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CATECHOL OXIDASE FROM GREEN OLIVES: PROPERTIES AND PARTIAL PURIFICATION

NOACH BEN-SHALOM*, VARDA KAHN*, EITAN HAREL† and ALFRED M. MAYER†

*Division of Food Technology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel; †Botany Department, The Hebrew University of Jerusalem, Israel

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Abstract—Catechol oxidase was extracted from an acetone powder prepared from green olive. The enzyme was purified 240-fold by ammonium sulphate fractionation followed by ion exchange chromatography and gel filtration. The enzyme was characterized by substrate specificity and response to inhibitors. Between 7 and 9 bands having catechol oxidase activity could be detected by gel electrophoresis and electrofocusing. The purified enzyme had an estimated MW of 42000. The enzyme was strongly inhibited by diethyldithiocarbamate. Inhibition by chloride was strongly dependent on pH. The enzyme did not oxidise monophenols.

INTRODUCTION

The browning reaction which results from mechanical injury during postharvest storage or processing of fruits and vegetables is a widespread phenomenon [1]. It is commercially undesirable in most cases because of the unpleasant appearance and the concomitant development of off-flavour. The main enzyme causing browning reactions is catechol oxidase (*o*-diphenol: O_2 oxido reductase EC 1.14.18.1). Catechol oxidase has been studied in many fruits [2–6] and other plant tissues and exhibits a multiplicity of forms upon gel filtration [7, 8], acrylamide gel electrophoresis [2, 7, 9–11], and isoelectric focusing [11–13]. The multiplicity may be caused by variation in the degree of aggregation of subunits [7–9] or to the presence of true isoenzymes [10].

Catechol oxidase is present in some tissues as a soluble enzyme (e.g. peaches [5], pears [6] and mushrooms [9]), and in other cases it is located in a particulate fraction (e.g. sugar cane leaves [8], grapes [4] and apples [14]). The enzyme may occur in latent forms which can be converted to the active form by urea [10, 15], sodium dodecylsulphate (SDS) [10, 15, 16], or by treatment with trypsin [10, 17, 18]. Since it is a copper enzyme, it is inhibited markedly by diethyldithiocarbamate (dieca) and cyanide [4, 5, 19–21]. It is also inhibited by high concentrations of halides [22–24] and some compounds containing carboxyl groups [6, 23, 25–27].

In green olives, brown spots appear immediately after mechanical injury. Preliminary work showed that blanching the olive before mechanical injury occurs can prevent the browning, indicating the probable enzymic nature of the reaction. It was therefore decided to study the catechol oxidase from green olives. In the following we will describe its partial purification and report on some of its properties.

RESULTS

Catechol oxidase could be extracted from acetone powder prepared from green olives by various buffers. Of the buffers tested, optimal solubilization was obtained by extraction with 0.1 M NaPi buffer, pH 7, for 0.5 hr while stirring at 0°. When this suspension was centrifuged at 20000 *g* for 30 min, 65–75% of the total activity was

Table 1. Effect of SDS on the extraction of catechol oxidase from acetone powder

Extraction medium	Fraction	Protein (μg/ml)	Activity (units/ml) pH 5	Activity (units/ml) pH 6.5	Specific activity (units/mg protein) pH 5	Specific activity (units/mg protein) pH 6.5
Control, buffer only	Crude suspension	450	600	150	1330	330
	Pellet	250	130	85	520	340
	Supernatant	225	400	60	1780	270
	Supernatant + 0.1% SDS	225	430	540	1777	2400
Buffer + 0.1% SDS	Crude suspension	600	1200	1000	2000	1660
	Pellet	200	130	115	650	575
	Supernatant	400	1000	800	2500	2000

100 mg acetone powder were suspended in 30 ml buffer NaPi 0.1 M, pH 7.6, with or without (control) 0.1% SDS, and stirred for 30 min at room temperature. Aliquots of each suspension were tested for catechol oxidase activity, the remainder was centrifuged for 30 minutes at 20000 *g*, and activity in the pellet and in the supernatant was determined.

found in the supernatant (Table 1). The acetone powder could be stored for long periods at −18° with little loss of activity. Addition of 0.5% polyclar AT or PVP to the acetone powder at a ratio of 2.5:1 (w/w) had little effect on the extracted activity while addition of a large excess of polyclar AT or PVP was inhibitory.

