV. Enzyme diluent ; 20 mM potassium phosphate, 0.1% bovine serum albumin, pH7.5

Preparation of Enzyme Solution
Dilute the enzyme suspension to approx. 5 U/mL with the enzyme diluent.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I 27.60mL
   Solution II 0.90mL
   Solution III 1.50mL
2. Incubate at 30 \(^\circ\)C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (\(\Delta\text{Abs}_{340}\)) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \(\frac{(\Delta\text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01}\) x d.f.

Specific activity (U/mg protein) = \(\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}\)

\text{d.f.} \quad \text{; dilution factor}
\text{6.22 ; millimolar extinction coefficient of NADH (cm}^2/\mu\text{mol)}
\text{*Protein concentration ; determined by the Bradford's method}
Fig. 1  pH profile

\[
\begin{align*}
\text{△ acetate, ○ phosphate,} & \\
\mathbf{●} \text{ Tris-HCl, △ Glycine-KOH}
\end{align*}
\]

Fig. 2  pH stability

treated for 24 hr at 4 °C in the following buffer solution (0.1 M): △ acetate, ○ phosphate, ● Tris-HCl, △ Glycine-KOH

Fig. 3  Thermal stability

treated for 15 min in 25 mM potassium phosphate buffer pH 7.5, with or without 3.2 M ammonium sulphate (AmS).

Fig. 4  Thermal stability

treated in 25 mM potassium phosphate buffer pH 7.5 at ○ 40 °C, □ 50 °C, ● 60 °C without ammonium sulphate.
Fig. 5  Storage Stability

ammonium suiphate suspension (ca. 1300U/mL)
store at 4 °C (○) or 10 °C (□).
GLUCOKINASE 2 (GlcK2)

[EC 2. 7. 1. 2]

from Recombinant E.coli

ATP + D-Glucose ↔ ADP + D-Glucose 6-phosphate

SPECIFICATION

State: Lyophilized
Specific activity: more than 350 U/mg protein
Contaminants: (as GlcK2 activity = 100 %)
  - Glucose-6-phosphate dehydrogenase < 0.01 %
  - Phosphoglucomutase < 0.01 %
  - 6-Phosphogluconate dehydrogenase < 0.01 %
  - Hexose-6-phosphate isomerase < 0.01 %
  - Glutathione reductase < 0.01 %

PROPERTIES

Subunit molecular weight: ca. 32,000
Optimum pH: 9.0 (Fig. 1)
pH stability: 7.0 - 10.0 (Fig. 2)
Optimum temperature: 70 °C (Fig. 3, 4)
Thermal stability: No detectable decrease in activity up to 60 °C.
Michaelis constants: (60mM Tris-HCl buffer, pH 8.5, at 30 °C)
  - Glucose 0.1 mM
  - ATP 0.05 mM
Substrate specificity: D-Glucose 100 %
  - D-Mannose 20 %
  - D-Fructose 0 %

STORAGE

Stable at -20°C for at least one year

APPLICATION

The enzyme is useful for diagnostic reagent, for example, glucose determination or CK determination, and for the specific determination of glucose.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reactions.

\[
\text{ATP} + \text{Glucose} \xrightarrow{\text{GlcK2}} \text{ADP} + \text{Glucose 6-phosphate} \\
\text{Glucose 6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{Gluconolactone 6-phosphate} + \text{NADPH} + \text{H}^+
\]

Unit Definition
One unit of activity is defined as the amount of GlcK2 that forms 1 μmol of glucose 6-phosphate per minute at 30 °C.

