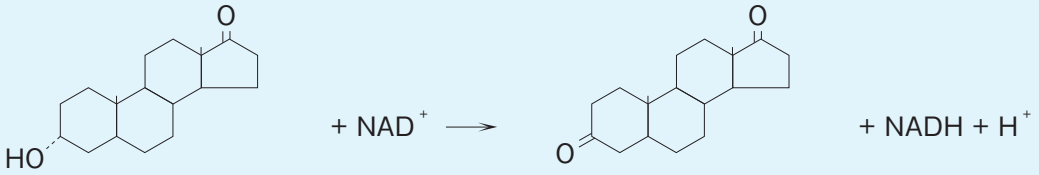
3α-HYDROXYSTEROID DEHYDROGENASE [3α-HSD Ⅱ]

from *Pseudomonas* sp.
(3 α -Hydroxysteroid: NAD^+ oxidoreductase, EC 1.1.1.50)



Androsterone　　3-Oxoandrosterone

Preparation and Specification

Appearance : White amorphous powder, lyophilized
Specific activity : More than 30 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 41 kDa (gel filtration)	
Isoelectric point	: pH 4.8 ± 0.2	
Michaelis constant	: Androsterone 2.1 × 10^{-4}M	
Optimum pH	: 8.0–10.0	Figure 1
pH stability	: 6.0–10.0 (37℃, 10 min)	Figure 2
Optimum temperature	: 50℃ (Phosphate buffer)	Figure 3
Thermal stability	: Stable at 50℃ and below (pH 8.0, 10 min)	Figure 4
Effect of metal ions	: See Table 2	
Effect of detergents	: See Table 3	
Inhibitor	: $MnCl_2$	

Applications for Diagnostic Test

This enzyme is useful for enzymatic cycling determination of **bile acid** when coupled with thio-NAD and NADH.

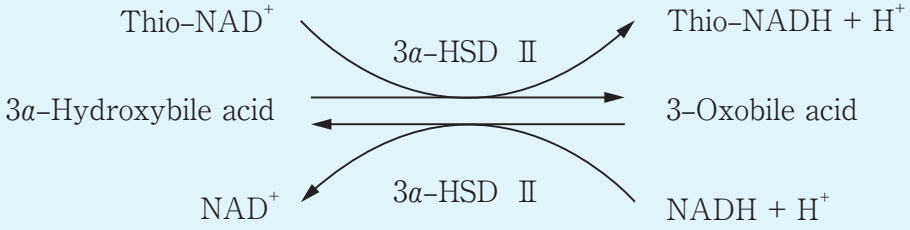


Table 1. Substrate specificity

Substrate (1mM)	Relative activity (%)
Cholic acid	100
Androsterone	131
Deoxycholic acid	115
Chenodeoxycholic acid	89.0
Glycocholic acid	103
Taurocholic acid	99.0
Taurodeoxycholic acid	128

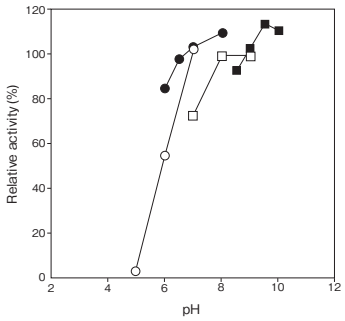
Table 2. Effect of metal ions on 3 α-HSD Ⅱ activity

Metal ion (10mM)	Relative activity (%)
None	100
NaCl	105
KCl	102
LiCl	101
$MgCl_2$	106
$MnCl_2$	16.0
$CaCl_2$	105

