Partial Characterization of Soluble Peroxidase in Pericarp of Litchi Fruit*

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Abstract Soluble peroxidase in pericarp of litchi (*Litchi chinensis* sonn. Cv. Heiye) fruit was extracted by phosphate buffer and purified by ammonium sulfate precipitation, ion exchange chromatography using DEAE Sephadex A-50 column, and gel filtration using Sephadex G-100 column. The specific activity of the purified enzyme increased 65.70 fold over the crude extract with 45.66% recovery. The effects of pH and temperature on activity of peroxidase(POD) were assayed. The K_m for H_2O_2 and 4-methylcatechol were determined by Lineweaver-Burk plots. Several compounds including phonelic compounds were used as the substrates of the enzyme for specificity study. The effects of various inhibitors on this peroxidsae were also assayed.

Key words litchi, peroxidase, purification, enzyme property, browning

Litchi, as a major fruit in the south of China with a high market value, is popular for its deliciously flavored, sweet, and juicy aril. Unfortunately, this luscious fruit has very poor storability as its rapidly pericarp browning, deterioration and decay. A lot of research work involved in the process of browning have been carried out in the past decades, among which enzymatic oxidation of phenolic compounds was suggested to be a key step. Since polyphenol oxidases were the first kind of enzymes to be discovered involved in browning of plant tissues, they have been detailedly studied in litchi fruit. But peroxidases, which are also a kind of oxido-reductases and can oxidize polyphenolic compounds[1], were ignored in the previous study of the mechanism of browning in litchi fruits. Recently, Tian *et al*.[2, 3] noticed that soluble peroxidases may play an important role in postharvest fruit browning.

Peroxidases (EC 1.11.1.7) are oxido-reductases, distributing in various tissues of most plant species. They are found to have multiple molecular forms and not only occur in cytoplasm and cell organelles, but also be associated with cell wall[4]. The isoenzyme compositions of free and bound peroxidases appear to differ molecularly and functionally[5]. Since then, it needs different methods and conditions to extract and purify soluble and bound peroxidases[6]. Concerning their suggested involvement in hormone balance, ethylene biosynthesis, cell development, membrane integrity and respiration control[7], soluble peroxidases were implicated in the metabolic control of ripening and senescence of fruit. For the study on their functions and properties, peroxidases were extracted from many plant resources and purified by various methods[6, 8~13]. But there was almost no report about the characterization of purified peroxidases in litchi pericarp. The objective of this study was to extract and purify soluble peroxidase from litchi pericarp in order to determine its enzymatic properties and explain its possible involvement in pericarp browning of litchi fruit.

1 Materials and methods

1.1 Fruit and reagents

Mature litchi (*Litchi chinensis* sonn. Cv. Heiye) fruits were harvested from an orchard of Maoming City, Guangdong Province, precooled immediately, and transferred to Beijing by air within 48 h of harvest. The pericarp was peeled off quickly and stored in liquefied nitrogen for later use. DEAE Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia. All other chemicals were analytical grade and all reagents were prepared in deionized water.

1.2 Preparation of acetone powder

After taken out of liquefied nitrogen and thawing for 2 h in nitrogen gas at 0℃, 93 g of litchi pericarp was cut into little pieces and homogenized with 10 g of polyvinyl polypyrrolidone (PVP) in 500 ml of cold acetone (-26℃). The slurry was filtered after most of the pigments were removed by repeated wash with cold acetone (-26℃). The wet debris cake was allowed to dry overnight at 2℃ to give 78 g total mass of powder.

1.3 Enzyme extraction

All following purification steps were carried out at 4℃ unless otherwise stated. 78 g of acetone powder was homogenized with 400 ml of 0.05 mol/L sodium phosphate buffer (pH 6.6). After soaked at 2℃ for 16 h, the slurry was filtered and washed 3 times with the same buffer to produce as more

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soluble enzyme as possible. All 600 ml of the filtrate was collected and centrifuged (HITACHI 20PR-52D, Japan) at 4 000 *g* for 20 min to make sure no unsolvable suspension particle in it.