Extraction of the enzyme with 0.1 M NaPi buffer, pH 7.6, containing neutral or cationic detergents such as Triton X-100 or cetyltrimethylammonium bromide, failed to improve solubilization. However, anionic detergents such as SDS and deoxycholate increased the activity of catechol oxidase in the supernatant. Catechol oxidase was extracted from the acetone powder at pH 7.6 in the absence or presence of 0.1% SDS and the activity was assayed at pH 5 and 6.5. Supernatant activity increased 2.5- and 13-fold at pH 5 and at pH 6.5, respectively, compared with supernatant extracted without SDS (Table 1). Further studies showed that the addition of 0.1% SDS to the control supernatant had no effect on catechol oxidase activity when assayed at pH 5, but at pH 6.5 it induced a 9-fold increase in enzyme activity. Thus, extraction in the presence of SDS releases more catechol oxidase to the supernatant and, if the assay is performed at pH 6.5, SDS also causes a marked enhancement of activity of the solubilized enzyme.

Extraction from acetone powder, in the presence of trypsin, also resulted in increased solubilized activity compared with the control.

Incubation of acetone powder for up to 60 min with Pi buffer, 0.1 M, pH 7.6 at 28° resulted in a drop in activity at pH 5 of *ca* 50%, while at pH 6.5 activity hardly changed. When the acetone powder was incubated under the same conditions in the presence of 25 μg/ml trypsin activity at pH 5 rose from 450 units/ml to over 600 units/ml. At pH 6.5 the increase was much greater, from 100 to 400 units/ml within 30 min. Addition of trypsin to the control supernatant prior to assaying resulted in increased activity at pH 6.5 but not at pH 5.

For routine purposes, the enzyme was extracted from the acetone powder with buffer only (see Experimental), in order to eliminate the compound effect of detergents and trypsin.

Ammonium sulphate fractionation

Olive catechol oxidase precipitated over a wide range of concentrations of ammonium sulphate (30–90% saturation). Less than 5% of the activity precipitated in the 0–30% fraction. The sp. act. in the dialysed 30–90% fraction (partially purified fraction) was 3.3-fold higher than that of the original suspension of acetone powder (Table 2).

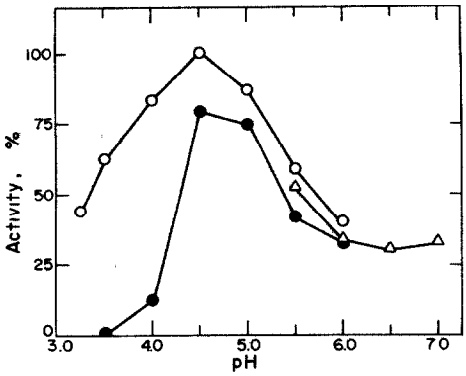

Fig. 1. The effect of pH on the activity of olive catechol oxidase. Enzyme source—partially purified fraction (30–90% saturation with $(NH_4)_2SO_4$). Substrate: 10 mM 4-methylcatechol. Buffers: 80 mM of NaOAc (○), Na citrate (●), or NaPi (△).

pH profile

The pH activity profile of the partially purified catechol oxidase was determined between pH 3.5 and 8 (Fig. 1). Maximal activity was at pH 4.5. Citrate ions appeared to inhibit enzyme activity, especially below pH 4.5.

Electrophoresis and electrofocusing of partially purified olive catechol oxidase

The partially purified fraction was resolved by acrylamide gel electrophoresis into 7 bands (a–g) having catechol oxidase activity, (Fig. 2a). The band designated as band e was the most intense. The same fraction was separated into 9 bands of activity (I–IX) when analyzed by gel electrofocusing between pH 3.5 and 10. The bands of strong activity were located at about pH 5, 5.6 and 7. Most of the other bands fell between pH 4.8 and 5.6 (Fig. 2).