Solutions
1. Buffer solution; 100 mM Tris-HCl, pH 9.0
2. ATP solution; 100 mM (0.605 g ATP disodium salt·3H₂O/(8.2 mL distilled water + 1.8 mL 1 N-NaOH))
3. MgCl₂ solution; 1 M (20.33 g MgCl₂·6H₂O/100 mL distilled water)
4. NADP⁺ solution; 22.5 mM [(0.172 g NADP+ monosodium salt or 0.177 g NADP+ disodium salt)/10 mL distilled water]
5. Glucose solution; 40 mM (0.072 g glucose (anhyd.)/10 mL distilled water)
6. Glucose-6-phosphate dehydrogenase (G6PDH); (from yeast. Roche Diagnostics K.K., No. 127 671) suspension in 3.2 M (NH₄)₂SO₄ solution (10 mg/2 mL) approx. 140 U/mg at 25 °C

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50 mM Tris-HCl buffer, pH 8.5.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  17.97 mL
   Solution II 1.20 mL
   Solution III 0.60 mL
   Solution IV 1.20 mL
   Solution V 9.00 mL
   Solution VI 0.03 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \( \frac{(ΔAbs₃₄₀) \times (3.00 + 0.01)}{6.22 \times 0.01} \) X d.f.

Specific activity (U/mg protein) = \( \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}} \)

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADPH (cm²/μmol)
*Protein concentration; determined by Bradford's method

REFERENCE
3. Tomita, K., Kamei, S., Nagata, K., Okuno, H., Shiraishi, T., Motoyama, A., Ohkubo, A., and
Yamanaka, M.; *ibid.*, 3, 11 (1987)
**Fig. 1  pH profile**

- △ acetate, ○ phosphate, ● Tris-HCl, ▲ carbonate

**Fig. 2  pH stability**

- treated for 24 hr at 4°C in the following buffer solution (0.1 M), : △ acetate, ○ phosphate, ● Tris-HCl, ▲ carbonate

**Fig. 3  Thermal stability**

- treated for 15 min. in 0.1 M Tris-HCl buffer, pH 8.9

**Fig. 4  Thermal stability**

- treated in 0.1 M Tris-HCl buffer, pH 8.9
  - ○ 60°C, □ 70°C, ● 80°C
Fig. 5  Thermal activity

defined as 100% at 70 °C
GLUCOSE DEHYDROGENASE (GlcDH2)

[EC 1.1.1.47]

from recombinant E. coli

D-Glucose + NAD(P)^+ ↔ D-Glucono-δ-lactone + NAD(P)H + H^+

SPECIFICATION

State: Lyophilized
Specific activity: more than 900 U/mg protein
Contaminants: (as GlcDH2 activity = 100 %)
NADH oxidase < 0.01 %

PROPERTIES

Molecular weight: ca. 126,000
Subunit molecular weight: ca. 31,500
Optimum pH: 8.5 (Fig. 1)
pH stability: 5.0 - 10.0 (with 3M NaCl) (Fig. 2)
Thermal stability: No significant decrease in activity up to 70 °C.
(with 3M NaCl and 0.1% BSA) (Fig. 3, 4)
Michaelis constants:
- D-Glucose: 3.7 mM
- NAD^+: 0.06 mM
- NADP^+: 0.02 mM
Substrate specificity (100 mM):
- D-Glucose: 100 %
- D-Maltose: 1.1 %
- D-Galactose: 0.1 %
- D-Xylose: 3.0 %
- D-Fructose: 0.3 %
- D-Mannose: 4.8 %
- D-Arabinose: 0 %
- Trehalose: 0 %
- D-Lactose: 1.3 %
- D-Sucrose: 0 %
- 2-Deoxy-D-Glucose: 100 %
- D-Glucose-1-Phosphate: 0 %
- D-Glucose-6-Phosphate: 0 %
- D-Sorbitol: 0 %

STORAGE

Stable at -20 °C for at least one year

APPLICATION

This enzyme is useful for determination of glucose.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{D-Glucose} + \text{NAD}^{+} \xrightarrow{\text{GlcDH2}} \text{D-Glucono-\delta-lactone} + \text{NADH} + \text{H}^{+}
\]

Unit Definition
One unit of activity is defined as the amount of GlcDH2 that forms 1 μmol of NADH per minute at 37 °C.

