Biosci. Biotechnol. Biochem., **64** (7), 1352–1358, 2000

The Characteristics and Applications of Recombinant Cholesterol Dehydrogenase

Koji KISHI,*,† Yoshifumi WATAZU,* Yoshiaki KATAYAMA,** and Hiroaki OKABE***

*International Reagents Corp., 1-1-2, Murotani, Nishi-ku, Kobe 651-2241, Japan
**Department of Clinical Chemistry, National Cardiovascular Center Hospital, 5-7-1, Fujishiro-dai, Suita 565-0873, Japan
***Department of Laboratory Medicine, School of Medicine, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Received November 8, 1999; Accepted February 24, 2000

Mass production of an r-CDH derived from *Nocardia* species was made possible by gene technology. (Horinouchi *et al.*, *Applied and Environmental Microbiology*, 57, 1386–1393 (1991)). However, the characteristics of the r-CDH have not been studied in detail and have not been improved enough for industrial use. We accordingly characterized both the native-CDH and the r-CDH prepared from *Streptomyces lividans*. Both CDHs were monomers with molecular masses of 37 kDa. The K_m of r-CDH was 2.50×10^{-3} M for cholesterol and 2.33×10^{-4} M for NAD. The activators of CDHs were TritonX-100 and cholate. TritonX-405, Ag^+, and Zn^{2+} inhibited both enzymes. The residual activity of native CDH after heat treatment was 32% (37°C, 60 min), while the r-CDH showed a residual activity of 87% (37°C, 60 min). The r-CDH is an enzyme with high substrate specificity for cholesterol as well as native CDH and higher thermal stability than native CDH.

We have developed a novel serum cholesterol assay using the r-CDH, which permits the direct measurement of cholesterol by measuring NADH reaction products. We conclude that this r-CDH enzyme is useful and can be used to measure cholesterol in a clinical chemistry setting.

Key words: cholesterol dehydrogenase; cholesterol oxidase; recombinant enzyme; serum

CDH oxidizes the 3-β-hydroxyl group of cholesterol to cholestenone with concomitant reduction of the coenzyme as follows: NAD or NADP to produce NADH or NADPH, Cholesterol + $NAD(P)^+ \rightarrow \Delta$-*4*-cholesten-*3*-one + NAD(P)H + H^+.

T. Akiba[1] separated and purified native CDH from *Nocardia* species in 1990. Since native CDH can be produced only in small amounts, Horinouchi *et al.*[2] in 1991 used a *Streptomyces* host-vector system[3] to produce r-CDH successfully on a large scale, but the characteristics of CDH has not been studied in detail.

On the other hand, we investigated and developed a diagnostic assay system by use of the enzyme protein. Serum cholesterol in the clinical laboratory is a very important marker for diagnosis and treatment of arteriosclerosis, which is one of the modern adult diseases. However, interference due to reduced substances, *e.g.* bilirubin and ascorbate, are inevitable in the conventional colorimetric assays for the measurement of cholesterol in human serum using cholesterol oxidase (COD, EC 1.1.3.6).[4] Cholesterol assays using CDH eliminate these COD interferences and permit the direct measurement of cholesterol using the absorbance at 340 nm of NADH reaction products.

In this report, we characterize both the native CDH and the r-CDH and use them to measure cholesterol with use of r-CDH in a clinical laboratory.

Materials and Methods

Enzyme activity. The activity of native and r-CDH was measured by the amount of NADH produced by the CDH-catalyzed oxidation of cholesterol to Δ-*4*-cholesten-*3*-one in a reaction mixture containing 1.5 mM NAD, 0.86 mM cholesterol, 0.12% TritonX-100, and 0.003 unit/ml CDH in 0.1 M Tris-HCl buffer at pH 8.5. One unit (U) of enzyme activity was defined as the amount of CDH that catalyzes the oxidation of 1 μmole of cholesterol per minute at 25°C.

