The Catalytic Mechanism of Tyrosine Phenol-Lyase from *Erwinia herbicola:* The Effect of Substrate Structure on pH-Dependence of Kinetic Parameters in the Reactions with Ring-Substituted Tyrosines

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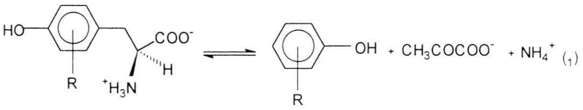
Apparently homogeneous tyrosine phenol-lyase (TPL) from *Erwinia herbicola* has been prepared by a new method. The pH-dependencies of the main kinetic parameters for the reactions of *Erwinia* TPL with tyrosine, 2-fluorotyrosine, 3-fluorotyrosine, 2-chlorotyrosine, and 3,4-dihydroxyphenylalanine (DOPA) have been studied. The pattern of pH-dependence of V_{max} depends on the nature of the substituent in the aromatic ring. For the substrates bearing small substituents (H, 2-F, 3-F) V_{max} values were found to be pH-independent. For 2-chlorotyrosine and DOPA V_{max} decreased at lower pH, the effect being described by equation with one pK_a. Generally two bases are reflected in the pH dependence of V_{max}/K_m. The first base, probably is responsible for the abstraction of α-proton, while the second one, interacts with the phenolic hydroxyl at the stage of binding. The reaction of TPL with DOPA differs from the reactions with other tyrosines by the requirement of an additional base which is reflected in the pH-profiles of both V_{max} and V_{max}/K_m. For the reaction of TPL from *Citrobacter intermedius* with DOPA only V_{max}/K_m values could be determined. The activity of *Citrobacter* enzyme towards DOPA is considerably less than that of *E. herbicola* enzyme, and its maximal value is attained at higher pH.

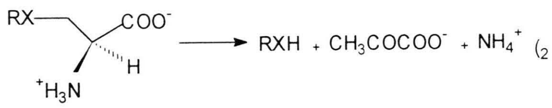
Introduction

A characteristic representative of pyridoxal-5-phosphate-(PLP)-dependent lyases, tyrosine phenol-lyase (TPL; EC 4. 1. 99. 2.) is active towards substrates of different types. It catalyzes the reversible β–elimination of L-tyrosine or its ring-substituted analogues (Yamada and Kumagai, 1975; Nagasawa *et al.,* 1981).



R = H, OH, alkyl, halide

It also acts on L-serine or L-cysteine derivatives *in vitro*, bringing about their irreversible decomposition (Yamada and Kumagai, 1975) :



X = O, S; R = H, alkyl, aryl

The enzyme is distributed mainly in enterobacteria (Enei *et al.,* 1972) but has also been found in some arthropods (Duffey and Blum, 1977; Duffey *et al.,* 1977). Homogeneous preparations of TPL have been obtained from the cells of *Citrobacter* species (Kumagai *et al.,* 1970; Demidkina *et al.,* 1984; Phillips *et al.,* 1987) and *Erwinia herbicola* (Kumagai *et al.,* 1972).

In experiments with mice (Meadows *et al.,* 1977) the purified TPL was shown to reduce plasma tyrosine levels which led to inhibition of growth of melanoma tumors.

The amino acid sequences for both enzymes have been deduced from the gene DNA sequences

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(Kurusu *et al.*, 1991; Iwamori *et al.*, 1992), and the extent of the sequence identity was found equal to 90% (Iwamori *et al.*, 1992). For *Citrobacter* TPL, a three-dimensional model has been constructed based on the amino acid sequence data and X-ray analysis (Antson *et al.*, 1993).

The generally accepted mechanism of TPL catalysis for tyrosine-type substrates (Faleev *et al.*, 1983; Palcic *et al.*, 1986; Kiik and Phillips, 1988) needs the participation of at least two basic groups, of which the first one abstracts the α–proton, while the second base assists in the tautomerization of the phenol group to cyclohexadienone by accepting the proton from the phenolic hydroxyl group. This tautomerization converts the aromatic moiety into a good leaving group to allow the following β–elimination stage. On the other hand, in the case of S-alkylcysteine-type substrates, the alkylthiolate is eliminated, which is a sufficiently good leaving group, and the participation of the second base does not seem necessary. This consideration has been substantiated by the studies of pH-dependence of the kinetic parameters of TPL reactions (Kiik and Phillips, 1988), which revealed two basic groups participating in the reaction of tyrosine, and one basic group for the reaction of S-methyl-L-cysteine.