Solutions
- **I** Buffer solution : 100 mM Tris-HCl, pH8.5 (at 25°C)
- **II** NAD⁺ solution : 100 mM (0.663 g NAD⁺ free acid/10 mL distilled water)
- **III** D-Glucose solution : 1 M (1.802 g glucose (anhyd.)/10 mL distilled water)
- **IV** NaCl solution : 5 M (2.92 g NaCl/10 mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 15 U/ml with 20 mM potassium phosphate buffer containing 1mg/mL BSA and 2 M NaCl, pH 6.5.

Procedure
1. Prepare the following reaction mixture and pipette 2.70 mL of reaction mixture into a cuvette.
   - Solution I : 17.22mL
   - Solution II : 0.50mL
   - Solution III : 2.00mL
   - Solution IV : 0.28mL
2. Incubate at 37 °C for about 3 minutes.
3. Add 0.015 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (\(\Delta\text{Abs}_{340}\)) in the linear portion of curve.

Calculation
\[
\text{Volume activity (U/mL)} = \left(\frac{\Delta\text{Abs}_{340} \times (2.70 + 0.015)}{6.22 \times 0.015}\right) \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

\(\text{d.f.} \); dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm²/μmol)
*Protein concentration ; determined by the absorbance at 280nm (Abs280), where 1 Abs280 = 1 mg/mL

REFERENCE
Fig. 1  pH profile

Fig. 2  pH stability

Fig. 3  Thermal stability

Fig. 4  Thermal stability

*O* phosphate,  ■ Tris-HCl,  ▲ glycine

*O* phosphate,  ● Tris-HCl,  ▲ glycine

treated for 24 hr at 4°C in the following buffer solution (0.1 M) containing 3M NaCl: △ acetate,  ● phosphate,  ▲ Tris-HCl,  ▲ glycine

treated for 15 min in 0.1M phosphate buffer, pH 6.5, containing 3M NaCl and 0.1% BSA

treated for in 0.1M phosphate buffer, pH 6.5, containing 3M NaCl and 0.1% BSA,  ○ 60°C,  □ 70°C,  ● 80°C
D-LACTATE DEHYDROGENASE (D-LDH)

[EC 1.1.1.28]

from *Microorganism*

D-Lactate + NAD⁺ ↔ Pyruvate + NADH + H⁺

*FOR PYRUVATE → LACTATE REACTION*

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 2,500 U/mg protein</td>
</tr>
<tr>
<td>Contaminants</td>
<td>(as D-LDH activity = 100 %)</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>GOT</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>GPT</td>
<td>&lt; 0.01 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>ca. 80,000</th>
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<tbody>
<tr>
<td>Subunit molecular weight</td>
<td>ca. 40,000</td>
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<tr>
<td>Optimum pH</td>
<td>7.5</td>
</tr>
<tr>
<td>pH stability</td>
<td>5.5 - 10.0</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.1</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>No detectable decrease in activity up to 40 °C.</td>
</tr>
<tr>
<td>Michaelis constants</td>
<td>(94 mM Potassium phosphate buffer, pH 7.5, at 30 °C)</td>
</tr>
<tr>
<td></td>
<td>Pyruvate 0.80 mM</td>
</tr>
<tr>
<td></td>
<td>NADH 0.18 mM</td>
</tr>
<tr>
<td>Stabilizers</td>
<td>(NH₄)₂ SO₄, BSA</td>
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<tr>
<td>Inhibitors</td>
<td>Zn²⁺, Cu²⁺</td>
</tr>
</tbody>
</table>

**STORAGE**

Stable at -20 °C at least one year
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{Pyruvate + NADH + H}^+ \xrightarrow{D-LDH} \text{D-Lactate + NAD}^+
\]

Unit Definition
One unit is defined as the amount of D-LDH that forms 1 μmol of NAD\(^+\) per minute at 30 °C.