† To whom correspondence should be addressed

Abbreviations: CDH, cholesterol dehydrogenase; native CDH, the native cholesterol dehydrogenase from *Nocardia* species; r-CDH, recombinant cholesterol dehydrogenase; CE, cholesterol esterase; COD, cholesterol oxidase; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

Enzyme purification. Production of r-CDH in the *Streptomyces lividans* TK54 strain[5] carrying the pIJ702[3] grown in 200 ml of medium containing 1.0% glucose, 2.0% meat extract, 1.0% yeast extract, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% antifoam (Adekanol LG126), 0.01% thiostreptone, and 0.2% KH_2PO_4 at pH 7.2 and 30°C in a 500-ml shaker. The *Streptomyces lividans* TK54 strain as a host and the pIJ702 as a vector were provided by D. A. Hopwood and T. Akiba. The *Streptomyces lividans* TK54 was transformed by the protoplast method.[6] After 24 h of the first cultivation, 200 ml of culture broth was inoculated in 20 liters of medium containing 1.0% glucose, 2.0% meat extract, 1.0% yeast extract, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% antifoam, 0.01% thiostreptone, and 0.2% KH_2PO_4 at pH 7.2. The cultivation was done in a 30-liter jar fermentor at 30°C for 24 h with agitation at 300 rpm and an air flow of 10 l/min. The broth was centrifuged at 12,000 × *g* for 20 min to harvest the cells.

Production of the native CDH started with cultivation of *Nocardia* species strain Ch2-1 (Ferm-PNo.6217),[2] which was provided by T. Akiba (Amano Pharmaceutical Co. Ltd., Nagoya, Japan), in 200 ml of medium containing 0.5% glucose, 0.5% meat extract, and 0.02% yeast extract at pH 7.2 and 30°C. After 24 h of this first cultivation, 200 ml of culture broth was inoculated in 20 liters of medium containing 0.5% cholesterol, 0.5% glucose, 0.5% meat extract, and 0.02% yeast extract at pH 7.2. The cultivation was done in a 30-liter jar fermentor at 30°C for 40 h with agitation at 200 rpm and an air flow of 10 l/min. The broth was centrifuged at 8,000 × *g* for 10 min to harvest the cells. The native and r-CDH containing bacterial cells were purified in the same way as described below.

(1) Extraction of crude enzyme solution. Five hundred grams (500 g) of the bacterial cell harvest was suspended in 5.0 liters of 10 mM phosphate buffer (pH 7.0) containing 0.1% TritonX-100, and the suspension was homogenized and extracted with a Dyno Mill KDL (Willy A, Bachofen Manufacturing Engineers, Switzerland). The homogenate was centrifuged at 12,000 × *g* for 15 min to obtain the translucent crude enzyme solution.

(2) Ammonium sulfate precipitation. Solid ammonium sulfate was added to the crude enzyme solution to 45% saturation with stirring. The mixture was centrifuged at 12,000 × *g* for 15 min. The ammonium sulfate precipitate fraction was collected and dissolved in 700 ml of 10 mM phosphate buffer (pH 7.0) containing 0.1% TritonX-100, and concentrated and desalted by ultrafiltration (Amicon YM-10, Millipore, U.S.A.).

(3) Ion chromatography. Five hundred grams (500 g) of DEAE-cellulose (Brawn) equilibrated with 10 mM phosphate buffer (pH 7.0) was mixed with the ammonium sulfate precipitate fraction for 30 min at 10°C. The DEAE-cellulose was collected by filtration on filter paper and washed with 1,600 ml of 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate. The CDH enzyme was eluted with 1,000 ml of 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate and 0.4 M sodium chloride. Solid ammonium sulfate was added to the eluate to 45% saturation, the mixture centrifuged at 12,000 × *g* for 15 min, and the ammonium sulfate precipitate was collected.

(4) DEAE-Sepharose chromatography. The ammonium sulfate precipitate was dissolved in 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate, and the solution was concentrated by ultrafiltration. The concentrated solution was adsorbed onto a 200-ml DEAE-Sepharose column (ϕ 4.6 × 12 cm, Pharmacia Biotech, Sweden) equilibrated with 10 mM phosphate buffer (pH 7.0). After being washed with 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate, the enzyme was eluted with 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate and 0.2 M sodium chloride. The elute was concentrated and desalted by ultrafiltration.

