ISOLATION AND SOME CHARACTERISTICS OF DIAMINE OXIDASE FROM ETIOLATED PEA SEEDLINGS

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The isolation of diamine oxidase from etiolated pea seedlings (*Pisum sativum* L.) is described. The new procedure involves precipitation of contaminating proteins by rivanol, salting out with ammonium sulfate, controlled thermal denaturation at 60°C, and chromatography on carboxymethyl-cellulose and DEAE-Sephadex in buffers containing 1 μM cupric sulfate. The final purification step is effected on a column of Sephadex G-200. The overall purification of the enzyme with respect to the crude pea extract is 400—700 fold and the yield is 50—60%. The average specific activity of the purest final preparation tested with putrescine as substrate was 43 U/mg. The preparation was homogeneous when subjected to gel filtration in column or on thin layer and showed a molecular weight of $1{\cdot}70-1{\cdot}78 \cdot 10^5$ daltons. Three minor enzymatically active zones of lower electrophoretic mobility in addition to the main enzyme zone were observed when the preparation was examined by disc electrophoresis in polyacrylamide gel. The optical properties of pea diamine oxidase are also described.

Diamine oxidases (histaminases, E.C. 1.4.3.6) have been found in man, animals, plants, molds, and bacteria and are believed to play an important role in the regulation of the level of histamine and polyamines. The examination of diamine oxidases has led to the conclusion that these enzymes are pyridoxal phosphate cuproproteins. The copper content of these proteins has been proved unambiguously in some cases[1-4], doubts, however, have been raised quite recently[5] as to the presence of pyridoxal phosphate.

Germinating pea seeds, especially those grown in the dark[6], represent the richest source of diamine oxidase known. Attempts aimed at the determination of the nature of the coenzyme have been unsuccessful[7-9] in spite of the fact that the enzyme is inhibited by carbonyl reagents and is related in many other respects to hog kidney diamine oxidase[3,4] or to plasma amine oxidase[5,10]. The fragmentary knowledge of the composition of the pea enzyme as well as the possibility of its practical use in the membrane-bound form for rapid oxidimetric determination of biogenic polyamines[11] call for efficient procedure of diamine oxidase isolation from natural sources. The purification procedures[1,12,13] used so far involve considerable losses of material in certain steps or yield unstable preparations[9]. During the years of our studies on this problem we introduced several new steps into the isolation procedure. The

method described here gives a good yield of a relatively stable and chromatographically homogeneous preparation whose characteristics are in good agreement with recorded data.

EXPERIMENTAL

Material

Rivanol lactate and acriflavine chloride were products of Spofa. Carboxymethyl-cellulose CM-23, diethylaminoethyl-cellulose DE-22, and cellulose phosphate P 11 were from Whatman. Sephadex G-150, G-200 (superfine), and DEAE-Sephadex A-50 (medium) were purchased from Pharmacia, Uppsala. Bio-gel A 1·5 m (medium) was from Bio-Rad Laboratories, California. Granulated hydroxyl apatite was prepared according to Mazin and Sulimov[14]. Crystalline bovine liver catalase was a product of Reanal, Hungary. Horse radish peroxidase (Lachema, Brno) had an activity of 100 purpurogalline units per mg. The set of protein standards Combithek II was purchased from Boehringer, FRG.

Isolation of Diamine Oxidase

Pea seeds (*Pisum sativum* L.) cv. *Liliput* or *Klarus* were allowed to soak 24 h in water and left germinate 6–8 days at 23°C in the dark. The roots were cut off and the cotyledons with aboveground parts were stored at −20°C until used further. All purification procedures were carried out at 4–5°C.

Extraction. One kg of germinating pea seeds free of roots was homogenized in Ultra-Turrax blender (10 000 rev./min), 10 min in 2 liters of 0·1M potassium phosphate buffer, pH 7, with cooling. The homogenate was squeezed through a nylon cloth and small portion of the filtrate was clarified by centrifugation for the determination of protein content.

Rivanol treatment. The extract cooled by ice was treated dropwise 10 min with vigorous stirring with a 4% solution of rivanol lactate (1 ml per every 80 mg of protein). The mixture was centrifuged 1 h at 3 000*g* and the sediment was discarded. The supernatant was decolorized by active charcoal (approximately 8 g) and again centrifuged.

