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Ascorbate oxidase from starfruit (*Averrhoa carambola*): preparation and its application in the determination of ascorbic acid from fruit juices

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Abstract

A study was conducted to utilize ascorbate oxidase (AAO) from very immature starfruit for the enzymatic determination of ascorbic acid (AsA) in colored samples. The enzyme preparation was carried out by a combination of $(NH_4)_2SO_4$ fractionation, DEAE-Toyopearl 650 M and ultrafiltration. A calibration curve for AsA was constructed by plotting the amount of AsA oxidized by the enzyme at a specified reaction time against the absorbance. The curve showed a linear relationship in the range of 0–100 μg ml^{-1} AsA used. Using the plot, the values of AsA in juice samples were determined and compared with the conventional 2,6-dichloroindophenol method.

Keywords: Ascorbate oxidase; Ascorbic acid; Starfruit; Averrhoa carambola

1. Introduction

The content of ascorbic acid (AsA) has been used as a fundamental objective marker for defining the commercial value of fruit and vegetable products. Methods for assaying AsA, 2,4-dinitrophenylhydrazine (DNP) and 2,6-dichloroindophenol (DIP) which were based on the reducing properties of AsA are practical but not always satisfactory for accurate determination of the vitamin in colored samples of plant and animal origins. Tono and Fujita (1984) reported the method to determine ascorbate oxidase (AAO) activity in fruits and Tsumura et al. (1993) made use of peroxidase for spectrophotometric determination of AsA. Both oxidases are two potential enzymes that can be used to oxidize AsA. The measurement is usually based on the principle where total conversion of AsA to dehydro-AsA by the enzymes is measured by total change in absorbance (Whitaker, 1991). The concentration of AsA can then be calculated by the Beer–Lambert Law or by constructing a calibration curve of absorbance versus AsA concentration.

However, one major obstacle to the wide usage of this method is the high costs of those purified enzymes and therefore, they are not economical to be used in the food analysis. Starfruit has been found to be a potential source of the enzyme. Apart from being used as a raw material for making jam, confectionary, jellies, refreshing drinks, juice concentrate, the fruit also contains a fairly high concentration of AAO. Moreover, it is not a seasonal fruit and therefore can provide a year-long raw material for the enzyme. This paper describes the preparation of AAO from starfruit for determining AsA content in colored foods and the results were compared with 2,6-dichloroindophenol method.

2. Materials and methods

2.1. Materials

Starfruits were obtained from Universiti Putra Malaysia. The fruits were kept at −20°C until further use. Juices such as blackcurrant, tomato and guava were obtained from retail markets. DEAE-Toyopearl 650 M

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was obtained from Tosoh Co., Tokyo, Japan. All other Chemicals used were laboratory grade reagents purchased either from BDH or Sigma Chemical Co.

2.2. Methods

2.2.1. Preparation of crude extract

Two kilograms of frozen starfruit was homogenized with 1.5 times its weight of ice cold (about 5°C) 0.1 M phosphate buffer (PB) pH 7. The homogenate was filtered through a layer of cotton cloth and the filtrate was centrifuged at 15 500 ***g*** for 30 min at 2°C. The supernatant was collected as the crude extract.

2.2.2. Purification procedure

2.2.2.1. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ corresponding to 20% saturation was added to the crude extract. After holding for 3 h, the precipitate developed was removed by centrifugation and the supernatant was brought up to 80% saturation with solid $(NH_4)_2SO_4$. Protein precipitating between 20 and 80% saturation was collected by centrifugation and dissolved in a minimum volume of 0.01 M PB, pH 7.0. After dialysis against four changes of the same buffer, the insoluble residue remaining was removed by centrifugation.

2.2.2.2. DEAE-Toyopearl 650 M. The dialyzed enzyme solution was applied to the anion-exchange column (3.2×16 cm) which had been equilibrated with 0.01 M PB pH 7 at a flow rate of 0.8 ml/min. The column was washed excessively with the equilibration buffer. AAO was eluted from the column by elution solution at different concentrations (0.05 and 0.1 M PB, pH 7, and 0.3 and 0.5 M NaCl in 0.01 M PB, pH 7) in a stepwise manner.