Heat inactivation

Experiments were performed to assess the thermal stability of the partially purified olive catechol oxidase. About half the activity was lost during 19 min at 75° or

Table 2. Purification of catechol oxidase from green olives

Step	Volume (ml)	Total protein (mg)	Total activity (10^3 units)	Specific activity (10^3 units/mg protein)	Yield (%)	Purification (fold)
1. Acetone powder suspension	200	255	300	1.2	100	
2. Supernatant	170	133	204	1.5	68	1.3
3. Dialysed 30–90% $(NH_4)_2SO_4$	1	17	72	4.2	24	3.3
4. Sephadex G-100 (peak B-fractions 29–32)	14	1.26	35	27.8	12	23.3
5. DEAE cellulose (peak fractions)	10	0.08	22.5	281	7.5	239

2 g acetone powders were used as the source of catechol oxidase.

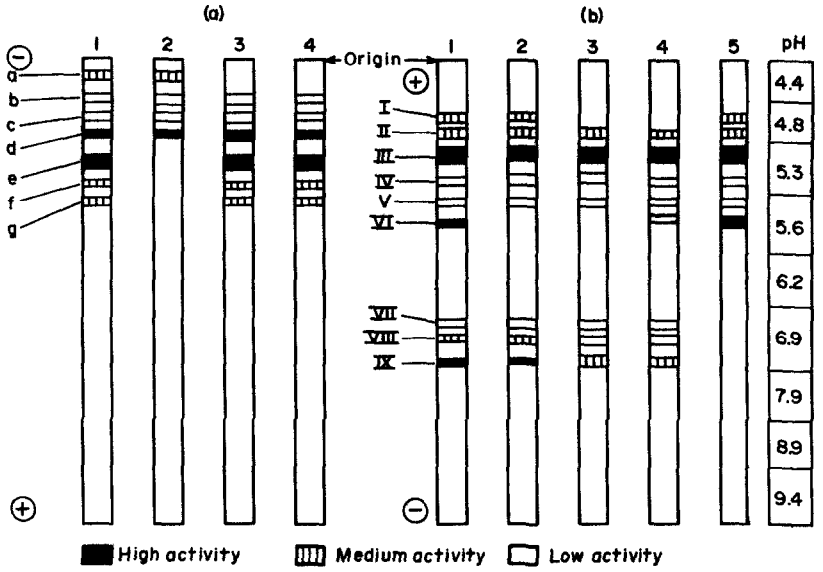

Fig. 2. Polyacrylamide gel electrophoresis and isoelectric focusing of olive catechol oxidase at different stages of purification. 1. 30–90% $(NH_4)_2SO_4$ fraction (25 μg protein/gel). 2. Peak A from Sephadex G-100 (60 μg protein/gel). 3. Peak B from Sephadex G-100 (4 μg protein/gel). 4. Peak tubes from DEAE cellulose column (0.5 μg protein/gel). 5. As in (1), but after incubation for 10 min at 70°. The activity of catechol oxidase applied to gels 1–5 was 50–75 units/gel. At the end of the run the gels were developed with caffeic acid.

15 min at 70°. Since it appeared from some experiments that the preparation might be heterogeneous with regard to heat inactivation, we tried to determine whether the multiple forms of the enzyme were differentially inactivated by heat. The enzyme preparation was heated for 10 min at 70° and the bands were then resolved by isoelectric focusing. The 3 fast moving bands disappeared under these conditions (Fig. 2b, gels 1 and 5). This phenomenon was not observed when the same samples were examined by acrylamide gel electrophoresis.

Gel filtration and ion exchange chromatography

The partially purified fraction was passed through a column of Sephadex G-100. The elution pattern revealed two peaks containing 90% of the activity applied to the column. One peak of activity (A) corresponded clearly to a peak in protein, while the second peak (B) was not accompanied by a peak of absorbance at 280 nm. The first peak (A), which was eluted with the void volume, contained 10% of the recovered activity and its sp. act. was lower than that originally applied to the column. The second peak (B) represented a 7-fold purification over the partially purified fraction (Table 2).

Analysis of the two peaks by gel electrophoresis indicated that peak A contained the 4 bands having the slower electrophoretic mobilities (a–d), while peak B contained most of the slow bands and all the fast ones (Fig. 2a). It was conceivable that peak A contained species which resulted from an aggregation of subunits. However we were unable to demonstrate the conversion of peak B to A by concentrating the former and rerunning the preparation on the same column. Comparison of the isoelectric focusing pattern of samples from each peak showed that peak B lacked bands I and VI (Fig. 2b).

