VARIATION OF SUPEROXIDE DISMUTASES DURING THE DEVELOPMENT OF THE FRUIT FLY CERATITIS CAPITATA L.

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SUMMARY : Superoxide dismutase levels were estimated in eggs, larvae and pupae of the fruit fly Ceratitis capitata, as well as in adult flies. No changes occur in the first three stages, but development of the adult fly is accompanied by a large increase in mitochondrial superoxide dismutase per gm of material, and a much smaller relative increase in the cytoplasmic enzyme.

Insects are the most abundant class of organisms in the animal kingdom, but they have not been thoroughly studied with respect to a wide variety of proteins, despite the multiple advantages offered by the various stages of development culminating in the adult insect. Lipid compositions and metabolism in the Mediterranean fruit fly Ceratitis capitata have been extensively studied (1, 2) as well as the changes occuring during the transitions eggs ⟶ larva ⟶ pupa ⟶ adult fly (3). In view of the low respiration rates of eggs, larva and pupa compared with the extremely high rate of consumption of oxygen in the active adult insect it was of interest to examine variations of a protective enzyme, superoxide dismutase (4), during the development of this insect. It may be noted that within the 24 hours following emergence the adult fly shows a 50 fold increase in the volume of mitochondria which is naturally related to the high utilisation of energy during flight. Since increase in oxidative processes could give rise to a related increase in the level of superoxide ion concentration, which would be extremely toxic for the cell and the organism, for example by uncontrolled oxidation of unsaturated lipids, we decided to study both the

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cytoplasmic and mitochondrial superoxide dismutases at the various stages of development of this fruit fly.

MATERIAL AND METHODS

Culture of insects. The Ceratitis capitata was cultured in the INIA-El Encin (Alcala de Henares - Spain) according to the method previously described (5). Insects were collected as follows :

Developmental stages of the insect studied	Age of collection
Eggs	12 - 24 hours
Larvae	2 - 3 days before larval - pupal apolysis.
Pupae	2 days after larval - pupal apolysis.
Pupae	6 to 1 hour before emergence of the adults.
Adult	1 - 2 days after emergence.

Insects were killed by immersion in liquid nitrogen immediately after collection and stored at - 20° C until required.

Homogenisation and preparation of extracts. Insects (2.5 g) were homogenised in 50 ml of 0.01 M potassium phosphate pH 7.0 containing 0.1 % streptomycin sulphate (in order to avoid protein degradation by the enteric bacteria contained in the insects), 0.1 % Triton X-100 and 2 - 3 drops of n-octanol, at maximum speed for 2 x 1.5 min in a Virtis blendor. The homogenate was centrifuged at 14,000 rpm (Centrifuge Sorvall RC 2 B, rotor SS - 34) for 15 min at 4°. The pellet was resuspended in 50 ml of the same buffer and homogenised again and centrifuged as before. To the clear combined supernatants was added KCl to a final concentration of 0.1 M and the solution was then heated at 54° for 3 min and cooled down to 4°. The suspension was clarified by centrifugation at 15,000 rpm (Sorvall RC 2 B, rotor SS - 34) for 20 min at 4°. The supernatant was five times concentrated by ultrafiltration using a PM-10 Amicon membrane at 2 bar.

Ethanol-Chloroform treatment was essentially as described by Mc Cord and Fridovich (4).

Ammonium sulphate fractionation. Solutions were brought to 25 % saturation with powdered ammonium sulphate, the precipitated material removed by centrifugation and the clear supernatant brought up to 85 % saturation by the addition of solid ammonium sulphate. The precipitated material was suspended in the minimum amount of 0.01 M potassium phosphate pH 7.0 and dialysed for 24 h with several changes of buffer. (This treatment was only used prior to the Sephadex column utilized for the separation of dismutases).

Preparative column of Sephadex G-100. A column (3.6 x 60 cm) of Sephadex

G-100 equilibrated in 0.05 M potassium phosphate pH 7.5 was used for the separation of different molecular weight dismutases from a concentrated extract (8 ml) of 150 grams of adult flies, after heat treatment and ammonium sulphate fractionation.

Assay of enzymatic activity in solutions. The standard assay was performed in 3.0 ml of 0.1 M glycine-NaOH buffer pH 9.0 containing 10^{-4} M EDTA, 10^{-5} M luminol, 10^{-5} M hypoxanthine and aliquots of superoxide dismutase. 5 µl of xanthine oxidase (1 mg/ml) were injected to start the reaction. Measurements of light intensity were carried out with the apparatus described previously (6). Under these conditions, 50 % light emission inhibition is considered as corresponding to 0.1 units of superoxide dismutase activity. In order to test inhibition of dismutases by CN^- ions, 2×10^{-3} M KCN was used in the reaction solution.

