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Inhibition of Potato Polyphenol Oxidase by Anions and Activity in Various Carboxylate Buffers (pH 4.8) at Constant Ionic Strength

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The activity of potato polyphenol oxidase (tyrosinase) toward DL-3,4-dihydroxyphenylalanine (K_M 5.39 mM) was studied using a variety of carboxylate buffers at a common pH and ionic strength. Enzyme activity, greatest in citrate and least in oxalate, correlated with increasing carboxyl concentration and molecular mass. The lower activity in oxalate was attributed to more effective chelation of a copper(II) form of the enzyme by the oxalate dianion. Sodium halide salts inhibited the enzyme. Although there was little difference in inhibition between sodium and potassium salts, the degree and type of inhibition was anion dependent; K_{is}, values for NaCl and KCl, (competitive inhibitors) were 1.82 and 1.62 mM, whereas Na_2SO_4 and K_2SO_4 (mixed inhibitors) had K_{is} and K_{ii} values in the 250 to 450 mM range.

Keywords: Tyrosinase, Polyphenol oxidase, Anionic inhibition, Buffer effects

INTRODUCTION

Polyphenol oxidase (tyrosinase), is a widely distributed oligomeric, copper-containing enzyme. It is responsible for browning reactions ranging from the oxidative darkening of damaged vegetables and fruits upon exposure to air, to skin pigmentation (synthesis of melanin). The activity of the enzyme can be separated into two reactions: *ortho*-hydroxylation of phenols to catechols (cresolase activity) and oxidation of catechols to *ortho*-quinones (catecholase activity).[1] This study considers only the latter activity.

The mechanism of the enzyme is fairly complex (Scheme 1), involving cycling among three enzymatic states, *deoxy*-, *oxy*-, and *met*-. The *oxy*-enzyme, $[Cu(II)]_2O\text{-}O$, is reversibly formed from the *deoxy*-form $[Cu(I)]_2$ upon binding molecular oxygen which oxidizes $[Cu(I)]_2$ and becomes bound peroxide. The *oxy*-enzyme can then bind and oxidize a number of different *ortho*-diphenolic compounds to the corresponding *ortho*-quinone plus water, leaving the *met*-enzyme $[Cu(II)]_2$. The latter form can then bind and oxidize another molecule of *ortho*-diphenolic compound to yield the *ortho*-quinone plus water

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$$[Cu(I)]_2 + O_2 \Leftrightarrow [Cu(II)]_2O\text{-}O$$
(*deoxy-*) (*oxy-*)

$$2H^+ + [Cu(II)]_2O\text{-}O + \textit{ortho}\text{-diphenol} \rightarrow$$
(*oxy-*)

$$[Cu(II)]_2 + \textit{ortho}\text{-quinone} + 2H_2O$$
(*met-*)

$$[Cu(II)]_2 + \textit{ortho}\text{-diphenol} \rightarrow [Cu(I)]_2 + \textit{ortho}\text{-quinone} + 2H^+$$
(*met-*) (*deoxy-*)

$$\text{Sum: } O_2 + 2\,\textit{ortho}\text{-diphenol} \rightarrow 2\,\textit{ortho}\text{-quinone} + 2H_2O$$

SCHEME 1 Proposed mechanism for polyphenol oxidase.[2]

and the *deoxy*-enzyme.[2] The dominant resting form of the enzyme is the *met*-enzyme.

Recently Cho and Ahn[3] saw weak inhibition of the potato enzyme by NaCl and reported that NaCl was an uncompetitive inhibitor of the enzyme with a K_{ii} of 0.3 M. In addition, they found that only KCl and $CuCl_2$, from a number of metal chloride salts, affected tyrosinase activity. Whereas 0.04 mM $CuCl_2$ stimulated activity about 2.6-fold, 0.4 M KCl reduced enzyme activity by 1.4-fold. They interpreted these results to mean that K^+ has a greater affinity for the enzyme than Cu^{2+} and readily displaces Cu^{2+} from the enzyme.[3] Effects of Cl^- from $ZnCl_2$, $NiCl_2$, $AlCl_3$, $CoCl_2$, $CaCl_2$, $HgCl_2$, $MnCl_2$, all tested up to 1.0 M, were not detected relative to that seen with NaCl and KCl. Moreover, the fact that Cl^- in the former salts failed to inhibit, whereas both NaCl and KCl acted as inhibitors, was not addressed.

Here we report experiments that demonstrated that the catecholase activity of potato tyrosinase was affected by the nature of the buffer in which reactions were carried out. Studies done at constant pH and ionic strength showed that activity was correlated to the concentration of carboxyl groups and also to molecular mass. Activity in oxalate was much less than that in citrate, the best of the buffers. This was true whether ionic strengths were adjusted with NaCl (a strong inhibitor) or Na_2SO_4 (a weak inhibitor or stimulator at the ionic strength used in the reactions). The mode of inhibition of tyrosinase by NaCl and KCl was predominantly competitive, whereas Na_2SO_4 and K_2SO_4 were mixed inhibitors. We found no difference between the sodium and potassium salts and thus, no cationic effect on activity.