Precipitation with ammonium sulfate. Solid ammonium sulfate was added to the supernatant to give 70% saturation and the liquid was stirred for 30 min in the cold. The precipitate was collected by centrifugation (3 000*g*, 1 h), resuspended in 40 ml of 0·1M potassium phosphate buffer, pH 6·4 and dialyzed against the same buffer overnight.

Heat denaturation. The dialyzed solution of crude diamine oxidase was placed in a water bath 65°C warm and rapidly heated to 55–58°C with stirring. The solution was then transferred to another bath heated at 60°C and maintained at this temperature 5 min. Subsequently the solution was cooled in an ice bath and the precipitate together with the remaining charcoal removed by 30-min centrifugation at 5 000*g*. The supernatant was concentrated osmotically in dialyzing tubing against polyethylene glycol (Aquacid III), to a volume of approximately 20 ml. The concentrated solution was dialyzed 48 h against 20 mM potassium phosphate buffer, pH 6, containing 1 μM $CuSO_4$.

*Fractionation on carboxymethyl-cellulose.** The dialyzed enzyme from 4 batches of initial preparation was applied onto a column of CM-23 cellulose (2·6 cm × 40 cm) equilibrated in 20 mM potassium phosphate buffer, pH 6. The proteins not adsorbed were washed off with the same buffer and diamine oxidase was eluted by a linear gradient developed with 200 ml of 20 mM phosphate, pH 6, and 200 ml of 0·1M phosphate, pH 6·4. The pink colored enzyme fractions contained in the active peak were pooled, concentrated by ultrafiltration through a membrane (Amicon Corp.) to a volume of several ml and dialyzed against 20 mM potassium phosphate buffer, pH 7, containing 1 μM $CuSO_4$.

*Removal of inert proteins.** The enzyme solution from the preceding chromatography step was applied onto a column (2·6 cm × 35 cm) of DEAE-Sephadex or DE-22 cellulose equilibrated in 20 mM potassium phosphate buffer, pH 7. The same buffer was used for elution. The active fractions were pooled, concentrated to 4 ml by ultrafiltration and applied onto a column of Sephadex G-200 (1·6 cm × 90 cm), equilibrated in 20 mM phosphate buffer, pH 7. The specific activity of the most active fractions eluted by the equilibration buffer was 40—44.

*Chromatography on hydroxylapatite.** The dilute diamine oxidase solution from the preceding step was applied onto a column of granulated hydroxylapatite (2·6 cm × 20 cm), equilibrated in 20 mM potassium phosphate buffer, pH 7. Elution was effected by a linear gradient developed with 250 ml of 20 mM potassium phosphate buffer, pH 7, and 250 ml of 0·2M phosphate, pH 7. The pink colored fractions showing the highest specific activity were pooled and stored at −20°C.

Methods

Diamine oxidase activity was measured at 30°C by an oxygen electrode[15]. The reaction mixture (3 ml) was saturated with air and contained 0·1M potassium phosphate buffer, pH 7, 10 mM putrescine dihydrochloride, 0·1 ml of ethanol, and 50 μg of catalase. The *o*-aminobenzaldehyde method[16] was used for serial determinations; the value of $\varepsilon_{430} = 2370\ M^{-1}\ cm^{-1}$ (ref.[17]) was used for the calculation of activity units from absorbance values. Both methods gave identical results. One enzyme activity unit (U) is defined as the quantity of enzyme catalyzing the uptake of 1 μmol of oxygen or the formation of 1 μmol of Δ^1-pyrroline in 1 min at 30°C and pH 7. The specific activity is given in units per mg of protein.

For a rapid assessment of diamine oxidase activity in fractions from column chromatography, the following spot test based on the principle of the *o*-aminobenzaldehyde method was used: one drop of the effluent was mixed with one drop of the reagent containing saturated *o*-aminobenzaldehyde solution in 0·1M phosphate buffer, pH 7, with the admixture of 10 mM cadaverine dihydrochloride. The rate of development of the orange color[17] is a measure of the enzyme concentration.

Protein content of pea extract and of crude diamine oxidase preparations was determined by the biuret method; the turbidity was subtracted after the addition of solid KCN (ref.[18]) and bovine serum albumin was used as a standard. The protein content of the column effluent was determined[19] from absorbance at 260 and 280 nm.