In this paper the pH-profiles for the reactions of *E. herbicola* TPL with a number of tyrosine-type substrates: tyrosine, 2-fluorotyrosine, 3-fluorotyrosine, 2-chlorotyrosine, and 3,4-dihydroxyphenylalanine (DOPA) have been studied. The results demonstrate that although the requirement for two bases being reflected in the pH-dependencies is general, the pattern of the pH-profile depends on the substituent in the aromatic ring, and the origin of this effect probably is associated with the steric parameters. In addition, *E. herbicola* TPL was obtained by a new method which differs from the traditional one (Kumagai *et al.*, 1972) by a more simple composition of the culture medium used for the cell growth, and fewer steps and higher yield in the purification procedure.

Experimental Procedures

Materials and methods

The lactate dehydrogenase (LDH) from rabbit muscle, PLP and NADH were purchased from United States Biochemical Co. (Cleveland, Ohio). TPL from *C. intermedius* was obtained as described by Demidkina *et al.* (1984). S-(*o*-nitrophenyl)-L-cysteine (SOPC) was prepared from L-cysteine and *o*-fluoronitrobenzene (Phillips *et al.*, 1989). 3-Fluoro-L-tyrosine was prepared by the enzymatic synthesis (Phillips *et al.*, 1990). 2-Fluoro-L-tyrosine and 2-chloro-L-tyrosine were prepared as described by Faleev *et al.* (1995).

Cell growth

E. herbicola cells (ATCC 21434) were grown on a medium containing 0.1% $MgSO_4$ x $7H_2O$, 0.01% pyridoxine hydrochloride, 0.5% potassium lactate, 0.5% $(NH_4)_2SO_4$, 0.001% $FeSO_4$, 2.3% K_2HPO_4 x $3H_2O$, 0.34% K_2HPO_4 and 0.2% L-tyrosine, pH 7.0. Incubation was carried out in flasks at 30°C for 24 hr with reciprocal shaking. The cells were harvested by centrifugation and washed with distilled water. Normally about 4 grams of wet cells were obtained per 1 liter of the medium.

Enzyme assay

Activities were determined by reaction of 0.623 mM S-(*o*-nitrophenyl)-L-cysteine (SOPC) in 50 mM potassium phosphate buffer, pH 8.0, containing 0.1 mM PLP in total volume of 0.6 ml at 30°C. One unit of activity was defined as the amount of enzyme which catalyzes the decomposition of 1 micromol of SOPC per minute under these conditions. The rate of reaction of *E. herbicola* TPL with SOPC was greater than the rate of reaction with L-tyrosine by the factor of 3.04 under the standard conditions. Protein determination during the purification procedure was performed by the method of Lowry *et al.* (1952) , and, for the pure enzyme, by direct measurement of the absorbance at 280 nm, using an E value of 0.808 ml x mg^{-1} x cm^{-1} (Kumagai *et al.*, 1972).

Purification of TPL

All operations throughout the purification procedure were carried out at 3–6 °C in 0.1M potassium phosphate buffer, pH 7.6, containing 0.1mM PLP and 0.2 mM dithiothreitol.

i) Preparation of the cell extract

The cell paste was suspended in the standard buffer to give a suspension of 0.25 g per 1 ml which

Table I. Purification of TPL from *E. herbicola*. The activities were determined by the reaction with S-(*o*-nitrophenyl)-L-cysteine (SOPC).

Purification step	Total activity [units]	Total protein [mg]	Specific activity [units/mg]	Yield [%]
Cell extract	547	2126	0.27	100
Protamine sulfate treatment	551	1968	0.28	96
Ammonium sultafe fractionation	425	944	0.45	74
Sephadex A-50 treatment	258	66	3.9	45

subsequently was subjected to ultrasonic disintegration (15–20 kcycles) for 30 min, and cell debris was removed by centrifugation.

ii) Protamine sulfate treatment

Protamine sulfate, as a 5% (w/v) solution in water, was added to the cell extract in the quantity equal to 5% of the total amount of protein, and the precipitate formed was removed by centrifugation.

iii) Fractionation by ammonium sulfate

Ammonium sulfate was added to the clear supernatant to 30% saturation, the precipitate was separated by centrifugation and discarded. Then, the solution was brought to 60% saturation with ammonium sulfate, the resultant precipitate was collected by centrifugation, dissolved in the standard buffer and dialysed against it overnight.

