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CHARACTERIZATION OF POLYPHENOL OXIDASE FROM WHITE CHERRY FRUIT *(Starks gold)*

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Abstract
Polyphenol oxidase (PPO) from white cherry fruit (starks gold) was extracted and purified through $(NH_4)_2SO_4$ precipitation, dialysis and ion exchange chromatography. The enzyme showed two peaks with PPO activity on Toyopearl 650 M column, which were denoted as isoenzyme A and isoenzyme B. A 3.9 fold purification of isoenzyme A with a recovery of 34.9 % and 76.7 fold purification of isoenzyme B with a recovery of 54.3 % were achieved. pH optima of isoenzyme A and B were 4.5 and 4.98, respectively. The temperature optima for enzyme activity were found to be 20 °C for isoenzyme A and 30°C for isoenzyme B. The affinity of isoenzyme B for catechol as substrate was higher than that of isoenzyme A. Activation energies and Z values were found to be 22.1°C (r^2= 0.883) and 98.5 kj/mol (r^2= 0.878) for isoenzyme A and 13.9 °C (r^2= 0.990) and 157.1 kj/mol (r^2= 0.989) for isoenzyme B, respectively. Inhibitory effect of L-cysteine and sodium disulphide differed from each other.

Keywords: White cherry, polyphenol oxidase, enzymatic browning, kinetics, thermal inactivation

BEYAZ KİRAZ MEYVESİ *(Starks gold)* POLİFENOL OKSİDAZININ KARAKTERİZASYONU

Özet
Beyaz kiraz meyvesinden (starks gold) polifenol oksidaz enzimi ekstrakte edilmiş ve amonyum sülfat çökeltmesi, diyaliz ve iyon değişim kromatoğrafisi ile saflaştırılmıştır. Toyopearl 650 M kolon kromatoğrafisi sonucunda PFO aktivitesi gösteren izoenzim A ve izoenzim B ile belirtilmiş iki pik elde edilmiştir. İzoenzim A % 34.9 verimle 3.9 kat, izoenzim B % 54.3 verimle 76.7 kat saflaştırılmıştır. İzoenzim A ve B'nin optimum pH değerleri sırası ile 4.5 ve 4.98 olarak bulunmuştur. Optimum sıcaklık değerinin izoenzim A için 20 °C ve izoenzim B için 30 °C olduğu saptanmıştır. İzoenzim B'nin kateşol substratına ilgisi izoenzim B'den daha yüksektir. Aktivasyon enerjisi ve Z değerleri izoenzim A için 22.1 °C (r^2= 0.883) ve 98.5 kj/mol (r^2= 0.878), izoenzim B için 13.9 °C (r^2= 0.990) ve 157.1 kj/mol (r^2= 0.989) bulunmuştur. L-sistein ve sodyum disülfitin inhibitör etkisinin birbirinden farklı olduğu belirlenmiştir.

Anahtar kelimeler: Beyaz kiraz, polifenol oksidaz, enzimatik esmerleşme, kinetik, termal inaktivasyon

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INTRODUCTION

Enzymatic browning of foods occurs during handling, storage and processing of fruits and vegetables, which impairs the sensory properties and marketability of the product and also lowers the nutritional value due to associated changes in colour, flavour and softening (due probably to the action of pectic enzymes) (1-2). It is estimated that over 50% losses in fruit occur as a result of enzymatic browning (3). Oxidative browning is catalysed by polyphenol oxidase (PPO) (EC 1.14.18.1). PPO, which is widely distributed in the plant and animal kingdoms, is a copper-containing enzyme. Polyphenol oxidases (PPO) (EC 1.14.18.1) are very important enzymes in food industry due to their involvement in the enzymatic browning of edible plants. In the presence of molecular oxygen, PPO catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (4). There have been numerous studies on PPO from various sources, e.g. taro and potato (5) cherry laurel (6), banana (7), grape (8). Existence of PPOs in multiple forms (isoforms) was found in various plant sources. Multiple forms detected in such plants as apple, banana, dog-rose, grape, kiwifruit, lettuce, mushroom, peach, pineapple, potato, spinach, strawberry and sweet potato. Such forms of PPO are recognized by their distinguishable differences in physical, chemical or enzymatic properties such as electrophoretic mobility, temperature and pH optimum, substrate specificity and pI (9).

