Performance Characteristics of Cholesterol Oxidase for Kinetic Determination of Total Cholesterol

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The enzymatic method for cholesterol determination can use either an endpoint or a kinetic method. Not much is known concerning the properties (K_m and V_{max}) of the commercial enzyme for the kinetic method. We measured the K_m and V_{max} of *Brevibacterium*, *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* cholesterol oxidase. *Brevibacterium* gave the highest K_m value (230.3×10^{-4} M), followed by *Streptomyces* (2.17×10^{-4} M), *Cellulomonas* (0.84×10^{-4} M), and *Pseudomonas* (0.61×10^{-4} M). The K_m values and the linearity obtained from *Streptomyces* (2.6 mmol/L), *Pseudomonas* (2.1 mmol/L), or *Cellulomonas* (2.1 mmol/L) were too low. Dichlorophenol isomers,

acting as inhibitors, increased the enzyme's K_m . The addition of 3,4-dichlorophenol raised the K_m of *Streptomyces* from 2.17×10^{-4} to 24.89×10^{-4} M. The linearity was increased from 2.6 to 13.0 mmol/L. The high K_m of *Brevibacterium* resulted in an insensitive reaction and low cholesterol linearity (7.8 mmol/L). An increase in the sample-to-reagent ratio from 1:100 to 1:10 enhanced the reaction rate and the linearity from 7.8 to 20.7 mmol/L. We suggest that *Brevibacterium* and *Streptomyces* cholesterol oxidase (with the addition of 3,4 dichlorophenol) are good sources for serum cholesterol determination by the kinetic method. J. Clin. Lab. Anal. 19:247–252, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Serum cholesterol is a common biochemical marker for the clinical diagnosis and prognosis of atherosclerosis, coronary heart disease, and other lipid disorders. According to the Expert Panel of the National Cholesterol Education Program (NCEP), increased blood cholesterol is closely correlated with an increased risk of coronary heart disease, and adults should be routinely screened for elevated cholesterol (1).

Enzymatic cholesterol determination uses cholesterol esterase to hydrolyze cholesterol esters that produce free cholesterol, which is oxidized to cholest-4-ene-3-one and hydrogen peroxide by cholesterol oxidase (2). Hydrogen peroxide is catalyzed to create a colored product. For the measurement of product, the endpoint method is

usually performed. The kinetic method offers advantages over the endpoint assay, such as short analysis time, less interfering substances, and no requirement for sample blank measurement (3,4).

There are several sources of cholesterol oxidase, such as that isolated from *Nocardia erythropolis*, *Streptomyces*, *Mycobacteria*, *Brevibacterium*, *Schizophyllum*, *Pseudomonas fluorescens*, and *Cellulomonas* (2,5–9).

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Deeg and Ziegenhorn (9) found that *Nocardia* cholesterol oxidase was unsuitable for kinetic cholesterol determination because its K_m ($1-69 \times 10^{-6} \text{ M}$) was too low (6,7) and lacked competitive inhibitors to elevate the K_m value (9). They concluded that *Streptomyces* was the only suitable enzyme for the kinetic method.

In this study we investigated the performance characteristics of *Streptomyces*, *Pseudomonas fluorescens*, *Brevibacterium*, and *Cellulomonas* enzyme for the analytical determination of cholesterol by the enzymatic kinetic method.

MATERIALS AND METHODS

Apparatus

We determined the reaction rates of cholesterol oxidase using the Vitalab Selectra analyzer (E. Merck, Darmstadt, Germany). We estimated the K_m and $V_{\max}$ values from the Enzfitter program (Elsevier-Biosoft, Cambridge, UK) by calculating from the saturation curves and Lineweaver-Burk plots.

Chemicals

All sources of cholesterol oxidase, peroxidase (horse-radish), sodium cholate, and Brij 35 solution (30% W/V) were obtained from Sigma Chemical Co. (St. Louis, MO). All dichlorophenol isomers were obtained from Aldrich Chemical Inc. (Milwaukee, WI), and 4-aminophenazone was obtained from BDH (Dorset, UK).