(5) Blue-Sepharose chromatography. The concentrated DEAE-Sepharose eluate solution was put on a 100 ml Blue-Sepharose column (ϕ 3.6 × 10 cm, Pharmacia Biotech, Sweden) equilibrated with 10 mM phosphate buffer (pH 7.0). After being washed with 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate and 0.1 M sodium chloride, the column was developed with a linear gradient of NaCl (0.1 to 1 M) in 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium cholate. Active fractions were collected and concentrated by ultrafiltration.

(6) Cellulofine GC700m gel filtration chromatography. The Blue-Sepharose active fraction was filtered through a 0.2 μm membrane filter and put on Cellulofine GC700m column (ϕ 3.0 × 100 cm, Chisso, Japan) and eluted with 10 mM phosphate buffer (pH 7.0) containing 0.1M sodium chloride and 0.1% sodium cholate as the mobile phase. Fractions containing CDH activity were collected and concentrated by ultrafiltration. A summary of the native and r-CDH purification is shown in Table 1. The native-CDH was purified about 676-fold in terms of specific activity with a recovery of 11.4%. The r-CDH was purified about 132-fold in terms of specific activity with a recovery of 22.9%. Two homogeneous enzyme preparations showed the specific activity of 41 U/mg. The enzyme preparations gave a single band on SDS-PAGE by the method of Laemmli[7] (Fig. 1). The

Table 1. Summary of Purification

Procedures	r-CDH					Native-CDH				
	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification (Fold)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification (Fold)
Crude homogenate	18,100	59,200	0.31	100.0	1.0	3,200	53,300	0.06	100.0	1.0
$(NH_4)_2SO_4$ ppt.	15,840	19,800	0.80	87.5	2.6	2,620	21,900	0.12	81.9	2.0
DEAE-Cellulose (Brown)	12,600	1,050	12.00	69.5	38.7	1,940	2,170	0.89	60.6	14.8
DEAE-Sepharose	9,900	475	20.80	54.7	67.1	1,080	253	4.30	33.8	71.6
Blue-Sepharose	7,560	204	37.10	41.8	119.7	830	38	21.80	25.9	363.3
Cellulofine GC700m	4,140	101	41.00	22.9	132.2	365	9	40.60	11.4	676.7

characteristics of these purified enzymes were examined for the measurement determination of cholesterol.

Assay method for serum cholesterol. We devised an extension of the CDH assay for free cholesterol to permit the measurement of serum cholesterol using r-CDH. Cholesteryl esters are first converted to free cholesterol by hydrolysis with cholesterol esterase (CE) (EC 3.2.1.13). The resulting total cholesterol is measured by determing the amount of NADH produced by the r-CDH catalyzed oxidation of cholesterol.

Fresh human serum was tested as samples.

Two assay solutions were prepared to permit the preparation of independent step blank solutions.

The CE reagent was composed of 6.0 mM NAD, 0.2% sodium deoxycholate, 0.3% TritonX-100, 3 U/ml of CE (derived from *Pseudomonas* species, Asahi Chemical Industry Co., Ltd., Japan) and 20 mM dichlorohydrazinium (for trapping ketone on cholestenone[8]) in 0.1 M Tris-HCl buffer, pH 9.0.

The r-CDH reagent was composed of 30 U/ml of r-CDH in 0.02 M phosphate buffer, pH 7.0.

A 100 μl of human serum for a test sample or 100 μl of saline for a control sample and 3.0 ml of the CE reagent were mixed and incubated at 37°C for 5 min. One ml of the r-CDH reagent was added, mixed well, and incubated at 37°C for an additional 5 min. The absorbance of NADH produced in the 5 min after mixing with the r-CDH reagent was measured at 340 nm against distilled water for the test sample [A1] or the saline control [B1].

The test sample blank [A2] was prepared by adding 1.0 ml of 0.02 M phosphate buffer, pH 7.0 instead of 1.0 ml of the r-CDH reagent to the test sample. The saline control blank [B2] was prepared by adding 1.0 ml of 0.02 M phosphate buffer, pH 7.0 instead of 1.0 ml of the r-CDH reagent to the saline control sample.

A Hitachi UV-3210 spectrophotometer (Hitachi Co. Ltd., Japan) was used for the absorbance measurements. The cholesterol concentration was calculated according to the following formula.