Disc-electrophoresis in polyacrylamide gel was carried out by the method of Davis[20] with the exception that the spacer gel layer was omitted. The separation was carried out in Tris–glycine buffer, pH 8·3 and in β-alanine–acetic acid buffer, pH 4·3 (ref.[21]), 3 h at a current of 5 mA per tube (gel column height 7·5 cm). The proteins were stained 1 h with 1% solution of amido black 10 B

* The equilibration and elution buffers contained 1 μM cupric sulfate.

in 7% acetic acid. The background was destained electrophoretically in 7% acetic acid. Diamine oxidase was detected by dipping the gels in the following reagent solution: 8 ml of 0·1M phosphate buffer, pH 7, 1 ml of 0·1M putrescine dihydrochloride, 1 ml of 0·2% dianisidine in methanol, and 0·1 mg of horse radish peroxidase. The enzyme zones were stained brown during 10–60 min. The stained gels were stored in 4% formaldehyde.

Molecular weight of diamine oxidase was determined by the standard technique of gel filtration at 4°C in 50 mM potassium phosphate buffer, pH 7, containing 0·1M sodium chloride; columns (1·6 cm × 100 cm) of Biogel A-1·5 m or Sephadex G-150 and thin layers (20 cm × 40 cm) of Sephadex G-200 were used. The protein zones in the paper replica of the thin layer were stained with 0·25% solution of Coomassie brilliant blue in methanol–glacial acetic acid (9 : 1, v/v). Ovalbumin, bovine serum albumin, aldolase, catalase, and ferritin were used as reference proteins.

Spectrophotometric measurements were made in 1 cm quartz cells in Cary 118 C spectrophotometer.

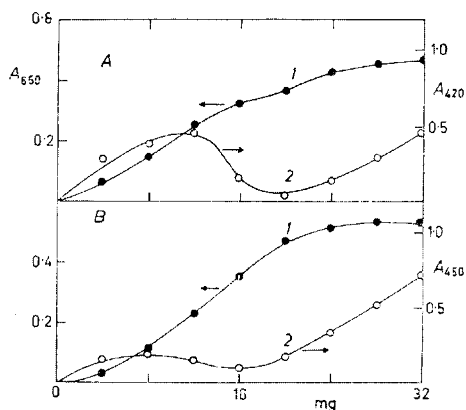

FIG. 1

Determination of Optimum Quantity of Acridine Dye for Precipitation of Contaminating Proteins

The dilute extract of pea cotyledons (41·5 mg of protein in the case of rivanol –*A*, 38 mg in the case of acriflavine –*B*) was treated with various quantities of the dye and the intensity of the turbidity was measured turbidimetrically at 650 nm *1*; volume 100 ml. The quantity of the free dye in the filtrate diluted with water 1 : 1 or 1 : 19 was determined photometrically at 420 and 450 nm, respectively (*2*); abscissa quantity of dye. See text for details.

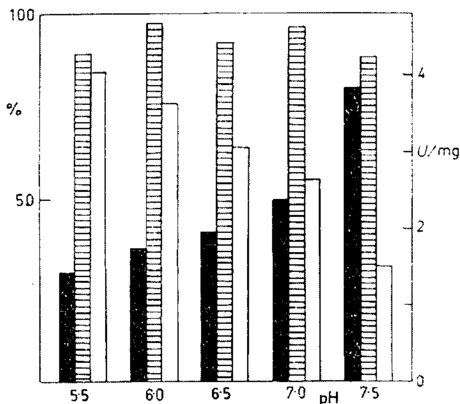

FIG. 2

Effect of pH on Purification of Pea Diamine Oxidase by Controlled Heat Denaturation

The enzyme from the aboveground parts of pea, purified by fractionation with ammonium sulfate (30–60%, specific activity 1·35 at 25°C) and dialyzed was heated 10 min at 60°C in 67 mM potassium phosphate buffer at different pH. The protein content of the supernatant (full columns) and enzymic activity (hatched columns) are expressed in % of control not treated thermally. Empty columns indicate specific activity.

RESULTS

Interaction of pea proteins with acridine dyes. It was shown in preliminary experiments that rivanol and acriflavine, two antiseptics, neither precipitate pea diamine oxidase nor inhibit its activity when used at a low concentration. The optimal quantity of the precipitating agent necessary for an efficient removal of the contaminating proteins was determined as follows: 0·1 – 1·0 ml of 4% rivanol was diluted with water to 98 ml and 2 ml was added of the supernatant obtained by centrifugation of pea cotyledon homogenate. The measurement of turbidity at 650 nm showed that turbidity markedly increases with the increasing concentration of the dye up to a weight ratio of protein to rivanol equal 2 : 1 where the filtrate of the mixture contains the minimal quantity of the free dye measured photometrically (Fig. 1). Larger rivanol additions are without any significant effect on the turbidity development. A similar dependence was observed also with acriflavine (Fig. 1). When the above ratio of protein to the acridine derivative was used in preparative-scale experiments, 75 – 80% of the soluble proteins of the pea extract was precipitated and 80 – 90% of enzymatic activity was retained in the solution.