2.2.2.3. Ultrafiltration. Clear fractions containing AAO activity were combined and concentrated using ultrafiltration (Toyo Advantec Stirred Cell, molecular weight cut-off 10 000) to a volume of 10 ml (protein concentration 0.62 mg ml^{-1}) and kept at −20°C in the presence of 20% glycerol. It can be stored for more than one year without a significant loss of activity. For activity and AsA measurement, the concentrated AAO solution required 200–250 times dilution with 0.01 M phosphate buffer, pH 7.

2.2.3. Enzyme activity and protein assay

AAO activity was measured according to the spectrophotometric method of Tono and Fujita (1984). The assay mixture contained 0.5 ml of 0.8 mM AsA in 1% metaphosphoric acid (HPO_3), 1.5 ml of McIlvaine buffer, pH 6 (0.2 M disodium hydrogen phosphate–0.1 M citric acid) and 0.5 ml of AAO of appropriate dilution. After incubation at 35°C for 5 min, the reaction was terminated by adding 2.5 ml of 2% HPO_3. For the control, the enzyme was added in the presence of 2% HPO_3. One unit of enzyme activity was defined as that causing an increase of 0.01 in absorbancy/min at 243 nm. Protein was assayed by Hartree's method (1972) with bovine serum albumin as the standard. The protein content of column eluates was estimated by measuring absorbance at 280 nm

2.2.4. Characterization of enzyme

2.2.4.1. Optimum pH. The optimum pH was determined by assessing AAO activity in McIlvaine buffer ranging from pH 3 to 8. The conditions of the assay were otherwise similar to that given above.

2.2.4.2. Heat stability. Heat stability in McIlvaine buffer at the optimum pH was studied from 30 to 70°C. For the determination, 2 ml of AAO solution in a test tube with thermometer was incubated in a temperature-controlled water bath. When temperature of the solution reached the required temperature, 0.5 ml of the solution was withdrawn from the test tube at 30 min. The remaining activity of the enzyme was then determined.

2.2.5. Profile of AAO-catalyzed AsA oxidation

The reaction was initiated by adding 0.5 ml AAO solution (30 units or 3 μg protein) into a reaction mixture containing 0.5 ml AsA solution (final concentration 1.6×10^{-4} M) and 1.5 of McIlvaine buffer pH 5.0. After incubation at 30°C for 5, 10, 15, 20 and 30 min, each reaction mixture was terminated by adding 2.5 ml of 2% HPO_3. For the control (zero minute reaction), the enzyme was added in the presence of 2% HPO_3. The height of the absorbance peak (in mm) for each reaction mixture was recorded using double-beam spectrophotometer. The difference of the absorbance peak (in mm) was then calculated by subtracting the values of each reading at different incubation periods from the control.

2.2.6. Calibration curve and AsA determination

A mixture of 0.5 ml of each working standard solution ranging between 0 and 100 μg ml^{-1} or sample diluted in 1% HPO_3, 1.5 ml McIlvaine buffer pH 5.0 and 0.5 ml of diluted AAO solution (30 units or 3 μg protein) was incubated at 30°C for 20 min. After incubation, the reaction was terminated by adding 2.5 ml of 2% HPO_3 (II). For the control, the enzyme was added in the presence of 2% HPO_3 (I). The difference of the absorbance (I–II) was measured at 243 nm. Absorbance reading was taken between the range of 0 and 1 unit or otherwise further dilution with 2% HPO_3 would be needed. Determination of ascorbic acid by titration against 2,6-dichloroindophenol was performed on 2–5 ml aliquots of sample essentially as outlined by the Association of Official Analytical Chemists (1975).

3. Results and discussion

3.1. Analysis of AAO activity profile in starfruit

Table 1 shows the concentration of AAO activity at different maturity stages of starfruit. The highest activity was found in the early stages of growth, i.e. very immature fruit (3 weeks after fruit sets). The specific activity decreased as the fruit became more mature. A similar observation was also reported for satsuma mandarin fruit (Saari, Fujita, Yamaguchi, & Tono, 1996). The very high concentration of AAO at the early stage has been implicated with its involvement in the growth process in the dividing cell (Lin & Varner, 1991). On the basis of this result, very immature starfruits weighing between 10 and 15 g were chosen as the source of AAO.

