Performance Characteristics of Cholesterol Oxidase for Kinetic Determination of Total Cholesterol

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ABSTRACT

Not much is known concerning the properties (Michaelis constant, K_m and maximum velocity, V_{max}) of commercial enzyme for the kinetic cholesterol method. We measured the K_m and V_{max} of *Brevibacterium* (E1), *Streptomyces* (E2), *Pseudomonas fluorescens* (E3), and *Cellulomonas* (E4) cholesterol oxidase. The apparent K_m of four enzymes in cholesterol reagents with and without the addition of dichlorophenol isomers, 5 mmol/L are shown in the Table. *Brevibacterium* gave the highest K_m value (230.3x10^{-4} M), followed by *Streptomyces* (2.17X10^{-4} M), *Cellulomonas* (0.84x10^{-4} M) and *Pseudomonas* (0.61x10^{-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. In order to increase the enzyme's K_m, we studied the effect of six dichlorophenol isomers, acting as inhibitors. Likewise, 2,6-dichlorophenol significantly raised the apparent K_m value of *Streptomyces*. Addition of 1, 3, 5 and 7 mmol/L of dichlorophenol increased the K_m of the enzyme from 2.17x10^{-4} M to 4.26x10^{-4}, 11.29x10^{-4}, 24.89x10^{-4} and 31.77x10^{-4} M, respectively. Up to 2 mmol/L 2,6-dichlorophenol, 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 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.

The four cholesterol oxidases are different. The K_m of *Brevibacterium* is high while those of *Streptomyces, Pseudomonas fluorescens* and *Cellulomonas* are low. *Brevibacterium* in a sample to regent ratio of 1:10 and *Streptomyces* with the addition of 2,6-dichlorophenol in a sample to reagent ratio of 1:100 may be considered as appropriate sources of cholesterol oxidase useful for the kinetic cholesterol assay in human serum.

	E1	E2	E3	B 1
Without dichlorophenol	**230.3**	**2.17**	**0.61**	**- + 5 1**
With the addition of:				
/) 3 * aphenoli l	208.1	2.27	0.67	0.53
/) 0 * aphenoli l	161.6	2.08	0.67	- + 1 /
/) 2 * aphenoli l	190.0	2.25	0.69	0.51
/) 1 * aphenoli l	223.5	2.15	0.63	- + 1 2
0) 2 * aphenoli l	217.7	3.92	0.66	0.33
0) 1 * aphenoli l	299.1	/ 1 + 5 6	0.99	0.37

INTRODUCTION

Enzymatic cholesterol assay uses cholesterol esterase to hydrolyze cholesterol esters producing free cholesterol, which is oxidized by cholesterol oxidase (COD) (1). Hydrogen peroxide is catalyzed to create a colored product, which is usually measured by the endpoint method. The kinetic method may offer advantages over the endpoint assay, such as short analysis time, less interfering substances and no requirement for sample blank measurement (2,3).

There are several sources of COD, such as those isolated from *Nocardia erythropolis, Streptomyces, Brevibacterium, Pseudomonas fluorescens,* and *Cellulomonas*. Deeg and Ziegenhorn (8) found *Nocardia* COD unsuitable for the kinetic cholesterol determination because its K_m was too low. They also lacked competitive inhibitors to elevate the K_m value (8). They concluded that *Streptomyces* was the only enzyme suitable for the kinetic method.

This study investigates the performance characteristics of *Streptomyces, Pseudomonas fluorescens, Brevibacterium* and *Cellulomonas* enzyme for cholesterol determination by the kinetic method.

MATERIALS AND METHODS

1. Apparatus

We determined reaction rates of COD by using the Vitalab Selectra analyzer (E. Merck Dramstadt, Germany).

2. Reagents

Cholesterol oxidase K_m. We prepared the reagents containing 10000 U/L of peroxidase, 3 mmol/L of sodium cholate, 4-aminoantipyrine, 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. Four sets of working reagents were prepared by adding each enzyme, 250 U/L. To study the effect of 2,6-dichlorophenol on K_m of *Streptomyces*, the set of working reagents was prepared containing 2,6-dichlorophenol of 0, 1, 3, 5 or 7 mmol/L.

Cholesterol standard. We prepared the cholesterol standards , 25.9, and 50.0 mmol/L in absolute ethanol containing Triton X-100 (20% v/v).

3. Procedures

Cholesterol oxidase K_m. The parameters of Vitalab Selectra were set as following: Reaction mode: kinetic, Delay: 12 sec, Wavelength: 505 nm, Sample and Reagent volumes: *Streptomyces* (3 and 297 µL), *Pseudomonas fluorescens* (3 and 297µL), *Brevibacterium* (30 and 270µL), or *Cellulomonas* (2 and 398 µL), Factor: 73.529 (calculated from inverse of the quinonemine milimolar extinction coefficient of 13.6). We performed the initial rates of enzyme by multiplying the absorbance changed per minute with factor. The K_m, and V_{max} of each COD were calculated by using the Enzfitter program (Elsevier Biosoft, Cambridge CB2 1LA,UK).

Linearity. The method parameters were set as mentioned above, except the sample and the 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.