MATERIALS AND METHODS

Enzymes and Chemicals

Russet potatoes were used for partial purification of tyrosinase, following the protocol of Cho and Ahn[4] through the ammonium sulfate step, except that homogenization was in 12.5 mM citric acid and 25 mM sodium phosphate buffer, pH 4.8. After the final centrifugation step, the pellet was dissolved in the same buffer, again centrifuged and frozen at −20 °C. There was no detectable loss of activity over several weeks. Mushroom tyrosinase, used to verify the NaCl inhibition, was purchased from Sigma-Aldrich Co. (T7755), St. Louis MO, USA. All chemicals were of the highest purity commerically available.

Enzyme Assay

Enzymatic assays were routinely carried out using a Shimadzu UV-1201 spectrophotometer by monitoring the increase in absorbance at 475 nm, corresponding to the formation of the semi-stable product of DL-DOPA oxidation, dopachrome.[5] Enzyme reactions were performed with varying concentrations of DL-DOPA at 25 °C

in 3.0 mL that contained 12.5 mM citric acid and 25 mM sodium phosphate (pH 4.80) in addition to the substrate, enzyme, and any other addition. Phosphate was omitted from the buffer study and all buffer concentrations were 12.5 mM, with ionic strengths adjusted to 0.061 (that of the citrate-phosphate buffer at pH 4.80) with either NaCl or Na_2SO_4. A stock solution of 30 mM substrate was used to give final concentrations from 2 to 24 mM DL-DOPA. Independent experiments of initial velocities versus relative enzyme concentration were performed to show the range of enzyme concentration that yielded linearity with saturating ($10\,K_M$) substrate. The enzyme was used in a final dilution in the reaction mixture of at least 1:240 from the initial stock solution. Reactions were monitored over a period of several minutes, with initial velocities calculated as $v_o =$ (A_{475} after 2 min) – (A_{475} after 1 min), converted to μmol min^{-1} using the 3600 $M^{-1}\,cm^{-1}$ extinction coefficient for dopachrome, reported by Duckworth and Coleman.[1] A unit of tyrosinase activity is defined as 1.0 μmol product formed per minute.

Determination of K_{is} and K_{ii} values

Separate solutions of 1.5 M NaCl, 1.5 M KCl, 2 M Na_2SO_4 and 0.8 M K_2SO_4 were prepared in buffer. These were added to the enzyme reactions in final concentrations ranging from 5.0 to 16.7 mM for the chloride salts, 41.7 to 333.3 mM for Na_2SO_4 and 16.7 to 133.3 mM for K_2SO_4. The data were plotted using the Lineweaver-Burk equation ($1/v_o$ *vs* $1/[S_o]$) and K_M, K_{is}, and K_{ii} values were determined by linear regression of the data. Dixon plots ($1/v_o$ *vs* [inhibitor$_o$]) for each inhibitor were used to verify the mode of inhibition and the K_{is} values were again determined using linear regression.

Preparation of Buffers at Constant pH and Ionic Strengths

Commercially available alkyl and alkenyl carboxylic acids were obtained and used to prepare a series of 12.5 mM buffer solutions all at pH 4.80 and an ionic strength of 0.058 ± 0.003. Ionic strengths were calculated from the equation $I = 0.5\sum c_i Z_i^2$, where c is the molar concentration of the ion and Z is the charge on the ion. The Henderson-Hasselbalch equation was used to calculate the respective concentrations of protonated carboxyl, [-COOH], and deprotonated carboxylate, [$-COO^-$], forms of the carboxylic acids, based on their respective pK_a's.[6] From the sum of the carboxylate concentrations, the necessary amount of NaCl needed to achieve an ionic strength of 0.061 (that of the citrate-phosphate buffer routinely used) was added. NaOH was then used to adjust the pH to 4.80. A summary of these calculations and additions is shown in Table I.

TABLE I Activity of potato tyrosinase in a variety of alkyl and alkenyl carboxylate buffers at pH 4.80 and an ionic strength $\cong 0.061$ *I*. The pK_a values in the Table were obtained from reference 6. pH's were adjusted to 4.80 using NaOH and either a Corning Model 7 or Orion Model 720A pH meter standardized to pH 4.00. The sodium ion concentrations are assumed as they are based on an ionic strength of 0.061 and are therefore dependent on the accuracy of the pK_a's

Buffer	pK_a	Concentration (mM)				Activity (μmol min^{-1})
		-COOH	$-COO^-$	Na^+	Cl^-	
Acetate	4.76	5.890	6.60	61.00	54.40	0.675
Adipate	4.42	3.680	8.82			
	5.41	9.970	2.53			
	Σ	13.650	11.40	62.10	47.50	0.916
Citrate	3.09	0.240	12.30			
	4.75	5.890	6.61			
	5.41	10.000	2.46			
	Σ	16.130	21.37	62.20	37.5	1.986
Fumarate	3.10	0.025	12.25			
	4.60	4.840	7.66			
	Σ	4.865	19.91	68.70	33.4	0.795
Malate	3.40	0.498	12.00			
	5.20	8.940	3.56			
	Σ	9.438	15.56	67.44	39.0	0.904
Oxalate	1.23	$\cong 0$	12.50			
	4.19	2.470	10.00			
	Σ	2.470	22.50	65.30	45.8	0.325
Succinate	4.19	2.470	10.00			
	5.48	10.300	2.16			
	Σ	12.770	12.16	64.04	45.8	1.482