Solutions
I  Buffer solution; 100 mM Potassium phosphate buffer, pH 7.5
II  Sodium pyruvate solution; 100 mM (100 mg sodium pyruvate/10 mL distilled water)
III  NADH solution; 13.1 mM (0.100 g NADH disodium salt·3H\(_2\)O/10 mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 3 to 5 U/mL with 50 mM potassium phosphate buffer containing 1 mg/mL BSA, pH 7.0.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  28.00mL
   Solution II  1.20mL
   Solution III  0.80mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs\(_{340}\)) in the linear portion of curve.

Calculation
\[
\text{Volume activity (U/mL)} = \frac{(\Delta\text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

Specific activity (U/mg protein) = \[
\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)*}}
\]

d.f.  ; dilution factor
6.22  ; millimolar extinction coefficient of NADH (cm\(^2\)/μmol)
*Protein concentration ; determined by Bradford’s method
Fig. 1  pH profile

- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 2  pH stability

- treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
- △ acetate,
- ○ phosphate,
- ● Tris-HCl,
- ■ Gly-KOH

Fig. 3  Thermal stability

- treated for 15 min in 0.1 M potassium phosphate buffer, pH 7.0

Fig. 4  Thermal stability

- treated in 0.1 M potassium phosphate buffer, pH 7.0
- ○ 40 °C, □ 45 °C, ● 50 °C
MALATE DEHYDROGENASE (MDH)

[EC 1. 1. 1. 37]

from Microorganism

L-Malate$^+$ NAD$^+$ $\leftrightarrow$ Oxaloacetate $+$ NADH $+$ H$^+$

FOR OXALATE $\rightarrow$ MALATE REACTION

SPECIFICATION

State : Lyophilized
Specific activity : more than 1,200 U/mg protein
Contaminants : (as MDH activity = 100 %)
  GOT < 0.01 %
  GPT < 0.01 %
  NADHoxidase < 0.01 %
  Glutamate dehydrogenase < 0.01 %
  Fumarase < 0.01 %

PROPERTIES

Molecular weight : ca. 72,000
Subunit molecular weight : ca. 36,000
Optimum pH : 9.0 (Fig. 1)
pH stability : 5.5 - 11.0 (Fig. 2)
Thermal stability : No detectable decrease in activity up to 50 °C. (Fig. 3, 4)
Michaelis constants : (90mM Tris-HCl buffer, pH 9.0, at 30 °C)
  Oxaloacetate 0.027 mM
  NADH 0.014 mM

STORAGE

Stable at -20 °C for at least six months

APPLICATION

This enzyme is useful for enzymatic determination of L- malate and of glutamate oxaloacetate transaminase in clinical analysis.
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}^+ 
\]

Unit Definition
One unit of activity is defined as the amount of MDH that forms 1 μmol of NAD\(^+\) per minute at 30 °C.

Solutions
I. Buffer solution ; 200 mM Tris-HCl, pH 9.0
II. Oxaloacetate solution ; 15 mM (0.020 g oxaloacetate/10 mL distilled water)
III. NADH solution ; 13.1 mM (0.100 g NADH disodium salt·3H\(_2\)O/10 mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 3 to 5 U/mL with 100 mM Tris-HCl buffer, pH 9.0.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   - Solution I 13.50 mL
   - Solution II 1.00 mL
   - Solution III 0.57 mL
   - H\(_2\)O 14.93 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs\(_{340}\)) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \(\frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01}\) X d.f.

