fatty acids in one candy bar was high, whereas in another comparable type bar the same fatty acids were low. This indicates that various manufacturers used different types of oils and/or mixtures of oils in varying proportions in their formulations.

The total polyunsaturated fatty acids were generally fairly low when compared to the amount of saturated fatty acids (Table I). In most instances the *cis,cis*-methylene polyunsaturated fatty acids calculated as trilinolein were considerably lower than the total polyunsaturated fatty acids, indicating that hydrogenated fats and oils are widely used by candy bar manufacturers.

The cholesterol found in some of the candy bars is probably due to the milk or milk products used in their manufacture since the labels do not declare any animal fat. Coconut, palm kernel, and palm oils and cocoa butter can contribute to the cholesterol content since they contain small amounts of cholesterol and they are listed on the ingredient labels. Sitosterol, a plant sterol, was found in all the candy bars, indicating that vegetable oils are used in their formulations.

Over 50% of the ingredients in the candy bars are carbohydrates (found by difference), ranging from 52.6 to 86.4 g/100 g.

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Polyphenol Oxidase of Dates

Shin Hasegawa* and V. P. Maier

Polyphenol oxidase was purified from an extract of Deglet Noor dates by $(NH_4)_2SO_4$ precipitation, followed by two successive DEAE-cellulose columns, which resulted in a 510-fold increase in specific activity. The purified preparation contained no monophenolase activity and catalyzed the oxidation of only *o*-dihydroxyphenols. The enzyme had maximal activity over a wide pH range, 4.5–6.5, and was relatively heat stable. KCN and thiourea were potent inhibitors. Its substrate specificity was tested on 22 phenols.

Browning is one of the most important quality changes associated with ripening of dates. The color darkens during postharvest processing and storage. Although a broad range of colors is acceptable, color uniformity within a single package or among packages is an important quality factor for consumer acceptance.

Maier and co-workers (Maier and Schiller, 1959, 1960, 1961a,b; Maier and Metzler, 1965a,b; Maier et al., 1964a) have demonstrated three different systems involved in browning of Deglet Noor dates: (1) sugar browning, (2) enzymic oxidative browning of polyphenols, and (3) oxidative browning of tannins. They have shown that the enzymic oxidation of polyphenols is responsible for browning of the fruit during ripening and also contributes somewhat to browning during processing and the early stages of storage.

Catechins and dactylifric acid are the principal polyphenolic enzymic browning substrates present in dates, particularly green dates (Maier and Metzler, 1965b; Maier et al., 1964b). Soluble and insoluble procyanidin tannins are also present, but they are not enzymic browning substrates (Maier and Metzler, 1965a). The concentration of polyphenols decreases steadily during fruit ripening and storage as browning proceeds (Maier and Metzler, 1965a).

Because of the occurrence of caffeoylshikimic acids (dactylifric acids) in dates rather than caffeoylquinic acids (chlorogenic acids) and the importance of polyphenol oxidase in browning of the fruit, we have purified polyphenol oxidase and studied its properties, particularly its substrate specificity.

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EXPERIMENTAL SECTION

Materials. The dates (Deglet Noor variety, *Phoenix dactylifera* L.) used for isolation of polyphenol oxidase were grown at the U.S. Date and Citrus Station, Indio, CA. Samples were harvested at an early red stage and kept at −20 °C until used.

Extraction and Purification of Polyphenol Oxidase. Seven dates taken randomly from storage were pitted, sliced into small disks, and blended in 200 mL of 0.1 M potassium phosphate buffer at pH 7.0 containing 0.5% poly(vinylpyrrolidone). The resulting mixture was centrifuged at 20000*g* for 10 min, and the supernatant was dialyzed against running H_2O for 16 h at 2 °C. The dialysate was then brought to 0.9 saturation with $(NH_4)_2SO_4$