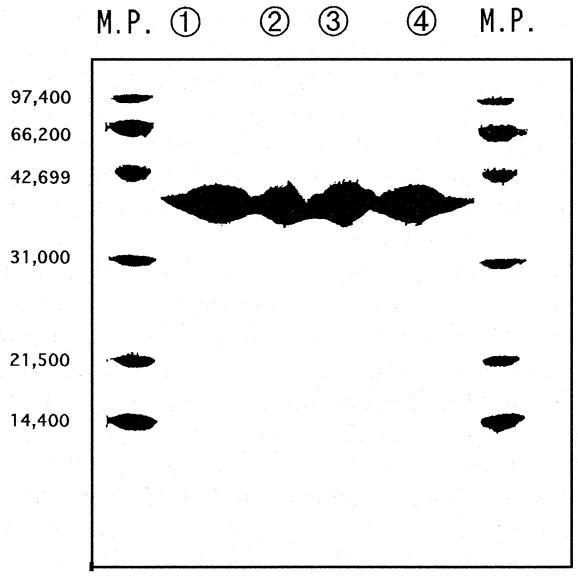

Fig. 1. Analytical SDS-Polyacrylamide Gel Electrophoresis of Purified Recombinant and Native Cholesterol Dehydrogenase from *Nocardia* Species.

Lanes ① and ④, native CDH; Lanes ② and ③, r-CDH; M.P., molecular weight markers. Each protein was electrophoresed on a polyacrylamide gel containing 0.1% SDS. Molecular weight markers were SDS-PAGE MW Standards-Low (Bio-Rad Laboratories, U.S.A.). The arrow indicates the position of tracking dye, Coomassie Brilliant Blue.

$$\text{Concentration (mg/dl)} = \{(A1 - B1) - (A2 - B2)\} \times TV \times 10^3 \times 386.66/(\varepsilon \times SV \times 10)$$

Where:

A1: Absorbance of the test sample at 340 nm

B1: Absorbance of the saline control sample at 340 nm

A2: Absorbance of the test sample blank at 340 nm

B2: Absorbance of the saline control blank at 340 nm

TV: Total volume of the reaction solution (4.1 ml)

SV: Sample volume (0.1 ml)

ε: Molar extinction coefficient of NADH at 340 nm (6.30×10^3 $M^{-1} \cdot cm^{-1}$)[9]

386.66: Molecular weight of cholesterol (g/mole).

Results

Characteristics of the enzymes

One ml of a 10 U/ml solution of native- or r-CDH was added to 30 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.86 mM cholesterol, 1.5 mM NAD, and 0.12% TritonX-100 and incubated at 37°C for 20 min. As controls, the NAD was omitted, or the CDH solution was replaced by distilled water and incubated similarly. These incubated solutions were extracted three times with *n*-hexane.[10]

The extracts were dried by evaporation, re-dissolved in 1 ml of *n*-hexane, and chromatographed on a thin layer silica gel (Merck, Germany) using 20% *n*-hexane-$CHCl_3$ as the mobile phase. The native and r-CDH converted cholesterol to a single product in the presence of NAD. The R_f of this product agreed with that of a standard sample of *Δ-4*-cholesten-*3*-one (Sigma Chemicals Co., U.S.A.).

Molecular weight and isoelectric point

The molecular weight of the native and r-CDH was obtained using SDS polyacrylamide gel electrophoresis. As shown in Fig. 1, both CDHs show the same molecular weight of 37 kDa. Isoelectric focusing in a density gradient was done using various concentrations of sucrose by Vesterberg's method.[11] Electrophoresis was done at 400 V and 5°C for 48 h using Pharmalyte 3–10 (Pharmacia Biotech, Sweden) as the carrier ampholyte. The pH of each fraction (1.8 ml) indicated that the isoelectric points (p*I*) of native-CDH and r-CDH were 4.54 and 4.59, respectively.

pH activity and stability

In a buffer containing *N*-(*2*-acetamido)-*2*-aminoethanesulfonic acid (ACES), Tris and *N*-cyclohexyl-*2*-aminoethanesulfonic acid (CHES), both types of enzymes are active at pH 6 or above with a peak activity at around pH 9. To observe the stability at various pHs, the enzyme preparations were incubated at 37°C for 60 min in a 0.1 M solutions of each buffer and the residual CDH activity was measured. The r-CDH was stable at pHs ranging from 6.0–8.5, and the native CDH was stable at pHs ranging from 6.5–7.5 (Fig. 2).