3.2. Purification of AAO

Fig. 1 shows the elution profile of AAO activity on DEAE-Toyopearl 650 M column. A large amount of unbound proteins was removed by excessive washing with 0.01 M phosphate buffer, pH 7 as indicated by absorption at 280 nm. Stepwise elution of the column with different buffer concentrations separated the enzyme into several peaks. The emergence of such a profile could have been due to the existence of multiple forms of AAO in starfruit. Suzuki and Ogiso (1973) obtained three to seven different electrophoretic mobilities of AAO in pea seedling roots. Sekiya, Hamade, Kimura, Mizuno, and Shimose (1990) found three AAO forms isolated from cucumber with isoelectric points of 8.3, 8.5 and 8.7. For AAO of starfruit, the highest and bulk of activity were eluted off the column in the low salt concentration of buffer (0.05 M phosphate buffer). The weakly bound AAO collected in the fraction is likely equivalent to the basic AAO of cucumber reported by Sekiya et al. (1990). Minor concentrations of other AAO isozymes were more tightly bound to the column and could only be eluted with buffer containing much higher salt concentrations. Therefore, the bulk fraction of AAO activity of starfruit was easily separated from impurities and colored components. Summary of typical purification of AAO from starfruit is summarized in Table 2. Approximately 6 mg of AAO was obtained with a yield of 43%. The stepwise elution purified the enzyme to a 79-fold increase in specific activity. About 2000 samples can be analyzed with the amount of protein obtained from 2 kg of very immature starfruit.

Table 1
Ascorbate oxidase activity of starfruit at different maturity

Maturity stages	Weeks	AAO activity (unit/mg protein)
Very immature fruit	3	137.1
Immature fruit	6	62.6
Mature green fruit	9	15.9
Ripe fruit	12	2.7

Enzyme extracts were prepared from 100 g of starfruit as described under Materials and methods. The results of two preparations were averaged. AAO activity and protein were determined in triplicate. One unit of AAO activity was defined as an increase in 0.01 absorbancy min^{-1} at 243 nm.

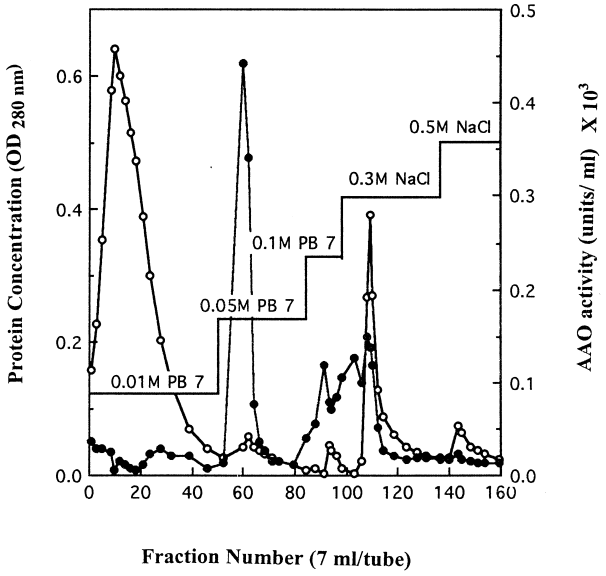

Fig. 1. Elution profile of crude AAO on DEAE-Toyopearl 650 M. Protein (180 mg) in 200 ml was applied onto the column (3.2×16 cm). Elution was by stepwise increase of concentrations of phosphate buffer, pH 7, and NaCl dissolved in 0.01 M phosphate buffer pH 7. Protein concentration —○—; AAO activity —●—.

Table 2
Purification of ascorbate oxidase from starfruit for ascorbic acid determination procedure

Procedure	Volume (ml)	Total activity (unit×10^3)	Total protein (mg)	Specific activity (unit per mg protein)	Purification fold	Recovery (%)
Crude extract	780	143	1115	128.3	1.0	100
$(NH_4)_2SO_4$ fractionation (20–80%)	220	129	207	623.2	4.9	90
DEAE-Toyopearl 650 M	91	69	6.8	10 147.1	79.1	48
Ultrafiltration	10	61	6.2	9838.7	76.7	43

3.3. Absorption spectrum of AAO

Fig. 2 shows the absorption spectrum of the concentrated AAO with a characteristic peak at about 600 nm. The profile is similar to those reported for purified AAO of cucumber (Sekiya et al., 1990), zucchini squash (Lee & Dawson, 1973) and from microorganisms (Murao et al., 1992). This justifies that the enzyme contained copper as its prosthetic entity. Lee and Dawson (1973) proposed that the prosthetic copper influences the catalytic efficiency of the enzyme.