1.4 $(NH_4)_2SO_4$ precipitation

Solid $(NH_4)_2SO_4$ was added slowly with stirring to the centrifuged extract to 30% saturation. After standing for 2 h, the precipitate was excluded by centrifugation at 25 000 *g* for 20 min. More solid $(NH_4)_2SO_4$ was added to the supernatant up to 80% saturation. The precipitate was collected by centrifugation (25 000 *g*, 20 min) and washed by $(NH_4)_2SO_4$ solution of 80% saturation for 3 times. It was dissolved in a minimal volume of sodium phosphate buffer (0.005 mol/L, pH 6.6), and then dialyzed for 24 h against 3 L of the same buffer with 4 changes to give 125 ml of sample for further purification.

1.5 Ion exchange chromatography

The dialyzed sample was applied on DEAE Sephadex A-50 column (1.6 cm × 18 cm) previously equilibrated with 0.005 mol/L sodium phosphate buffer (pH 6.6). After then, the column was washed by the equilibration buffer and the adsorbed proteins were eluted at a flow rate of 0.2 ml/min with linear gradient ranging from 0.005 mol/L of sodium phosphate (pH 6.6) to 1.0 mol/L of NaCl in the same buffer. The fraction volume was 4 ml and the absorbency of each fraction was read at 280 nm for protein detection, while the peroxidase activity was assayed as well. Fractions containing peroxidase activity were collected and applied on a same previously equilibrated DEAE Sephadex A-50 column again. All chromatography conditions were the same with the first time except the elution solution had a linear gradient ranging from 0.005 mol/L phosphate buffer (pH 6.6) to 0.5 mol/L but not 1.0 mol/L of NaCl. Fractions containing peroxidase activity were pooled and concentrated to 4.5 ml of sample by dialysis against PEG-20 000 powder.

1.6 Gel filtration

After dialyzed for 24 h against 500 ml of 0.02 mol/L sodium phosphate buffer (pH 6.6) with 4 changes, a 4.5 ml of sample resulting from ion exchange chromatography was applied to a column of Sephadex G-100 (1 cm × 30 cm) previously equilibrated with 0.02 mol/L sodium phosphate buffer (pH 6.6) and subsequently eluted with the same buffer at a flow rate of 0.1 ml/min. Each fraction had a volume of 1 ml and its absorbency was read at 280 nm for protein detection. The peroxidase activity of each fraction was assayed as well. Fractions containing peroxidase activity were pooled and stored at 2 ℃ as purified peroxidase for subsequent study.

1.7 Assay of enzyme activity and protein determination

The assay of enzyme activity was carried out at 25 ℃ by spectrophotometic method in a 3-ml cuvette. A 3 ml of reaction mixture was buffered by 0.02 mol/L sodium phosphate buffer (pH 6.8), consisting of 0.05 mol/L of H_2O_2, 0.05 mol/L of 4-methylcatechol and 100 μl of peroxidase solution with the concentration which gave linear response over a period of 2 min. The change in absorbency at 470 nm was followed every second by spectrophotometer (Shimadzu UV-160, Japan). One unit of peroxidase activity was defined as the amount of enzyme which cause an increase of one absorbency unit per minute under the described assay conditions. Protein concentration was determined by Coomassie brilliant blue G-250 using BSA as a standard[14].

2 Results and discussion

2.1 Extraction and initial purification

The preparation of an acetone powder was carried out before buffer extraction in order to remove endogenous substrates of peroxidase. The existence of these endogenous substrates may make the assay of enzyme activity imprecise, and they can combine with the peroxidase protein in the crude extract, which may produce pseudo-peak with peroxidase activity when applied to ion exchange chromatography and gel filtration. Soluble peroxidase of litchi pericarp can be easily extracted by buffer of low molarity, and since no difference in specific activity was found when several buffer molarities were used ranging from 0.05 to 0.2 mol/L[6], the concentration of 0.05 mol/L was used in this extraction. $(NH_4)_2SO_4$ precipitation could remove most non-protein compounds in the crude extraction and proteins with far different properties from peroxidase. The precipitate gained by $(NH_4)_2SO_4$ of 30% saturation had almost no peroxidase activity, and after the compounds containing most of the peroxidase activity was precipitated by $(NH_4)_2SO_4$ of 80% saturation, only a little peroxidsae activity was found in the supernatant.