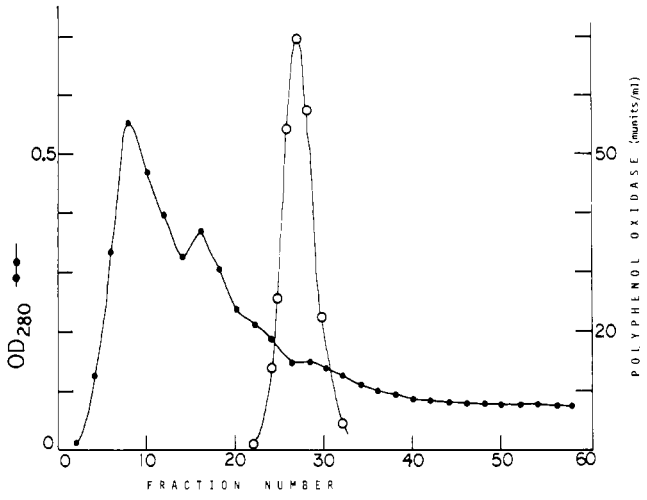

Figure 1. Chromatogram of date polyphenol oxidase on DEAE-cellulose. The column (2.5 × 13 cm) was eluted with a linear gradient of potassium phosphate buffer at pH 7.0 as described under Experimental Section. (●) Protein; (○) polyphenol oxidase.

by addition of solid salt. The precipitate was pelleted by centrifugation at 20000*g* for 15 min, collected, and dissolved in a minimum portion of 0.01 M potassium phosphate buffer at pH 7.0. The solution was then dialyzed against 0.01 M potassium phosphate buffer at pH 7.0 for 3 h.

The dialysate was loaded on a 2.5 × 13 cm column of DEAE-cellulose which had been equilibrated with 0.01 M potassium phosphate buffer at pH 7.0. The column was eluted with a linear gradient formed between 250 mL of 0.05 M potassium phosphate buffer at pH 7.0 and 250 mL of 0.5 M potassium phosphate buffer at pH 7.0. The effluent was collected in 4-mL fractions. Fractions containing enzyme activity were combined, dialyzed against 0.01 M potassium phosphate buffer at pH 6.0, and fractionated again on a 1.5 × 17 cm column of DEAE-cellulose previously equilibrated with 0.01 M potassium phosphate buffer at pH 6.0. The column was eluted with a linear gradient consisting of 200 mL of 0.01 M potassium phosphate buffer at pH 6.0 and 200 mL of 0.2 M potassium phosphate buffer at pH 6.0. The effluent was collected in 5-mL fractions.

Assay Methods. Protein concentration was measured by the procedure of Lowry et al. (1951). Polyphenol oxidase activity was determined by measuring the initial rate of decrease in absorbance at 326 nm. Unless otherwise stated, activity was assayed in 4 mL of a reaction mixture consisting of 0.1 M citrate–phosphate (1:1) buffer at pH 5.0, 1×10^{-4} M chlorogenic acid, and 1–10 milliunits of enzyme. The reaction was carried out at 23 °C in a standard silica cuvette with a 1-cm light path. One unit of polyphenol oxidase activity is defined as the amount which catalyzes the oxidation of 1 μmol of chlorogenic acid per min under the conditions used. For substrate specificity tests, the activity was measured colorimetrically at 430 nm. Reaction mixtures consisted of 0.1 M citrate–phosphate (1:1) buffer at pH 5.0, 5×10^{-3} M of various substrates, and 5 milliunits of the enzyme in 4.0 mL.

RESULTS AND DISCUSSION

Purification of Polyphenol Oxidase. Typical elution patterns for the two-step purification of polyphenol oxidase isolated from Deglet Noor dates are shown in Figures 1 and 2. The purification steps are summarized in Table I. The overall purification resulted in a 510-fold increase in specific activity over the original extract, and the recovery was 32%. The final preparation, fractions 86–94 (Figure 2), was used for characterization of the enzyme.