Optimum temperature and stability

The heat stability and optimum temperature of each CDH were examined. A ten U/ml CDH solution in 0.1 M ACES buffer (pH 7.0) was prepared and incubated at various temperatures for 15 min. The residual activity of the native-CDH was 32% at 37°C and 15% at 40°C, while the r-CDH showed a residual activity of 87.5% at 37°C and 78.8% at 40°C (Fig. 3). The optimum reaction temperature was examined using a 0.1 M Tris-HCl buffer (pH 8.5). Both enzymes showed their highest activity at 30°C.

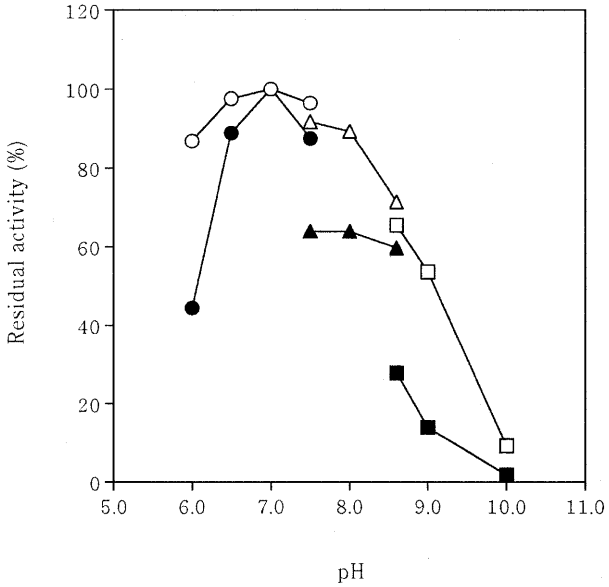

Fig. 2. Effects of pH on the Stabilities of the Purified Recombinant and Native-Cholesterol Dehydrogenase.

The purified recombinant enzyme was incubated for 60 minutes at 37°C in a 0.1 M ACES buffer (○), a 0.1 M Tris buffer (△), and a 0.1 M CHES buffer (□). The purified native enzyme was incubated for 60 minutes at 37°C in a 0.1 M ACES buffer (●), a 0.1 M Tris buffer (▲), and a 0.1 M CHES buffer (■). Residual activities of both the enzymes were assayed at pH 7.0.

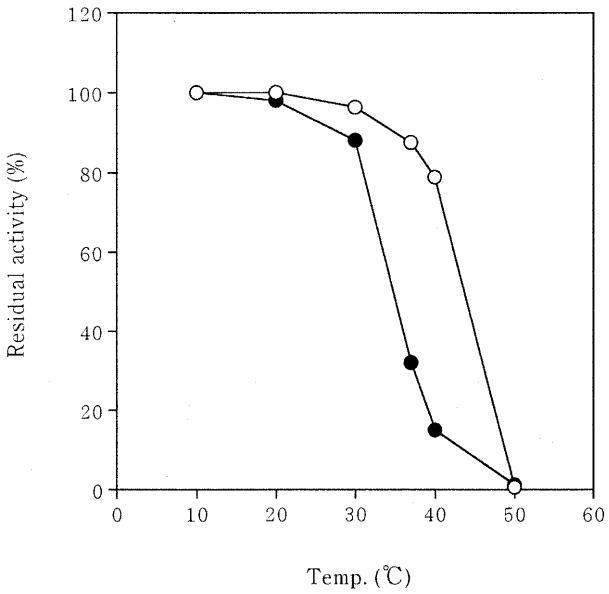

Fig. 3. Effects of Temperature on the Stabilities of the Purified Recombinant and Native-Cholesterol Dehydrogenase.

The purified recombinant enzyme (○) and the purified native enzyme (●) were incubated for 15 minutes in a 0.1 M ACES buffer (pH 7.0) at various temperatures. Residual activities of both the enzymes were assayed at 10°C.