RESULTS

Buffer Effects

Autooxidation of dopachrome is decreased at lower pH,[5] and therefore assays were done at pH values below 5.5. Various anions, including aromatic carboxylates, are known inhibitors of tyrosinase.[1,3,7] Further, citrate is frequently used as a buffer or buffer component when studying tyrosinase activity at lower pH. Therefore, we examined the effects of a number of alkyl and one alkene carboxylate buffers at a constant pH (4.80) on tyrosinase activity. Because many enzymes are affected by ionic strength,[8] all buffer solutions were adjusted to a constant ionic strength of 0.061 by addition of either NaCl or Na_2SO_4. At the ionic strength selected, Cl^- is inhibitory whereas inhibition by Na_2SO_4 is negligible (Figure 1) or even stimulatory at low concentrations (data not shown).

The best correlation between activity and any variable we examined was with carboxyl concentration ([-COOH] – Figure 2A, $R^2 = 0.757$; data from Table I); the next best correlation was with molecular mass (Figure 2B, $R^2 = 0.615$, data from Table I). Carboxylate concentration ([-COO^-] – $R^2 = 0.087$), and total anion concentration ([Cl^-] + [-COO^-] – $R^2 = 0.016$) did not appear to correlate with tyrosinase activity.

Because NaCl at an ionic strength of 0.061 is an inhibitor, whereas Na_2SO_4 is not, the above experiments were repeated for oxalate, acetate, succinate, and citrate buffers with Na_2SO_4 used to adjust to the same common ionic strength. This experiment, performed in duplicate each time, was done at two enzyme concentrations and also at two different pH values (Table II).

On the basis of these experiments it was concluded that citrate-phosphate buffer was both effective and optimal at the desired pH's. In agreement with others,[1,7] the absence or pre-

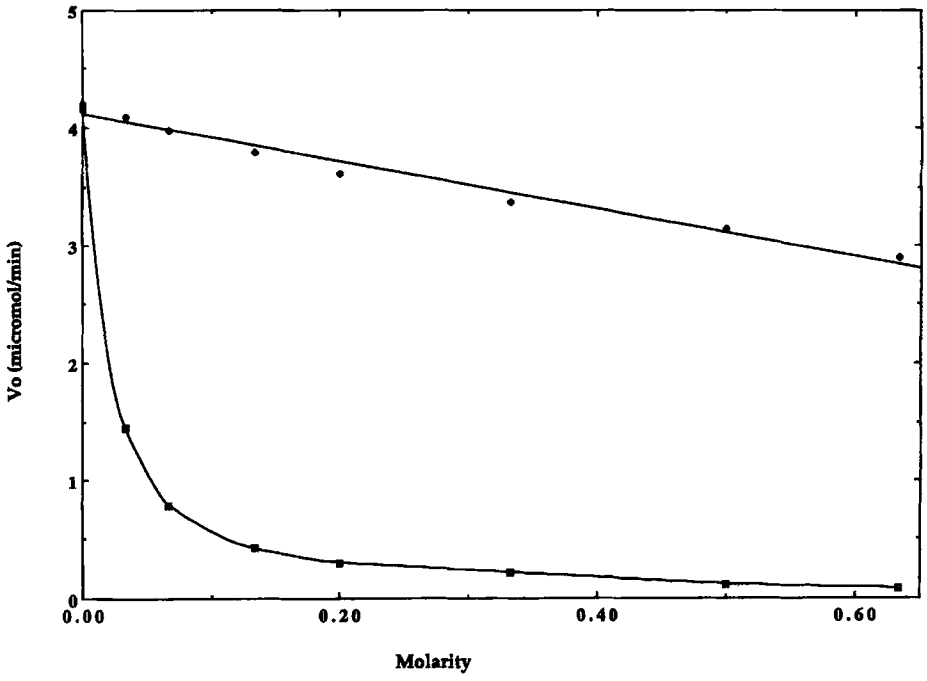


FIGURE 1 Effects of NaCl and Na_2SO_4 on potato tyrosinase at pH 4.80. Reaction mixtures were as described in Methods and contained the final concentrations of the salts shown. □ – NaCl, ◆ – Na_2SO_4.

