Analytical Methods

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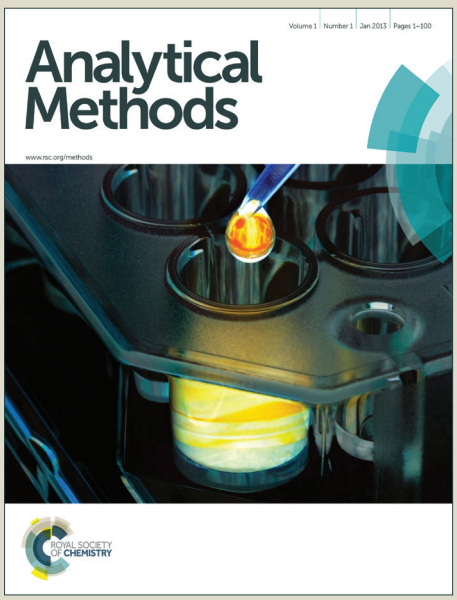

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A New Spectrophotometric Assay for Measuring Pyruvate Dehydrogenase Complex Activity: A Comparative Evaluation

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Pyruvate dehydrogenase complex (PDHc) plays a key role in pyruvate decarboxylation, the transformation of pyruvate into acetyl-CoA. In this study, a new assay for measuring PDHc activity has been developed. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is adopted as the electron acceptor in this method, and the absorbance change caused by the reduction of MTT by hydroxyethyl-TPP, the catalyzed product of pyruvate dehydrogenase (PDH, E1), within a certain period of time is measured to reflect the PDHc activity. For a further impartially evaluation on the characteristics of the newly developed assay, a series of comparative studies was also conducted with several commonly used assays on PDHc activity, including the conventional spectrophotometric assay of NADH accumulation, the p-iodonitrotetrazolium violet (INT) - coupled assay, the 2, 6-dichlorophenolindophenol (2, 6-DCPIP) assay, and the potassium ferricyanide assay. Results have proved that the spectrophotometric assay using MTT is highly sensitive and low-cost, with little interference from the sulfhydryl compound. In conclusion, the newly established assay is applicable for activity measurement not only in purified PDH and PDH in purified PDHc, but also in crude PDH solution prepared under certain conditions, hence the assay can indirectly reflect the PDHc activity.

Introduction

As an important member in the α-ketoacid dehydrogenase complex family, pyruvate dehydrogenase complex (PDHc) is found in mitochondria from a wide range of organisms, including microorganisms, mammals and higher plants. PDHc consists of pyruvate dehydrogenase (PDH, E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3), as well as pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase, which are responsible for the activity modulation. Besides, protein X of unknown function and some cofactors are also component of the complex. As one of three apoenzymes in PDHc, PDH is a thiamine pyrophosphate (TPP) - dependent decarboxylase, which catalyzes hydroxyethyl-TPP generation via pyruvate decarboxylation. In the subsequent step, with the effect of E2, hydroxyethyl group is oxidized into an acetyl group, following by a translocation to lipoamide, the cofactor of E2. For the activity in allosterism of E1, TPP and Mg^{2+} are essential cofactors. [1-2] Related studies have shown that PDHc plays an indispensable part in the glucose metabolism, which is vital for living organisms. [3-4] Therefore, it is of great importance to explore accurate yet convenient assays for measuring PDHc activity.

To date, assays for measuring PDHc activity have been reported were almost established in

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1950's to 1960's. According to the basic principle they originated, these methods can be divided into two categories: spectrophotometry and radioisotope method. The standard spectrophotometry to determine the formation rate of reduced coenzyme I (NADH) is applicable only for PDHc with high purity. [5-7] Another limitation to this method should be ascribed to the negative feedback inhibition by the product of the PDHc catalyzed reaction and the high cost of necessary reagents. Other commonly used spectrophotometric assays for measuring PDHc activity include the ferricyanide assay and the 2, 6-dichlorophenolindophenol (2, 6-DCPIP) assay. [5-8] As for the radioisotope method, it is mainly used for E1 enzyme activity measurement in the crude enzyme solution by measuring the amount of ^{14}C-labeled CO_2 generated.[9]

It is undoubted that measuring PDHc activity is critical in the study of its nature, gene cloning, separation, purification, identification and other related research topics. Unfortunately, there are deficiencies from various aspects in existing methods. Therefore, we report in this article the phenazine methosulfate-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (PMS-MTT) assay, for an accurate measurement of the PDHc activity. MTT is employed as an electron acceptor in the assay to measure the enzyme activity by detecting the absorbance change of the PMS-MTT complex at 566 nm. By comparison with various established methods, a conclusion is reached that the PMS-MTT assay can effectively measure the PDHc activity while keep away from drawbacks of other methods.

Materials and methods

Materials- Reagents and buffer

Pyruvate dehydrogenase complex (PDHc) from pig's heart, pyruvate dehydrogenase (PDH) from lactic acid bacteria, CoASNa, NADH, TPP, MTT, PMS, $MgCl_2$, pyruvic acid were all purchased from Sigma-Aldrich, and all other chemicals are of analytical grade.

The homogenate buffer was prepared by a mixture of 50 mM tris-HCl (pH 8.0), 0.7 M sucrose, 2 mM EDTA, 57 mM β-mercaptoethanol, and 0.5% (W/V) BSA. Buffer B refers to the 50 mM potassium phosphate buffer (pH 8.0).

Enzyme pretreatment

Both the PDH from lactic acid bacteria and PDHc from pig's heart were diluted to a certain concentration with 50 mmol potassium phosphate buffer (pH 7.1) containing 20% glycerol. No sulfhydryl protecting agent was added in the enzyme pretreatment process in order not to affect the enzyme activity measurement. In the following crude PDH preparation, all centrifugation were carried out at 4 ℃ unless otherwise stated, and the ultrosonication were conducted at 40% of full intensity for every 10 s with an interval of 20 s in between.

Preparation of the crude PDH from pea mitochondria

Full pea seeds were soaked in water overnight, and then evenly distributed on the sieve. Pea seeds were grown in dark with water spray for 4 to 5 times a day to keep the seed moisturized. The sprouts were collected and their roots were removed until their height reached 20 to 30 cm, and then every 100 g sprouts were rinsed with deionized water before being homogenated in 100 ml precooled homogenization buffer. The filtrate after homogenization was centrifuged at 2, 000 g for 20 minutes, and then the supernatant was again centrifuged at 30, 000 g for 40 minutes. The precipitates thus obtained was resuspended in the homogenization buffer, and washed twice under the same condition described above in DI water and once in cold acetone, subsequently. Mitochondria, the final precipitate obtained, is stored at -20 ℃.[10] Before activity measurement, the lyophilized

mitochondria powder was resuspended in buffer B to reach a concentration of 20 mg/ml, before being broken with ultrasonication in ice-bath for 40 minutes. Then after centrifugation at 20, 000 g for 30 minutes, the supernatant was used for enzyme activity measurement.

Preparation of crude PDH from *E. coli*

E. coli was cultivated in LB medium at 30 ℃ overnight before centrifugation at 8, 000 g and the collected bacteria was washed once with buffer B. Bacteria cells from every 100 ml culture medium was resuspended in 15 ml buffer B, prior to disruption with a discontinuous ultrasonication, i.e., every 10-second ultrasonication at an intensity of 40% followed by a 20-seconds pause, in ice-bath for 40 minutes. After centrifugation at 20, 000 g for 30 min, the supernatant was used for enzyme activity measurement.

Measuring PDHc activity

For each of the following methods in PDHc activity measurement, at least ten tests were performed on different samples, and data shown in the result part represent the mean of ten tests.

