Fast Determination of the Quality of Cyt c Products Through the Mimic Peroxidase Activity Assaying

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Citation

Abstract
As cytochrome c (Cyt c) contained heme cofactors that could perform the oxidation-reduction reactions, or Cyt c could play the role of peroxidase, the assaying of its mimic peroxidase activity was used to determine the quality of Cyt c products. When the substrate was in excess, that the initial velocity of enzymatic reaction was remarkable linear proportional to Cyt c concentration was demonstrated. The regression equations of three purified products between Cyt c concentration and their catalytic speed (absorbance at 426 nm) were measured and compared to that of the standard Cyt c using their slope or catalytic speed, which was used to determine the quality of Cyt c products. If the quality index of the standard was 1 (100%), the quality index of three Cyt c products was 66.46% (sample 1), 49.46% (sample 2) and 40.43% (sample 3, semi-finished purified product) respectively. The experimental results demonstrated that this test was a rapid and microanalysis method that was available to judge the quality of Cyt c products.

1. Introduction

Cytochrome c (Cyt c) is an activator for cellular respiration in organisms, belonging to the medicine to improve cell metabolism [1-3]. So it is mainly used for emergency and auxiliary treatment of tissue hypoxia in clinical [4-6]. The determination of Cyt c activity is very important for judge the quality of Cyt c products during the production of Cyt c medicine. The traditional method to determine the activity of Cyt c based on the rate Cyt c can be restored as the enzyme, mainly from Paul [7] in 1948, has long been adopted, and not changed. The researchers also try to make improvements to the method, but there are still some limitations [8, 9]. Theoretically, for assessing the quality of the finished Cyt c products or purified Cyt c, it could be performed by measuring the purity or bioactivity of Cyt c products. However, whatever for the purity or the activity, the determination methods are relatively cumbersome, large sample consumption and also higher cost. An effective and rapid method is needed for judging the quality of Cyt c products during the process of Cyt c medicine.

As Cyt c contains heme cofactors [10-12] that can perform the oxidation-reduction reactions, so it can play the role of peroxidase [13]. The decomposition of hydrogen peroxide by Cyt c catalysis could make o-phenyldiamine become the 2, 3-Diaminophenazine [14], the catalyzed reaction is as the following.

![Reaction Diagram](1)
This reaction (1) can be expressed as \( \text{M} + \text{N} \xrightarrow{\text{E}} \text{P} + \text{Q} \). When the other conditions are fixed, the reaction rate equation is as follows,

\[
\frac{d[N]}{dt} = k \ [M] \ [N] \ [E] \tag{2}
\]

In the initial reaction, the concentration of M and N are made in excess, so the reaction rate depends on the concentration of catalyst Cyt c (E) \[15\],

\[
\frac{d[N]}{dt} = k_1 \ [E] \tag{3}
\]

When the absorbance (A) of product P or Q is known, the concentration of Cyt c can be calculated. The reaction is terminated at a particular time (t), the following reaction equation can be established,

\[
A = k_1 \ \varepsilon \ L \ [E] \ t = k_2 \ [E] \ t = k_3 \ [E] \tag{4}
\]

Equation (4) explains the proportional relationship between the enzymatic reaction rate and enzyme concentration. In equation (1), the maximum optical absorption of product 2, 3-diaminophenazine is at 426 nm \[14, 16\], so the standard curve can be produced according to the reaction of equation (1) and the absorbance at 426 nm for determining the contents of Cyt C. In comparison to the standard curve, the quality of Cyt c products can be judged by measuring their catalytic rate in the same content.

Based on the mechanism of Cyt c as a mimic enzyme that can play the role of a peroxidase, several Cyt c products were prepared in this study and the practice for determining the quality of Cyt c product was performed. Therefore, the aim of this study is to probe or establish an available method for quickly and effectively determining the quality of Cyt c products.