2.2 Peroxidase purification

Figure 1 showed the result of the chromatography

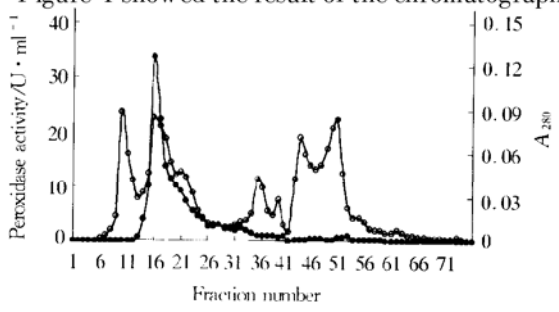

Fig. 1 The First DEAE Sephadex A-50 column chromatography of litchi pericarp peroxidase

Column size: 1.6 cm × 18 cm. Equilibration buffer: 0.005 mol/L of sodium phosphate buffer (pH 6.6). Elution buffer: linear gradient ranging from 0.005 mol/L of sodium phosphate (pH 6.6) to 1.0 mol/L of NaCl in the same buffer. Flow rate: 0.2 ml/min. Fraction volume: 4 ml. ●—●: peroxidase activity; ○—○: A_{280}.

of the final extract of litchi pericarp on DEAE Sephadex A-50 column after $(NH_4)_2SO_4$ precipitation. There were several protein peaks, but only one of them displayed peroxidase activity. This result was similar to that found in papaya fruit[6], soybean[10] and peach fruit[15], but different from that found in tomato[8]. The pool of fractions with peroxidase activity was applied on DEAE Sephadex A-50 column again because unexpected protein peaks were too close to the peroxidase of peak, and a slower ascending gradient of salt concentration of the elution buffer can detach them from each other better. The result of the second ion exchange chromatography was shown in figure 2.

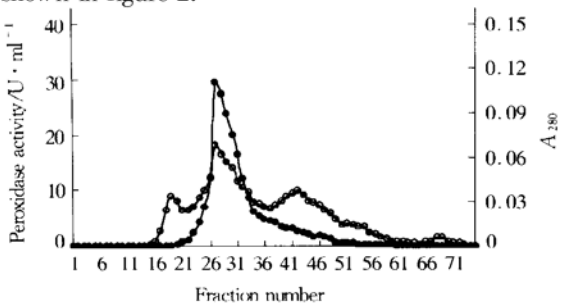

Fig. 2 Second DEAE Sephadex A-50 column chromatography of litchi pericarp peroxidase

Column size: 1.6 cm × 18 cm. Equilibration buffer: 0.005 mol/L of sodium phosphate buffer (pH 6.6). Elution buffer: linear gradient ranging from 0.005 mol/L of sodium phosphate (pH 6.6) to 0.5 mol/L of NaCl in the same buffer. Flow rate: 0.2 ml/min. Fraction volume: 4 ml. ●—●: peroxidase activity; ○—○: A_{280}.

Further purification involved gel filtration on Sephadex G-100. Inner proteins, having similar electric properties but different molecular mass and structure as compared with peroxidase, were removed. The result of the gel filtration presented in figure 3. Only one peak was assayed to contain peroxidase activity, indicating the presence of only one enzyme species or more than one species of somewhat similar moleculuar mass.

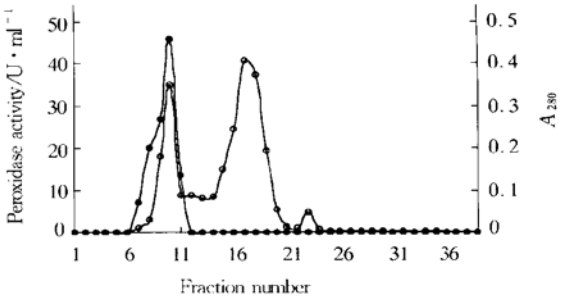

Fig. 3 Sephadex G-100 column gel filtration of litchi pericarp peroxidase

Column size: 1 cm × 30 cm. Equilibration and elution buffer: 0.02 mol/L of sodium phosphate buffer (pH 6.6). Flow rate: 0.1 ml/min. Fraction volume: 1 ml. ●—●: peroxidase activity; ○—○: A_{280}.

