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Application of Cabbage Peroxidase for Glucose Assay

Inshik Park, Sunok Kho and In Nam

Dept. of Food Science and Nutrition, Dong-A University, Pusan 604-714, Korea

Abstract

Cabbage contained high peroxidase activity among tested plant sources. The cabbage peroxidase can replace horseradish peroxidase to assay glucose with glucose oxidase. The amount of glucose can be determined quantitatively by glucose oxidase-cabbage peroxidase. The optimum pH and temperature for enzymatic glucose determination by glucose oxidase-cabbage peroxidase were 6.0 and 35~45℃, respectively. The glucose assay was inhibited by addition of various metal salts such as mercuric chloride, lead acetate, silver nitrate, ammonium molybdate, sodium tungstate, and cupric sulfate. The relationship between absorbance and amount of glucose was linear up to 8.33 mM glucose in the assay mixture under the assay conditions.

Introduction

Glucose has been quantitatively determined by two stage enzymatic reactions. β-Glucose is oxidized to gluconic acid by glucose oxidase, with the concurrent release of hydrogen peroxide. The hydrogen peroxide produced oxidizes reduced chromogen such as o-dianisidine to the oxidized chromogen and water.[1-4]

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-gluconic acid}$$

$$H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2H_2O + D$$

The quantity of oxidized dye can be accurately measued and is a direct measure of the glucose originally present. The great advantage of this method is that it is specific for glucose, and no other carbohydrate will react. This method is very accurate, reliable and fast. The complete test for glucose is available commercially, in the form of test strip impregnated with glucose oxidase, peroxidase and a dye, as individual test kits or packed for use in an automated system which includes an immobilized glucose oxidase.[5] The peroxidase used in this method has been from horseradish. It was well characterized and accordingly commercially available. We have investigated the new peroxidase source containing relatively high activity, and found that cabbage posses high peroxidase compared to other plant sources. In this report, we present the use of cabbage peroxidase as an enzyme reagent for glucose replacing horseradish peroxidase.

Materials and Methods

Materials

Glucose, o-dianisidine hydrochioride were purchased from Sigma Chemical Co. Various plants including cabbage were obtained from a local market. Various plant peroxidases were prepared by addition of equal volume of water to the plants followed by homogenizing them in a mixer at high speed for a min. They were them centrifuged at 1,200g for 30 min, and the supernatants were used

as crude enzyme preparation.[6] All other reagents used were of analytical grade.

Enzyme purification

The glucose oxidase was from *Aspergillus niger*, which was isolated from soil in our laboratory. Crude glucose oxidase was applied to DEAE-Sephacel, which was equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The active glucose oxidase was eluted by washing the column with linear gradient of 0-0.5 M NaCl in the equilibration buffer. The crude cabbage peroxidase was applied to CM-Sepharose CL-6B, which was equilibrated with 10 mM Tris-HCl buffer (pH 7.0). The active peroxidase from cabbage was eluted by washing the column with linear gradient of 0-0.5 M NaCl in the equilibration buffer. It was further purified by using gel filtration on Sepharose CL-6B, which was equilibrated with 10 mM Tris-HCl (pH 7.0).

Glucose determination

In the standard glucose assay, the final concentration of reagents were glucose 0.1 M ; o-dianisidine hydrochloride, 0.43 mM ; Na-phosphate buffer, pH 6.0, 0.2 M. The total assay volume was 3 ml and the temperature of incubation is 45℃. The assay was initiated by adding 20 μl of cabbage peroxidase and 0.4 ml of glucose oxidase. The assay was performed for 20 min and stopped by adding 0.1 ml of 1.7 M NaOH. The resulting color formation by oxidized o-dianisidine was measured at 460 nm.

Peroxidase Assay

In the standard peroxidase assay, the final concentration of reagents were hydrogen peroxide, 0.85 mM ; o-dianisidine hydrochloride, 0.24 mM ; Na-acetate buffer, pH 5.5, 0.2 M ; The total assay volume was 3 ml and the temperature of incubation was 45℃. The assay was initiated by adding 20 μl of peroxidase to the assay mixture. It was performed for a min and stopped by adding 0.1 ml of 1.7 M NaOH. The resulting oxidized o-dianisidine was measured at 460 nm.