Fractions 29–32 (peak B) from the Sephadex G-100 column (35000 activity units and 1.26 mg protein) were subjected to ion exchange chromatography on a DEAE-cellulose column. Catechol oxidase was eluted as a single peak at about 0.15 M Na_2SO_4, ahead of the bulk of protein. This represented a 240-fold purification over the starting material (Table 2). The electrophoretic pattern was identical to that of peak B from the Sephadex G-100 column.

The steps used to purify olive catechol oxidase are summarized in Table 2.

Substrate specificity

Tyrosine, *p*-cresol or quinol were not oxidized by olive catechol oxidase at any stage of its purification. *o*-dihydroxy phenols with a carboxyl group (protocatechuic acid) and trihydroxy phenols such as gallic acid or pyrogallol were not oxidized.

K_m and V_{max} values for some phenolic substrates are shown in Table 3. Of the substrates tested, 4-Me catechol was oxidized most readily. The K_m values varied among

Table 3. Oxidation of various substrates by olive catechol oxidase

Substrate	K_m (mM)	V_{max} (μmol O_2 min/mg protein)
4-Methylcatechol	3.3	2.91
Chlorogenic acid	5.0	2.15
Catechol	8.3	1.48
D-catechin	10.0	0.25
DL 3,4-dihydroxyphenylalanine	10.0	0.23
3,4-dihydroxyphenylethylamine	10.5	0.14

Enzyme source: peak B from Sephadex G-100 column (step 4, Table 2). K_m and V_{max} were determined from Lineweaver–Burk plots. Activity was determined at pH 5, using 0.1 M Na citrate buffer.

these substrates by a factor of four only, while the V_{max} values showed a 20-fold range of variation. No differences were observed in the relative activities toward substrates by the enzyme at the different stages of purification. Moreover, no marked differences were noted in the rate of oxidation of caffeic acid, 4-Me catechol and chlorogenic acid by the various bands separated by electrophoresis.

Molecular weight

An estimate of 42000 for the MW of olive catechol oxidase was calculated from its elution volume on Sephadex G-100 (peak B,) after calibration of the column with proteins of known MW [28].

Inhibitors

The effect of some inhibitors on olive catechol oxidase is shown in Table 4. The enzyme was strongly inhibited by 2-mercaptoethanol (0.03 mM) and sodium bisulfite 0.06 mM) but not by 10 mM EDTA or 50 mM DL-cysteine. The most potent inhibitor was dieca, with 50% inhibition at 5 μM. Benzoic acid appeared to inhibit olive catechol oxidase competitively, with a K_i of 0.2 mM.

The data in Table 4 demonstrate that halogen ions were relatively poor inhibitors of the enzyme. The inhibition at 5 μM. Benzoic acid appeared to inhibit electronegative potential, and independent of the accompanying cation. The extent of inhibition of olive catechol oxidase by chloride ion was strongly dependent on pH; the concentration required for 50% inhibition increased with increasing pH (Table 4).

The inhibition of the enzyme by chloride ion as a function of pH was studied using catechol oxidases from three sources. It was found that avocado and olive catechol oxidase, which have low pH optima (about pH 5), were more effectively inhibited by Cl^- than mushroom tyrosinase which has a higher pH optimum (pH 6.5).

DISCUSSION

The partial purification of olive catechol oxidase necessitated the preparation of an acetone powder which ensured removal of oil and endogenous phenols. The use of SDS improved the extraction from the acetone powder and activated the extracted enzyme. Activation by SDS has been reported previously for catechol oxidase from sugar beet leaves [18] and mushroom [16] at neutral pH, and at slightly acid pH for catechol oxidase from broad bean [29]. Trypsin activation of catechol oxidase has been reported for sugar beet and spinach [13, 17], but its pH dependence has not been previously described. It appears that both trypsin and SDS affect the activity of olive catechol oxidase by shifting its pH optimum toward pH 6.5.

The pH optima which have been reported for catechol oxidases are usually around pH 4.5, pH 7 or both [2, 5, 6, 30, 31]. Maximum activity of olive catechol oxidase using 4-methylcatechol as the substrate was observed at pH 4.5 (Fig. 1). Exposure of catechol oxidase to a pH shock [32], detergents (Table 1) or trypsin changes the dependence of enzyme activity on pH. The variability in the pH optimum of the enzyme from various sources may be due to species specificity. It is, however, possible that the reported variability is a result of changes in the enzyme protein, induced by the isolating techniques or by endogenous factors present in the tissue from which the enzyme was isolated.