Standard NADH assay using CoA

The method is based on the determination of the formation of NADH, the common catalyzed product of three enzymes in the PDHc, which can result an increase in the absorbance at 340 nm. The protocol is slightly modified with reference to methods reported by Schwartz et al. and Nemeria.[5,7] A 3 ml reaction system contains 50 mmol potassium phosphate buffer (pH 7.4), 1.0 mmol $MgCl_2$, 0.05 mmol CoASNa, 3.0 mmol cysteine hydrochloride, 0.2 mmol TPP, 2.0 mmol β-NAD, 2.0 mmol sodium pyruvate and enzyme. In the control system, enzyme was replaced by an equal volume of water, while others remained unchanged.

Iodine nitro tetrazolato-violet (INT) coupled assay

This method is based on the increase in the absorbance at 500 nm, which is caused by the INT reduction by NADH, the common catalyzed product of three enzymes in the PDHc. The protocol is slightly modified with reference to methods reported by Hinmant et al.[11] A 3 ml reaction system contains 0.6 mM INT and 6.5 μM PMS in addition to the reaction system of the coenzyme A method. Taking into account the effect of the sulfhydryl protecting agents and CoA to the method, two control systems were employed, control A started by addition of enzyme without sodium pyruvate, control B started by addition of CoASNa without enzyme. [12,13]

E1 activity measurement in the PDHc- 2, 6-DCPIP assay

In this assay, 2, 6-DCPIP is employed as the electron acceptor. By detection of the decrease in its absorbance at 600 nm, E1 activity is measured by examination of the amount of 2,6-DCPIP reduced by hydroxyethyl-TPP, the product of E1. The protocol followed that reported by Nemeria et al with certain modification.[7] A 3 ml reaction system contains 50 mmol potassium phosphate buffer (pH 7.1), 1 mmol•L^{-1} $MgCl_2$, 0.2 mmol•L^{-1} TPP, 0.1 mmol•L^{-1} DCPIP, and 2.0 mmol•L^{-1} sodium pyruvate. In four control groups employed, one of following components, enzyme, TPP, $MgCl_2$, and sodium pyruvate, was intentionally deleted, respectively. The reaction was started by addition of enzyme and the control group without TPP was taken as the reference. 1 U was defined as the amount of 1 mmol•L^{-1} 2,6-DCPIP reduced by 1 mg of the enzyme within 1 minute.

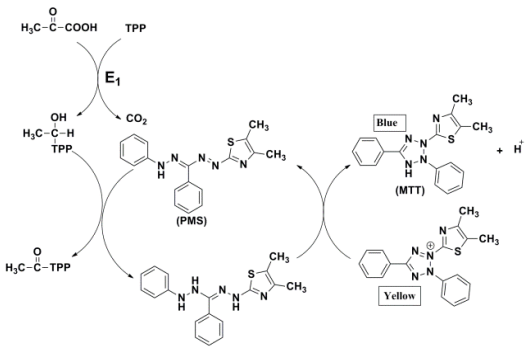
Potassium ferricyanide assay

In this assay, E1 activity is measured by the decrease in absorbance at 420 nm, which stands for the reduction of potassium ferricyanide by hydroxyethyl-TPP, the product of E1. The protocol followed that reported by Schwartz、Reed and Maldonado with slight modification.[5,6] A 3 ml reaction system contains 20 mmol Tricine buffer (pH 7.7), 1.0

mmol $MgCl_2$, 0.2 mmol TPP, 1.8 mmol potassium ferricyanide, 2.0 mmol sodium pyruvate, and the enzyme. Two replacements were made in the control group with 0.6 mmol potassium ferricyanide and inactivated enzyme while other components kept unchanged.

PMS-MTT assay

As shown in Scheme 1, in existence of the substrate, sodium pyruvate, and the cofactor, $MgCl_2$ and TPP, PMS was employed as the electron transport complex and MTT the electron acceptor. PDHc activity is measured by examination of the increase in the absorbance at 540 nm to 640 nm, which was exhibited by MTT reduced by hydroxyethyl-TPP, the catalyzed product of E1. Based on the absorbance scan of the product (Fig. 1), it was found that maximum absorbance appeared in 560 nm, and thus 560 nm was used to evaluate the PDH activity in further experiments with the PMS-MTT assay.



Scheme 1. Principle of the PMS-MTT assay.

In detail, a 3 ml reaction system contains 50 mmol potassium phosphate buffer (pH 7.1), 1 $mmol \cdot L^{-1}$ $MgCl_2$, 0.2 $mmol \cdot L^{-1}$ TPP, 0.5 $mmol \cdot L^{-1}$ MTT, 6.5 $mmol \cdot L^{-1}$ PMS, and 2.0 $mmol \cdot L^{-1}$ sodium pyruvate. In order to illustrate it is sodium pyruvate that causes the reduction of MTT and explore the requirement of PMS-MTT assay to cofactors, one of following components, enzyme, TPP, $MgCl_2$, and sodium pyruvate, was intentionally deleted, respectively, from each of four control groups. The reaction was started by addition of enzyme into the experimental group and control groups with single factor deleted (excluding the group

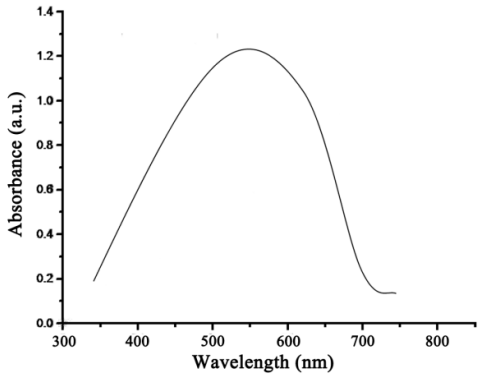


Figure 1. Visible spectra of the final product in determination PDHs activity with the PMS-MTT assay.

without enzyme) and by addition of PMS to the control without enzyme. In the first 10 minutes after reaction started, continuous examination on the change in absorbance was conducted, eliminating the possible influence on MTT reduction by any other factor. Because no change was found in OD_{566} of the control group without TPP, this group was taken as the reference to results of other groups. Hinman's method was used for reference in the definition on the unit of enzyme activity.[11] An extinction coefficient identified by experiments, 18.65 $mM^{-1} \cdot cm^{-1}$, was employed to define the enzyme activity in the PMS-MTT assay. It was found in our experiment if NADH, the reductive reagent, is kept a certain amount, the extinction coefficient will stay the same even MTT is excess. Based on this fact, it is reasonable to establish a linear relationship between the reduced amount of MTT and the extinction coefficient when certain amount of MTT is reacted with excess NADH. Thus, 18.65 $mM^{-1} \cdot cm^{-1}$, was obtained as the extinction coefficient in the PMS-MTT assay.

Determination of the influence on PMS-MTT assay and 2, 6-DCPIP assay with the addition of β-mercaptoethanol

Impact of the sulfhydryl protecting agent is detected from almost all assays in PDHc activity measurement. Hence it is necessary to

unearth what influence *β*-mercaptoethanol might have on different assays. The comparison was carried out by replacing the enzyme solution with *β*-mercaptoethanol at a final concentration of 50μmol•L^{-1} while keeping all other components unchanged in complete reaction systems of the 2,6-DCPIP assay and the PMS-MTT assay, respectively. And then the time-dependent change in absorbance within five minutes started from the addition of *β*-mercaptoethanol was recorded.

Results and Discussion

Standard curve

Linear relationship between the amount of reduced MTT and OD_{566}. A linear relationship between the amount of reduced MTT and OD_{566} (Fig. 2, R^2=0.999) was obtained by reaction of excess NADH, the reductive reagent, and MTT at a certain amount. In addition, the extinction coefficient did not change as the amount of PMS changed. Based on the fitting equation to data from Figure 2, y =0.01865x (SD=0.02353, p<0.01), the extinction coefficient of reductive MTT was calculated to be 18.65 mM^{-1}•cm^{-1}.