Reagents

Cholesterol oxidase K_m

We prepared the reagent base A by dissolving 10,000 U/L of peroxidase, 3 mmol/L of sodium cholate, 0.5 mmol/L of 4-aminophenazone, 20 mmol/L of phenol, and 15 ml/L of Brij 35 solution (30% W/V) in phosphate buffer 0.1 mol/L, pH 7.0. To prepare the working reagent to study the K_m , we added each enzyme (250 U/L) to the reagent base A.

Effect of dichlorophenol isomers on K_m of enzyme

We prepared the reagent base B by adding 5 mmol/L of 2,3- (B1), 2,4- (B2), 2,5- (B3), 2,6- (B4), 3,4- (B5), or 3,5-dichlorophenol (B6) to the reagent base A. Four sets of working cholesterol reagents containing each cholesterol oxidase were prepared by adding each enzyme (250 U/L) to reagent bases B1–B6.

Effect of 3,4 dichlorophenol concentration on K_m of *Streptomyces*

Reagent base C was prepared by adding 250 U/L of *Streptomyces* to the reagent base A. To prepare the

working cholesterol reagents, we added 3,4-dichlorophenol at concentrations of 0, 1, 3, 5, or 7 mmol/L to the reagent base C.

Cholesterol standard

We prepared the stock standards (25.9 and 50.0 mmol/L) by dissolving the appropriate amount of cholesterol (Sigma grade 99%) in an ethanol diluent (absolute ethanol containing Triton X-100, 20% v/v). For the K_m study, the stock standard (50 mmol/L) was diluted with the ethanol diluent to obtain cholesterol concentrations ranging from 2.5 to 50.0 mmol/L. For the linearity study, the stock standard (25.9 mmol/L) was diluted with the diluent to obtain cholesterol concentrations ranging from 0.5 to 25.9 mmol/L.

Procedures

Cholesterol oxidase K_m

The parameters for Vitalab Selectra were set as follows: reaction mode: kinetic; delay: 12 sec; wavelength: 505 nm; sample and reagent volumes: *Streptomyces* (3 and 297 μL), *Pseudomonas fluorescens* (3 and 297 μL), *Brevibacterium* (30 and 270 μL), and *Cellulomonas* (2 and 398 μL); and factor: 73.529 (calculated from the inverse of the quinonemine millimolar extinction coefficient of 13.6). We determined the initial rates of each enzyme reaction by multiplying the absorbance changed per minute with factor. The K_m , and $V_{\max}$ of each cholesterol oxidase were calculated from the relationship between substrate concentrations and the rate using the Enzfitter program.

Linearity

The Vitalab Selectra's parameters were set as described above, except that the sample and the working cholesterol reagent volumes used were 3 and 300 μL , respectively. To determine the suitable ratio of sample to *Brevibacterium* reagent, three sample volumes (3, 15, and 30 μL) were used to achieve ratios of 1:100, 1:20, and 1:10, respectively. We determined the cholesterol in working standards. The absorbance changes per minute vs. cholesterol concentration were plotted. The linearity of cholesterol was evaluated using NCCLS EP6-A guidelines (10).

RESULTS

K_m of Various Cholesterol Oxidase Enzymes

Streptomyces, *Pseudomonas fluorescens*, and *Cellulomonas* sources of cholesterol oxidase with and without various dichlorophenol isomers reached saturation at 0.5 mmol/L of cholesterol. *Brevibacterium* did not reach

the steady state at the cholesterol concentrations used in the reaction mixture (0–5 mmol/L). The addition of 2,4-, 3,4-, and 3,5-dichlorophenol decreased the rate of *Brevibacterium*, *Streptomyces*, and *Pseudomonas fluorescens* cholesterol oxidase reaction, whereas 2,3-, 2,5-, and 2,6-dichlorophenol increased the reaction rate (Table 1). For *Cellulomonas*, the reaction rates were slightly increased by the addition of all dichlorophenol isomers.