Fig. 1. SDS – PAGE electrophoresisis of TPL from *E. herbicola* performed by the method of Laemmli (1970). Left: Protein standards (molecular weight) from the bottom to top: 1) lysozyme (14.4 kDa); 2) soybean trypsin (21.5 kDa); 3) carbonic anhydrase (31.0 kDa); 4) ovalbumin (45.0 kDa); 5) bovine serum albumin (66.2 kDa); 6) phosphorylase B (95.2 kDa); 7) β-galactosidase (116.25 kDa); 8. myosin (200.0 kDa). The protein standards were from Bio-Rad. Right: The purified preparation of TPL (10 μg).

iv) DEAE- Sephadex A 50 treatment

To 5.5 ml of the dialysed protein solution, containing 425 mg of protein with specific TPL activity of 0.45 units/mg, was added a gel of DEAE-Sephadex A–50 containing 2.0 g of dry sorbent equilibrated with the standard buffer. The mixture was stirred for 5 min., then it was poured into a funnel with a fritted glass filter, and the supernatant removed with gentle suction. The gel was repeatedly washed with the standard buffer by adding fresh portions and removal of the liquid with suction until the latter contained no protein. The gel was then washed in the same way with the standard buffer containing 0.2 M KCl, which resulted in elution of TPL. The enzyme was precipitated from the combined eluates by adding ammonium sulfate to 60% saturation. The enzyme was a single band on SDS- polyacrylamide gels (Fig 1). Stored at 4° C in 60% saturated ammonium sulfate, it retained its activity during several weeks. The results of a typical purification procedure are summarized in Table I.

Kinetic measurements

The rates of pyruvate formation from tyrosine analogues were measured at 30° C using the coupled assay with LDH and NADH as described by Kiik and Phillips (1988). In experiments with DOPA, the solutions containing all the components except TPL were placed in the sample cell, while the mixtures of all the components including TPL were placed in the reference cell and the increase of absorption at 340 nm was measured. The dependence of kinetic parameters on pH was studied in 0.1 M triethanolamine-phosphate buffers containing 0.1 M KCl. The values of V_{max} and K_m were obtained by fitting the initial velocity vs. substrate concentration data to the Michaelis-Menten equation using a nonlinear least-squares program (ENZFITTER). The pH-dependencies of kinetic parameters were fitted to equations:

$$\log Y = \log c - \log(1 + [H^+]/K_1) \quad (3)$$

$$\log Y = \log c - \log(1 + [H^+]/K_1 + [H^+]^2/K_1K_2) \quad (4)$$

or

$$\log Y = \log c - \log(1 + [H^+]/K_a + K_b/[H^+]) \quad (5)$$

by using the FORTRAN programs of Cleland (1979) adapted to run on IBM-compatible personal computers.

Results

The dependencies of the main kinetic parameters on pH for the reactions of *Erwinia* TPL with various substrates were examined in the range of pH from 6.5 to 9.5. The results are summarized in Figs 2–3 and in Table II. For the reaction with L-tyrosine we obtained practically the same pH-dependence for V_{max}/K_m as was observed previously (Kiik and Phillips, 1988) for the partially purified TPL from the cells grown on the traditional medium. It is described by two pK_as of 7.19 +/- 0.09 and 8.15 +/- 0.07. Rather unexpectedly, we have now found the value of V_{max} is independent of pH, whereas it decreased below a pK_a of 7.41