Results and Discussion

Comparison of peroxidase activity

Since peroxidase has been used as an enzyme reagent to assay compounds which release hydrogen peroxide by respective oxidases, it would be useful to select new source contaning high peroxidase activity. Table 1 exhibits the relative peroxidase activities from various plant sources. Among tested plant sources, cabbage contained the highest peroxidase activity. Since glucose is oxidized to gluconic acid with liberating hydrogen peroxide by glucose oxidase, it can be easily measured quantitatively by using glucose oxidase-cabbage peroxidase system.

Effect of incubation time on glucose assay

The time dependent glucose assay was performed by using 0.3 m mole of glucose in the standard

Table 1. Comparison of peroxidase activity from various plant sources

Sources	Absorbance(460 nm)
Apple	0.09
Korean radish	1.18
Potato	0.11
Carrot	0.22
Cucumber	0.13
Squash	0.18
Onion	0.23
Pear	0.07
Korean cabbage	0.17
Strawberry	0.09
Tomato	0.07
Orange	0.12
Cabbage	1.51
Sweet potato	0.49

The reaction was performed at pH 5.5 and temperature of 45℃ for one min, and 20 μl of enzyme was used.

assay mixture as described in Methods. The relationship between reaction time and absorbance at 460 nm was linear up to 20 min at 45℃ as shown in Fig.1. Therefore, the glucose assay by glucose oxidase-cabbage peroxidase has been performed for 20 min.

Effect of pH on glucose assay

The effect of pH on glucose assay by glucose oxidase-cabbage peroxidase is shown in Fig.2. The optimum pH for glucose assay was 6.0. The pH optimum of glucose oxidase from *Aspergillus niger* was 5.5,[7] and that of cabbage peroxidase is 4.0-5.5.[8] However, the optimum pH for glucose assay was shifted to pH 6.0. This seems due to the fact that glucose oxidase uses β-glucose only. The mutarotation from α-glucose to β-glucose is most active at neutral pH, and is dependent upon phosphate ions. The optimum pH for glucose assay by glucose oxidase and horseradish peroxidase was also same as that by cabbage peroxidase.[8] The buffers (0.2 M) used were as follow : pH 3, Na-glycine ; pH 4.0-5.5, Na-acetate ; pH 6-6.5, Na-phosphate ; pH 7.0-8.0, Tris-HCl ; pH 9.0-10.0, Na-glycine ; pH 11.0, Na-phosphate.

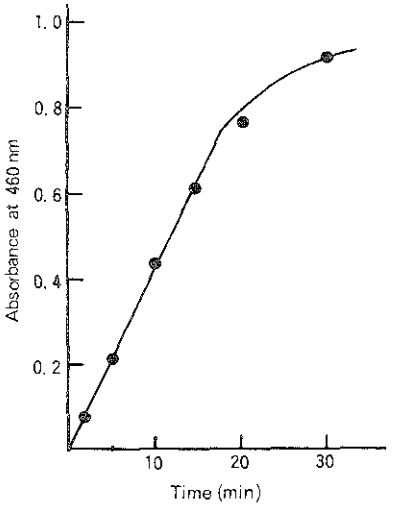

Fig. 1. Effect of incubation time of glucose oxidase-cabbage peroxidase assay.

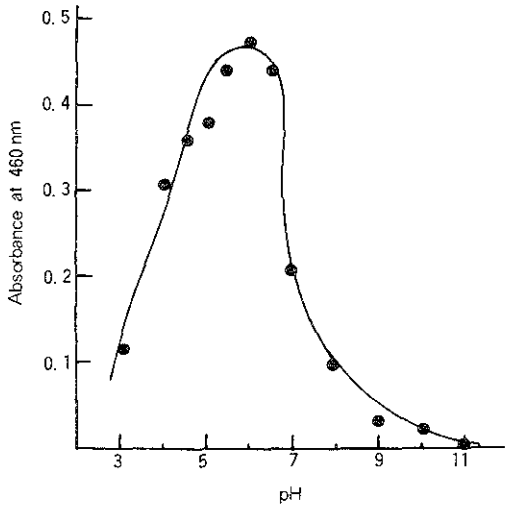

Fig. 2. Effect of pH on glucose oxidase-cabbage peroxidase assay.