Enzymatic browning catalyzed by PPO is generally considered to be detrimental to food quality from both organoleptic and nutritional points of view. Polyphenol oxidase-catalyzed browning reactions in fruits and vegetables during handling and processing impairs the sensory properties and marketability of the product and also lowers the nutritional value. An understanding of the essential factors controlling the action of PPO is necessary in an attempt to inhibit or control PPO activity in fruit and vegetables during processing. Therefore, it is important to control the PPO activity, as well as to determine its characteristics associated with the variety. No such research has been carried out on PPO from white cherry. The present work was undertaken to study purification and characterization of PPO from white cherry.

MATERIAL AND METHODS

Materials

White cherries used in this study were obtained from Eregli region of Turkey and frozen at -25 °C until used. Catechol, triton X-100, sodium metabisulfite, polyvinylpyrolidone (PVPP), ascorbic acid and commasie brillant blue (CBB) were purchased from Merck (Darmstadt, Germany). Polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), L-cysteine, citric acid, acetone, and cellulose membrane (76 x 49 mm) were purchased from Sigma-Aldrich (St. Louis, USA). Toyopearl DEAE-650 M was purchased from Supelco (Montgomeryville, USA). All chemicals were of analytical grade.

Enzyme Extraction

The frozen berries were deseeded and then 300 g of pulps were homogenized in 400 mL of cold acetone (-25 °C) containing 3.33 g of polyethylene glycol, using a pre-chilled Waring blender (Model HGBTWTS3, Torrington, Connecticut, USA) for 2 min at low speed. The slurry was vacuum filtered through filter paper. The residue was re-extracted with 200 mL of cold acetone. This procedure was repeated until a white powder was obtained. The resultant acetone powder was dried overnight at room temperature and stored at -25 °C (10).

Acetone powder (10g) was homogenized for 40 sec in 300 mL of 0.1 M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid, 0.1% polyvinylpyrolidene, 0.5% Triton X-100 and 1 mM PMSF, using Waring blender. After the homogenate was magnetically stirred for 3 h at 4 °C, it was centrifuged at 10000 x g for 45 min at 4 °C. The resulting supernatant was subjected to ammonium sulphate precipitation. The fraction precipitated at 90% saturation was separated by centrifugation at 10000 x g for 45 min at 4 °C. The precipitate was dissolved in a small amount of 10 mM phosphate buffer, pH 6.8, and dialyzed overnight at 4 °C in the same buffer (11).

Ion Exchange Chromatography

For ion exchange chromatography, the dialysate was applied to a column (2.5x30 cm) filled with DEAE-Toyopearl 650 M, balanced with 10 mM phosphate buffer, pH 6.8. The column was eluted with same buffer at the flow rate of 0.5 mL/min and linear gradient of phosphate buffer concentration from 10 to 200 mM. Fractions in 4 mL were collected in which the protein level and PPO activity towards catechol as substrate were monitored. The fractions which showed PPO activity were combined and were used as enzyme source in the following experiments.

Protein Determination

Protein contents of the enzyme extracts were determined according to Bradford method using bovine serum albumin as a standard (12).

Assay of Enzyme Activity

PPO activity was determined in 1.0 mL assay mixtures in a spectrophotometer (Shimadzu UV-1700, Kyoto, Japan) fitted with a thermostatted cuvette by measuring the increase in absorbance at 410 nm at 30 °C. The initial rate was calculated from the slope of the linear part of the absorbance -time curve. Unless otherwise stated, the standard reaction mixture consisted of 0.1 mL of enzyme solution and 0.9 mL of catechol in 0.2 M citric acid buffer (pH 4.50) for isoenzyme A and pH 4.98 for isoenzyme B). In all experiments, control experiments without enzyme were conducted and no significant oxidation of substrate was observed during the short period employed to measure PPO activity. One unit of enzyme activity was defined as the amount of enzyme that caused an increase of 0.001 in the absorbance value per min under the assay conditions (7).

pH Optima

PPO activity was determined in a pH range of 3.04-5.80 in 0.2 M citric acid buffer and 6.30 in 0.2 M phosphate buffer. PPO activity was assayed, using the standard reaction mixture but changing the pH of the buffer. PPO activity was calculated in the form of percent residual PPO activity at the optimum pH. The optimum pH obtained for this enzyme was used in all the other experiments.