Specific activity (U/mg protein) = \(\frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}\)

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NAD (cm\(^2\)/μmol)
*Protein concentration ; determined by Bradford’s method
Fig. 1  pH profile
\[ \triangle \text{acetate, } \circ \text{phosphate, } \bullet \text{Tris-HCl, } \blacksquare \text{Gly-KOH} \]

Fig. 2  pH stability
\[ \triangle \text{acetate, } \circ \text{phosphate, } \bullet \text{Tris-HCl, } \blacksquare \text{Gly-KOH} \]

Fig. 3  Thermal stability
treated for 15 min in 0.1 M Tris-HCl buffer, pH 9.0
\[ \circ 50 ^\circ \text{C, } \blacksquare 55 ^\circ \text{C, } \bullet 60 ^\circ \text{C} \]

Fig. 4  Thermal stability
treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
\[ \triangle \text{acetate, } \circ \text{phosphate, } \bullet \text{Tris-HCl, } \blacksquare \text{Gly-KOH} \]
MUTAROTASE (MRO)

[EC 5.1.3.3]

from Microorganism

α-D-glucose ↔ β-D-glucose

SPECIFICATION

State : Lyophilized
Specific activity : more than 120 U/mg protein
Contaminants : (as MRO activity = 100 %)
NADH oxidase : < 0.01 %

PROPERTIES

Subunit molecular weight : ca. 39,500
Optimum pH : 7.0 - 9.0 (Fig. 1)

pH stability : 3.5 - 10.0 (Fig. 2)
Thermal stability : No detectable decrease in activity up to 50 °C. (Fig. 3, 4)

STORAGE

Stable at -20 °C for at least one year

APPLICATION

This enzyme is useful for enzymatic determination of glucose.
ASSAY

Principle
Acceleration of the glucose dehydrogenase reaction by Mutarotase is measured according to the following reactions.

\[
\begin{align*}
\text{sucrose} & \rightarrow \beta-\text{Fructosidase} \rightarrow \alpha-\text{glucose} + \text{fructose} \\
\alpha-\text{glucose} & \rightarrow \text{MRO} \rightarrow \beta-\text{glucose} \\
\beta-\text{glucose} + \text{NAD}^+ & \rightarrow \text{GlucoseDH} \rightarrow \text{glucono-lactone + NADH + H}^+
\end{align*}
\]

Unit Definition
One unit of activity is defined as the amount of Mutarotase that forms 10μmol of NADH per minute at 25 °C.

Solutions

I  HEPES buffer; 50 mM (1.19 g HEPES / 100 mL distilled water, adjust pH to 7.5 with NaOH)
II Sucrose solution; 16.7 mM (57 mg Sucrose / 10 mL distilled water)
III NAD\(^+\) solution; 100 mM (0.663 g NAD\(^+\) free acid / 10 mL distilled water)
IV Glucose dehydrogenase solution; 3 kU/mL (GlcDH2, Nipro Corp. / 20 mM potassium phosphate containing 2M NaCl, pH6.5)
V  \(\beta\)-Fructosidase solution; \(\geq\)30 kU/mL (100 mg Invertase from baker’s yeast, Sigma-Aldrich I4504 / 1 mL distilled water)

Preparation of Enzyme Solution
Dissolve the lyophilized enzyme with distilled water and dilute to 0.7 to 1.4 U/mL with the enzyme diluent (20 mM potassium phosphate pH7.3 containing 1mg/mL BSA).

Procedure
1. Prepare the following reaction mixture and pipette 2.70 mL of reaction mixture into a cuvette.
   - Solution I 19.90mL
   - Solution II 1.00mL
   - Solution III 0.60mL
2. Add 0.015 mL of the enzyme solution into the cuvette and mix.
3. Incubate at 25 °C for about 3 minutes.
4. Add 0.06 mL of the Solution V into the cuvette and mix.
5. Read absorbance change at 340nm per minute (\(\Delta\text{Abs1}\)) in the linear portion of curve.
6. Run the procedure 1 to 5 with the enzyme diluent instead of the enzyme solution (\(\Delta\text{Abs2}\)).