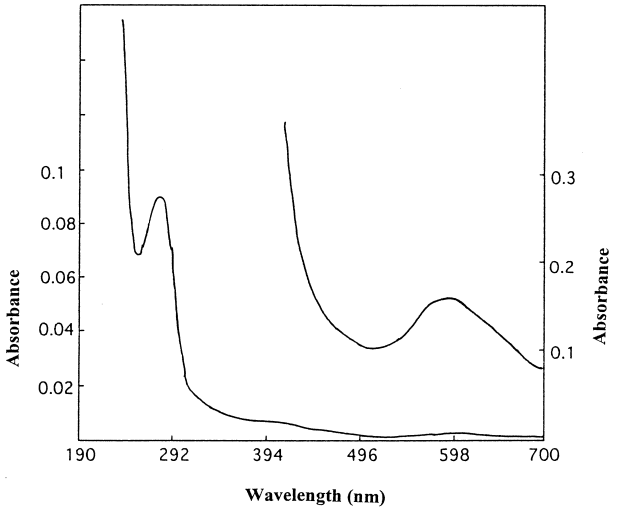

Fig. 2. Absorption spectrum of purified ascorbate oxidase in 0.01 M phosphate buffer, pH 7. Concentrated enzyme from the ultrafiltration step was applied onto Sephadex G-100 gel filtration. Active fractions were pooled and concentrated using ultrafiltration The protein concentration measured in the visible range was 3.0 mg ml^{-1}.

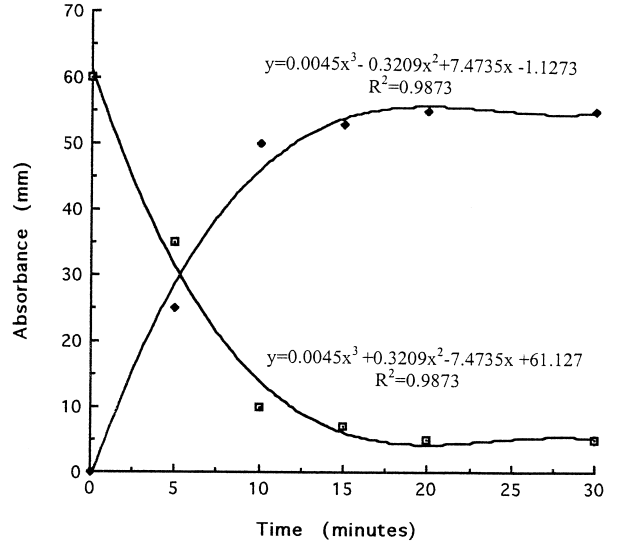

Fig. 3. Ultraviolet absorption spectra (in mm) and the difference in absorbance, ΔA_{243nm} (in mm) during starfruit AAO-catalyzed AsA oxidation. The reaction time was carried out at 0, 5, 10, 15, 20 and 30 min. Absorbance ⊡, — polynomial (absorbance), absorbance difference ◆, — polynomial (absorbance difference).

3.4. Effect of pH and temperature

The effects of pH and temperature on the AAO activity were studied in order to get information for the best reaction conditions of the assay system. The activity of AAO of starfruit was maximal at around pH 5–6. This result is slightly broader than the range between pH 6 and 6.5 reported by Sekiya et al. (1990) and Maccarrone, D'Andrea, Salucci, Avigliano, and Finazzi (1993) for the purified AAO of cucumber and zucchini, respectively. Temperature stability of AAO was found below 45°C for 30 min incubation. This is within the range reported for the enzyme from Cucurbitaceae which had a temperature stability between 32 and 52°C (Saari et al., 1995). On the basis on the above results, the following assay system was conducted in MacIlvaine

Table 3
AsA contents in various colored samples[a] determined by starfruit AAO-catalyzed AsA oxidation (SACAO) method and DIP method

Sample	SACAO method (mg% ± SD)	DIP method[b] (mg% ± SD)
Blackcurrant juice	60 ± 0.14	66 ± 0.32
Tomato juice	19 ± 0.27	23 ± 0.41
Guava juice	22 ± 0.09	25 ± 0.11

Values reported are the averages of three determinations. SD, standard deviation.