Temperature Optima

The activity of PPO was determined at 4.50 pH for isoenzyme A and 4.98 pH for isoenzyme B at temperatures ranging from 10 °C to 70 °C. 0.9 mL of catechol solution in buffer was heated to the appropriate temperature in a water bath. After equilibration of the reaction mixture at the selected temperature, 0.1 mL of the enzyme solution was added and the enzyme activity was measured. PPO activity was calculated in the form of percent residual PPO activity at the optimum temperature.

Kinetic Parameters

In order to determine Michaelis constant (K_m) and maximum velocity (V_m), PPO activities were measured using catechol as substrate at various concentrations (6.25-100 mM). K_m and V_m values of the enzyme were calculated from a plot of 1/V vs. 1/S by the method of Lineweaver and Burk.

Thermal Inactivation Kinetics

Thermal inactivations of PPOs were studied at the selected temperatures for various times (5, 10 and 15 min at 60, 65 and 75 °C) using screw-cap tubes. The screw-cap tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of a 0.5 mL aliquot of enzyme solution. The enzyme samples were removed from water bath after pre-set times and were immediately transferred to ice bath to stop thermal inactivation. After the sample was cooled in ice bath, 0.1 mL of the heated enzyme solution was mixed with 0.9 mL of catechol and the residual activity (A) was determined spectrophotometrically. A non-heated enzyme sample was used as blank (A_o). The percentage residual activity was calculated by comparison with the unheated sample. First order inactivation constant (k_D) was calculated from the slope of the natural logarithm (ln) of A/A_o vs. time graph. Half-lives of the enzymes ($t_{1/2}$) were calculated by using the following equation: $t_{1/2} = 0.693/ k_D$.

Decimal reduction time (D value) was estimated from the relationship between k_D and D value: $D = \ln (10)/k_D$. The Z value, which is the temperature increase required for a one-$\log_{10}$ reduction (90% decrease) in D value was determined from a plot of $\log_{10}$D versus temperature. The slope of the graph is equal to 1/Z value. The energy of activation

of denaturation (E_a) was calculated by multiplying the slope of Arrhenius plot (i.e. natural logarithm of k_D values vs. reciprocal of absolute temperatures (1/T)) with universal gas constant, R (kJ/mol.K) (7).

Effects of Inhibitors

Inhibitors examined were L-cysteine and sodium metabislufite. The reaction mixture contained 0.8 mL of catechol in 0.2 M citric acid buffer, 0.1 mL inhibitor at a final concentration of 0.01, 0.1, 1.0 mM and 0.1 mL enzyme solution. Percentage inhibition was calculated using the following equation: Inhibition (%) = $[(A_o - A_i)/A_o)].100$, where, A_o is the initial PPO activity (without inhibitor) and Ai is the PPO activity with inhibitor.

RESULTS AND DISCUSSION

Extraction and Purification

It has been reported that some plant PPOs are membrane-bound. Therefore, use of detergents is required to solubilize the enzyme. Phenol compounds interfere with purification of proteins from plants. They cross-link proteins by hydrogen bonds and covalent interactions. Furthermore, homogenization of the plant tissues initiates enzymatic browning which results in the formation of quinones. The quinones may also form covalent linkages that may not be reversible. Use of phenol-absorbing polymers, such as polyethylene glycol (PEG) or PVPP and use of reducing agents such as ascorbic acid are commonly applied in order to overcome these problems (13-14).

PPO was purified from white cherry fruits using a DEAE-Toyopearl 650M column. A summary of extraction and purification is given in Table 1. Following ammonium sulfate precipitation, the dialyzed enzyme extract was applied to Toyopearl 650 M column, yielding two peaks with PPO activity which were denoted as isoenzyme A and isoenzyme B (Fig. 1). Existence of PPOs in multiple molecular forms (isoforms) was found in various plant sources (9). In most cases, PPO appeared to exist in multiple forms which differed by one or more characteristics such as latency, catalytic behaviour, molecular mass, isoelectric point, immunological specificity and hydrophobicity (15). In an apple extract, four active PPO molecular forms were found in an early work by Harel et al. (16) and Harel and Mayer (17). Oba et al. (18) reported that the banana contains two active PPO isoforms. Flurkey and Jen (19) observed three forms of PPO from peach, Wesche-Ebeling and Montgomery (20) found two active PPO isoforms from banana.