Throughout all of the purification processes, the rate of yield and purification fold were calculated for each step and the result was shown in table 1. Total peroxidase activity of 45.66% was recovered with 65.7-fold increase of specific activity.

Table 1 Purification of peroxidase from litchi pericarp

Procedure	Total activity/U	Specific activity/U·mg^{-1}	Purification fold	Yield/%
Buffer extract	8308.72	23.74	1	100
$(NH_4)_2SO_4$(30% ~ 80% saturation)	7601.74	56.87	2.40	91.49
First DEAE Sephadex A-50	6741.77	277.22	11.68	81.14
Second DEAE Sephadex A-50	4793.11	554.58	23.36	57.69
Sephadex G-100	3793.48	1559.42	65.70	45.66

2.3 Enzyme properties

2.3.1 Heat stability: Peroxidases are remarkable of their heat stability. Most kinds of enzymes in plant body can not keep activity for a long time even at a not too high temperature. It's already a good record for polyphenol oxidase in yam tubers to keep most of the activity for only 30 min at 40 ℃, and have a half-life of only 10 min at 60 ℃[16]. But as to the peroxidase produced in this study (Figure 4a), no activity was lost in 40 min, and more than 95% activity was hold for 2 h with incubation at 40 ℃. When incubated at 60 ℃, it was found to have a half-life of 50 min. This peroxidase can be allowed to stay at 25 ~ 30 ℃ for two weeks without no losing of activity. The results indicated that peroxidase in litchi pericarp had a good heat stability. The optimum reaction temperature of this peroxidase was 30 ℃.

2.3.2 Effect of pH: The effect of pH on the peroxidase activity was examined between pH 1 ~ 10.5, using 0.1 mol/L of KCl-HCl buffer for pH values ranging from 1 to 2, citric acid-NaH_2PO_4 buffer for pH values ranging from 3 to 5.6, sodium phosphate buffer for pH values ranging from 6.0 to 7.8, and sodium carbonate buffer for pH values ranging from 8.3 to 10.5. The results showed a broad pH profile ranging from 3.6 to 8.3 (Figure 4b). The optimum pH of peroxidase in litchi pericarp was 6.8, which was far higher than that in other plant materials[6, 9].

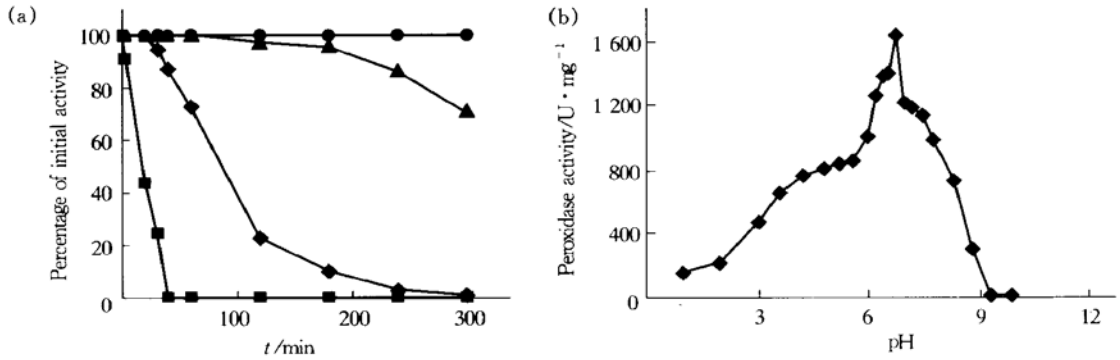

Fig. 4 Effect of temperature and pH on peroxidase activity

(a) temperature. ●—●: 30 ℃; ▲—▲: 40 ℃; ◆—◆: 60 ℃; ■—■: 80 ℃. (b) pH.