Calculation
\[
\text{Volume activity (U/mL)} = \frac{(\Delta\text{Abs1} - \Delta\text{Abs2}) \times (2.70 + 0.015 + 0.060)}{6.22 \times 0.015 \times 10} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm\(^2\)/μmol)
10 ; conversion factor
*Protein concentration ; determined by the absorbance at 280nm (Abs280), where 1 Abs280 = 1 mg/mL
至適pH

残存活性(%)
P H
 acetate MES PIPES HEPES Tris-HCl Bicine Glycine-KOH Buffer

Fig. 1 pH profile

Fig. 2 pH stability

Fig. 3 Thermal stability

Fig. 4 Thermal stability

acetate, MES, PIPES, HEPES, Tris-HCl, Bicine, Glycine-KOH

treated for 24 hr at 4 °C in the following buffer solution (0.1 M) containing 0.1 % BSA;
acetate, phosphate, Tris-HCl, Glycine-KOH

treated for 15 min in 0.1 M potassium phosphate buffer pH 6.5, 0.1 % BSA at 40 °C, 50 °C, 60 °C.
PHENYLALANINE DEHYDROGENASE (PheDH)

[EC 1.4.1.20]

from *Thermoactinomyces intermedius*

L-Phenylalanine + NAD$^+$ + H$_2$O ↔ Phenylpyruvate + NH$_4^+$ + NADH

**SPECIFICATION**

<table>
<thead>
<tr>
<th>State</th>
<th>Ammonium sulphate suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>more than 30 U/mg protein</td>
</tr>
<tr>
<td>Contaminants (as PheDH activity = 100 %)</td>
<td></td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>&lt; 0.01 %</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>&lt; 0.01 %</td>
</tr>
</tbody>
</table>

**PROPERTIES**

| Molecular weight | ca. 380,000 |
| Subunit molecular weight | ca. 40,000 |
| Optimum pH       | 11.5        |
| pH stability     | 5.0 - 10.0  |
| Thermal stability| No detectable decrease in activity up to 50 °C, |
| Michaelis constants | (200 mM Gly-KCl-KOH buffer, pH 11.0, at 30 °C) |
| L-Phenylalanine   | 0.66 mM     |
| NAD$^+$           | 0.05 mM     |

**Substrate specificity**

| L-Phenylalanine | 100 % |
| L-Tyrosine      | 7.6 % |
| L-Methionine    | 1.5 % |

**STORAGE**

Stable at 0 to 4 °C for at least six months (Do not freeze)
ASSAY

Principle
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{L-Phenylalanine} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{PheDH}} \text{Phenylpyruvate} + \text{NH}_4^+ + \text{NADH}
\]

Unit Definition
One unit of activity is defined as the amount of PheDH that forms 1 μmol of NADH per minute at 30 °C.

Solutions
I  Buffer solution ; 400 mM Gly-KCl-KOH, pH 11.0
II L-Phenylalanine solution ; 100 mM (0.165 g L-phenylalanine/10 mL distilled water)
III NAD⁺ solution ; 100 mM (0.663 g NAD⁺ free acid/10 mL distilled water)

Preparation of Enzyme Solution
Dilute the ammonium sulphate suspension of enzyme to 2 to 6 U/mL with 10 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl.

Procedure
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   Solution I  15.00 mL
   Solution II 3.00 mL
   Solution III 0.15 mL
   H₂O 11.85 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340nm per minute (ΔAbs₃₄₀) in the linear portion of curve.

Calculation
Volume activity (U/mL) = \frac{(ΔAbs₃₄₀) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}

Specific activity (U/mg protein) = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm²/μmol)
*Protein concentration ; determined by Bradford's method

REFERENCE
Fig. 1  pH profile

- ■ Gly-KCl-KOH

- ○ phosphate, △ acetate,

Fig. 2  pH stability

- Gly-KCl-KOH treated for 15 min in 10 mM potassium phosphate buffer, pH 7.2

- ○ 50 °C, □ 60 °C, ● 70 °C

Fig. 3  Thermal stability

- △ acetate, ○ phosphate,

Fig. 4  Thermal stability

- Gly-KCl-KOH treated for 24 hr at 4 °C in the following buffer solution (50 mM):