Activators and inhibitors

Activators and inhibitors of r-CDH and native CDH were examined. Both enzyme were activated by detergents with a hydrophile-lipophile balance (HLB)

Table 2. Comparison of r-CDH, Native CDH and COD from *Nocardia* Species

	r-CDH	Native CDH	COD[a)]
Molecular weight	37,000	37,000	ND
Subunit	Monomer	Monomer	ND
Opt. pH	>9.0	>9.0	7.0
Opt. Temp.	30°C	30°C	ND
p*I*	4.59	4.54	ND
pH stability	6.0~8.5	6.5~7.5	ND
Thermal stability	87% (37°C, 60 min)	32% (37°C, 60 min)	50°C
K_m (M)			
Cholesterol	2.50×10^{-3} M	2.63×10^{-3} M	1.4×10^{-5} M
NAD	2.33×10^{-4} M	2.13×10^{-4} M	—
Substrate specificity (%)			
Cholesterol	100.0	100.0	100
β-Sitosterol	51.6	49.1	105
Stigmasterol	30.3	30.0	34
Ergosterol	50.0	50.0	60
Pregnenolone	14.1	10.1	82
Cholate	0.0	0.0	ND
Deoxycholate	0.0	0.0	ND
Taurocholate	0.0	0.0	ND
Testosterone	0.0	0.0	ND
Pregnanediol	0.0	0.0	24
Dehydroepiandrosterone	0.0	0.0	1
Lanosterol	0.0	0.0	ND
Metal requirement (%)			
None	100	100	ND
$MnCl_2$	99	99	ND
$MgCl_2$	93	93	ND
$CaCl_2$	100	100	ND
$FeCl_2$	96	96	ND
$LiCl_2$	96	96	ND
$NiCl_2$	48	48	ND
$ZnCl_2$	0	0	ND
$AgNO_3$	0	0	ND
Activator			
	TritonX-100	TritonX-100	ND
0.1% Cholate	130	130	ND
Inhibitor			
0.1% TritonX-405	35	35	ND

a): Data from reference 14. ND: No data.

of less than 13 (TritonX-100) and inhibited by detergents with HLB of higher than 16 (TritonX-405). Various metal salts were added to the reaction mixtures at a concentration of 1.0 mM, and their effect on enzyme activity was examined. Ag^+ and Zn^{2+} inhibited both enzymes.

Substrate specificity

The substrate specificity of r-CDH and native-CDH for various compounds that have the steroid skeleton was examined. Both enzymes acted on cholesterol. Deoxycholate, taurocholate, testosterone, pregnanediol, dehydroepiandrosterone or lanosterol were not oxidized by these enzymes. The coenzyme requirement was also examined. NAD was found to be the best coenzyme for both enzymes.

Michaelis constant (K_m) of CDH

The K_m of CDHs for cholesterol were measured in a reaction mixture containing 1.5 mM NAD, cholesterol (0.48–2.90 mM), 1.87% TritonX-100 and 0.006 U/ml CDH in 0.1 M Tris-HCl buffer at pH 8.5. The K_m of CDHs for NAD were measured in a reaction mixture containing NAD (0.135–0.675 mM), 0.86 mM cholesterol, 1.2% TritonX-100 and 0.006 U/ml CDH in 0.1 M Tris-HCl buffer at pH 8.5. The K_m of native-CDH and r-CDH for cholesterol were 2.50×10^{-3} M and 2.63×10^{-3} M and for NAD were 2.13×10^{-4} M and 2.33×10^{-4} M.

Nucleotide sequence of the r-CDH gene

The nucleotides were sequenced automatically on the Model 373A DNA Sequencing System with Dye Primer and Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, U.S.A.). Nucleotide sequence of the r-CDH gene on plasmid isolated from *Streptomyces lividans* TK54 was coincide with that of the *Nocardia* CDH gene in the report by S. Horinouchi *et al.*[2)]

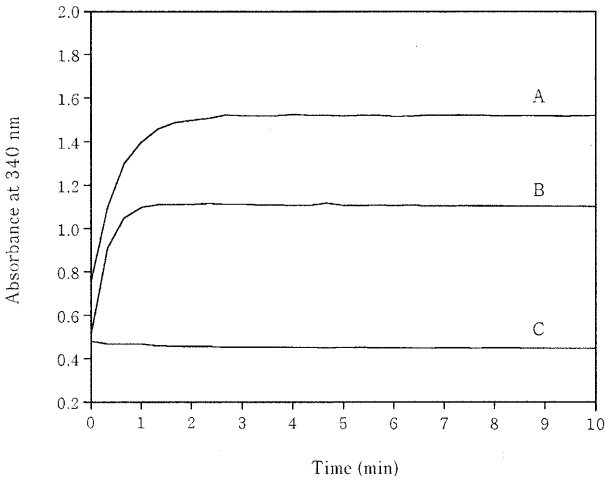

Fig. 4. Reaction Course of Human Serum Samples (A and B) and Saline (C) after Mixing with CDH Reagent.