The purification achieved with isoenzyme A was 3.9 fold with a recovery of 48.7 % and that with isoenzyme B was 76.7 fold with a recovery of 54.3%. Yang et al. (21) employed DEAE-Toyopearl 650 M to purify PPO from banana pulp and they achieved a purification fold of 9.3 with recovery of % 40.7. Paul and Gowda (22) obtained PPO with 4.8 fold purification from field bean seed using DEAE-Sephacel chromatogtaphy. Gawlik-Dziki et al. (23) employed DEAE-Sephadex A-50 to purify PPO from butter lettuce and they achieved a purification of 8.0 fold with a recovery of 20.9%. Shi (24) who studied PPO from tobacco leaves achieved a purification level of 39 fold using DEAE-Sephadex A-50 chromatogtaphy.

pH Optima

Enzyme activity is strongly affected by the pH of the medium because amino acid side chains in the catalytic centre may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionization, and io-

Table 1 Purification of PPO from white cherry

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg protein)	Purification (fold)	Recovery (%)
Isoenzyme A Crude extract	250	24	593000	24708.3	1.00	100.0
$(NH_4)_2SO_4$ precipitation and dialysis	40	5.8	206960	35682.8	1.44	34.9
DEAE-Toyopearl 650M	28	3.0	289016	96338.7	3.9	48.7
Isoenzyme B Crude extract	250	24	551000	22958.3	1.00	100.0
$(NH_4)_2SO_4$ precipitation and dialysis	40	5.8	445760	76855.2	3.3	80.9
DEAE-Toyopearl 650M	28	0.17	299432	1761364.7	76.7	54.3

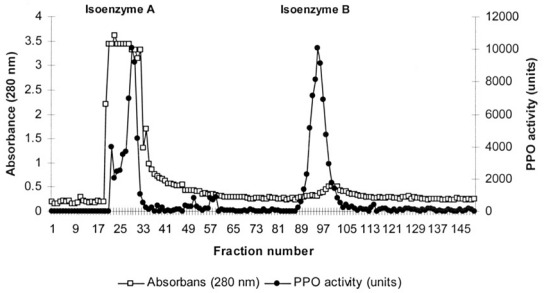

Fig. 1. Elution pattern of white cherry PPO on Toyopearl 650 M. In order to remove unadsorbed proteins, the column was washed with 10 mM pH, 6.8 phosphate buffer until the 60th fraction after which gradient elution was started.

nized side chains may play an essential role in maintaining protein structure (25). PPO activity as a function of pH was determined in a pH range of 3.46–6.30, and the results are depicted in Fig. 2. As can be seen from the graph, the two isoenzymes exhibited similar pH activity profile. As the pH increased from 3.04 to 4.5, the enzyme activity of isoenzyme A increased, with the maximal activity occurring at pH 4.5, after which the activity started to decline. The enzyme activity of isoenzyme B increased with increasing pH from 3.04 to 4.98, with the maximal activity occurring at pH 4.98, after which the activity dropped sharply.

The pH optimum for PPO is found to be dependent on the enzyme source, substrate and extraction methods used. Ding et al. (26) reported an optimum pH value of 4.50 for loquat PPO using chlorogenic acid as substrate. Selles-Marchart et al. (27) who studied loquat PPO reported optimum pH values of 6.5 for soluble form and of 4.0 for latent form, using 4-tert butylcatechol as

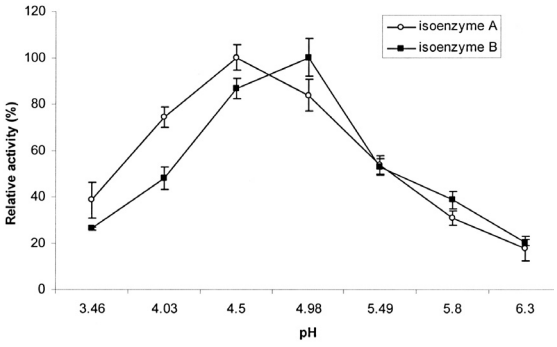

Fig. 2. Activity of white cherry PPO as a function of pH. Each data point is the mean of three determinations. The vertical bars represent standard deviations.