Controlled heat denaturation. Diamine oxidase is considerably unstable in crude pea extracts. The enzyme activity dropped to 18% after 10 min heating at 55°C and pH 7. By contrast, the enzyme partly purified by fractionation with ammonium sulfate could be heated in the pH-range 5·5 – 7 up to 20 min at 55 – 60°C without a substantial loss of activity. The purification degree strongly depends on the pH of the medium. We found optimal conditions at pH around 6 where the content of contaminating proteins decreased after 10-min heating at 60°C approximately by 60% and the resulting specific activity was two- to three-times higher (Fig. 2). The activity losses in preparative-scale experiments were 6 – 12%.

Chromatographic fractionation. The partially purified enzyme after the heat treatment adsorbed to cellulose phosphate at pH 6·4 (ref.[13]), was eluted by a linear gradient developed with 20 mM phosphate buffer, pH 6·4, and 0·1M potassium phosphate buffer, pH 7, in the front part of the main peak. The specific activity of diamine oxidase increased 6 – 10 times yet the enzyme was not free of contaminating proteins even after chromatography had been repeated. The losses went up to 60% when the addition of the cupric salt to the elution buffers was omitted.

The best results with carboxymethyl-cellulose were obtained when the column was equilibrated in 20 mM potassium phosphate buffer at pH 6 and diamine oxidase was eluted by a linear gradient of buffers containing cupric sulfate (final concentration 1 μM). The enzyme emerged at the hind shoulder of the main protein peak (Fig. 3). The pooled active fractions yielded approximately 90% of the enzyme applied; the specific activity was 14 – 17. Rechromatography increased specific activity to 19 – 24 yet even the preparation thus obtained was contaminated with other

proteins. The latter were removed by anion-exchange chromatography on DEAE--Sephadex or DEAE-cellulose where diamine oxidase is not adsorbed (*cf.* also refs[2,22]). The specific activity increased to 31–34 after this step. The additional purification of diamine oxidase achieved by chromatography on Sephadex G-200 and granulated

TABLE I

Isolation of Partly Purified Pea Diamine Oxidase

The values given in the Table are means obtained with two typical enzyme preparations. Cotyledons (1 kg) of pea cv. *Klarus* (A) or seedlings (1 kg) of pea cv. *Liliput* free of roots (B) served as starting material.

Procedure	Variant	Volume ml	Total protein g	Total activity U	Specific activity U/mg	Purification	Yield %
Extraction	A	2 190	48·49	3 054	0·063	1·0	100
	B	2 630	36·95	4 033	0·109	1·0	100
Rivanol treatment	A	2 611	10·83	2 422	0·224	3·6	79·3
	B	2 603	9·55	3 227	0·338	3·1	80·0
70% Saturation with ammonium sulfate, dialysis	A	148	6·09	2 567	0·422	6·7	84·1
	B	197	6·95	3 107	0·447	4·1	77·0
Heat denaturation (5 min at 55–60°C)	A	118	3·08	2 278	0·740	11·7	74·6
	B	165	3·04	2 630	0·865	7·9	65·2

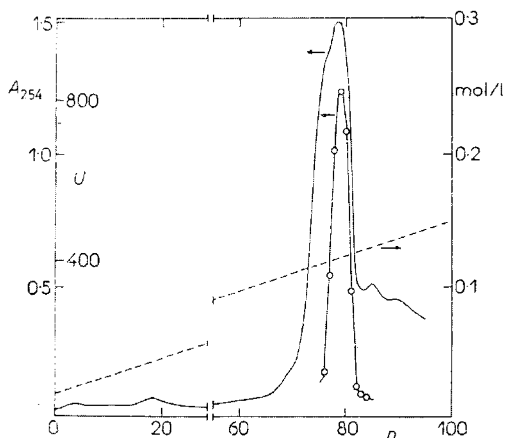

FIG. 3

Elution Profile of Partly Purified Pea Diamine Oxidase on Cellulose CM-23 Column

The enzyme (82 ml, 2 800 U, specific activity 1·3) was placed onto a 2·6 cm × 35 cm column equilibrated in 20 mM potassium phosphate buffer at pH 6. Elution was effected by a linear gradient developed with 200 ml of 20 mM equilibrating buffer and 200 ml of 0·2M potassium phosphate buffer at pH 6·4 (broken line). Fraction volume 3·3 ml/10 min. Full line absorbance at 256 nm, ○ enzymic activity in fraction.