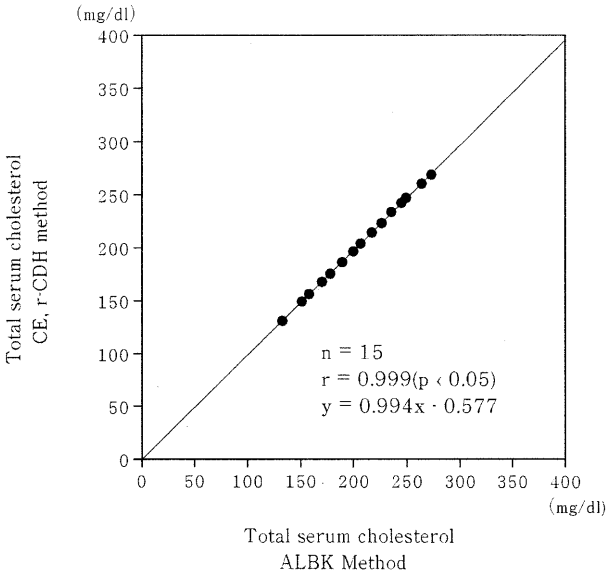

Fig. 5. The Correlation between the Total Serum Cholesterol Measured by the CE, r-CDH Method and the ALBK Method.

Assay of serum cholesterol

The course of reaction after mixing with 30 U/ml r-CDH reagent is shown in Fig. 4. The reaction of CDH was complete in 3.5 min and the final NADH produced was stable for at least 10 min. The correlation of this assay with the Abell-Levy-Brodie-Kendall (ALBK) method[12,13] is shown in Fig. 5. The correlation between the methods was excellent.

Discussion

As shown in Table 2, the substrate specificity of both the r-CDH and native-CDH were much the same as COD from *Nocardia* species.[14] However, the K_m of COD for cholesterol was lower than that of CDH.

The various properties of r-CDH were observed. r-CDH and native-CDH are similar in their physicochemical properties such as molecular weight, K_ms for substrate and coenzymes, isoelectric point, optimal pH, and optimal temperature. However, the r-CDH was superior to native CDH with respect to heat and pH stability. It was supposed that the reason was caused by the difference of primary protein structure, higher order protein structure, or contamination by small amount of protein such as protease and so on between native CDH and r-CDH. But, the various properties of r-CDH and native-CDH were shown in this paper, their primary and higher order protein structures are not clarified yet. Therefore, the clarification of primary and higher order protein structures are needed. It has been reported that improved heat stability can be obtained by manipulation of the primary structure while maintaining reactivity to the substrate,[15] applied protein engineering and gene technology such as suppresser variation by heat treatment,[16] changing hydrophobic interaction,[17] and other methods.[18,19] These reports suggest the possibility that an r-CDH with better heat stability can be developed. The finding of the improved heat stability is very interesting because it can increase its industrial usefulness.

Similar to the native CDH, r-CDH showed high substrate specificity for cholesterol. Using this property in combination with CE, we constructed an assay for serum cholesterol. The results obtained by this new method agreed well with those determined by the ALBK method. We conclude that this r-CDH enzyme is useful and can be used to measure cholesterol in a clinical chemistry setting.

Acknowledgments

The authors thank Mr. Tetsunori Akiba (Amano Pharmaceutical Co. Ltd., Japan) for the useful suggestions concerning enzyme purification and cultivation of the native *Nocardia* species strain and the recombinant *Streptomyces lividans* strain in this study. Dr. Masakazu Nakamura (Member of U.S. National Cholesterol Reference Method Laboratory Network) provided skillful technical data on serum cholesterol measurement. We also thank Dr. C. Heldebrant (Alpha Therapeutic Corp., U.S.A.) for assistance during the preparation of this manuscript.