Effect of temperature on glucose assay

The glucose assay was performed at various temperature from 25℃ to 60℃. The optimum temperature was 35-45℃ as shown in Fig. 3. Since the cabbage peroxidase was stable even at 60℃ when incubated for 30 min,[8] the decrease of absorbance above 45℃ seems due to the inactivation of glucose oxidase used. The optimum temperature for glucose assay by glucose oxidase and horseradish pe-

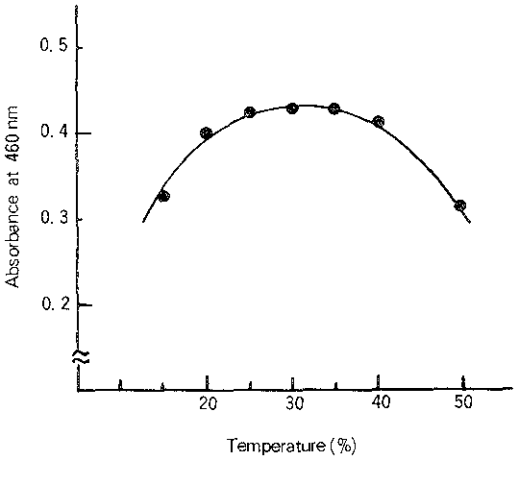

Fig. 3. Effect pf reaction temperature on glucose oxidase-cabbage peroxidase assay.

roxidase was 40℃,[8] which is similar to the glucose assay by glucose oxidase and cabbage peroxidase.

Effect of metal salts

The effects of added metal ions on the glucose assay were determined by addition of 10 mM of various metal ions to the standard assay mixture (Table 2). The glucose assay was inhibited by Mo^{6+}, W^{6+}, Pb^{2+}, Ag^{+}, and Cu^{2+} ions. The inhibition to glucose assay by these metal ions seems due to inhibition of these metal ions to the either glucose oxidase or cabbage peroxidase.[9]

Glucose standard curve

Fig.4 shows the glucose standard curve by glucose oxidase and cabbage peroxidase. The glucose standard curve can be used as a standard for glucose determination up to 8.33 mM glucose concentration. The rate limiting step of glucose assay by this method is glucose oxidase reaction due to its high Km. Therefore, it is necessary to use glucose oxidase possessing low Km and high Vmax to increase the sensitivity of glucose assay by this method.

Table 2. Effect of added metal ions on glucose assay by glucose oxidase-cabbage peroxidase

Metal salts(10 mM)	Relative activity(%)
None	100.0
Mercuric chloride	45.4
Manganese chloride	103.6
Lead acetate	37.6
Silver nitrate	13.0
Ammonium molybdate	8.8
Zinc chloride	103.8
Magnesium chloride	101.4
Sodium arsenate	90.2
Sodium tungstate	34.4
Cupric acetate	2.6
Calcium chloride	96.8

The amount of glucose used was 0.3 m mole, and assayed at 45℃ for 20 min.

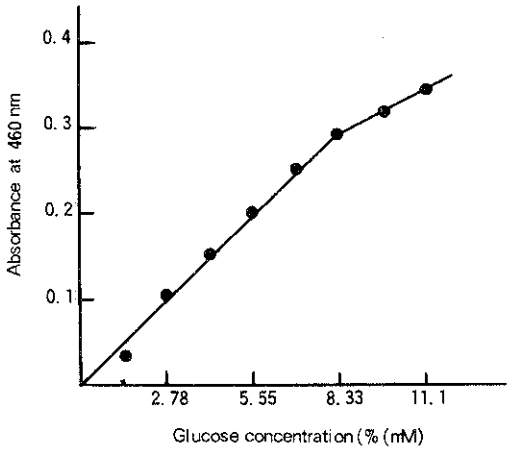

Fig. 4. Effect of glucose concentration on glucose oxidase-peroxidase assay.

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양배추 Peroxidase의 포도당 분석에의 이용

박인식 · 고선옥 · 남인

동아대학교 식품영양학과

요 약

양배추에는 분석된 식물체 시료중에서 가장높은 peroxidase활성을 함유하였다. 양배추 peroxidase는 양고추냉이 peroxidase를 대체하여 glucose oxidase와 더불어 포도당을 정량적으로 분석하는데 이용가능하였다. 양배추 peroxidase와 glucose oxidase를 사용한 포도당 분석의 최적조건은 pH 6.0 및 온도 35−45℃였다. 포도당 분석은 수은, 납, 은, 몰리브덴, 텅스텐 및 구리이온에 의해서 저해되었다. 양배추 peroxidase와 glucose oxidase를 이용한 포도당분석의 표준곡선은 포도당 농도가 8.33mM 이하에서는 흡광도(460mM)와 포도당 농도와의 직선적인 관계를 보였다.