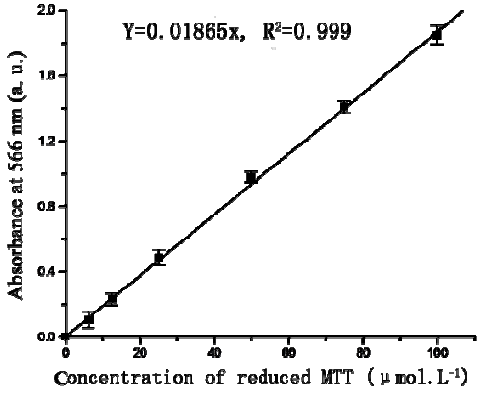


Figure 2. Standard curve of the reduced MTT by its absorbance at OD_{566}.

Linear relationship between the amount of DCPIP and OD_{600}

It was also disclosed that there is a linear relationship between the amount of DCPIP and OD_{600}. Based on data from Fig. 3 and the corresponding fitting equation, y = 0.01239x (SD=0.00744, p<0.01), the extinction coefficient of DCPIP was calculated to be12.34 mM^{-1}•cm^{-1}.

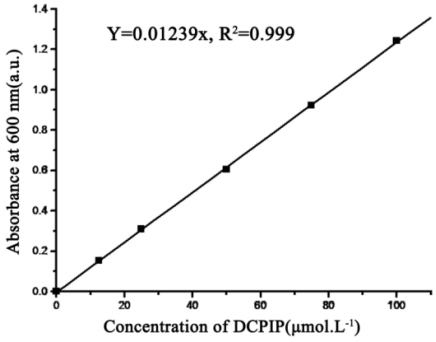


Figure 3. Standard curve of the DCPIP by its absorbance at OD_{600}.

Measurement of the Activity of PDHc from pig's heart- Standard NADH assay

It is shown in Figure 4 that the absorbance reached the maximum within one minute after the reaction started, and then it dropped until a plateau of relative stable value. The underlying reason might be that the product of PDH (E1), the enzyme for the rate-limiting step, is easily depleted when E2 and E3 are in existence, making the enzymatic reaction equilibrium move to the right and thus the initial rate of reaction reaches the maximum within a very short time. Furthermore, the subsequent drop of the reaction rate should be ascribed to the negative feedback inhibition by the product of the PDHc catalyzed reaction. Previous studies such as that conducted by Berg A have indicated that both ratios of NAD^+ to NADH, and CoA to acetyl-CoA , are important influence factors on the PDHc activity. [5-8, 14]

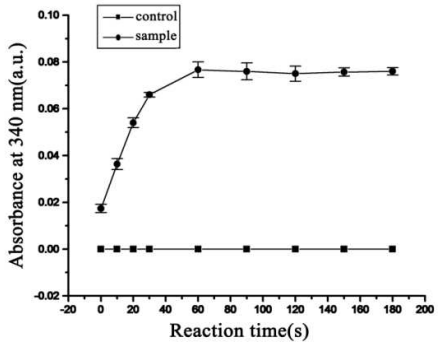


Figure 4. Time-dependant absorbance change in the measurement of PDHc activity by the standard NADH assay.

INT coupled assay

Relatively big changes in absorbance (Fig. 5) were discovered in two control groups within the measurement period, illustrating a high background absorbance in this assay. Comparatively, the absorbance change in the control group without enzyme was lower than that in the control group with enzyme, from which we suggest that this difference might be ascribed to the reaction between impurities in the enzyme and interferents in the system, e.g. the thiol protecting agent. Moreover, absorbance change in the control group with enzyme was even larger than that in the complete reaction system, while there was no significant difference in the absorbance change between control groups and the experimental group, demonstrating that the INT coupled assay is not applicable to measure crude enzyme solution activity.

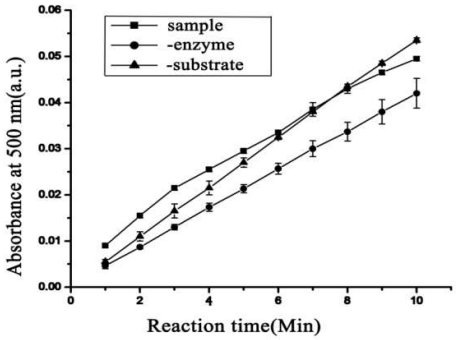


Figure 5. Time-dependant absorbance change in the measurement of PDHc activity by the INT coupled assay.

Measuring the activity of PDH from lactic acid bacteria and from PDHc in pig's heart-2, 6-DCPIP assay

The activity measurement result of PDH from lactic acid bacteria is shown in Figure 6. No obvious time-dependant change in absorbance was observed in three control groups without TPP, sodium pyruvate, and enzyme, respectively. In addition, there was no significant difference among results from these three control groups. In both the control group without $MgCl_2$ and the experimental group, changes in absorbance turned out to appear a linear increase within the measurement period, with a slope of 0.0146 (R^2=0.9874) for the control group without MgCl2 and a slope of 0.0785 (R^2=0.9828) for the experimental group. The much smaller slope of the control group, which is only 18.65% of that of the experimental group, confirming the significant difference ($p<0.01$) between the absorbance change between sample and control. Results proved the applicability to measure the activity of PDH from lactic acid bacteria.

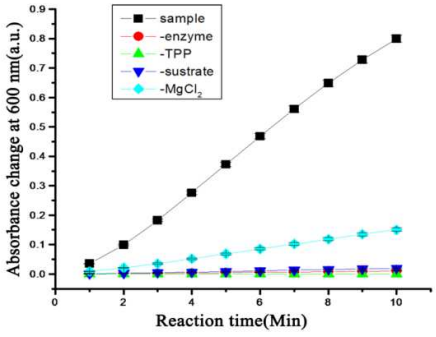


Figure 6. Time-dependant absorbance change in the activity measurement of PDH from lactic acid bacteria by the 2, 6-DCPIP assay.

In the result of activity measurement of PDH from PDHc in pig's heart (Fig. 7), there was obvious change in the absorbance, especially within the initial reaction period, in the experimental group and all control groups except the one without enzyme. Furthermore, it is unlikely that the absorbance change were

linear with reaction time in this case, which is significant different ($p<0.01$) with the control group without enzyme. Comparatively, absorbance change in the experimental group was the largest, proving this assay could be used to measure the activity of PDH from PDHc in pig's heart, with more or less influence from some interferents.

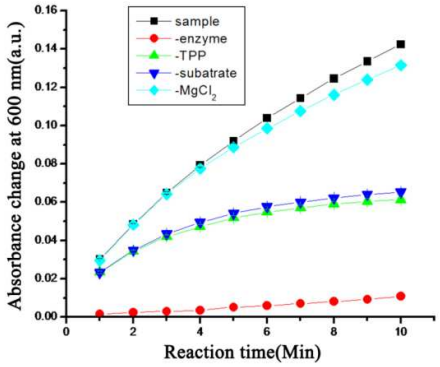


Figure 7. Time-dependant absorbance change in the activity measurement of PDH from PDHc in pig's heart by the 2, 6-DCPIP assay.

Potassium ferricyanide assay

It was found that total OD_{420} was still negative until 30 minutes after the reaction started, and further study disclosed that total OD_{420} was kept unchanged after one hour (data not indicated). Hence we consider this assay to be of poor sensitivity in measuring PDH activity.

PMS-MTT assay

The activity of PDH from lactic acid bacteria measured by the PMS-MTT assay (Fig. 8) were almost the same as that measured by the 2, 6-DCPIP assay. The slope of 0.0442 (R^2=0.9944) for the control group without $MgCl_2$ was 42.55% of the slope for the experimental group (k=0.1039, R^2=0.9938). The relatively larger slope of the experimental group than that of the result measured by the 2, 6-DCPIP assay revealed the different requirement of Mg^{2+} in these two assays.