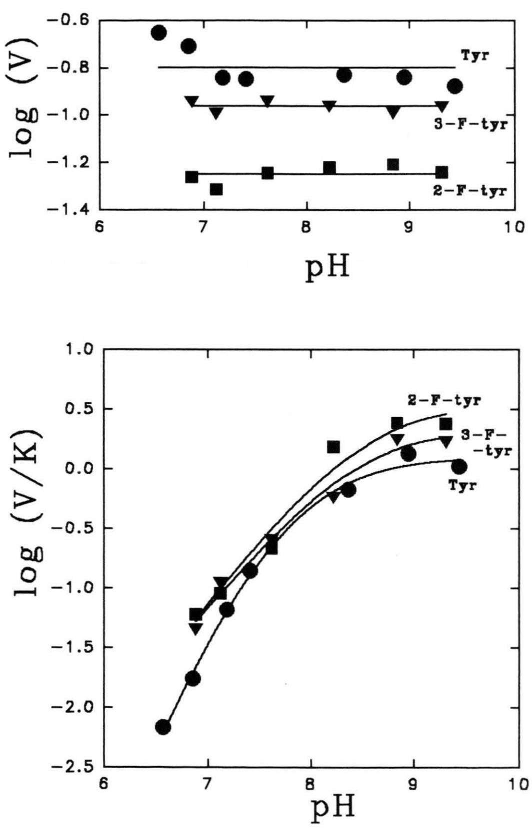


Fig. 2. The pH dependencies of the kinetic parameters for the reactions of *E. herbicola* TPL with tyrosine, 2-fluorotyrosine and 3-fluorotyrosine. The reactions were run in total volume of 0.635 ml. The amounts of TPL added were: 0.0397 SOPC units in experiments with tyrosine and 3-fluorotyrosine; and 0.0858 units in experiments with 2-fluorotyrosine. The curves for *V/K* for tyrosine and 2-fluorotyrosine are from a fit using Eqn. (4). The curve for 3-fluorotyrosine is from a fit using Eqn. (3).

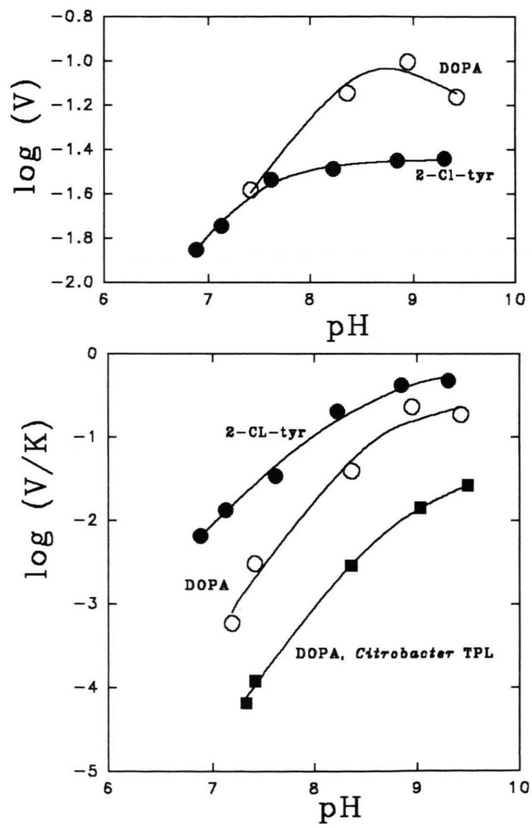


Fig. 3. The pH dependencies of the kinetic parameters for the reactions of TPL with 2-chlorotyrosine and DOPA. The reactions were run in total volume of 0.63 ml. The amounts of TPL added were 0.0397 SOPC units in experiments with DOPA , and 0.0686 SOPC units in experiments with 2-chlorotyrosine. The curves for *V* are from a fit of data using Eqn. (3) in the case of 2-chlorotyrosine , and from a fit using Eqn. (5) in the case of DOPA; the curves for *V/K* are from a fit using Eqn. (4).

Table II. The dependence of kinetic parameters on pH for the reactions of TPL from *E. herbicola* with various substrates.

Substrate	k_{cat} Type of equation pK_a (S.E.)	k_{cat} Max. value Sec^{-1} (S.E.)	k_{cat}/K_m Type of equation pK_a (S.E.)	k_{cat}/K_m Max. value $M^{-1} \cdot sec^{-1}$ (S.E.)
Tyrosine	pH-independent	1.33 (0.28)	two pK_a (Eqn. (4)) $pK_{a1} = 7.19$ (0.09) $pK_{a2} = 8.15$ (0.07)	10400 (600)
2-Fluorotyrosine	pH-independent	0.22 (0.02)	two pK_a (Eqn. (4)) $pK_{a1} = 6.2$ (0.7) $pK_{a2} = 8.61$ (0.13)	13650 (1770)
3-Fluorotyrosine	pH-independent	0.86 (0.04)	one pK_a (Eqn. (3)) $pK_a = 8.47$ (0.05)	16700 (1460)
2-Chlorotyrosine	one pK_a (Eqn. (3)) $pK_a = 7.08$ (0.25)	0.178 (0.003)	two pK_a (Eqn. (4)) $pK_{a1} = 6.6$ (0.2) $pK_{a2} = 8.73$ (0.09)	3350 (415)
DOPA	bell-shaped (Eqn. (5)) $pK_a = 7.96$ (0.14) $pK_b = 9.67$ (0.37)	0.95 (0.25)	two pK_a (Eqn. (4)) $pK_{a1} = 8.04$ (0.27) $pK_{a2} = 8.83$ (0.32)	2380 (760)