RESULTS

1. Km of various COD enzymes

The *Brevibacterium* COD gave the highest K_m value (230.3x10^{-4} M), followed by the K_m of *Streptomyces* (2.17x10^{-4} M), *Pseudomonas fluorescens* (0.61x10^{-4} M) and *Cellulomonas* (0.84x10^{-4} M). Table 1 When 2,6-dichlorophenol increased the K_m of *Streptomyces*. The apparent K_m value was increased from 2.17x10^{-4} M to 4.26x10^{-4}, 11.29x10^{-4}, 24.89x10^{-4} and 31.77x10^{-4} M at the concentration of 1, 3, 5 and 7 mmol/L was added, respectively, whereas the V_{max} was not changed (around 60x10^{-3} M/min). On the contrary other isomers failed to produce an effect on the K_m of *Streptomyces* (Table 1). All dichlorophenol did not significantly alter *Brevibacterium* and *Pseudomonas fluorescens* K_m values. For *Cellulomonas* enzyme, the apparent K_m values were decreased but the V_{max} were increased by the addition of various dichlorophenol isomers. Furthermore, the V_{max} of the four enzymes were increased by 2,6-dichlorophenol.

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.

Choesterol Oxidase Source	Kinetic Property of Enzyme K_m (10^{-4} M)	V_{max} (10^{-3} M/min)
***Brevibacterium*, 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
***Streptomyces* , 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**
***Pseudomonas*, 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
***Cellulomonas*, 250U/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

2. Linearity of cholesterol

Figure 1 shows the assay linearity obtained from *Brevibacterium* (1A), *Streptomyces* (1B), *Pseudomonas* (1C), and *Cellulomonas* COD (1D). Using the sample to *Brevibacterium* 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 and reagent ratio to 1:20 and 1:10. The linearity was extended up to 10.4 and 20.7 mmol/L, respectively. The reportable range of cholesterol obtained from *Streptomyces, Pseudomonas* 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. Addition of 2 mmol/L 2,6-dichlorophenol (5 mmol/L) in *Streptomyces* reagent extended the linearity from 2.6 mmol/L to 13.0 mmol/L (Figure 1B).

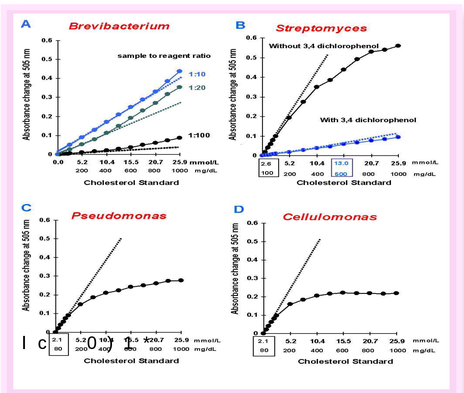

Figure 1. Linearity of cholesterol standard obtained from the working cholesterol reagent containing Brevibacterium (A), Streptomyces (B), Pseudomonas fluorescens (C), and Cellulomonas cholesterol oxidase (D), 250U/L.

DISCUSSION

Brevibacterium COD gave the highest K_m value, which is too high for clinical determination of cholesterol by conventional analyzers. The reaction rate is low (Figure 1 A) and insensitive when compared to cholesterol reactions obtain from the other sources (Figure 1B). Increasing the sample concentration would extend the linearity (Figure 1A). A sample to reagent ratio of 1:10 appears the best.

The K_m values of *Streptomyces, Pseudomonas fluorescens* and *Cellulomonas* COD were too low. The linearity obtained from these reagents was unsuitable to detect cholesterol normally present in human serum (Figure 1). In order to increase the K_m of enzyme, we studied the effect of six dichlorophenol isomers on each enzyme. Likewise, 2,6-dichlorophenol significantly raised the apparent K_m value of *Streptomyces* raising it more than 11 fold. Moreover, increasing its concentrations gave the higher K_m and the same V_{max} of *Streptomyces*. Therefore, our study confirms the results of Deeg and Ziegenhorn that 2,6-dichlorophenol is an ideal competitive inhibitor for *Streptomyces* enzyme (8). We selected at 5 mmol/L as a more optimal concentration, which increased the linearity from 2.6 to 13.0 mmol/L.

The addition of all dichlorophenol isomers gave slightly decreased in the K_m values of *Cellulomonas* enzyme (Table 1). Moreover, their isomers seem to be the activator of enzyme. Our results also showed that 3,4-dichlorophenol might be an activator of the four enzymes.

The K_m values of *Streptomyces, Pseudomonas* and *Cellulomonas* COD were low, except for that of *Streptomyces*, which was increased by 2,6-dichlorophenol. *Pseudomonas fluorescens* and *Cellulomonas* COD are not suitable for the kinetic cholesterol determination in human serum because of their too low Km values and limited linearity (0.5 to 2.1 mmol/L).

CONCLUSION

The properties of COD isolated from *Brevibacterium, Streptomyces, Pseudomonas fluorescens* and *Cellulomonas* are different. 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, could be used to elevate K_m value of *Streptomyces*. We suggest that *Brevibacterium* in a sample to regent 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 as appropriate sources of COD useful for the kinetic cholesterol assay in human serum.

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