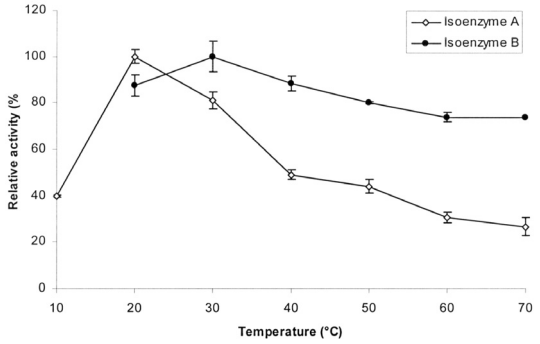

Fig. 3. Activity of white cherry PPO as a function of temperature. Each data point is the mean of three determinations. The vertical bars represent standard deviations.

substrate. Orenes-Pinero et al. (28) reported an optimum pH value of 5.0 for quince PPO, using *p*-cresol as substrate. Other reported values include 6.50 for banana peel PPO (14), 4.20 for grape PPO (29), 5.70 for broccoli PPO (30), and 7.5 for avocado PPO (31).

Temperature Optima

Temperature affects both the velocity of enzymatic reactions and stability of the enzyme. Temperature have also effects on the equilibria of all association /dissociation reactions, solubility of substrates and ionization of prototropic groups in the catalytic centre of the enzyme and enzyme-substrate complex (25). Effect of temperature on PPO activity was investigated in the range 10-70 °C and the results are depicted in Fig. 3. As seen in Fig. 3, the two isoenzymes exhibited quite different temperature activity profile. The optimum temperature for activity for isoenzyme A was 20°C and that for isoenzyme B was 30 °C. The isoenzyme B retained more than 80% activity between the temperatures studied. The activity of isoenzyme A, however, dropped sharply with increasing temperature after the optimum temperature.

Different optimum temperatures for PPO obtained from various sources have been reported in the literature. Yang et al. (21) reported an optimum temperature of 30 °C for banana peel PPO using dopamine as substrate. Other reported values include 25 °C for grape PPO (29), 30-35 °C for soluble PPO from loquat and 70 °C for latent form (27), 30 °C for banana PPO (7). The optimum temperatures obtained in this study compare well with the reported values.

Kinetic Parameters

Substrate specificity of PPOs varies widely, depending on the source and purity of the enzyme. The affinity of PPO for a particular substrate may even vary among multiple forms of a particular preparation. It is often suggested that the preferred substrate is the most abundant phenolic, which is not always the case. Catechins, cinnamic acid esters, 3,4-dihyroxy phenylalanine (DOPA) and tyrosine are the most important natural phenolic substrates of PPO in fruits and vegetables. Caffeoyltartaric and p-coumaroyltartaric acid are the major substrates for grape PPO (13,32).

The kinetic parameters were measured at pH 4.5 and 20 °C for isoenzyme A and pH 4.98 and 30 °C for isoenzyme B, using catechol as substrate. The Km and Vmax values were calculated using Lineweaver-Burk plot. The Km value for isoenzyme A was 107.0 mM and that for isoenzyme B was 30.3 mM. Km value is a measure of affinity of the enzyme for the substrate, with smaller values representing greater affinity (33). The affinity of isoenzyme A was lower than that of isoenzyme B. The maximum reaction velocity (Vmax) for isoenzymes A and B were found to be 2.01 and 1.26 OD410/min, respectively. The literature provides information on a number of different Km values, e.g. 8.5 for banana PPO (7), 12.52 mM tea leaf PPO (34), 67.9 mM for taro and 6.8 mM for potato (5). As can be seen, affinity of PPOs from various sources varies widely.