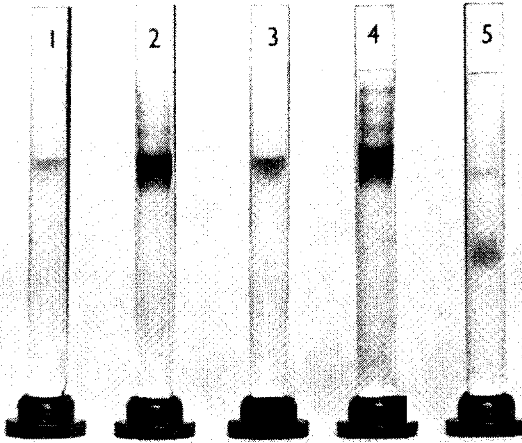

FIG. 4

Disc-Electropherograms of Pea Diamine Oxidase

Electrophoresis was carried out in 7·2% polyacrylamide gel at pH 8·3 and 5 mA per tube. Cathode at the top. The gels were stained for proteins (1, 3, 5) and for enzymic activity (2, 4). Gel 5 shows the enzyme which has been treated with a mixture of 1% dodecyl sulfate, 1% mercaptoethanol, and 6M urea. Quantity of enzyme used 8 μg (1, 2) and 16 μg (3—5).

hydroxylapatite was not substantial. The results of the whole isolation procedure, giving a 50–60% yield of the enzyme, are summarized in Table I and II.

Characterization of final preparation. Diamine oxidase moved as a single, pink-colored band on the hydroxylapatite and Sephadex G-200 column. The purest preparation was relatively stable at temperatures close to 0°C when concentrated or repeatedly frozen and thawed. The preparation lost approximately 15% of its activity when equilibrated with deionized water and lyophilized, yet the lyophilisate was soluble and remained active even after several months of storage at 4°C. When the enzyme solution was dialyzed against a concentrated solution of ammonium sulfate, the enzyme separated as a pink colored precipitate.

The absorption spectrum of diamine oxidase of specific activity 44 displayed a single wide band in the visible region centered at 495 nm and a sharp protein band at 280 nm in the ultraviolet region. The absorbance ratios at characteristic wavelengths showed the following values: $A_{280}/A_{260} = 1{\cdot}840$, $A_{495}/A_{280} = 0{\cdot}013$ (Mann[1] reports $A_{500}/A_{280} = 0{\cdot}017$). The millimolar extinction coefficients were determined

TABLE II

Purification of Pea Diamine Oxidase by Column Chromatography

Column packing and dimensions	Applied enzyme			Eluted enzyme			Yield in each step[a] %	Purification in each step
	total activity U	total protein mg	specific activity U/mg	total activity U	total protein mg	specific activity U/mg		
CM-23 cellulose (2·6 cm × 40 cm)	12 270	8 707·0	1·4	11 250	676·1	16·6[b]	91·7	11·8
DEAE-Sephadex (2·6 cm × 35 cm)	5 180	271·4	19·1	4 465	144·6	30·9[c]	86·2	1·6
Sephadex G-200 (1·6 cm × 90 cm)	7 332	215·0	34·1	7 284	190·0	38·3	99·3	1·1
Hydroxylapatite (2·6 cm × 20 cm)	4 807	110·0	43·7	3 601	88·7	40·6	74·9	—

[a] Average value from total active peak. [b] A specific activity of 19·1 was obtained after rechromatography of the same material. [c] The specific activity increased to 34·1 after rechromatography on DE-22 cellulose.

with the weighed lyophilized preparation. The following values were obtained with the enzyme solution in 0·1M phosphate buffer at pH 7 (molecular weight 1·84 . 10^5 daltons, ref.[13]) ε_{495} = 3·14 mM^{-1} cm^{-1}, ε_{280} = 238·9 mM^{-1} cm^{-1}, and ε_{260} = 150·3 mM^{-1} cm^{-1}. It follows from the measurement that the content of the enzyme protein determined from the absorbance values at 280 and 260 nm (ref.[19]) should be divided by a factor of 1·40.