Brevibacterium had the highest K_m value (230.3×10^{-4} M), followed by the K_m 's of *Streptomyces* ($2.17 \times$

TABLE 1. The apparent K_m and V_{max} of *Brevibacterium*, *Streptomyces*, *Pseudomonas fluorescens* and *Cellulomonas* cholesterol oxidase in cholesterol reagents with and without the addition of dichlorophenol isomers, 5 mmol/L

Cholesterol oxidase source	Kinetic property of enzyme	
	K_m (10^{-4} M)	V_{max} (10^{-3} M/min)
<i>Brevibacterium</i> , 250 U/L		
Without dichlorophenol	230.3	237.94
With the addition of dichlorophenol:		
2,6-	208.1	436.47
2,3-	161.6	199.11
2,5-	190.0	231.18
2,4-	223.5	225.36
3,5-	217.7	177.26
3,4-	299.1	196.57
<i>Streptomyces</i> , 250 U/L		
Without dichlorophenol	2.17	61.47
With the addition of dichlorophenol:		
2,6-	2.27	121.70
2,3-	2.08	72.95
2,5-	2.25	81.93
2,4-	2.15	59.21
3,5-	3.92	55.59
3,4-	24.89	60.49
<i>Pseudomonas</i> , 250 U/L		
Without dichlorophenol	0.61	28.27
With the addition of dichlorophenol:		
2,6-	0.67	51.17
2,3-	0.67	39.49
2,5-	0.69	34.69
2,4-	0.63	27.27
3,5-	0.66	21.16
3,4-	0.99	29.46
<i>Cellulomonas</i> , 250 U/L		
Without dichlorophenol	0.84	13.44
With the addition of dichlorophenol:		
2,6-	0.53	32.01
2,3-	0.42	26.40
2,5-	0.51	25.05
2,4-	0.45	19.30
3,5-	0.33	14.99
3,4-	0.37	17.84

10^{-4} M), *Pseudomonas fluorescens* (0.61×10^{-4} M), and *Cellulomonas* (0.84×10^{-4} M). The addition of 3,4-dichlorophenol and 3,5-dichlorophenol increased the K_m of *Streptomyces* from 2.17×10^{-4} to 24.89×10^{-4} and 3.92×10^{-4} M, respectively. The apparent K_m value of *Streptomyces* was increased from 2.17×10^{-4} M to 4.26×10^{-4} , 11.29×10^{-4} , 24.89×10^{-4} , and 31.77×10^{-4} M when 3,4-dichlorophenol at concentrations of 1, 3, 5, and 7 mmol/L, respectively, was added, whereas the V_{max} was not changed ($\sim 60 \times 10^{-3}$ M/min). In contrast, 2,6-dichlorophenol, 2,3-dichlorophenol, 2,5-dichlorophenol, and 2,4-dichlorophenol failed to produce an effect on the K_m of *Streptomyces* (Table 1). The addition of 3,4-dichlorophenol increased the K_m of *Brevibacterium* enzyme (299.1×10^{-4} M) and *Pseudomonas fluorescens* (0.99×10^{-4} M), but the other isomers of dichlorophenol did not significantly alter their K_m values. For *Cellulomonas* enzyme, the apparent K_m values were decreased but the V_{max} values were increased by the addition of various dichlorophenol isomers. Furthermore, the V_{max} values of the four enzymes were increased by the addition of 2,6-dichlorophenol.