2. Materials and Methods

2.1. Materials

Pig heart was from the local market. Cyt c as the standard sample was purchased from Sigma. Permutite, AmberliteIR-50-NH₄, hydrogen peroxide, o-phenylenediamine, trichloroacetic acid (TCA), sodium chloride, ammonium sulfate and all other chemicals for buffers were of analytical or chemical grade and were purchased from Guangzhou Chemical Co. Ltd. (Guangzhou, China).

2.2. Preparation of Cyt c Products

The pieces of fresh pig heart tissue were repeatedly frozen and thawed three times after having been frozen at \(-18\)°C for three days, 200 g of them mixed with 800 mL deionized water with pH 4.0 was homogenized and extracted by stirring slowly for 60 min. After the sample solution was adjusted to pH 6.0, it was centrifuged at 3500 rpm for 10 min to obtain the supernatant. After being adjusted to pH 7.2 for precipitating the other proteins, this supernatant was filtered through four layers of gauze on funnel. The filtrate was mixed with the permutite according to 20/1 (V/W) and stirred slowly for 40 min, then the permutite became red, which explained that the Cyt c were adsorbed into the permutite\[17, 18\]. The supernatant was discarded.

The red permutite was put into a column to make a “chromatograph column”, which was washed fully with deionized water, 0.2% sodium chloride and again deionized water respectively at a flow rate of 60 mL per hour. The eluting solution 30% ammonium sulfate was used to elute the column and the red elution was collected. Solid ammonium sulfate was added to the red eluent with saturation of 67% for precipitating the other proteins, the supernatant was obtained after centrifuged at 3500 rpm for 10 min and was mixed with 20% TCA according to 100/8 (supernatant/TCA) to precipitate the Cyt c protein. When the red Cyt c was precipitated, the centrifugation at 3500 rpm for 10 min was done immediately and the Cyt c precipitated was obtained. These proteins were dissolved with deionized water and were dialyzed against deionized water. The Cyt c solution sample was concentrated and dried by vacuum cold drying apparatus (Heto, High Technology of Scandinavia, Denmark). This dried product was the semi-finished purified Cyt c product.

For further purification, the semi-finished purified product dissolved in deionized water was loaded to a small and long Ion Exchange Chromatography column (0.5×10 cm) made by AmberliteIR-50-NH₄. Deionized water was as an equilibrium solution and 0.06 mol/L Na₂HPO₄-0.4 mol/L NaCl as an eluant. The speed of sample loaded and eluted was less than 0.2 mL per min. The purified product of Cyt c was obtained after dialyzed against deionized water and dried by vacuum cold drying apparatus.

2.3. Determination of the Quality of Cyt c Products

Preparation of the standard Cyt c sample, 50 mg of Cyt c that was purchased from Sigma was dissolved in 20 mL water. Three different samples of purified Cyt c product, among which one was the semi-finished purified product marked as sample 3, were made with the same method as that as the standard Cyt c sample. Two milliliter of 20 mol/L o-phenylenediamine was put in a cuvette path length 1 cm, a certain amount of the standard Cyt c or Cyt c products was added in this cuvette and after 1 mL of 20% hydrogen peroxide was blended, the O.D. was measured at the time of 1 minute reaction. The Cyt c sample solution of 5, 10, 15, 20, 25, 30 µL were adopted respectively in these tests.

Every experiment was repeated three times. Mathematical statistics was performed to the obtained experimental data. The regression equations of three purified products between Cyt c concentration and absorbance were made using the software Excel and their slopes or catalytic speeds were compared to that of the standard Cyt c.
3. Results and Discussion

The measurement results of all reactions for probing the quality of different samples of Cyt c product was shown in Table 1. According to Table 1, the relationship lines between the content of Cyt c and their absorbance was shown in Figure 1.

Table 1. Absorbance (O.D. 426) of standard and samples with different Cyt c concentration at the time of 1 minute reaction (n=3).