When the molecular weight of diamine oxidase was examined by gel chromatography on columns of Sephadex G-150 or Bio-gel A – 1·5 m, the enzyme moved between catalase and aldolase and the value obtained from the linear part of the calibration curve corresponded to 1·7–1·78 . 10^5 daltons. Similarly, the enzyme moved close to aldolase in a thin layer of Sephadex G-200 and its molecular weight corresponded to 1·7 . 10^5 daltons. When subjected to disc-electrophoresis at pH 8·3 and 4·3, the enzyme moved as one strong band, identical to the band stained non-specifically for proteins. No inactive contaminants were detected. Three weak, enzymatically active bands of lower electrophoretic mobility (Fig. 4) were detected on the electropherograms at higher enzyme concentrations. When diamine oxidase was exposed to a mixture containing 1% dodecyl sulfate, 1% mercaptoethanol, and 6M urea, 3 h at room temperature or 5 min at 100°C, the enzyme remained fully soluble yet lost its activity and its electrophoretic mobility considerably increased (Fig. 4).*

DISCUSSION

The first step of the now classical isolation procedure introduced by Mann[12] is the precipitation of the contaminating proteins present in the pea extract by a mixture of chloroform and ethanol, a step which is paralleled by a 40% loss of diamine oxidase activity[9]. Subsequent fractionation with ammonium sulfate and repeated precipitation by acetic acid led to an almost 300-fold purification of diamine oxidase; the average yield was 42% (ref.[12]). The latter introduction of chromatography on hydroxylapatite and cellulose anion exchangers increased the total purification degree 880 times[1] and the specific activity determined manometrically with putrescine as substrate at 25°C was 48–55 U/mg. Werle and coworkers[9] modified Mann's procedure[12] and were able to achieve a 1000–1200-fold purification on kieselguhr columns yet the enzyme preparation obtained rapidly lost almost one half of its activity. Recently diamine oxidase from pea epicotyls has been purified by Mc Gowan and Muir[13]. These authors treated the crude extract with protamine sulfate, fractionated with ammonium sulfate and ethanol, and chromatographed on a cellulose phosphate column. They achieved a 419-fold purification, yet the losses during final rechromatography only were 69%. The specific activity determined spectrofluorometrically with benzylamine as substrate (1·005 U, 25°C) corresponds to 17·5–23 U/mg calculated[2,13] in terms of putrescine oxidation. The preparation was homogeneous in the ultracentrifuge and on disc-electrophoresis and the spectra of its solutions showed absorption maxima at 490 and 279 nm with a ratio of A_{280}/A_{260} = 1·84.

* See insert facing p. 2992.

The isolation procedure used in this study yielded a relatively stable enzyme preparation of identical optical and electromigration characteristics. The overall purification of the final preparation is 400–700-fold and varies with individual batches of material. With regard to the almost double original specific enzymic activity in extracts of the pea cultivars used, this value is comparable to the high purification degrees achieved by Mann[1] and Werle and coworkers[9]. The specific activity of the final preparation is lower than that obtained by Hill and Mann[2]; this fact could be accounted for by different methods used for the purity checks. The high yields of the individual purification steps, achieved by the addition of the cupric salt to the buffers, seem to exclude the possibility of enzyme modification during this new isolation process. In our opinion, if the addition of the cupric salt is ommitted activity is lost during chromatography on cation-exchange columns as a result of partial removal of copper from the enzyme molecule. It has been known that pea diamine oxidase can be freed of copper, *e.g.* by dialysis against diethyldithiocarbamate[1,2].

The molecular weight of the enzyme determined is in good agreement with the value of $1.84 \pm 0.06 \cdot 10^5$ daltons calculated from the sedimentation constant[13]. However, even the purest preparations obtained by us showed enzyme heterogeneity when the gel electropherograms were stained specifically. Since the minor fractions showed as a rule a lower electrophoretic mobility, they represent most likely different association forms of diamine oxidase. The association of hog kidney diamine oxidase in the absence of oxygen resulting in a product having a four times higher molecular weight than the original enzyme has been reported quite recently[23]. Pea diamine oxidase is supposed to be composed of two subunits of molecular weight around 96 000 daltons[2,13]. The possible existence of subunits seems to be favored in our experiments by the observed increase of electrophoretic mobility after the enzyme protein has been treated with compounds splitting oligomeric protein systems. The problem of subunit structure of pea diamine oxidase calls, however, for a more detailed investigation.

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