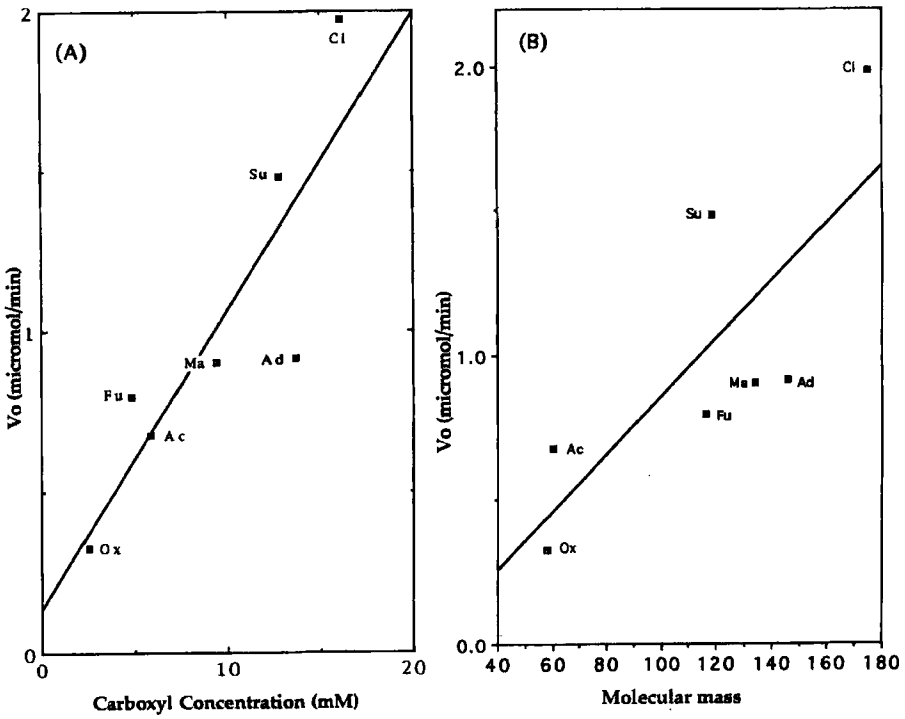


FIGURE 2 Correlation of activity of potato tyrosinase in a variety of buffers at pH 4.80 and 0.061 *I* with [-COOH] (A) and molecular mass (B). The data plotted used the information in Table I. Ci, citrate; Su, succinate; Ad, adipate; Ma, malate; Ac, acetate; Fu, fumarate; Ox, oxalate.

TABLE II Activity of potato tyrosinase at two different enzyme concentrations and pH values as a function of carboxyl group concentration at an ionic strength of 0.061 as adjusted with Na_2SO_4. R^2 values for the two experiments were 0.766 (pH 4.80) and 0.654 (pH 5.09–5.19)*

Buffer	Trial 1			Trial 2		
	pH	[-COOH] (mM)	μmol min^{-1}	pH	[-COOH] (mM)	μmol min^{-1}
Oxalate	4.80	2.470	0.114	5.15	1.24	0.030
Acetate	4.80	5.890	0.133	5.10	3.92	0.139
Succinate	4.80	12.770	0.151	5.09	10.30	0.148
Citrate	4.80	16.130	0.247	5.19	11.30	0.155

*The rates of activity shown are the means of duplicate assays. Error spread between duplicates for trial 1 ranged from 0 (acetate) to 5% (oxalate) and for trial 2 from 0 (citrate) to 36% (acetate).

sence of phosphate, mainly the monoanion at pH 4.8, had no observable effect. (The activities reported in the above Tables were in the absence of phosphate, whereas those which follow were in its presence.)

In aqueous solutions oxalate has a much stronger affinity for free copper(II) than any of the other carboxylate buffers ($K = 6.92 \times 10^4$ at 0.1 *I*). Under the same conditions *K* values for the other carboxylates vary from 68 to 398.[9] However, there is no clear correlation between the magnitude of the binding constant and the activities shown in Table I. To establish that the interaction depended upon carboxylate rather than simply electronegative oxygen, both glyoxal (10–80 mM) and ethylene glycol

(4.17 and 150 mM) were tested as potential inhibitors. No inhibition was observed in any of these trials.

Effects of Sodium Halides and $NaClO_3$ on the Activity of Tyrosinase

Because of the greater inhibition by NaCl, compared to that of Na_2SO_4 (Figure 1), it was of interest to examine the effects of other halides on tyrosinase activity. The results of a survey the sodium halide series is shown in Figure 3. Also shown in the Figure is the effect of $NaClO_3$ on activity.

Inhibition by NaCl, KCl, Na_2SO_4 and K_2SO_4

For the following trials, assays, with five different concentrations of substrate were each measured in the absence and presence of four concentrations of inhibitor. Because V_{max} is not a constant but varies with total enzyme concentration, studies with each inhibitor were done consecutively, with the same enzyme dilution,