Thermal Inactivation

Thermal inactivation of white cherry PPO was studied in the range 60-75 °C for 5, 10 and 15 min. The inactivation parameters are summarized in Table 2. The heat inactivation of the isoenzymes followed first order kinetics. There were differences between the two enzymes in terms of thermal stability. Increasing the temperature from 60 to 70 °C resulted in a decrease in $t_{1/2}$ values of both isoenzymes. Some of the reported PPO half-life values include between 7.3-85.6 min between 60-75 °C for banana PPO (7) 2.6-49.5 min between 65-80 °C grape PPO (29) 4.5 and 31.6 min at 75 °C for Ravat and Niagara grapes (35) respectively.

D values of isoenzyme A and isoenzyme B ranged between 72-203.8 min and 30.5-160 min at the temperatures studied (Table 2), respectively. Rapeanu et al. (36) reported *D* values for grape PPO between 133-11.5 min at temperatures of 55-65 °C. Z and E_a values were calculated as 22.1 °C (r^2= 0.883) and 98.5 kj/mol (r^2= 0.8776) for isoenzyme A and 13.9 °C (r^2= 0.990) and 157.1 kj/mol (r^2= 0.9886) for isoenzyme B, respectively. Some of the reported E_a values are 82.8 kJ/mol for pineapple puree PPO (37), 219 kJ/mol for grape PPO (38), 37.8-49.2 kJ/mol for two cherry laurel cultivars (6). E_a values obtained in this study are within the range of reported values. Reported Z values include 13.02 °C for grape PPO (39) and 14.2 °C for banana (7).

Effect of Inhibitors

Effects of cysteine and sodium disulfite on white cherry PPO activity were studied at various concentrations using catechol as the substrate. The results were reported as percentage inhibition in Table 3. The effects of inhibitors varied in a dose dependent manner. The inhibition of the isoenzyme A and B differed from each other. Isoenzyme B was more sensitive to the inhibitors tested. The mode of action of inhibitors differs from each other. Metabisulfite is a reducing agent, which can either reduce *o*-qionones to colorless diphenols, or react irreversibly with *o*-qionones to form stable colorless products. L-cysteine is a thiol compound, which is a strong nucleophile and suppresses enzymatic browning mainly via formation of colorless addition products with *o*-quinones (13). In a study carried out by Ünal (7), it was found most effective inhibitors for banana PPO were sodium metabisulphite and ascorbic acid. Gawlik-Dziki et al. (23) found that most potent inhibitor for broccoli PPO was sodium sulfate.

Table 2 Inactivation parameters of white cherry PPO

Temperature	Isoenzyme A				Isoenzyme B			
(°C)	*k* (min^{-1})	r^2	$t_{1/2}$ (min)	D (min)	*k* (min^{-1})	r^2	$t_{1/2}$ (min)	*D* (min)
60	0.0113	0.946	61.3	203.8	0.0144	0.876	48.1	160.0
65	0.0137	0.957	50.6	168.1	0.0286	0.949	24.2	80.6
70	0.0320	0.960	21.7	72.0	0.0754	0.959	9.2	30.5

Table 3 Effect of inhibitors on white cherry PPO activity

Inhibitor	Concentration (mmol L^{-1})	Inhibition* (%)	
		Isoenzyme A	Isoenzyme B
Cysteine	0.01	7.3 ± 4.7	13.7 ± 11.9
	0.10	37.7 ± 10.4	25.0 ± 4.5
	1.00	87.7 ± 1.7	100.0 ± 0.0
Sodium metabisulfite	0.01	11.4 ± 9.2	0.0
	0.10	72.6 ± 2.0	84.7± 0.6
	1.00	76.7 ± 5.0	100.0 ± 0.0

*Each value is the mean of three determinations ± standard deviations.

CONCLUSION

PPO from white cherry was partially purified through ammonium sulfate precipitation and ion exchange chromatography which yielded two peaks with PPO activity. Existence of PPOs in multiple isoforms has been reported in various plant sources. Some of the biochemical properties of both of the peaks were determined. They had different pH activity profiles and thereby different pH optima, isoenzyme A at pH 4.50 and isoenzyme B at pH 4.98. The temperature optima for the isoforms were found to be 20 and 30 °C. The affinity of the isoenzymes for catechol differed. The two isoenzymes displayed different heat resistance and sensitivity towards various inhibitors.

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