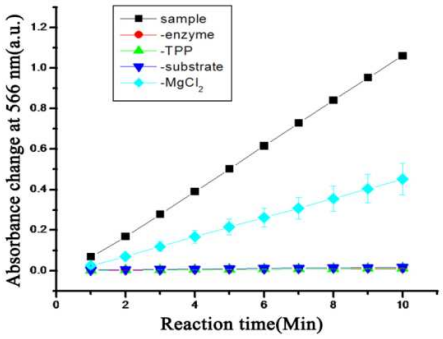


Figure 8. Time-dependant absorbance change in the activity measurement of PDH from lactic acid bacteria by the PMS-MTT assay.

For the activity measurement of PDH from PDHc in pig's heart by the PMS-MTT assay (Fig. 9), the slope of 0.0054 (R^2=0.9892) for the control group without $MgCl_2$ was 29.19% of the slope for the experimental group (k=0.0185, R^2=0.9764). Meanwhile, the absorbance obtained by the PMS-MTT assay was larger than that obtained by the 2, 6-DCPIP assay within the first 10 minutes of the reaction, demonstrating a higher sensitivity of the PMS-MTT assay. Two set of results both verified that the substrate-dependant absorbance change can be well detected by the PMS-MTT assay.

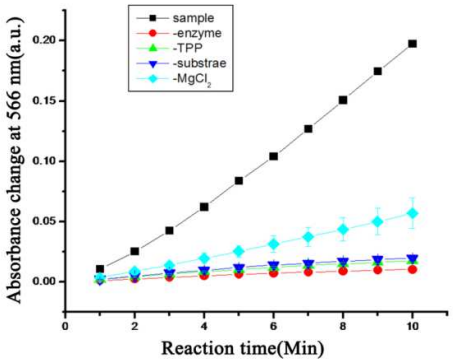


Figure 9. Time-dependant absorbance change in the activity measurement of PDH from PDHc in pig's heart by the PMS-MTT assay.

Activity measurement by PMS-MTT assay of crude PDH

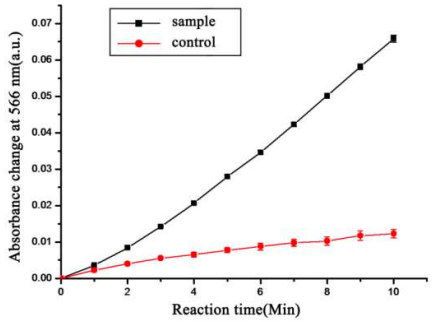


Figure 10. The activity measurement of crude PDH from pea mitochondria by PMS-MTT assay.

Crude PDH from pea mitochondria: Result of activity measurement by PMS-MTT assay of crude PDH in the homogenate from pea mitochondria is shown in Figure 10. It could be observed that within ten minutes from the beginning of reaction, the absorbance change of the experimental group is linearly proportional to the reaction time, with significant difference ($p<0.01$) from the result of the control group. Although the non-centrifuged crude homogenate could make the reaction mixture blue, which is the same as the color appeared in the PDH catalyzed reaction, the activity measured was not high because the mixture was opaque. In addition, there was almost no absorbance change of the supernatant after ultrasonication. It can be inferred that better enzyme preparation method should be established in PDHc activity measurement because the membrane protein must be kept intact with all activity preserved after its dissociation from the mitochondria membrane. However, we still believe that activity of crude PDH from pea mitochondria could be measured by PMS-MTT assay.

Crude PDH from *E. coli*

It is found in Figure 11 that within five minutes from the beginning of reaction, the absorbance change of the experimental group is linearly proportional to the reaction time, with a larger slope than that after five minutes, indicating an easier inactivation of crude enzyme by oxidization. From five minutes to ten minutes after the reaction started, there exhibited more obvious ($p<0.01$) substrate-dependant absorbance change in the experimental group than in the control group, the reaction system without enzyme. Hence we propose that the PMS-MTT assay is applicable in the activity measurement of crude PDH from *E. coli*.

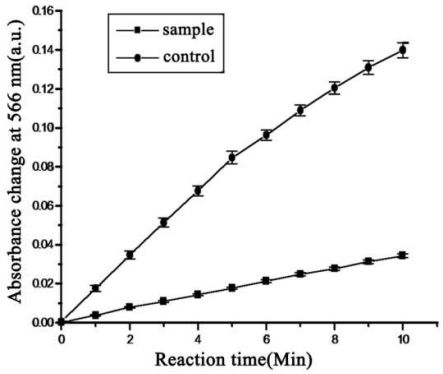


Figure 11. The activity measurement of crude PDH from *E. coli* by PMS-MTT assay.

Comparison of the effect of β-mercaptoethanol on two assays

An intense reaction would take place between 2, 6-DCPIP and 50 μmol•L^{-1} β-mercaptoethanol, resulting an average absorbance change of 0.556 within five minute (Fig. 12). While the absorbance change led to by β-mercaptoethanol of the same concentration in the PMS-MTT assay was only 0.013, indicating that β-mercaptoethanol hardly react with MTT. Further studies showed that obvious effect of β-mercaptoethanol will not occur until its concentration was far beyond the concentration needed by the enzymatic reaction.

On the contrary, the result of activity measurement of PDH from PDHc in pig's heart by the 2,6-DCPIP assay showed a non-linear relationship between the absorbance change and time (Fig. 7), which could be attributed to the apparent effect of certain amount of β-mercaptoethanol in PDHc from

pig's heart, although the final concentration of β-mercaptoethanol in the reaction system was only 20 μmol•L^{-1}.

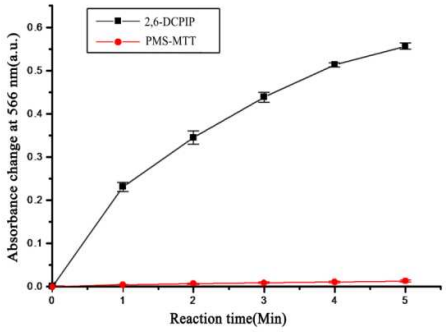


Figure 12. Time-dependant absorbance change of the 2, 6-DCPIP assay and the PMS-MTT assay.

Drawbacks and limitations of existing assays

Existing assays for measuring PDHc activity can be divided into two main categories, spectrophotometry and radioisotope method. Dynamic measurement is employed in the radioisotope method, making it less handy than the spectrophotometry. In addition, the use of unstable radioactive substance becomes another drawback of this method, a cause for the environmental pollution. Unfortunately, commonly used spectrophotometric assays for measuring PDHc activity are also far from perfect. In the standard NADH method, PDHc activity has been proved to be easily affected by both ratios of NAD^+ to NADH, and CoA to acetyl-CoA .[5,16-20] Result from this study (Fig. 4) again confirmed the negative feedback inhibition from the product is the inherent drawback of the standard NADH method.

While the high background absorbance of the INT-coupled assay has been substantiated by results from both Hinman, the first investigator of the method, and our study (Fig. 5).[11] We propose that this drawback is highly possible due to the reaction between the enzyme and some interferents in the system, e.g. the thiol protecting agent. Meanwhile, the poor PDHc activity of the sample used in our study and the concurrent high disturbance from other reagents might also the reason for the measurement failure by the INT-coupled method.

According to our analysis, there are several disadvantages of the improved method based on the standard NADH assay proposed by Guo et al.[21] First, the substrate concentration cannot be high (0. 015 μmolL^{-1} in the report) because the assay is realized by measuring the decrease in substrate, hence the linear change in the enzymatic reaction can be only found within a very short time of the initial reaction. Second, it is inapplicable to determine the continuous change in enzymatic reaction because the absorbance could only be read after the termination of the reaction. Thirdly, any operation error would lead to considerable influence on the result because of the small total volume (0.25 ml) of the reaction system, which makes the assay quite unstable.