2.3.3 Enzyme kinetic study: The primary double reciprocal plots of the initial velocities at pH 6.8 using H_2O_2 and 4-methylcatechol as substrates for the purified peroxidase showed series of lines crossing at a single point below the x-axis (Figure 5a, b), exhibiting the typical behavior of ordered mechanism of two-substrate reaction. Secondary plots of the intercepts of the primary plot against the concentration of another substrate gave out the parameters by which the Michaelis constants (K_m) were calculated (Figure 5c, d). The K_m values of H_2O_2 and 4-methylcatechol were 33.39 mmol/L and 44.13 mmol/L, respectively.

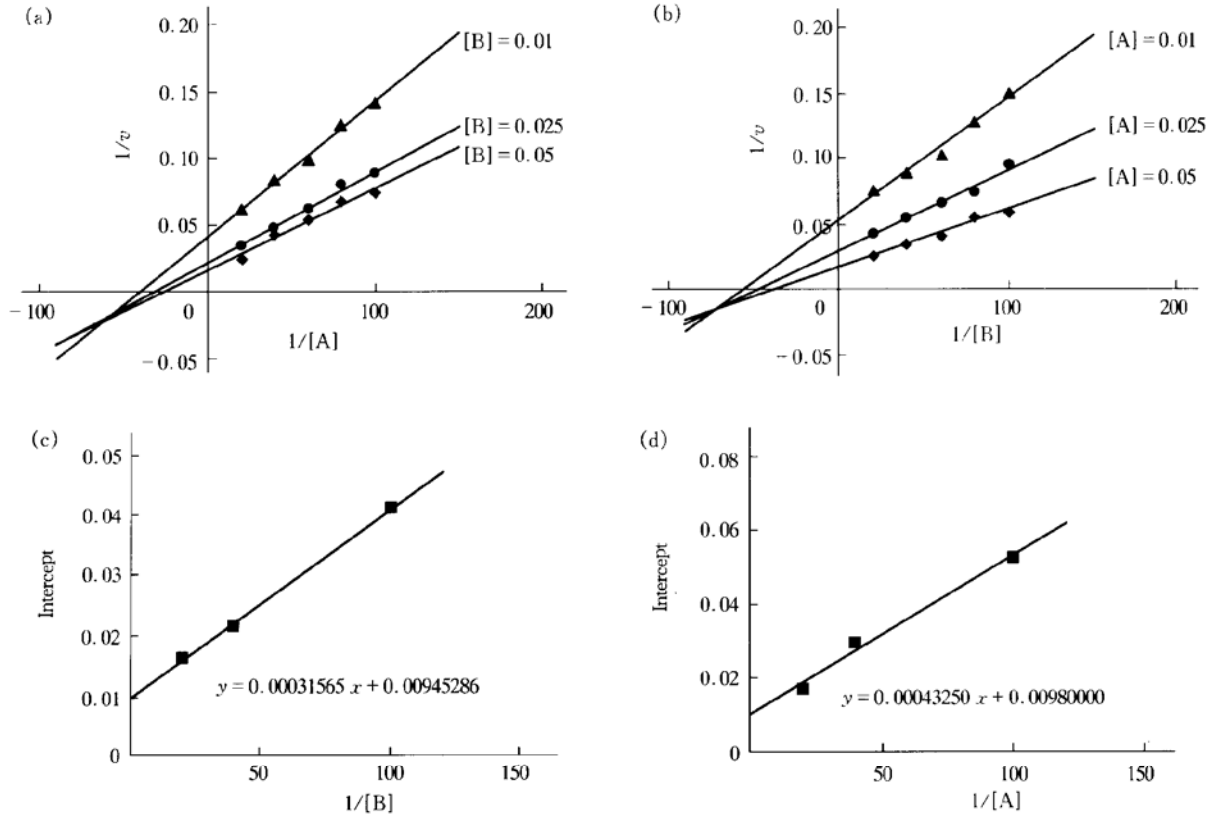

Fig. 5 Lineweaver-Burk plots of peroxidase in litchi pericarp

(a) $1/v$ against $1/[A]$; (b) $1/v$ against $1/[B]$; (c) intercept of lines in (a) against $1/[B]$; (d) intercept of lines in (b) against $1/[A]$. v, velocity in moles of product reducing per min per l of enzyme. [A], concentration of 4-methylcatechol in mol/L. [B], concentration of H_2O_2 in mol/L.