The isoelectric point of the major isoenzyme of olive catechol oxidase activity is between pH 4.4 and 5.6 (Fig. 2). Similar values were reported for catechol oxidase from potato tubers [13] and pear leaves [33].

Olive catechol oxidase, even at a relatively advanced stage of purification (Fig. 2) appeared in multiple forms after gel electrophoresis or gel electrofocusing. The same phenomenon has been observed in tissues such as cherry [31], banana [34], mushroom [35] and grape [10]. Catechol oxidase from avocado was also resolved by gel filtration on Sephadex G-100 into two fractions but no differences were observed in the isoenzyme patterns of the two fractions [11]. It is possible therefore that the multiple forms of olive catechol oxidase are true isoenzymes but the possibility that they result at least in part from various degrees of aggregation of subunits cannot be ruled out.

The degree of purification of the enzyme achieved here, about 240-fold, is somewhat less than that achieved for the enzyme from apples [3]. The estimate of MW (42000) is somewhat higher than the values reported for

Table 4. Inhibitors of olive catechol oxidase

Inhibitor	Concentration required for 50% inhibition (M)
KBr	1.3×10^{-1}
NaCl, KCl or NH_4Cl	2.8×10^{-2}
NaF	1.4×10^{-3}
8-Hydroxyquinoline	1.3×10^{-3}
Thiourea	8.0×10^{-4}
Salicylaldoxime	4.3×10^{-4}
Benzoic acid	2.0×10^{-4}
$NaHSO_3$	6.2×10^{-5}
2-Mercaptoethanol	2.9×10^{-5}
Dieca	5.1×10^{-6}

Enzyme source: peak B from Sephadex G-100 column. (step 4, Table 2). 10 μl enzyme (containing 0.9 μg protein), were preincubated with the inhibitors for two min at 26° in 2.5 ml of 0.12 M Na citrate buffer, pH 5. Reaction was initiated by the addition of 0.5 ml of 30 mM 4-methylcatechol.

Table 5. Inhibition of catechol oxidase by NaCl at various pH values

pH	Olive catechol oxidase	Avocado catechol oxidase	Mushroom tyrosinase
	Concentration of NaCl at 50% inhibition (mM)		
4.5	6	6	100
5	34	23	500
6	500	360	2100

Olive catechol oxidase: 30–90% $(NH_4)_2SO_4$ fraction (100 μl containing 5 μg protein and 30 activity units). Avocado catechol oxidase (11): 40–75% $(NH_4)_2SO_4$ fraction (100 μl containing 0.4 μg protein and 28 activity units). Mushroom tyrosinase (Grade III, Sigma; 100 μl containing 10 μg protein and 25 activity units). Enzyme was preincubated at 26° in the presence of 2.5 ml 0.1 M NaOAc buffer (OH 4.5 or 5), or 0.1 M NaPi, pH 6, containing various NaCl concentrations. The reaction was initiated after 2 min incubation by addition of 0.5 ml of 30 mM 4-methylcatechol.

the monomer of catechol oxidases from apples [7], mushrooms [9, 36] and leaves of sugar cane [8]. The substrate specificity of the enzyme, its apparent K_m values toward various substrates (Table 3) and its response to inhibitors resemble those reported for catechol oxidases from other sources [5, 12, 14, 24]. The strong inhibition by dieca suggests that it is indeed a copper enzyme. The competitive inhibition by benzoic acid is also in accord with that reported for other catechol oxidases [2, 25].