References

1) Akiba, T., The method for preparation of NAD(P)-dependent cholesterol dehydrogenase. Japanese patent, 90-18064 (1990).
2) Horinouchi, S., Ishizuka, H., and Beppu, T., Cloning, nucleotide sequence, and transcriptional analysis of the NAD(P)-dependent cholesterol dehydrogenase gene from a *Nocardia* sp. and its hyperexpression in *Streptomyces* spp. *Applied and Environmental*

Microbiology, **57**, 1386–1393 (1991).
3) Hopwood, D. A., T. Kieser, Wright, H. M., and Bibb, M. J., Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. *J. Gen. Microbiol.*, **129**, 2257–2269 (1983).
4) Allain, C. C., Poon, S., Chan, C. S. G., Richmond, W., and Fu, P. C., Enzymatic Determination of Total Serum cholesterol. *Clin. Chem.*, **20**, 470–475 (1974).
5) Katz, E., Thompson, C. J., and Hopwood, D. A., Cloning and expression of the tyrosinase gene from *Streptomyces* antibioticus in *Streptomyces lividans*. *J. Gen. Microbiol.*, **129**, 2703–2714 (1983).
6) Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrempf, H., Gentic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foudation, Norwich, United Kingdom (1985).
7) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
8) Dutcher, J. D. and Wintersteiner, O., Studies on the Wolff-Kishner reduction of steroid ketones. *J. Am. Chem. Soc.*, **61**, 1992–2000 (1939).
9) McComb, R. B., Bond, L. W., Burnett, R. W., Keech, R. C., and Bowers Jr., Determination of the molar absorptivity of NADH. *Clin. Chem.*, **22**, 141–150 (1976).
10) Cooper, G. R., Smith, S. J., Duncan, I. W., Mather, A., Fellows, W. D., Foley, T., Frantz, I. D. Jr., Gill, J. B., Grooms, T. A., Hynie, I., Laessig, R., LoBasso, F. A., Martin, J., Naito, H., Newman, H. A., Sideman, L., Turner, J. H., and Williams, D., Interlaboratory testing of the transferability of a candidate reference method for total cholesterol in serum. *Clin. Chem.*, **32**, 921–929 (1986).
11) Vesterberg, O., Isoelectric focusing of proteins. *Method in Enzymology*, **22**, 389–412, Academic Press Inc., New York (1977).
12) Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.*, **195**, 357–366 (1952).
13) Duncan, I. W., Mather, A., and Cooper, G. R., The procedure for the proposed cholesterol reference method. Centers for Disease Control, Atlanta, GA (1982).
14) Brooks, C. J. W. and Smith, A. G., Cholesterol oxidase: further studies of substrate specificity in relation to the analytical characterisation of steroids. *Journal of Chromatography*, **112**, 499–511 (1975).
15) Ganter, C. and Pluckthun, A., Glycine to alanine substitution in helices of glyceraldehyde-3-phosphate dehydrogenase: effects on stability. *Biochemistry*, **29**, 9395–9402 (1990).
16) Dao-Pin, S., Baase, W. A., and Matthews, B. W., A mutant T4 lysozyme (Val 131→Ala) designed to increase thermostability by the reaction of strain within an α-helix. *Protein: Structure, Function and Genetics*, **7**, 198–204 (1990).
17) Argos, P., Rossmann, G. M., Grau, M. U., Zuber, H., Frank, G., and Tratschin, D. J., Thermal stability and protein structure. *Biochemistry*, **18**, 5698–5703 (1979).
18) Doi, N. and Yanagawa, H., Screening of conformationally constrained random polypeptide libraries displayed on a protein scaffold. *Cell. Mol. Life Sci.*, **54**, 394–404 (1998).
19) Hayashi, Y., Numata, K., Yamagishi, A., Yutani, K., Sakurai, M., Tanaka, N., and Oshima, T., A stable intermediate in the thermal unfolding process of a chimeric 3-isopropylmalate dehydrogenase between a thermophilic and a mesophilic enzymes. *Protein Sci.*, **5**, 511–516 (1996).