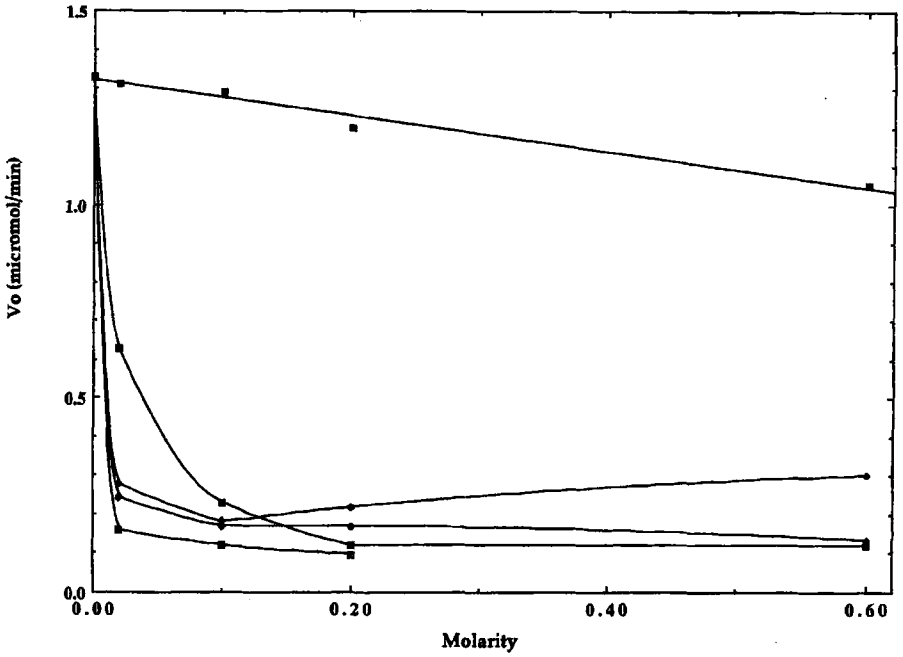


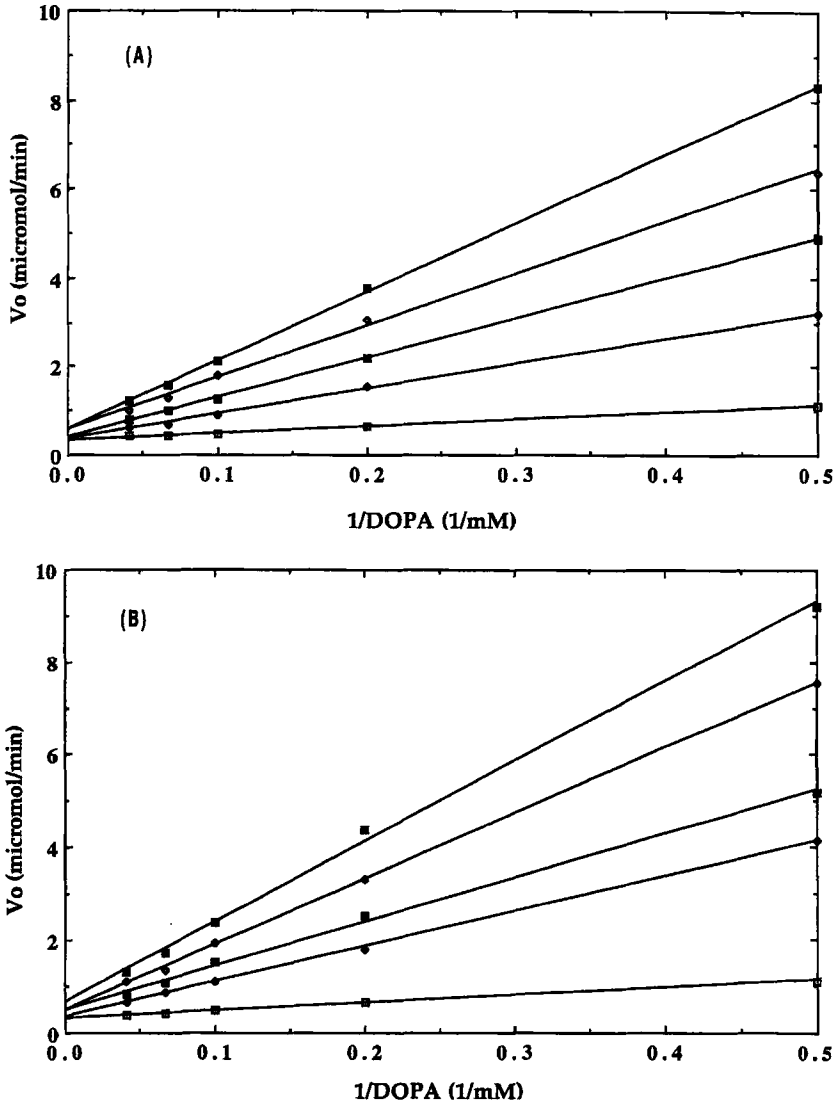
FIGURE 3 Survey of the effects of sodium halide salts and $NaClO_3$ on potato tyrosinase at pH 4.80 and a constant ioic strength of 0.061 adjusted with Na_2SO_4. (■) $NaClO_3$; (◘) NaBr; ◇ NaI; ◆ NaCl; □ NaF.

TABLE III Kinetic parameters of polyphenol oxidase activity on DL-DOPA as determined from the Lineweaver-Burk plots. Errors are listed as ± one standard deviation. The average $K_M = 5.39 \pm 0.57$ mM and the average $V_{max} = 3.25 \pm 0.36$ μmol min^{-1}.