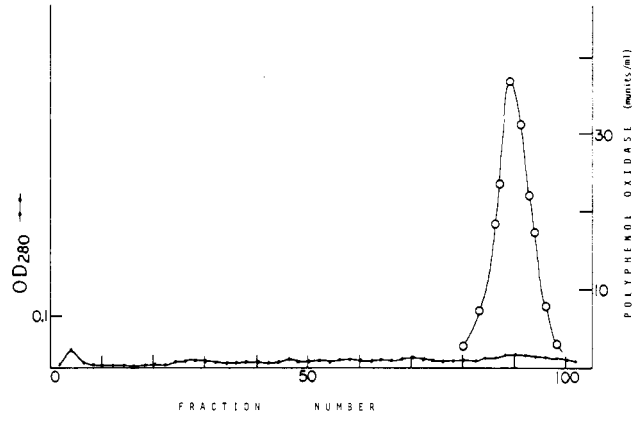

Figure 2. Chromatogram of date polyphenol oxidase on DEAE-cellulose. The column (1.5 × 17 cm) was eluted with a linear gradient of potassium phosphate at pH 6.0. (●) Protein; (○) polyphenol oxidase.

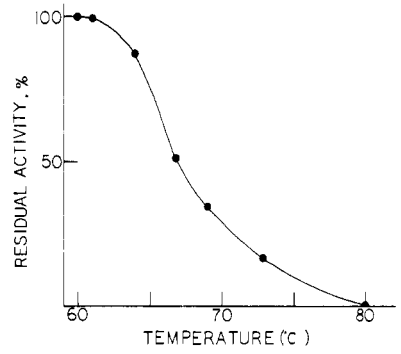

Figure 3. Effect of heat on stability of date polyphenol oxidase. Enzyme solutions, pH 5.0, were heated at various temperatures for 10 min, and the residual activities were determined by the standard procedure.

Table I. Purification of Date Polyphenol Oxidase

treatment	total vol, mL	act., milli-units	pro-tein, mg	sp act., milli-units/mg	purifn, *x*-fold	recov-ery, %
extract	217	2886	95.48	30.2	1	100
$(NH_4)_2$-SO_4 ppt	15	2280	75.00	30.4	1	79
first column	47	2115	3.01	1500	50	73
second column	30	924	0.06	15400	510	32

Properties of Polyphenol Oxidase. The activity of polyphenol oxidase at various hydrogen ion concentrations showed that the enzyme has its optimum activity over a broad pH range, 4.5–6.5.

Date polyphenol oxidase was relatively heat stable (Figure 3). We determined this stability by heating the enzyme (2 milliunits) in 0.1 M citrate–phosphate buffer (pH 5.0) solutions at different temperatures for 10 min and measuring residual activity under the standard conditions. Treatment at 61 °C had no effect on activity, treatment at 67 °C caused a 50% loss, and treatment at 80 °C caused a 100% loss. The control enzyme preparation for these tests was held at 0 °C before its activity was assayed.

KCN and thiourea, common inhibitors of oxidases, were potent inhibitors of date polyphenol oxidase (Table II).

Substrate Specificity. The results of tests on substrate specificity are summarized in Table III. Date polyphenol oxidase did not catalyze the oxidation of mono-

Table II. Effects of Inhibitors on Date Polyphenol Oxidase Activity[a]

inhibitors	concn, M	act., ΔOD_{326}/min	inhibn, %
none		9.2×10^{-3}	
KCN	5×10^{-4}	0	100
	5×10^{-5}	3.8×10^{-3}	58.7
thiourea	1×10^{-3}	5.1×10^{-3}	44.5
	1×10^{-4}	7.0×10^{-2}	24.0

[a] Reaction mixtures contained 0.1 M citrate–phosphate buffer at pH 5.0, 5×10^{-4} M chlorogenic acid, 2 milliunits of enzyme, and various concentrations of the inhibitors in 4.0 mL.