2.3.4 Specificity: Plant peroxidases were suggested to be less specific in substrate than that in animal and microbe[1]. A number of compounds were tested in this study of specificity of the peroxidase, including a wide range of some o-diphenols, monophenols, and m-diphenols (Table 2). Since polyphenol oxidases, having no ability to oxidize monophenols and m-diphenols[17], were found to be a key role in the

browning of fruit, it was rational to be suggested that peroxidases also play an important role in the browning of litchi pericarp.

Table 2 Substrate specificity

Substrates[1)]	λ_{assay}/ nm	Activity/ U·mg^{-1}
Catechol	398	1572. 34
4-Metylcatechol	460	1648. 71
DL-DOPA	434	465. 38
P-Caresol	310	117. 26
Hydroquinone	398	1008. 66
Resorcin	398	973. 62
Gallic acid	400	718. 49
2, 6-Dimethyoxidphenol	470	337. 5
Ferulic acid	287/ 310	78. 3
3, 3-Diaminobenzidine	450	1684. 57

[1)] No activity was obtained with quercetin, tyrosine, pyrogallol or vanillin.

2. 3. 5 Inhibitor study: Table 3 showed the effects of various inhibitors on the peroxidase activity with 4-metylcatechol and H_2O_2 as substrates. Like many other enzymes, in peoxidase litchi pericarp can be inhibited by thiourea which can change the structure of enzyme protein. EDTA inhibited the activity of this enzyme because it can chelate the Fe^{3+} in the active center of peroxidase molecule. $NaHSO_4$ and Na_2SO_4 were wildly used in post harvest treatment in order to delay the browning of litchi pericarp . From

Table 3 Effects of various inhibitors on peroxidase activity

%

Inhibitors	Inhibition percentage (at 0. 05 mmol/ L of inhibitor concentration)	Inhibition percentage (at 0. 1 mmol/ L of inhibitor concentration)	Inhibition percentage (at 0. 5 mmol/ L of inhibitor concentration)
Thiourea	14. 3	36. 7	83. 4
EDTA	8. 5	16. 5	53. 6
β-Mercaptoethanol	41. 7	43. 5	48. 7
Sodium metabisulphite	5. 0	8. 7	46. 5
Sodium Dietheyldithiocarbamate	7. 3	36. 5	66. 8
$NaHSO_4$	61. 5	74. 3	96. 2
Na_2SO_4	53	76. 5	94. 7
Sucrose	0	0	8. 6
$FeSO_4$	64. 3	81. 5	93. 0

common point of view, in such treatment, it was the inhibition of polyphenol oxidases in litchi pericarp that slowed the browning process. But this study displayed remarkable inhibition of $NaHSO_4$ and Na_2SO_4 on peroxidase activity. This might give a new explain for the application of $NaHSO_4$ and Na_2SO_4 in litchi post-harvest treatment to prevent the fruit from browning. It has been reported that ammonium salts stimulate the peroxidase activity in horseradish[18] and papaya[6]. A same effect was found in this study. NaCl had no inhibition effect on litchi peroxidase activity even at a high concentration over 0. 5 mol/ L. If the involvement of soluble peroxidase in the browning process of litchi pericarp can be cofirmed, this study may provide some new enzymatic inhibitors for post-harvest treatment to delay such browning process.