Parameter	Inhibitor			
	NaCl	KCl	Na_2SO_4	K_2SO_4
K_M (mM)	4.59	5.35	5.77	5.83
V_{max} (μmol min^{-1})	3.01	3.25	3.73	2.89
K_{is} (mM)	1.82 ± 0.07	1.62 ± 0.17	417.03 ± 81.03	444.65 ± 92.28
K_{ii} (mM)	–	–	676.28 ± 209.77	288.47 ± 1244.4

over a time span of less than two hours, on the same day. This gave a set of five curves for a single inhibitor, which were plotted using the Lineweaver-Burk and Dixon replot ($1/v_o$ *vs* $[I_o]$ – not shown) methods. In each case the data were fit by linear regression (Figure 4A–4D). The K_M values were found from the data set of 0 mM inhibitor. These values were then used to solve for the K_{is} and K_{ii} values for each concentration of inhibitor, which were then averaged. The results are compiled in Table III.

The effects of NaCl were also studied using commercially obtained tyrosinase purified from mushrooms. As was the case with the enzyme from potato, inhibition at the higher concentrations of DOPA was competitive and three K_{is} measurements averaged to 0.67 mM ± 0.05. The K_M obtained was 1.64 mM, roughly an order of magnitude greater than that found at a similar pH for catechol with the mushroom enzyme by Duckworth and Coleman (0.294 mM at pH 5.08).[1]



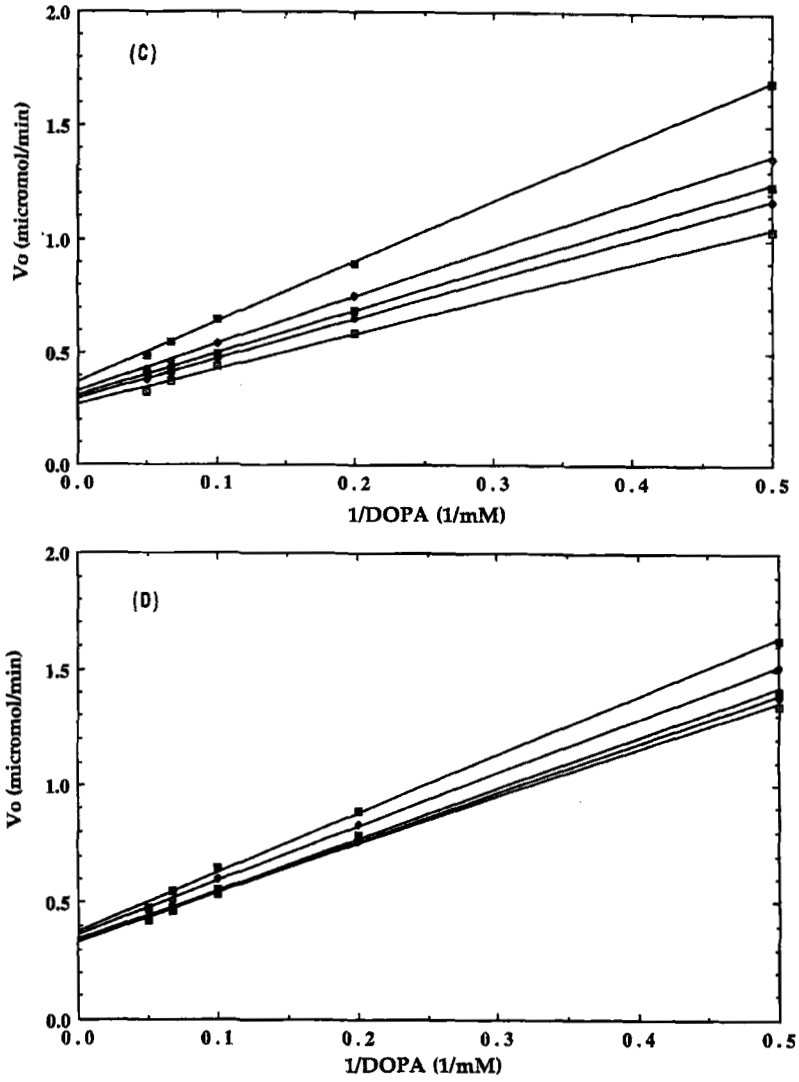


FIGURE 4 The inhibition of potato tyrosinase by sodium and potassium salts. All trials were done in citrate-phosphate buffer (12.5 mM and 25 mM, respectively) at pH 4.80. (A), NaCl: increasing concentrations of NaCl from bottom to top, 0.00, 5.00, 8.33, 12.50, 16.67 mM, ($R^2 = 0.9987$, 0.9983, 0.9999, 0.9980, 0.9997). (B), KCl: increasing concentrations of KCl from bottom to top, 0.00, 5.00, 8.33, 12.50, 16.67 mM, ($R^2 = 0.9998$, 0.9996, 0.9977, 0.9998, 0.9980). (C), Na_2SO_4: increasing concentrations of Na_2SO_4 from bottom to top, 0.00, 41.67, 83.33, 166.67, 333.33 mM, ($R^2 = 0.9982$, 0.9998, 0.9997, 0.9999, 0.9997). (D), K_2SO_4: increasing concentrations of K_2SO_4 from bottom to top, 0.00, 16.67, 33.33, 66.67, 133.33 mM, ($R^2 = 0.9964$, 0.9997, 0.9987, 0.9997, 0.9986). The data were fit by linear regression lines using Microsoft Excel 2000 with R^2 values given in order of increasing concentration of inhibitor. Kinetic parameters are shown in Table III.