Table 3. Effect of detergents on 3 α-HSD Ⅱ activity

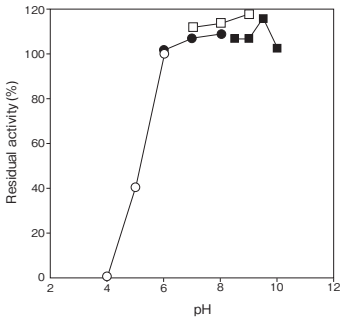
Detergent (0.1%)	Relative activity (%)
None	100
Triton X-100	71.0
Triton X-305	71.0
Triton X-114	71.0
Adekanol SO-120	104
Adekanol NP-720	75.0
Adekanol B-795	78.0
Emulgen B-66	75.0
Emulgen 911	76.0
Emulgen 709	84.0
Emulgen 810	50.0
Emulgen 109P	112
Rheodol 460	71.0
Rheodol TWL-103	72.0

Fig.1 pH Optimum



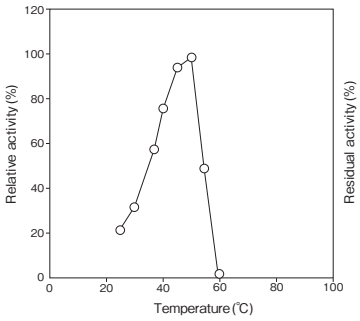
○ : 3,3-Dimethylglutarate-NaOH buffer
● : Phosphate buffer
□ : Tris-HCl buffer
■ : Glycine-NaOH buffer

Fig.2 pH Stability



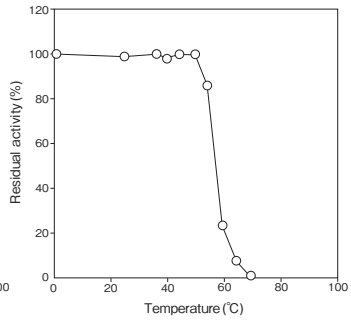
37℃, 10min
○ : Acetate buffer
● : Phosphate buffer
□ : Tris-HCl buffer
■ : Glycine-NaOH buffer

Fig.3 Optimum Temperature



pH 8.0
20 mM Phosphate buffer

Fig.4 Thermal Stability



pH 8.0, 10 min.
20 mM Phosphate buffer

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Assay

■ Principle

The assay is based on the increase in absorbance at 550 nm as formazan dye is formed in the following reactions:

$$\text{Cholic acid} + NAD^+ \xrightarrow{3\alpha\text{-HSD Ⅱ}} \text{3-Oxocholic acid} + NADH + H^+$$

$$NADH + H^+ + NTB \xrightarrow{DI} NAD^+ + NTBH_2$$

NAD: Nicotineamido adenine dinucleotide,
NTB: Nitrotetrazolium blue
DI: Diaphorase

■ Unit definition

One unit is defined as the amount of enzyme which oxidizes 1 μmole of cholic acid to 3-oxocholic acid per minute at 37℃ under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture

10 mM NAD solution	0.05 ml
0.25% (W/V) NTB solution	0.05 ml
100 U/ml DI solution	0.025 ml
2% (W/V) Triton X-100 solution	0.10 ml
0.2 M Tris-HCl buffer pH 8.0	0.10 ml
Distilled water	0.175 ml

1): 100 U/ml DI solution
Dissolve 100 U of DI with 1 ml of 10 mM Tris-HCl buffer pH 8.0.

2. Substrate solution (20 mM Androsterone)
Dissolve 23 mg of androsterone with 4 ml of methanol.
3. Reaction stopper
0.5% (W/V) Sodiumdodecyl sulfate (SDS) solution
4. Enzyme dilution buffer
10 mM Tris-HCl buffer pH 8.0
5. Reagents
NAD: NACALAI TESQUE, INC. #24334-84

NTB: Dojindo Laboratories　#344–02033
DI: Asahi Kasei Pharma Corporation　#T–06
Triton X–100: The Dow Chemical Company
Androsterone: Sigma Chemical Co. #A–9755
SDS (Sodium Dodecyl Sulfate) :
NACALAI TESQUE, INC. Extra pure #31606–75

■ **Enzyme solution**

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. Dilute it with enzyme dilution buffer to adjust the concentration as required.