As for the 2, 6-DCPIP assay, it is only applicable for measuring PDHc activity when there is no thiol protecting agent or any other strong reducing substance in the enzyme. Otherwise the assay would not work.

In this study, it is found that the sensitivity of the potassium ferricyanide assay is extremely poor, which is in accordance with conclusions from other reports on this assay.[1, 6] In these reports, hour instead of minute was used as the unit of time to define the enzyme activity. Actually, reactions among reagents in the system and some other uncertain factors may all affect PDHc activity measurement. In this report, it is for the convenience of comparison in several assays and for easier distinction between the enzymatic reaction and influence of sulfhydryl protecting agent or other interference factors that the result was shown in the absorbance change but not in the unit of enzyme activity.

Advantages of the PMS-MTT assay-Reliable stability

Normally, sulfhydryl protecting agent is

required in enzyme extraction or preservation for the maintenance of enzyme activity, because thiol or reactive hydroxyl are common components in the active center of enzyme. However, the addition of sulfhydryl protecting agent was proved in our study to be an interference factor for some assays of PDHc activity, such as the 2, 6-DCPIP assay and the INT-coupled assay. On the same condition, little interference was found when MTT was employed as the electron acceptor, providing a different point of view from the conclusion made by Hinman and Schwab that sulfhydryl protecting agent might be essential for the best measurement on enzyme activity.[11,22] Relatively, the addition of sulfhydryl protecting agent within certain range of amount in the extraction or preservation of enzyme with PMS-MTT assay could barely affect the enzyme activity, resulting a very small standard deviation (data not shown here) among repeatedly parallel measurements. Hence, better stability is regarded as the first advantage of the PMS-MTT assay over other assays.

When all other reaction conditions, including the unit of enzyme activity, were kept unchanged besides using different electron acceptor, within a same time period for PDHc measurement, the absorbance change in the MTT assay is much more obvious than that in the 2, 6-DCPIP assay. In addition, the extinction coefficient of the reductive MTT (18.65 mM^{-1} cm^{-1}) was larger than that of the 2, 6-DCPIP (12.34 mM^{-1} cm^{-1}). Moreover, when potassium ferricyanide was adopted as the electron acceptor instead of 2, 6-DCPIP or MTT, there was very little absorbance change within a period as long as ten minutes, demonstrating a very slow change in the reaction, and thus proved that the potassium ferricyanide assay was the least sensitive. This result was also supported by findings of other scholars who employed the potassium ferricyanide assay in measuring PDHc activity, in which they have to use 'hour' instead of 'minute' as the time unit to define the PDHc activity.[5, 20] Actually, both reactions among reagents and other possible interferents might affect the enzyme activity measurement. Consequently, the PMS-MTT assay on measuring PDHc activity is more sensitive.

Low cost is another advantage of the PMS-MTT assay in that the price of reagents used in the assay is much more inexpensive and easy to be commercially available than both CoA and NAD^+, which are required in the standard NADH assay.

The PMS-MTT assay is applicable for the activity measurement not only in purified PDH and PDH in purified PDHc, but also in the crude PDH solution prepared under certain conditions, and the assay can indirectly reflect the PDHc activity. Since the PDH-catalyzed process is the rate-limiting step in three enzymatic reactions of the whole PDHc, it is reasonable to reflect the PDHc activity by determination of the amount of MTT reduced by hydroxyethyl-TPP and avoid the measurement of NADH, which could be unstable in the enzymatic reaction and thus make the result inaccurate. In detail, 1 mole of hydroxyethyl-TPP, the product of E1 catalyzed reaction could reduce 1 mole of MTT. If E2 and E3 exist simultaneously, the combined action of three enzymes could produce 1 mole of NADH.

Currently used assays on measuring PDHC activity are either easy to be inhibited by the product (the standard NADH assay), or could not reflect the activity accurately (the INT coupled assay), in this case, it is a better choice to employ the PMS-MTT assay to indirectly reflect the PDHc activity, so that the drawbacks of current assays could be overcome.

There is only one thing need to be noticed that given the activity of PDH is too low and thus elongate the reaction time, the redox reaction of MTT, which can take place in air in the

existence of PMS, will make slight change in the absorbance.

To provide a clear view, the comparison between advantages and drawbacks of the PMS-MTT assay and conventional techniques are listed in Table 1.

Methods	Drawbacks	advantages
The standard NADH method	the negative feedback inhibition from the product	
The INT-coupled assay	the reaction between the enzyme and some interferents in the system	
The 2, 6-DCPIP assay	Poor sensitivity	reaction time can be long
The PMS-MTT assay	the redox reaction of MTT might interfere the absorbance if the activity of PDH is too low	reliable stability; more sensitive absorb change; low cost; wide range of use.

Table 1. A comparison between the PMS-MTT assay and conventional techniques.

Acknowledgement

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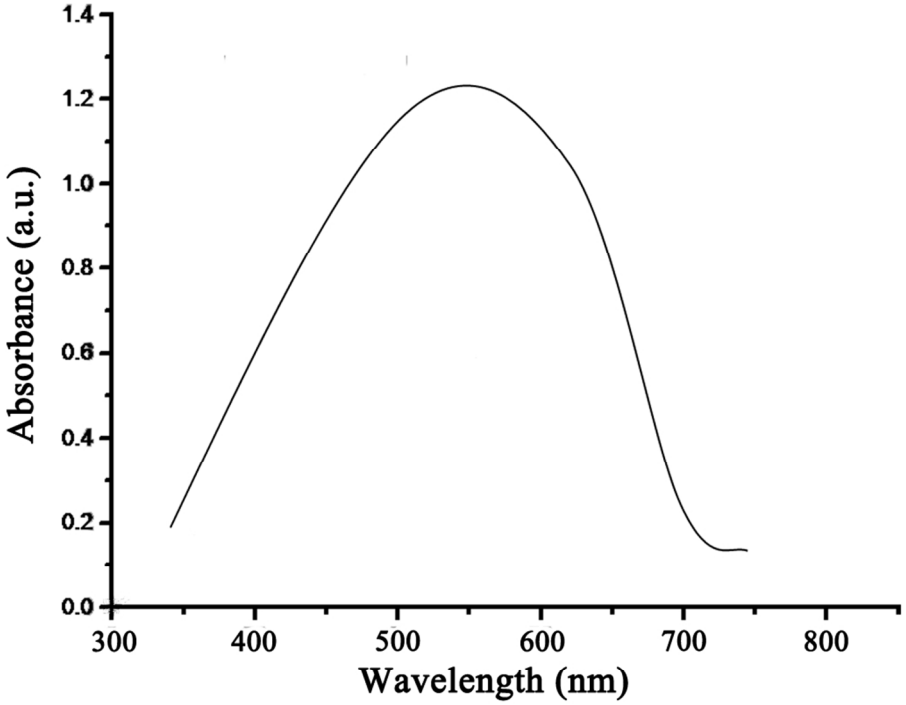