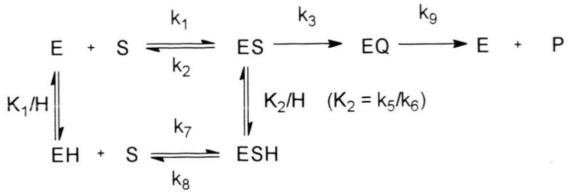
(Kiik and Phillips, 1988). The susceptibility of TPL preparations to reaction conditions, and, sometimes, to some uncontrollable factors was noticed by Palcic *et al.* (1986). The data of Kumagai *et al.* (1972) evidence for the existence of several forms of *Erwinia* TPL, different in their chromatographic behaviour. This seems a probable clew to the observed difference in kinetic properties of the enzymes prepared by different methods, although the decisive conclusion is premature. The V_{max} values were also found to be pH-independent for the reactions of 3-fluorotyrosine and 2-fluorotyrosine, while for 3-chlorotyrosine and DOPA V_{max} decreased at lower pH. For 2-chlorotyrosine the effect was described by equation with one pK_a (Eqn. 3). In the case of DOPA the data were better fit by a bell-shaped dependence (Eqn. 5). The pH-profiles of V_{max}/K_m for tyrosine-type substrates, except 3-fluorotyrosine, are described by equation with two pK_as (Eqn. 4). In the case of 3-fluorotyrosine a pK_a of 8.47 +/- 0.08 was observed in the V_{max}/K_m pH-profile, the data being fit by equations with either one or two pK_as. The use of the equation with two pK_as led to a decrease in sigma value from 0.2 to 0.1, which implies a better fit, but the value of the second pK_a could not be determined because of a very big standard error: the pK_a value of 4.5 +/- 17.6 was obtained. To compare the behavior of homogeneous enzymes from different microbial sources towards DOPA we studied the action of TPL from *C. intermedius* on DOPA at various pH. Because of the high K_m values, we were able only to determine V_{max}/K_m values through the pH range. The dependence of V_{max}/K_m on pH (Fig. 2) is described by the equation with two pK_as: $pK_{a1} = 8.00$ +/- 0.05; $pK_{a2} =$ 9.36 +/- 0.09.

Discussion

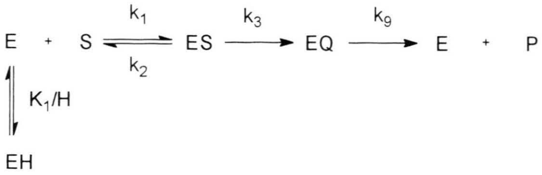
1. The substrate structure and the dependence of the main kinetic parameters on pH

For the analysis of the pH-dependencies of the kinetic parameters determined for the reactions of TPL with various substrates, we used the protonation mechanism described by Cleland (1977) (Scheme 1). According to this scheme all chemical transformations are included in k_3, and dissociation of all products is included in k_9.

SCHEME 1



SCHEME 2



The dependence of V_{max} on pH is determined by acid-base dissociation of a certain functional group (or groups) in the enzyme-substrate complex (K_2), while the analogous dependence of V_{max}/K_m is determined by dissociation of groups in the free enzyme (K_1). In terms of this general mechanism, the observed pK_a values for bases participating in proton abstraction may be perturbed in V_{max} and V_{max}/K_m pH-profiles by "forward" (k_3/k_9) and "backward" (k_3/k_2) commitments respectively.

When $k_5 = k_6 = k_7 = k_8 = 0$, the general scheme is reduced to the simpler one (Scheme 2); where substrate binds only with the correctly protonated form of the enzyme. In this case, V_{max} should be independent of pH, while the pK_a values observed in the pH-profile of V_{max}/K_m reflect the real pK_as (Cleland, 1977) in the free enzyme.