Linearity of Cholesterol

Figure 1 shows the linearity of the cholesterol assay using reagents containing *Brevibacterium* (Fig. 1A), *Streptomyces* (Fig. 1B), *Pseudomonas fluorescens* (Fig. 1C), and *Cellulomonas* (Fig. 1D) cholesterol oxidase. Using the sample to *Brevibacterium* cholesterol reagent ratio of 1:100, the absorbance change per minute was small and linear up to only 7.8 mmol/L. To increase the reaction rate and extend the linearity, we increased the sample-to-reagent ratio to 1:20 and 1:10. These changes in sample-to-reagent ratio extended the linearity up to 10.4 and 20.7 mmol/L, respectively. The ratio of 1:10 gave the higher sensitivity (slope of reaction curve as 0.0163). The reportable range of cholesterol obtained from *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* was 0.5 to 2.6 mmol/L, 0.5 to 2.1 mmol/L, and 0.5 to 2.1 mmol/L, respectively. The addition of 3,4-dichlorophenol (5 mmol/L) in *Streptomyces* cholesterol reagent extended the linearity of the method from 2.6 mmol/L to 13.0 mmol/L (Fig. 1B).

DISCUSSION

The K_m , a characteristic of a particular enzyme, represents the substrate concentration in moles per liter when the initial velocity is $1/2 V_{max}$. Its value depends on the system investigated, but does not depend on enzyme concentration (11). V_{max} is not a fundamental property of an enzyme, because it depends on the enzyme concentration. It has long been recognized that

Fig. 1. Linearity of the cholesterol standard obtained from a working cholesterol reagent containing 250 U/L of *Brevibacterium* (A), *Streptomyces* (B), *Pseudomonas fluorescens* (C), or *Cellulomonas* cholesterol oxidase (D).

the K_m value can be used to compare the binding of the same substrate with similar enzymes from different sources (12). The low K_m indicates that the substrate binding affinity is high (11).

To measure cholesterol by the kinetic method, the cholesterol concentration in a reaction mixture must be less than the K_m of cholesterol oxidase. We determined the K_m and V_{max} of enzymes isolated from *Brevibacterium*, *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas*. *Nocardia* and *Schizophyllum commune* were not included in our study because of their high cost and the lack of competitive inhibitors necessary to increase the K_m of the *Nocardia* enzyme (4). *Brevibacterium* cholesterol oxidase gave the highest K_m value. The substrate concentration (0.02 – $0.2 K_m$) used in the K_m determination was not in the appropriate substrate range (0.33 – $2.0 K_m$), as suggested by Segel

(12); therefore, we could only approximate its K_m value. To obtain the appropriate substrate range, the concentration of cholesterol standard should be 500 mmol/L, which is beyond the solubility of cholesterol in ethanol.

The K_m value of *Brevibacterium* (230.3×10^{-4} M) is too high for clinical laboratory determination of cholesterol by conventional analyzers. The absorbance change per minute of cholesterol reaction is low (Fig. 1A) and insensitive compared to cholesterol reactions obtained from the other sources (Fig. 1B–D); however, using a higher cholesterol concentration would extend the linearity. With a sample to the *Brevibacterium* reagent ratio of 1:20 and 1:10, the linearity of cholesterol was extended up to 10.4 and 20.7 mmol/L, respectively (Fig. 1A). A sample-to-reagent ratio of 1:10 appears to be best.

For *Streptomyces*, the K_m value of 2.17×10^{-4} M in our study was within the range (1.5 to 6.7×10^{-4} M) reported by other studies (13,14), but the K_m of *Pseudomonas fluorescens*, 0.61×10^{-4} M, was higher than previously reported (0.47×10^{-4} M) by the manufacturer (Amano Enzyme USA Co., Ltd., Lombard, IL). Slight differences in the K_m may be due to the differences among species. Moreover, the experimental conditions, such as pH, ionic strength, temperature, inhibitor, or activator, may affect the binding between the enzyme and substrate (12). The K_m value of *Cellulomonas* (0.84×10^{-4} M) is similar to that of *Pseudomonas fluorescens* (Table 1).