DISCUSSION

Polyphenol oxidase acting on DL-DOPA is easily monitored at A_{475} due to the sequence of non-enzymatic reactions that follow the initial enzymatically-catalyzed oxidation step. DOPA is oxidized to *o*-dopaquinone, following which the amino group of the product spontaneously undergoes an intramolecular 1,4-addition to the benzene ring, producing leukodopachrome. This is oxidized to dopachrome by another molecule of *o*-dopaquinone, which is reduced

back to DOPA.[10] Dopachrome is a chromophore, with a maximum absorption at 475 nm and a molar extinction coefficient of 3600 M^{-1} cm^{-1}.[1]

Although this assay method is useful, there are several drawbacks. A major disadvantage is that dopachrome is unstable in solution at pH values above 6.8, so measurements can only be made for a few minutes. In addition, the extinction coefficient of dopachrome is relatively low, so the assay has a low sensitivity.[11] Initially, we attempted to solve these problems by using a different assay, devised by Winder and Harris, that utilizes 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH), which traps the immediate product of the enzymatic reaction to yield a chromophore that absorbs maximally at 505 nm.[11] Advantages of this assay are that MBTH reacts with the immediate product of the enzymatic oxidation of DOPA and the extinction coefficient of the chromophore is high (~29,000 M^{-1} cm^{-1}), making it a more sensitive assay.[11] However, MBTH is fairly insoluble in water and is added to reactions as a solution in dimethylformamide.[11] We discuss this here because we found that this assay, which was developed using a higher pH and mammalian enzymes that have much lower K_M values than the potato enzyme, was unsuitable for our purposes because the concentration of DOPA in our assays was frequently greater than the maximum concentration of MBTH possible in the reaction mixture (2 to 24 mM as opposed to 6 mM). This raised the possibility that the MBTH was not able to trap all of the product and gave inaccurate results. Thus, we used the traditional dopachrome assay. The experiments were done, however, at low pH to stabilize the dopachrome product.[1]

One goal of the research described here was to examine the effects of various carboxylate buffers, some of which are known inhibitors of tyrosinase.[1,7] An additional goal was to resolve the type and magnitude of inhibition by choride and to determine whether there is a difference between the sodium and potassium salts, that is, a cation effect.[3] The buffer study, which was done at constant pH and ionic strength, showed that, of those we studied, tyrosinase had the greatest activity in citrate buffer. Phosphate buffer at pH 7.0 was used by Duckworth and Coleman[1] and Wilcox *et al.*[7] and is considered to not have any effect on tyrosinase activity. It has also been reported that succinate and pyrophosphate do not inhibit at 25 mM concentrations[12] and were the constituents of a series of buffers used by Duckworth and Coleman to study the effect of pH on oxidation of catechol.[1] Therefore, citrate, which in our hands gave higher activity than succinate (both at 12.5 mM), can be considered as non-inhibitory and, by analogy, the same can be said of the citrate-phosphate buffer.

In contrast to the significant correlation found between carboxylate inhibition and conjugation of the carboxylate with an aromatic ring (i.e., higher inhibition with benzoic acid derivatives, but much lower inhibition with phenylacetic acid)[7] the most significant correlation found in this series of carboxylates was a negative correlation with [-COOH] (i.e., lower activity with lower [-COOH]). However, the converse, that activity is lowest with higher [$-COO^-$], was not found. The range of activities seen likely reflects selective differences in the interaction of these carboxylates with the protein site to which copper ions are bound. The major difference in effectiveness between the series of benzoic acid derivatives[7] and the series of non-aromatic carboxylates studied here is probably that the former act more as substrate mimics than do the latter, and therefore bind with higher affinity to the enzyme at the dinuclear copper site. While we cannot make a direct comparison between the carboxylates we studied to the aromatic acids mentioned above, the latter appear to be at least a two orders of magnitude more effective.

Of all the buffers tested, tyrosinase activity was lowest in oxalate (least amount of carboxyl, lowest M_r, most carboxylate). In this regard, it is

of interest that oxalate is known to act as a natural inhibitor of tyrosinase from spinach leaves.[13] Based on its significant affinity for free copper(II) under conditions of ionic strength similar to those we used ($K = 6.92 \times 10^4$ at 0.1 I),[9] coupled with the fact that neither glyoxal nor ethylene glycol had any effect, it seems likely that this small dianion more effectively chelates the copper(II) in the active site of *met-* and/or *oxy-*form(s) of tyrosinase[7] because of its small size, geometry, and charge distribution relative to the other carboxylates.