- ◼ treated in 10 mM potassium phosphate buffer, pH 7.2

- ○ 50 °C, □ 60 °C, ● 70 °C
6-PHOSPHOGLUCONATE DEHYDROGENASE (DECARBOXYLATING) (6PGDH)

[EC 1. 1. 1. 44]

from *Microorganism*

6-Phospho-D-gluconate + NAD$^+$ ↔ D-Ribulose 5-phosphate + CO$_2$ + NADH + H$^+$

**SPECIFICATION**

State : Lyophilized
Specific activity : more than 40 U/mg protein
Contaminants : (as 6PGDH activity = 100 %)
  - Glucokinase  < 0.01 %
  - Phosphoglucomutase  < 0.01 %
  - Hexose-6-phosphate isomerase  < 0.01 %
  - Glutathione reductase  < 0.01 %

**PROPERTIES**

Molecular weight  : ca. 132,000
Subunit molecular weight  : ca. 33,000
Optimum pH  : 7.0 - 7.5  (Fig. 1)
pH stability  : 5.0 - 10.0  (Fig. 2)
Isoelectric point  : ca. 4.5
Thermal stability  : (50 mM MES-NaOH buffer, pH 6.8, containing 0.5 M KCl)
  - No detectable decrease in activity up to 40 °C.  (Fig. 3, 4)
Michaelis constants  : (80 mM Glycylglycine buffer, pH 7.5, at 30 °C)
  - 6-Phospho-D-gluconate  0.95 mM
  - NAD$^+$  0.32 mM
Stabilizer  : KCl, MgCl$_2$, Sorbitol, BSA
Activators  : Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, K$^+$, Na$^+$
Inhibitors  : Fructose 1,6-bisphosphate, Erythrose 4-phosphate, NADH

**STORAGE**

Stable at -20 °C for at least six months
**ASSAY**

**Principle**

The change in absorbance is measured at 340 nm according to the following reaction.

\[
6\text{-Phosphogluconate} + \text{NAD}^+ \xrightarrow{6\text{PGDH}} \text{Ribulose 5-phosphate} + \text{CO}_2 + \text{NADH} + \text{H}^+
\]

**Unit Definition**

One unit of activity is defined as the amount of 6PGDH that forms 1 μmol of NADH per minute at 30 °C.

**Solutions**

I. Buffer solution; 100 mM Glycylglycine-NaOH, pH 7.5
II. 6-Phospho-D-gluconate (6PG) solution; 100 mM (0.378 g 6PG trisodium salt·2H_2O/10 mL distilled water)
III. NAD\(^+\) solution; 50 mM (0.332 g NAD\(^+\) free acid/10 mL distilled water)
IV. MgCl\(_2\) solution; 1 M (20.33 g MgCl\(_2\)·6H_2O/100 mL distilled water)

**Preparation of Enzyme Solution**

Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 100 mM MES-NaOH buffer containing 1 mg/mL BSA, pH 6.8.

**Procedure**

1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   
<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24.6</td>
</tr>
<tr>
<td>II</td>
<td>3.0</td>
</tr>
<tr>
<td>III</td>
<td>2.1</td>
</tr>
<tr>
<td>IV</td>
<td>0.3</td>
</tr>
</tbody>
</table>

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs\(_{340}\)) in the linear portion of curve.