■ **Procedure**

1. Pipette accurately 0.5 ml of reaction mixture into a small test tube, then add 20 μl of enzyme solution into the same test tube and preincubate at 37℃.
   ※ In the case of a test blank, add 20 μl of enzyme dilution buffer in place of enzyme solution.
2. After 5 min, add exactly 25 μl of substrate solution and mix to start the reaction at 37℃.
3. At 5 min after starting the reaction, add 2.5 ml of the reaction stopper to stop the reaction.
4. Measure the absorbance at 550 nm.

Absorbance　sample : As
blank : Ab
△A = (As − Ab) ≦ 0.20 Abs

■ **Calculation**

$$\text{Activity (U/mg of powder)} = \frac{\triangle A/5}{16.7} \times \frac{3.045}{0.02} \times \frac{1}{X}$$

16.7 : millimolar extinction coefficient of $NTBH_2$ at 550 nm (cm^2/ μmole)
5 : reaction time (min)
3.045 : final volume (ml)
0.02 : volume of enzyme solution (ml)
X : concentration of the sample in enzyme solution (mg/ml)

Storage

Storage at −20℃ in the presence of a desiccant is recommended.

References

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3α–HSD Ⅱ活性測定法（Japanese）

Ⅰ．試薬液

1. 反応試薬混合液

10mM NAD 溶液	0.05 ml
0.25% (W/V) NTB 溶液	0.05 ml
100U/ml DI 溶液 [1)]	0.025 ml
2% (W/V) トリトン X–100 溶液	0.10 ml
0.2M トリス－HCl 緩衝液 pH8.0	0.10 ml
精製水	0.175 ml

1):100U/ml DI 溶液
DI 100 単位 (U) を 10mM トリス－HCl 緩衝液 pH8.0 1ml で溶解する。

2. 基質溶液 (20mM アンドロステロン溶液)
アンドロステロン 23mg を MeOH4ml で溶解する。
3. 反応停止液
0.5% (W/V) SDS 溶液
4. 酵素溶解希釈用液
10mM トリス－HCl 緩衝液 pH8.0
5. 試薬
NAD (ニコチンアミドアデニンジヌクレオチド):
ナカライテスク製　#24334–84
NTB (ニトロテトラゾリウムブルー):
同仁化学製　#344–02033
DI (ジアフォラーゼ):旭化成ファーマ製　#T–06
トリトン X–100:Dow Chemical 製
アンドロステロン:シグマ製　#A–9755
SDS (ドデシル硫酸ナトリウム):
ナカライテスク製　一級　#31606–75

Ⅱ．酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液で溶解して全容 20ml とする。
その液を酵素溶解希釈用液で適宜希釈する。

Ⅲ．測定操作法

1. 小試験管に反応試薬混合液 0.5ml を正確に分注し、後に酵素試料液 20 μl を正確に分注して 37℃で予備加温する。
   ※盲検は酵素試料液の代わりに酵素溶解希釈用液 20 μl を加える。
2. 5 分経過後、基質溶液 25 μl を正確に加えて混和し、37℃で反応を開始する。
3. 5 分経過後、反応停止液 2.50ml を正確に加えて混和し、反応を停止する。
4. 550nm における吸光度を測定する。
   求められた吸光度を試料液は As、盲検液は Ab とする。
   ΔA = (As − Ab) ≦ 0.20 Abs

Ⅳ．計算

$$\text{活性 (U/mg)} = \frac{\Delta A/5}{16.7} \times \frac{3.045}{0.02} \times \frac{1}{X}$$

16.7 : $NTBH_2$ の 550nm におけるミリモル分子吸光係数 (cm^2/ μmole)
5 : 反応時間 (min)
3.045 : 反応総液量 (ml)
0.02 : 反応に供した酵素試料液量 (ml)
X : 酵素試料液中の検品濃度 (mg/ml)