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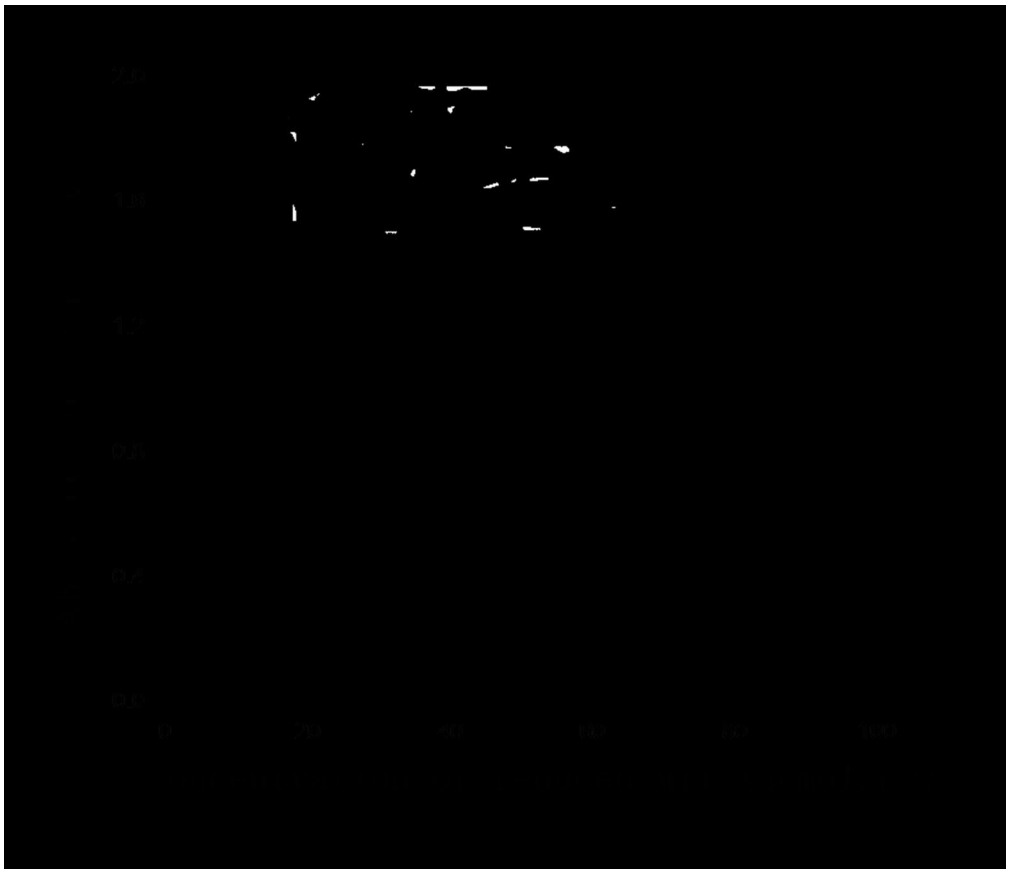

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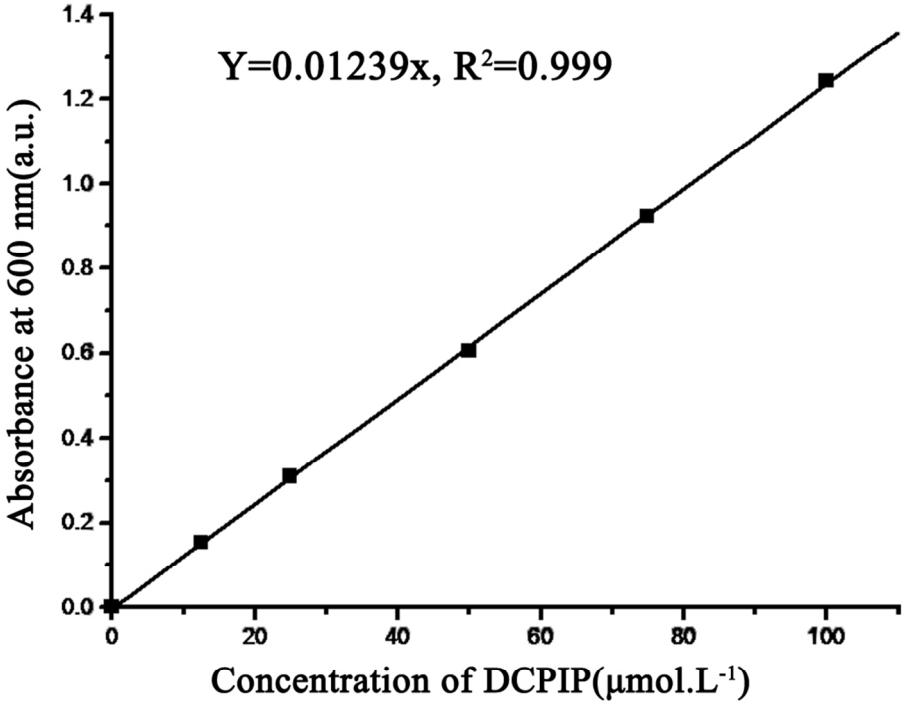

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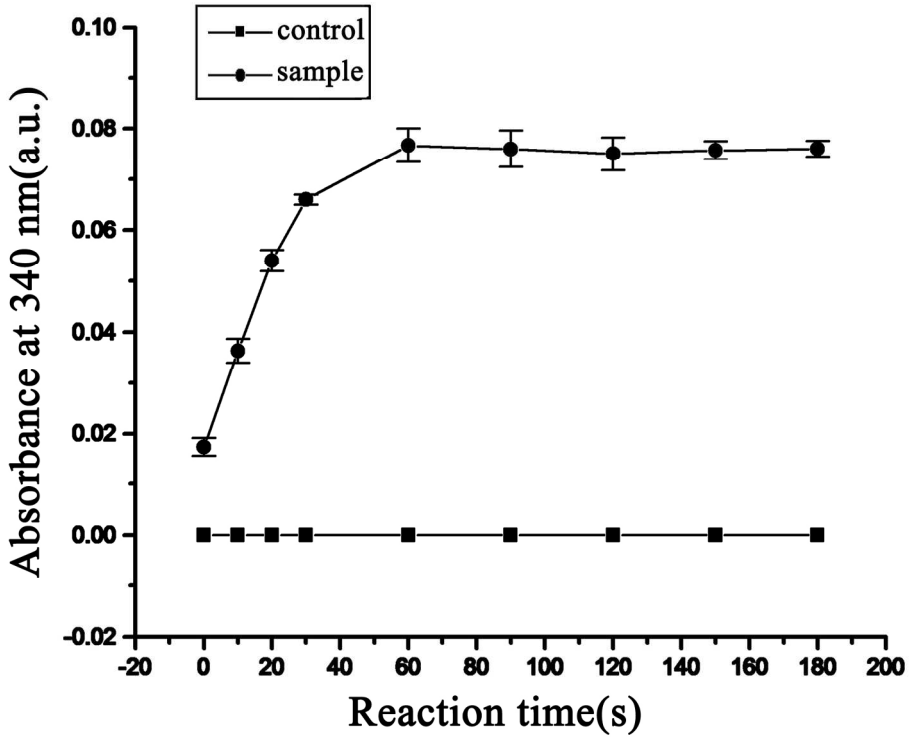

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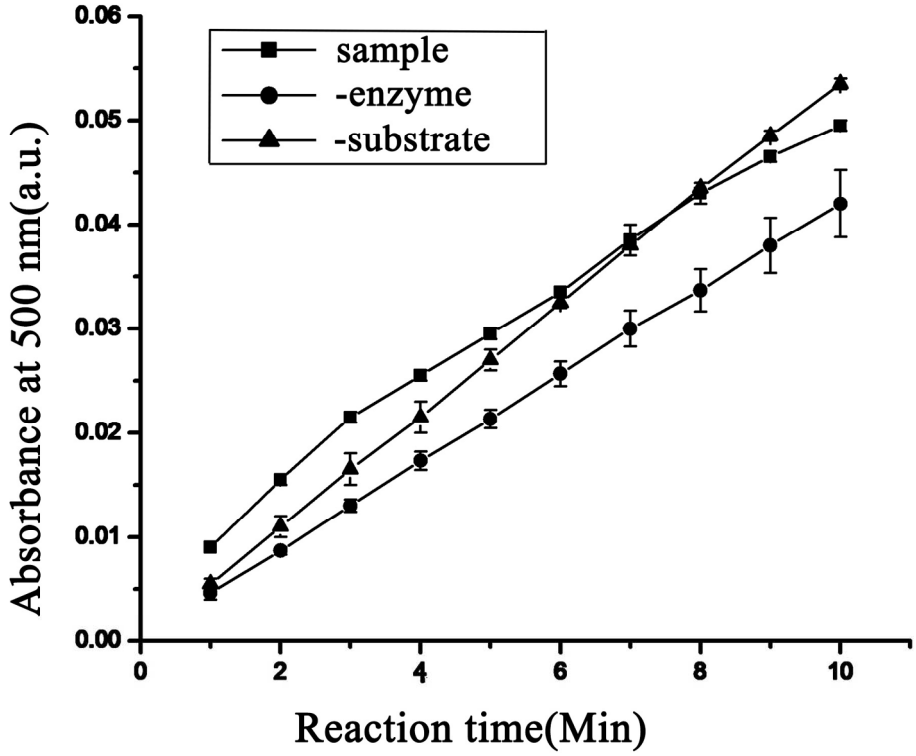