The K_m values of *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* were too low to achieve the first-order kinetic reaction over the clinically important range of serum cholesterol. The cholesterol linearities obtained from these reagents were only 2.6, 2.1, and 2.1 mmol/L, respectively (Fig. 1). These linearities were unsuitable for detecting cholesterol as normally present in human serum. To increase the K_m of the enzyme, we studied the effect of six dichlorophenol isomers on *Brevibacterium*, *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* (Table 1). Only 3,4-dichlorophenol significantly raised the apparent K_m value of *Streptomyces*, raising it more than 11-fold (2.17×10^{-4} to 24.89×10^{-4} M). Moreover, increasing the concentrations of 3,4-dichlorophenol gave a higher K_m and the same V_{max} as *Streptomyces*. Therefore, our study confirms the results of Deeg and Ziegenhorn (9) that 3,4-dichlorophenol is an ideal competitive inhibitor for *Streptomyces* enzyme (9). Because of the turbidity of the *Streptomyces* cholesterol reagent caused by 3,4-dichlorophenol at 7 mmol/L, we selected the 3,4-dichlorophenol concentration at 5 mmol/L as a more optimal concentration. Using 5 mmol/L of 3,4-dichlorophenol increased the linearity from 2.6 to 13.0 mmol/L as shown in Fig. 1B, which makes the enzyme suitable for measuring cholesterol in serum or plasma.

The characteristics of *Cellulomonas* enzyme differed from the other experimental enzymes. The addition of all dichlorophenol isomers produced slightly decreased K_m values (Table 1). Moreover, their isomers appeared to be the activator of *Cellulomonas* enzyme, since the reaction rates of the enzyme were increased. Our results also show that the reaction rates of all of the experimental cholesterol oxidases were increased by the addition of 2,6-dichlorophenol (Table 1). Therefore, 2,6-dichlorophenol may be an activator of the four enzymes.

The K_m values of *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* were low; however, the K_m value of *Streptomyces* was increased by the addition of 3,4 dichlorophenol. Cholesterol oxidase isolated from

Pseudomonas fluorescens and *Cellulomonas* are not suitable for kinetic cholesterol determination in human serum because of their too-low K_m values and limited linearity (0.5–2.1 mmol/L). Because the enzymes from *Brevibacterium* and *Streptomyces* (with the addition of 3,4 dichlorophenol) provide high K_m values and linearity up to high cholesterol concentrations, they are suitable for cholesterol assay by the kinetic method in human serum.

In recent years, different investigators have reported the determination of free and esterified cholesterol in food and biological fluid samples using the immobilized cholesterol oxidase sensors (15,16). Portable or implanted cholesterol oxidase type sensors require a linear response and a fast response time. It has long been recognized that the kinetic methods can quickly determine the analyte fast enough to obtain a steady response. Our results show that the detection limit depends on the source of the cholesterol oxidase. For *Brevibacterium*, the high K_m implies that the enzyme could tolerate a high cholesterol concentration, which would be encountered at sample-to-reagent ratio of 1:5 or 1:10 (close to the rate expected for an in vivo sensor), and thus cholesterol oxidase from *Brevibacterium* may be the best enzyme choice for in vivo sensors.

Streptomyces, *Pseudomonas fluorescens*, and *Cellulomonas* may be potentially good sources for cholesterol determination in biological materials (e.g., biological tissue extracts) with a low concentration of cholesterol, or for the remnant lipoprotein-cholesterol assay.

CONCLUSIONS

The cholesterol oxidase isolated from *Brevibacterium*, *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* exhibits different properties. The K_m of *Brevibacterium* enzyme is high, while those of *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* enzyme are low. However, 3,4 dichlorophenol, a competitive inhibitor, can be used to elevate K_m value of *Streptomyces*. We suggest that *Brevibacterium* in a sample-to-reagent ratio of 1:10 and *Streptomyces* (with the addition of 3,4-dichlorophenol) in a sample-to-reagent ratio of 1:100 may be considered to be appropriate sources of cholesterol oxidase for the kinetic cholesterol assay in human serum. Because the K_m values of *Pseudomonas fluorescens* and *Cellulomonas* cholesterol oxidase are too low, they are inappropriate sources for serum cholesterol determination by the kinetic method. These sources may be used if a suitable competitive inhibitor can be found that would raise the K_m value to a suitable level.

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