**Calculation**

\[
\text{Volume activity (U/mL)} = \frac{(\Delta\text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm\(^2\)/μmol)

*Protein concentration ; determined by Bradford’s method
Fig. 1  pH profile
△ acetate,  ○ phosphate, □ TEA-NaOH, ▲ GlyGly-NaOH, ● Tris-HCl

Fig. 2  pH stability
treated for 15 min in 50 mM MES-NaOH buffer, pH 6.8, containing 0.5 M KCl

Fig. 3  Thermal stability
treated for 24 hr at 4 °C in the following buffer solution (0.1 M):
△ acetate, ▲ GlyGly-NaOH, ● Tris-HCl, ■ Gly-KOH

Fig. 4  Thermal stability
treated in 50 mM MES-NaOH buffer, pH 6.8, containing 0.5 M KCl
○ 40 °C, □ 50 °C, ● 60 °C
SORBITOL DEHYDROGENASE (SorDH)

[EC 1.1.1.14]

from Microorganism

D-Sorbitol + NAD⁺ ↔ D-Fructose + NADH + H⁺

SPECIFICATION
State: Lyophilized
Specific activity: more than 30 U/mg protein
Contaminants: (as SorDH activity = 100 %)
   NADH oxidase <0.01 %

PROPERTIES
Molecular weight: ca. 68,000
Subunit molecular weight: ca. 26,000
Optimum pH: 11.0 (Fig. 1)
pH stability: 6.0 - 10.0 (Fig. 2)
Optimum temperature: 40 °C
Thermal stability: No detectable decrease in activity up to 35 °C. (Fig. 3, 4)
Michaelis constants: (100 mM Tris-HCl buffer, pH 9.0, at 30°C)
   D-Sorbitol 3.4 mM
   NAD⁺ 0.13 mM
Substrate specificity: D-Sorbitol 100 %
   Galactitol 27 %
   L-Iditol 42 %
   Xylitol 1 %
   D-Arabitol 0 %
   D-Mannitol 0 %
   D-Glucose 0 %
   D-Galactose 0 %
   Maltose 0 %

STORAGE
Stable at -20 °C for at least one year

APPLICATION
This enzyme is useful for determination of D-Sorbitol in clinical analysis and food analysis.
**ASSAY**

**Principle**
The change in absorbance is measured at 340 nm according to the following reaction.

\[
\text{D-Sorbitol} + \text{NAD}^+ \xrightarrow{\text{SorDH}} \text{D-Fructose} + \text{NADH} + \text{H}^+
\]

**Unit Definition**
One unit of activity is defined as the amount of SorDH that forms 1 μmol of NADH per minute at 30 °C.

**Solutions**

I  Buffer solution ; 100 mM Tris-HCl buffer, pH 9.0

II  NAD⁺ solution ; 20 mM (133 mg NAD⁺ free acid /10 mL distilled water)

III  D-Sorbitol solution ; 500mM (911 mg D-Sorbitol/10 mL 100 mM Tris-HCl buffer, pH 9.0)

**Preparation of Enzyme Solution**
Dissolve the lyophilized enzyme with distilled water and dilute to 5 to 10 U/mL with 50mM Tris-HCl buffer containing 1 mg/mL BSA, pH 8.0.

**Procedure**
1. Prepare the following reaction mixture and pipette 3.00 mL of reaction mixture into a cuvette.
   - solution I  24.00 mL
   - solution II 3.00 mL
   - solution III 3.00 mL
2. Incubate at 30 °C for about 3 minutes.
3. Add 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 340 nm per minute (ΔAbs₃₄₀) in the linear portion of the curve.

**Calculation**

\[
\text{Volume activity (U/mL)} = \frac{(\Delta \text{Abs}_{340}) \times (3.00 + 0.01)}{6.22 \times 0.01} \times \text{d.f.}
\]

\[
\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}
\]

d.f. ; dilution factor
6.22 ; millimolar extinction coefficient of NADH (cm²/μmol)
*Protein concentration ; determined by Bradford’s method
△ acetate, ○ phosphate, ● Tris-HCl, ▲ Gly-KOH, ■ Na₂HPO₄-NaOH

treated for 24 hr at 4 °C in the following buffer solution (0.1 M);
△ acetate, ○ phosphate, ● Tris-HCl, ▲ Gly-KOH, ■ Na₂HPO₄-NaOH

treated for 15 min in 0.1 M Tricine buffer, pH 8.0

○ 35 °C, □ 40 °C, ● 45 °C