<table>
<thead>
<tr>
<th>Cyt c V (µL)</th>
<th>W (µg)</th>
<th>Standard</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>12.5</td>
<td>0.451</td>
<td>0.308</td>
<td>0.315</td>
<td>0.216</td>
</tr>
<tr>
<td>10</td>
<td>25.0</td>
<td>0.669</td>
<td>0.479</td>
<td>0.419</td>
<td>0.291</td>
</tr>
<tr>
<td>15</td>
<td>37.5</td>
<td>0.894</td>
<td>0.609</td>
<td>0.520</td>
<td>0.421</td>
</tr>
<tr>
<td>20</td>
<td>50.0</td>
<td>1.161</td>
<td>0.760</td>
<td>0.639</td>
<td>0.486</td>
</tr>
<tr>
<td>25</td>
<td>62.5</td>
<td>1.358</td>
<td>0.921</td>
<td>0.779</td>
<td>0.583</td>
</tr>
<tr>
<td>30</td>
<td>75.0</td>
<td>1.598</td>
<td>1.085</td>
<td>0.873</td>
<td>0.680</td>
</tr>
</tbody>
</table>

Figure 1. Regression equations between absorbance and content of standard and samples Cyt c according to Table 1.

As shown in Fig. 1, the remarkable linear relationship between concentration and absorbance of Cyt c, especially for the standard Cyt c, was obtained in this measurement method. The correlation coefficient \( R^2 \) was 0.9990 for the standard, and the samples was 0.9988, 0.9971 and 0.9950, respectively, which explained the feasibility of this method. By comparison among the regression equations of these samples, the slope of the standard was the maximum 0.2305, and the slope of three samples was 0.1532, 0.1140 and 0.0932 respectively. From these slope of the lines, although the weight of samples including the standard Cyt c was the same, the catalytic reactions at different speeds were easily understood. Obviously, the slope was lower or the catalytic rate was slower, the quality of Cyt c was lower. Among the Cyt c products in this experiment, the quality of the sample 1 was the best and the sample 3 the semi-finished purified product was poorer than any other.

The gap of catalytic speed between the samples and the standard could be caused by that the purity of Cyt c protein in the samples was not enough as that of standard Cyt c, or the actual content of Cyt c in the samples were less than in the standard although they were in the same weight. The other one was that the activity of Cyt c protein in the samples had changed to weaker. Therefore, the gap of slope or catalytic speed between the samples and the standards reflected actually the comprehensive quality of the sample Cyt c products comparing to the standard. Obviously, the lower quality of these Cyt c products compared to the standard Cyt c was due to some cases arisen during the process of Cyt c extracting and purification, for example, denaturation or poor-purity of Cyt c, or maybe the process of Cyt c product adopted had some defects.
In this experiment, if the slope of standard Cyt c was 100, expressed by S2, the slope of the samples was expressed by S1, the comprehensive quality of every sample could be expressed as,

\[
\text{Quality index} = \frac{S1}{S2} \times 100\% \quad (5)
\]

By calculation, the quality index of three samples in this measurement was 66.46% (sample 1), 49.46% (sample 2) and 40.43% (sample 3, semi-finished purified product) respectively according to figure 1, was obviously lower than that of standard Cyt c. In fact, the quality index expressed in this method explained the function of Cyt c protein. So it could reflect the comprehensive quality or the actual quality of the Cyt c products.

This test was a rapid and microanalysis method, to eliminate systematic errors and repeat the experiment several times were demanded for measuring correctly and guaranteeing the accuracy of the experimental results. Obviously, this method had its advantages for determining the quality of Cyt c product.

4. Conclusions

Based on the initial velocity of mimic peroxidase catalysis of Cyt c, the regression equations of three purified products between Cyt c concentration and their catalytic speed (absorbance at 426 nm) were measured and compared to that of the standard Cyt c using their slope or catalytic speed. If the quality index of the standard was 1 (100%), the quality index of three Cyt c products was 66.46% (sample 1), 49.46% (sample 2) and 40.43% (sample 3, semi-finished purified product) respectively. The experimental results demonstrated that this test was available to judge the quality of Cyt c. This method possessed the property of rapid and microanalysis.

References


[8] Chen, Y. K., Huang, DY., Qiang, YF., Wang, MS.,(2003), Study on Quick Determination of Cytochrome C [J], Journal of Shanxi University, 26, 1, 64-66.