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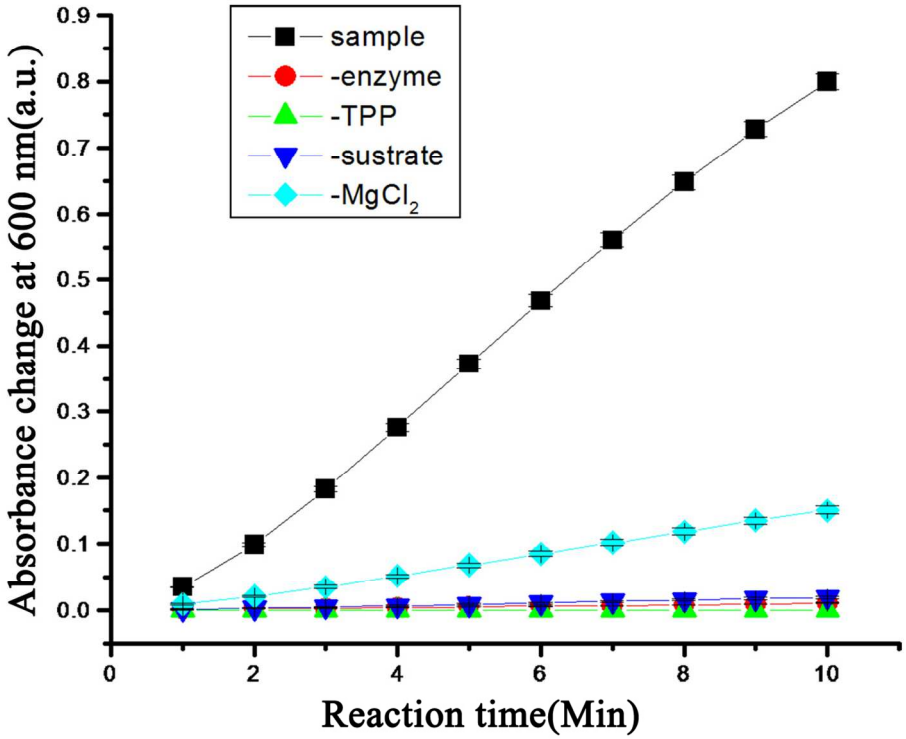

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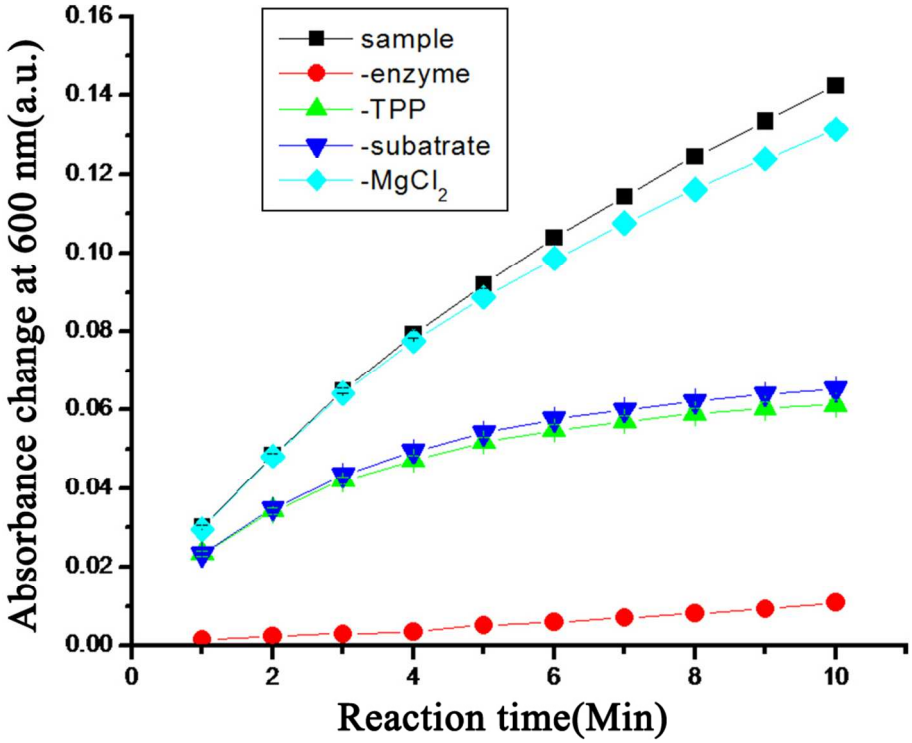

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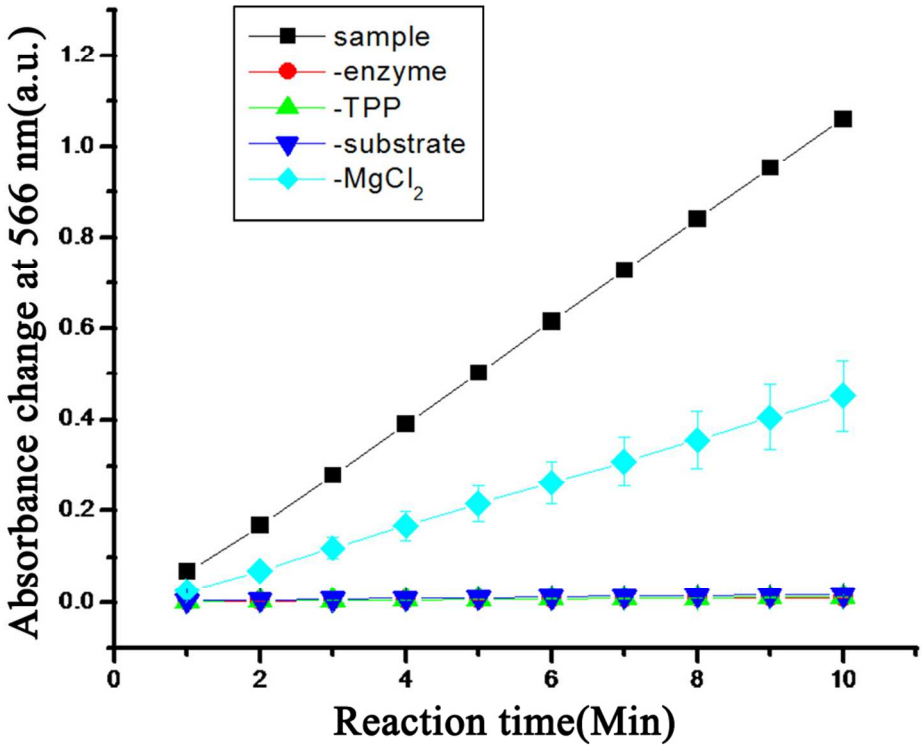