References

1 Boyer P D, Lardy H L, Myrbäk K M. The Enzymes. 2nd. New York: Academic Press, 1963. 227~ 231

2 Tian S P, Xu Y, Jiang A L, *et al*. Changes in enzymatic activity and quality attributes of late-mature peaches in response to controlled atmosphere conductions. Agricultural Science in China, 2002, **1**(2): 207~ 212

3 Tian S P, Xu Y, Jiang A L, *et al*. Physiological and quality responses of longan fruit to high O_2 or high CO_2 atmosphere in storage. Postharvest Biol Tech, 2002, **24**(3): 335~ 340

4 Raa J. Cytochemical localization of peroxidase in plant cell. Physiol Plant, 1973, **28**(1): 132~ 133

5 Adatthody K K, Racusen D. On the extraction of peroxidase isozymes from bean leaves. Can J Bot, 1967, **45**(12): 2237~ 2242

6 Silva E D, Lourenco E J, Neves V A. Soluble and bound peroxidases from papaya fruit. Phytochemistry, 1990, **29**(4): 1051~ 1056

7 Haard N F, Tobin C L. Patterns of soluble peroxidase in ripening banana fruit. J Food Science, 1971, **36**(5): 854~ 857

8 Brownleader M D, Ahemd N, Trevan M, *et al*. Purification and partial characterization of tomato extensin peroxidase. Plant Physiol, 1995, **109**(6): 1115~ 1123

9 Hamed R R, Maharem T M, Fatah M M A, *et al*. Purification of peroxidase isoenzymes from turnip roots. Phytochemistry, 1998, **48**(5): 1291~ 1294

10 刘 稳, 李 扬, 段新源, 等. 改良的大豆种皮过氧化物酶纯化方法及酶的底物氧化特性研究. 山东大学学报, 2001, **36**(1): 78~ 83
Liu W, LI Y, Duan X Y, *et al*. J Shandong Univ, 2001, **36**(1): 78~ 83

11 Kokkinakis D M, Brooks J L. Tomato peroxidase. Plant Physiol, 1979, **63**(1): 93~ 99

12 Shannon L M, Kay E, Lew J Y. Peroxidase isozymes from horseradish roots. J Biol Chem, 1966, **241**(9): 2166~ 2172

13 Lee T T. On extraction and quantitation of plant peroxidase isoenzymes. Physiol Plant, 1973, **29**(1): 198~ 203

14 Bradford M M. A rapid and sensitivity method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 1976, **72**(2): 248~ 254

15 Abeles F B, Biles C L. Characterization of peroxidases in lignifying peach fruit endocarp. Plant Physiol, 1991, **95**(2): 269~ 273

16 Anosike E O, Ayaebene A O. Purification and some properties of polyphenol oxidase from the yam tubers, *Dioscorea bulbifera*. Phytochemistry, 1981, **20**(12): 2625~ 2628

17 Mayer A M, Harel E. Polyphenol oxidases in plants. Phytochemistry, 1978, **18**(1): 193~ 215

18 Fridovich I. The stimulation of horseradish peroxidase by nitrogenous ligands. J Biol Chem, 1963, **238**(12): 3921~ 3927

荔枝果皮过氧化酶的纯化与性质研究*

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摘要 荔枝果皮采后褐变是影响这一重要热带水果经济价值的主要问题, 酚类物质的酶促氧化一直被认为是造成植物组织褐变的关键因素, 其中多酚氧化酶被研究得最多. 过氧化物酶在植物体中分布很广, 能够氧化多种底物, 在荔枝果皮中的含量也很高. 非结合性过氧化物酶已经被证明在果实的采后成熟与老化过程中参与多种过程. 在这项研究中, 用磷酸缓冲液提取荔枝果皮的非结合性过氧化物酶, 并通过硫酸铵沉淀, DEAE Sephadex A-50 离子交换柱层析以及 Sephadex G-100 凝胶过滤进行纯化. 对得到的酶溶液进行了酶学性质的研究, 发现荔枝果皮过氧化物酶具有较高的热稳定性和高的最适反应 pH 值 (6.8), 能够氧化许多底物尤其是单酚和各种多酚类物质, 反应抑制剂专一性与其他植物来源的过氧化物酶略有不同. 显示了过氧化物酶参与荔枝果皮褐变过程的可能性, 并为提高荔枝采后贮藏性提供了新的思路.

关键词 荔枝, 过氧化物酶, 纯化, 酶学性质

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