[a] The samples were centrifuged at 10 000 ***g*** (5°C) for 15 min and diluted 5 times with 1% HPO_3.

[b] 2,6-Dichloroindophenol.

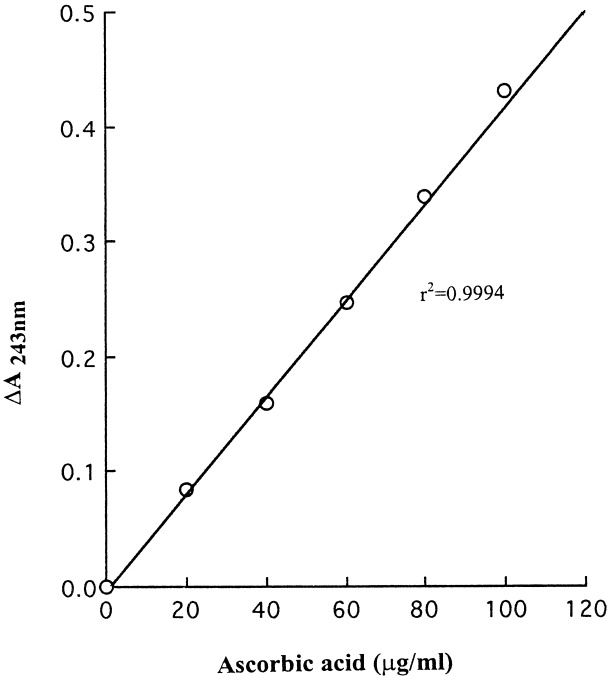

Fig. 4. Relation of AsA concentration to absorbance at 243 nm ($\Delta A_{243\ nm}$).

buffer pH 5 (as AsA is more stable in acidic pH) and at a temperature 30°C. At this temperature, AAO activity of starfruit remained constant for up to 60 min.

3.5. Profile of AAO-catalyzed AsA oxidation

The progress of AsA oxidation by the enzyme as a function of reaction time is shown in Fig. 3. As the enzymatic oxidation of AsA proceeds, the peak height (in mm) of the ultraviolet absorbance of the reaction mixture measured at 243 nm decreases. However, the difference in the peak height (in mm) increases with increasing reaction time. Complete oxidation of AsA as indicated by a plateau region was observed after 20 min incubation as there was no change in the height of the absorbance. This region is taken as the total difference in absorbance (A) and used as a basis for AsA determination.

3.6. Calibration curve and AsA determination

When data of AsA concentrations were plotted against absorbance differences (ΔA_{243nm}), these increases were found to be directly proportional to the concentrations of AsA utilized for up to 100 μg/ml (Fig. 4). Above the concentration, the curve tended to deviate from linearity. The correlation coefficient of the plot was typically 0.9994. This curve was then used to calculate the amount of AsA in colored samples.

Table 3 shows a comparison of AsA contents in colored juices estimated by the starfruit AAO-catalyzed method and by the conventional DIP method. Slightly lower AsA contents were obtained with the enzymatic method. A high specificity of the enzyme towards both L-AsA ($K_m = 1.04 \times 10^{-4}$ M) and D-AsA ($K_m = 2.71 \times 10^{-3}$ M) as well as sensitivity of the method used could be the reason for the relatively low values of AsA. In 2,6-dichloroindophenol method for determination of AsA, accuracy in titrating to the end point is vital to obtain a reproducible result. However, this is difficult for a colored sample even though it has been substantially diluted.

The above data suggested that starfruit AAO-catalyzed method is reliable for determining AsA content in colored juices. In fact, the preparation could overcome problems with the conventional 2,6-dichloroindophenol method. The method for the enzyme preparation yielded a sufficient purity to be used for AsA determination and a high recovery.

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