Table III. Substrate Specificity

substrate	relative act.[a]
p-cresol	0
L-tyrosine	0
o-, *m*-, and *p*-coumaric acid	0
catechol	71.2
hydroquinone	0
resorcinol	0
phloroglucinol	0
pyrogallol	5.6
2,5-dihydroxybenzoic acid	0
3,4-dihydroxybenzoic acid	0
4-methylcatechol	6.5
(+)-catechin	4.0
(−)-catechin	4.3
(+)-epicatechin	20.0
(−)-epicatechin	100.0
(−)-epicatechin gallate	12.0
(+)-gallocatechin	0
(−)-epigallocatechin gallate	0
dactylifric acid (3-*O*-caffeoylshikimic acid)	24.3
isodactylifric acid (4-*O*-caffeoylshikimic acid)	17.0
chlorogenic acid (3-*O*-caffeoylquinic acid)	10.0
caffeic acid	2.7

[a] Activity is relative to that of (−)-epicatechin oxidation.

phenols such as *p*-cresol, L-tyrosine, and *o*-, *m*-, and *p*-coumaric acids and hence had no monophenolase (cresolase) activity. It has been reported also that polyphenol oxidases isolated from peach (Wong et al., 1971), tea (Gregory and Bendall, 1966), and banana (Clayton, 1959) have no cresolase activity.

The enzyme attacked only *o*-dihydroxyphenols. Catechol (1,2-dihydroxybenzene) was an excellent substrate for the enzyme. The enzyme, however, did not attack hydroquinone (1,4-dihydroxybenzene), showing that like other polyphenol oxidases the enzyme attacks only *o*-dihydroxy groups.

Phloroglucinol (1,3,5-trihydroxybenzene) was not a substrate of the enzyme. As expected, pyrogallol (2,3-dihydroxyphenol) was attacked, but the rate was 0.08 times that of catechol. It is of interest that the enzyme did not attack 3,4-dihydroxybenzoic acid. Apparently, the carboxyl group at C-1 inhibited the activity. A methyl group attached to the same position, on the other hand, did not inhibit the activity completely, as evidenced by the fact that the enzyme attacked 4-methylcatechol. However, the rate of oxidation of 4-methylcatechol was approximately 0.09 times that of catechol.

One class of EtOAc-soluble polyphenols in Deglet Noor dates is the catechins (Maier and Metzler, 1965a). Among four stereoisomers tested, (−)-epicatechin was the best substrate for the enzyme, followed by (+)-epicatechin, (−)-catechin, and (+)-catechin. The hydroxy group at C-3 had a significant influence on the activity. When (−)-epicatechin gallate (gallic acid attached to the hydroxy group at C-3) was tested, the activity was 0.12 times that of (−)-epicatechin. The bulky gallate moiety apparently interferes with the affinity between the enzyme and the substrate. The enzyme did not attack (−)-epigallocatechin gallate.

The major EtOAc-soluble polyphenols in green dates are dactylifric acids, the three position isomers of monocaffeoylshikimic acid (Maier et al., 1964b). The apparent K_m values for dactylifric acid (3-*O*-caffeoylshikimic acid), isodactylifric acid (4-*O*-caffeoylshikimic acid), and chlorogenic acid (3-*O*-caffeoylquinic acid) were 4.1×10^{-5}, 7.8×10^{-4}, and 1.5×10^{-3} M, respectively. The V_m values for dactylifric acid and isodactylifric acid were respectively 2.4 and 1.7 times the V_m value for chlorogenic acid. These values clearly show the date polyphenol oxidase preferentially catalyzes the oxidation of polyphenolic compounds present in dates. Chlorogenic acid has been shown to be the best substrate for polyphenol oxidases of other plants (Pendharkar and Nair, 1974).

The Deglet Noor dates contained approximately 0.39–0.43 unit of polyphenol oxidase activity per fruit at the early red stage of ripening. The activity remained unchanged in dates harvested at the late ripe stage (about 0.4 unit/fruit). Because of its relatively high heat stability, the enzyme most likely stays active during postharvest treatments. The results of this study strongly support the previous findings by Maier and Metzler (1965a) that there are potential enzymic browning systems in dates throughout ripening and storage stages.

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