About 85% of resting tyrosinase is in the *met-*form,[14] which is known to bind exogenous ligands.[15] Carboxylate ligands that also contain an aromatic ring are known inhibitors of tyrosinase.[1,7,12] A series of these carboxylate compounds, in addition to acetate, was studied by Wilcox *et al.*[7] with respect to their binding to the *oxy-*, *met-*, and half-*met-*forms of the enzyme from *Neurospora crassa* in succinate buffer at pH 5.0. [Half-*met-*tyrosinase has one copper in the Cu(I) oxidation state and one in the Cu(II) oxidation state.] Inhibition appears to be through displacement of peroxide from the *oxy-*form.[7] The same group also studied the effects of CN^- and N_3^- on the enzyme, confirming earlier reports [16,17] and found that both acted as mixed inhibitors using L-DOPA as substrate (K_M, 1.04 mM). In contrast benzoate is a competitive inhibitor.[1,7] Wilcox *et al.* propose that benzoate, and by implication the other carboxylates they studied, must act as a competitive inhibitor with respect to L-DOPA by binding to the *oxy-*, *met-*, and *deoxy-*forms of the enzyme. Furthermore, CN^-, which is competitive with oxygen, binds only the *deoxy-*form, and N_3^-, which binds both the *met-* and *oxy-*forms, both act as mixed inhibitors.[7] In agreement with Duckworth and Coleman,[1] they propose a random, sequential mechanism for the enzyme. In contrast to these results found with the *Neurospora* enzyme using L-DOPA as substrate, Cho and Ahn, using catechol as substrate, found CN^- to be a competitive inhibitor (K_{is}, 32 mM) and azide to be an uncompetitive inhibitor (K_{ii}, 3.3 mM) of the oxidation of catechol.[3]

In our hands at pH 4.90 the enzyme from potato had a K_M for catechol of 1.63 mM, a value somewhat lower than that obtained by Cho and Ahn (4.11 mM) at pH 6.6,[4] whereas the K_M they reported for L-DOPA, 3.2 mM (also at pH 6.6), was less than we obtained (an average K_M of 5.39 mM) for DL-DOPA at pH 4.80.

Halide salts were remarkably effective inhibitors of tyrosinase, but complex ions such as ClO_3^- and SO_4^{2-} had little effect. The same is true for NO_3^-.[1] The effects of Cl^-, SO_4^{2-}, Na^+, and K^+ were examined in more detail. First, we performed a set of experiments to determine the mode of inhibition by NaCl. As shown by Lineweaver-Burk plots, regression lines nearly all intersected on the ordinate, indicating competitive inhibition. The values for K_{is} from each line are in good agreement [1.82 ± 0.07 (standard deviation) mM]. Similar results were found for KCl, with the average K_{is} value overlapping that for NaCl within one standard deviation of error. Chloride was nearly as effective an inhibitor as the majority of the aromatic carboxylates discussed above whose K_{is} values vary from 2 to 0.1 mM.[7] NaCl also acted as a competitive inhibitor of a more highly purified tyrosinase from mushroom, substantiating our results with the potato enzyme, even though the two enzymes have K_M values for DOPA that differ by about an order of magnitude. The mushroom enzyme is a tetrameric enzyme with four copper ions,[1] whereas the potato enzyme is reported to be a dimer with two copper ions.[3]

To separate the effects of the alkali metal cations from those of anions, we turned to sulfate salts. Preliminary studies showed that sodium sulfate was only slightly inhibitory even at a concentration of 0.2 M. Thus, any possible differences between the Na^+ and K^+ cations would not be masked by inhibition by the SO_4^{2-} dianion, as it might have been by chloride, an effective inhibitor. Inhibition by both sulfate salts was much less than that for the chloride salts

and was mixed rather than competitive. The K_{is} values for Na_2SO_4 and K_2SO_4 were 417 mM and 444 mM, respectively, and equal within the given error range; K_{ii} values had a much wider range.

In contrast to Cho and Ahn, who reported that inhibition by NaCl was completely uncompetitive,[3] in all cases of salt inhibition studied here, inhibition was either competitive or weakly mixed with $K_{is} \ll K_{ii}$. The reason for this discrepancy between their data and ours could be that the enzyme they used was one of two activities (isoenzymes) they observed. Other differences between their studies and those reported here are the pH at which reactions were done (4.8 as opposed to 6.6) and the substrate used (DL-DOPA *vs* catechol). Because we found no difference between sodium and potassium salts, it seems clear that inhibition was due only to the chloride anion. This conclusion is in agreement with other studies discussed above that demonstrated inhibition of tyrosinase by anions (cyanide, azide, benzoic acid).[1,7,16,17] Thus, the assertion that K^+ readily displaces Cu^{2+} from the enzyme, besides being chemically unreasonable, has neither experimental basis nor literature precedence.[3]

If one examines the catecholase model of Riley,[2] as outlined in Scheme 1, there are five forms of the enzyme present in a catalytic cycle (including ternary complexes of the *oxy*- and *met*-forms) each containing positively charged copper ions capable of binding anions. Thus, the inhibitor could bind to the copper ion(s) in such a way as to prevent *ortho*-diphenol or oxygen binding (competitive) or to hinder the final oxidation to *ortho*-quinone (uncompetitive). If both interactions occured mixed inhibition would result. Binding to the *deoxy*-form, which, in the absence of inhibitor, is rapidly converted to the dominant catalytic form, *oxy*-,[2] would decrease the amount of *oxy*- and result in decreased rates of reaction (i.e., competitive). It has been suggested that DOPA can bind to an allosteric site on tyrosinase or to otherwise exert an effect on its active site.[18] Therefore, while it seems reasonable that the enzyme could bind other effectors that could occasion the same result, all the observed anion inhibitions can be explained by direct interaction with the active site copper ions.

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