The experimental data are presented in Table II and in Figs 2 -3. The most interesting result, in our opinion, is the change of the pattern of pH profile of V_{max} with the change of the substituent in the aromatic ring. For the substrates bearing small substituents (tyrosine, 2-fluorotyrosine, 3-fluorotyrosine) the values of V_{max} were found to be pH-independent (Scheme 2 is realized). On the other hand, for 2-chlorotyrosine and DOPA , where substituents are larger, V_{max} decreases at lower pH. In the framework of the Scheme 1 the decrease in activity should be due either to protonation of a certain group in the enzyme-substrate complex, or to the binding of the substrate with the protonated form of the free enzyme (EH). It was shown by Nagasawa *et al.* (1981) for the reactions of TPL from *C. intermedius* with various ring-substituted tyrosines at constant pH of 8.0 that the relative rates decrease, and the values of K_m increase, with the increase in the van der Waals radii of the substituents , the effect being much stronger for the substituents in 3- than in 2-position. Based on these findings the assumption was made that the steric size of the substituent is an important factor in the interaction of the substrate with the enzyme (Nagasawa *et al.*, 1981). In elaboration of this concept the results obtained in the present work allow to conclude that the substrate substituents become in the active site close enough to the neighboring protein groups to affect the pattern of the enzyme-substrate interaction which is reflected in the pH-dependencies of the kinetic parameters. Evidence has been presented by Chen *et al.* (1995), that His 343 is at least partially responsible for the conformational change leading to the formation of the "closed" form of enzyme-substrate complex which is characterized by V_{max} being independent on pH. Therefore, it may be assumed that the larger substituents (2-Cl, 3-OH) in the substrate bring about an interference with His 343 or other residues, impeding the formation of the "closed" conformation.

For the reactions of tyrosine, 2-fluorotyrosine and 3-fluorotyrosine a base with the same pK_a of 8.6 +/- 0.3 is reflected in the pH-profile of V_{max}/K_m, and another base having a pK_a of 6.2–7.0 is observed in the same dependence for tyrosine and 2-fluorotyrosine. V_{max} for these substrates are pH-independent, thus the observed pKa values should be real. For the reaction of 2-chlorotyrosine the both basic groups are reflected in the pH-profile of V_{max}/K_m, and the group with the lower pK_a is reflected in the pH-dependence of V_{max}. These results basically agree with the concept of Kiik and Phillips (1988) that the base having the lower pK_a abstracts the proton from the α-position of the substrate while the second base, having the higher pK_a, may interact with the phenolic hydroxyl at the early stage of binding and accept the hydroxylic proton during the catalytic act.

2. DOPA reaction with TPLs from different microbial sources

The reaction of TPL with DOPA differs from the reactions with other substituted tyrosines by the requirement for a basic group with a pK_a of 8.00, which is reflected in the pH-profiles of both V_{max} and V_{max}/K_m. In the experimental conditions, the group responsible for the abstraction of the α–proton (pK_a 6.2 -7.0) should be available, so the group with pK_a of 8.00 probably is responsible for some additional aspect of mechanism which comes into play when DOPA is the substrate. At high pH DOPA reacts almost as well as tyrosine does (see Table II), thus probably some factor hindering the reaction is eliminated with the dissociation of this group. This factor may be related to the steric control of substrate specificity of TPL, which is especially severe for 3-substituted substrates (Nagasawa *et al.*, 1981; Faleev *et al.*, 1988).

The ability to catalyze synthesis of DOPA from ammonium pyruvate and catechol (Enei *et al.*, 1971; Yamada and Kumagai, 1975) is a very important property of TPL from the practical point of view. Examining this synthetic activity we have found (Faleev *et al.*, 1995) that cells of *E. herbicola* and *C. intermedius* differ significantly in their abilities to synthesize DOPA. To compare the behavior of homogeneous enzymes towards DOPA, we studied the action of *C. intermedius* TPL on DOPA at various pH values. Because of the high K_m values, we were able only to determine V_{max}/K_m values through the pH range. The dependence of V_{max}/K_m on pH (Fig. 3) is described by the equation with two pK_as: pK_{a1} = 8.00 +/- 0.05; pK_{a2} = 9.36 +/- 0.09. As in the case of *E. herbicola* enzyme, the pH-dependence for the reaction of *Citrobacter* TPL with DOPA is different from that for the reaction with tyrosine, where V_{max}/K_m values decrease below an average of two pK_a values of 7.8 (Kiik and Phillips, 1988).The activity of *Citrobacter* enzyme in experimental conditions is considerably less than that of *Erwinia* TPL and, as it follows from the pH-dependencies of V_{max}/K_m, its maximum value is attained at higher pH. Thus, one may conclude that the lower ability of *Citrobacter* cells to synthesize DOPA is due to the different kinetic properties of the enzyme itself. The observed drastic difference in catalytic properties seems remarkable in view of the very high extent (90%) of the sequence identity of TPL from *C. intermedius* and *E. herbicola* cells (Iwamori *et al.*, 1992).

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