The pH dependence of inhibition by chloride is shown by catechol oxidase from three distinct sources (Table 5). The difference between mushroom tyrosinase on the one hand and avocado and olive catechol oxidases on the other hand may be related to changes in the binding site for the substrate caused by a change in pH. Such changes, which are reflected in the pH optimum of the enzyme, can also influence the degree of inhibition by chloride. Inhibition of catechol oxidase by carboxylic acids [23, 25–27] and by halides [22–24] was shown to be strongly dependent on the pH: as the pH decreased, the inhibition increased. Robb *et al.* [23] suggested that this inhibition is due to the undissociated acid and that the pH probably affects the inhibitor rather than its binding site. We suggest that it is necessary to distinguish between the inhibition of catechol oxidase by carboxylic acids and by halide anions. Since the dissociation of the carboxylic group is strongly influenced by pH changes between 4 and 6, dependence of inhibition on pH would agree with the suggestion of Robb *et al.* [23]. However, this would not apply to the inhibition by halide ions. The anions are fully dissociated at physiological pH and dissociation is not influenced in the range of pH 4–7. We suggest that in the case of the halides, a 'complex' is formed between the copper and the anion which is stabilized as the pH decreases [37]. Carr [38] showed that the adsorption of chloride to proteins increases as the pH decreases. It was also shown by Nylen and Pettersson [39] that loss of the oxidase activity of the copper protein ceruloplasmin correlated with destruction of histidine residues. Since at pH 6 there is almost no inhibition of olive catechol oxidase by chloride and other halides and the pK of the imidazole group of histidine is around 6.5, it is possible that histidine, or some other group in the protein with a similar pK participates in the inhibition of catechol oxidase by halides. Further studies are required to clarify the mechanism of inhibition by these anions.

EXPERIMENTAL

Purification of the enzyme. Me_2CO powder was prepared from freshly picked, destoned green Manzanillo olives and stored at −20°. The powder was suspended in 0.1 M NaPi buffer, pH 7 (100 mg/10 ml), stirred for 30 min at 4°, and centrifuged at 20000 *g* for 30 min. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation, without pH adjustment. The fraction precipitating between 30 and 90% saturation with $(NH_4)_2SO_4$ was dissolved in 0.05 M NaPi buffer pH 6.5, dialysed against the same buffer, and cleared by centrifugation. This will be referred to as the partially purified fraction. The partially purified fraction (1 ml) was applied to a column of Sephadex G-100 (2.5 × 38 cm) and eluted with 0.05 M NaPi buffer, pH 6.5. Fractions (3 ml) were collected and assayed for catechol oxidase activity. Fractions 29–32 (peak B) from the Sephadex column were pooled, dialysed against 10 mM NaPi, pH 7.5, for 24 hr, and applied to the DEAE-cellulose column pre-equilibrated with 10 mM NaPi, pH 7.5. The column was washed with the same buffer and then eluted with a 600-ml linear gradient of Na_2SO_4 (0–0.5 M in 10 mM NaPi, pH 7.5. Fractions (1.5 ml) were collected and assayed for catechol oxidase activity.

Assay of enzyme activity. Catechol oxidase activity was assayed polarographically at 26° as described in ref. [14], using a Clark-type oxygen electrode. Unless otherwise specified, the reaction mixture (3 ml) contained 10 mM 4-Me catechol, 0.1 M Na citrate buffer, pH 5, and 25–100 µl of the enzyme preparation (containing 5–20 µg protein). The rate of the reaction was calculated from the initial linear portion of the trace of O_2 consumption. One unit of activity is defined as one scale unit/min, which is equivalent to 2.4 µl O_2/min.

Polyacrylamide gel electrophoresis. Performed essentially as described by Davis [40], using tubes of 5 mm i.d. filled to about 8 cm with 7.5% polyacrylamide in 0.37 M Tris–HCl, pH 8.9. The electrode buffer was 0.05 M Tris–borate, pH 9.2 [41]. Samples containing up to 60 µg protein were applied in 10% sucrose and run at 2.0 mA/tube. The gels were stained for catechol oxidase activity according to ref. [4], but *p*-phenylenediamine was replaced by 0.1% *m*-phenylenediamine.

Polyacrylamide gel electrofocusing. Carried out as described in ref. [42] in 7.5% polyacrylamide containing 1% ampholine, pH 3.5–10. The protein sample was applied in 10% sucrose and covered with a layer of sucrose and ampholine. The anode vessel contained 0.4% H_2SO_4 and the cathode vessel 0.4% ethanolamine. The sample was run toward the cathode at a current of 1.5 mA/tube for 5 hr. The gels were stained for catechol oxidase activity as described above under 'gel electrophoresis'. MW of catechol oxidase was estimated by gel filtration on a column of Sephadex G-100 calibrated with proteins of known MW. The column was eluted with 50 mM NaPi buffer, pH 6.5. Protein was determined by the method of ref. [43].

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