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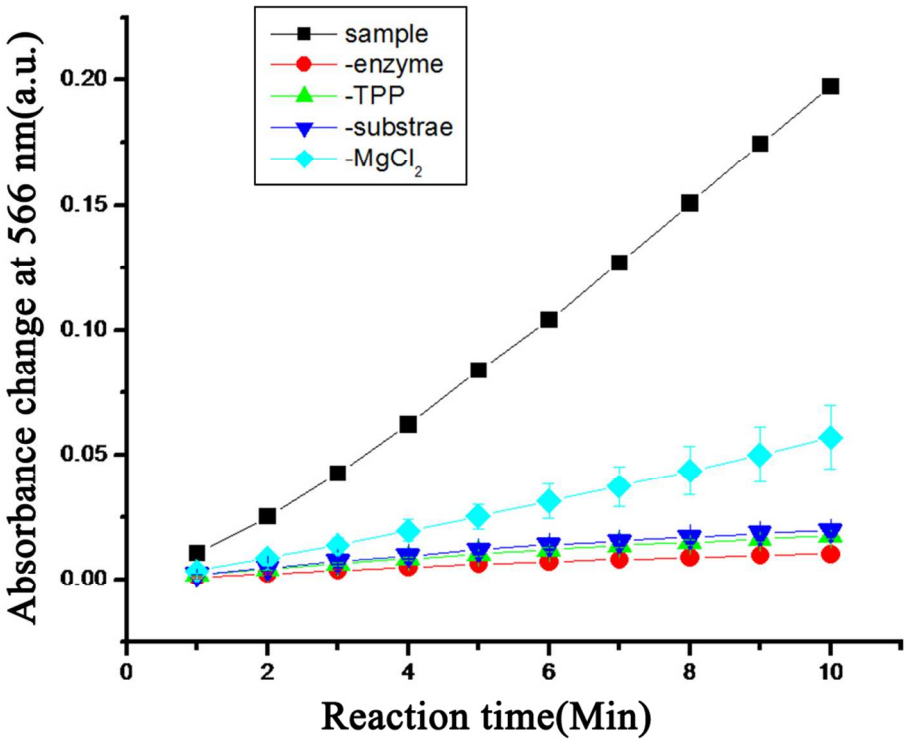

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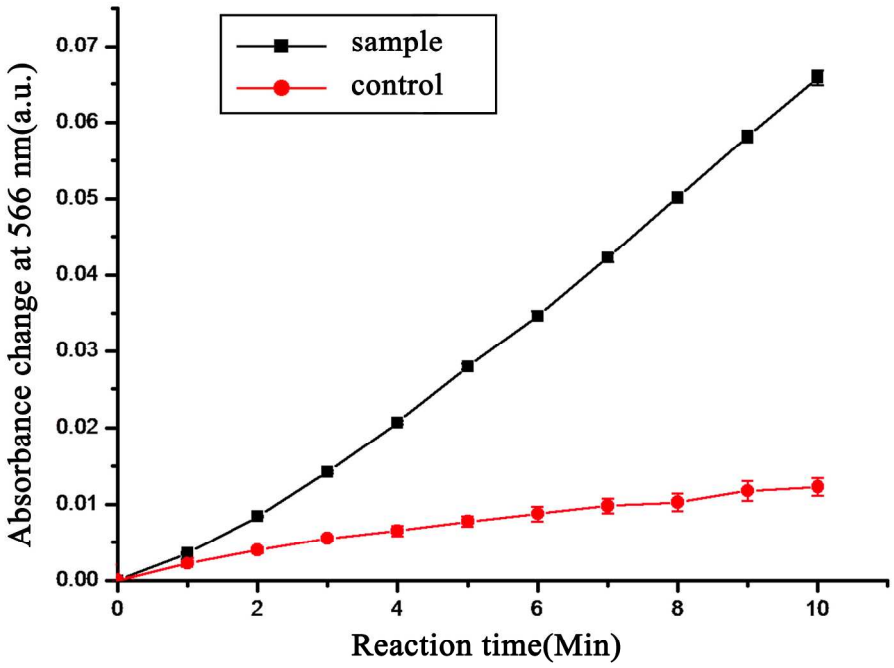

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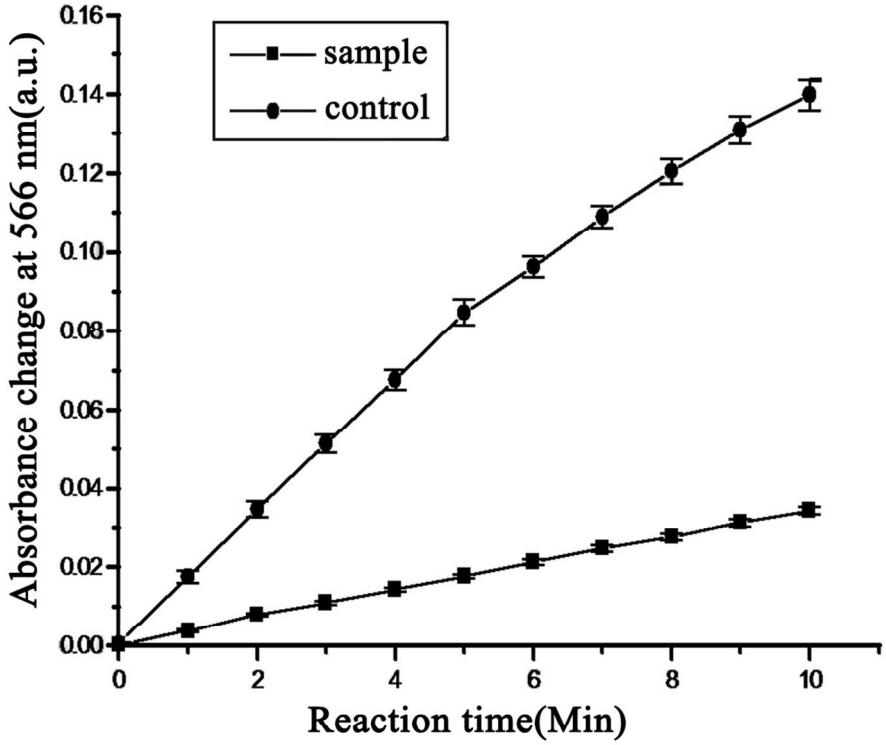

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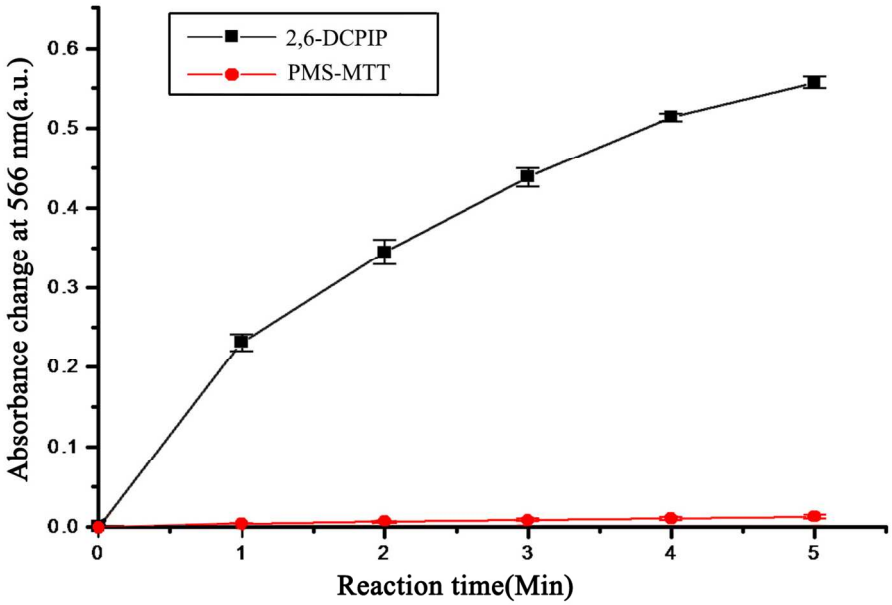

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We describe a new spectrophotometric assay for measuring pyruvate dehydrogenase complex activity, by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an electron acceptor.