Assay of enzymatic activity on polyacrylamide gels and plates. Immediately after electrophoresis or electrofocusing, the gels were stained for activity by the photochemical method (7). In order to test the presence of dismutases inactivated by CN^- ions, both the nitroblue tetrazolium and riboflavin solutions for staining containing 5×10^{-3} M KCN.

Acrylamide gel electrophoresis. Gel electrophoresis was carried out according to the procedure of Davis (8). Electrophoresis was performed in 7.5 % acrylamide gels with Tris-glycine buffer, pH 8.5 and a constant current of 4 mA per gel.

Electrofocussing. Enzyme samples were dialysed against 1 % aqueous glycine prior to application. Electrofocussing of samples and isoelectric point determinations were effected on a LKB-Multiphore 2117 apparatus.

Strips (0.5 x 1 cm) of Whatmann 3 MM paper were dipped in solutions of dismutase containing 400 to 800 units/ml of activity and applied to the anode of the LKB-PAG plates (anode solutions was 1 M H_3PO_4; cathode solution was 1 M NaOH). The plates were run for two hours maintaining 20 watts per plate constantly. After one hour the sample application strips were removed and electrofocussing continued. The pH along the plate was determined by cutting strips of gel every 0.5 cm, homogenising them in 1 ml of bidistilled water and after 1 hour the pH was determined in a pH-meter.

Measurements of sedimentation velocity were made with a Beckman Spinco model L2 - 65 B ultracentrifuge according to the method of Martin and Ames (9) with a linear 5 to 20 % (w/v) sucrose gradient. An SW 65 K rotor was used and centrifugation was for 21 hours at 50 000 rpm at 3° C with horse liver alcohol dehydrogenase (5.1 S) and horse myoglobin (2.0 S) as markers.

A Sephadex G-200 column (1.2 x 55 cm) equilibrated with 0.05 M Tris-HCl pH 7.5 containing 0.1 M NaCl was also used for separation of the two enzymes with respect to size. E. coli alkaline phosphatase, horse liver alcohol dehydrogenase, ovalbumin, human erythrocuprein, horse myoglobin and horse cytochrome c were used to calibrate the column.

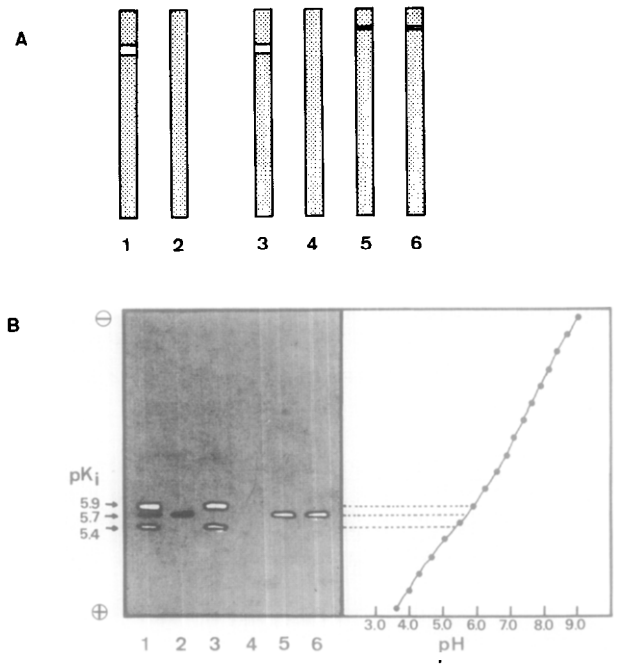

Fig. 1 A) acrylamide gel electrophoresis and B) electrofocalisation of superoxide dismutases from adult flies.
1. Extract (after heat treatment and concentration),
2. Plus 5 x 10^{-3} M KCN,
3. Cu enzyme after separation on Sephadex 100,
4. Plus 5 x 10^{-3} M KCN,
5. Mn enzyme after separation on Sephadex 100,
6. Plus 5 x 10^{-3} M KCN.

RESULTS AND DISCUSSION

Three superoxide dismutases are present in Ceratitis capitata at all stages of development. Two correspond to an erythrocuprein type of superoxide dismutase with a similar molecular weight (approximately 35 000 as indicated by gel filtration on Sephadex 200 and by sucrose gradient centrifugation). These enzymes have an R_F on acrylamide gel electrophoresis of 0.2 but differ slightly in isoelectric points with pIs of 5.9 for the major band (at least 80 - 90 % of the total copper SOD activity) and 5.4 for the minor band and hence can be separated by electrofocalisation on acrylamide gels (Fig. 1). These main components, like erythrocuprein, are inhibited by cyanide but are not destroyed on incubation in chloroform - ethanol mixtures.

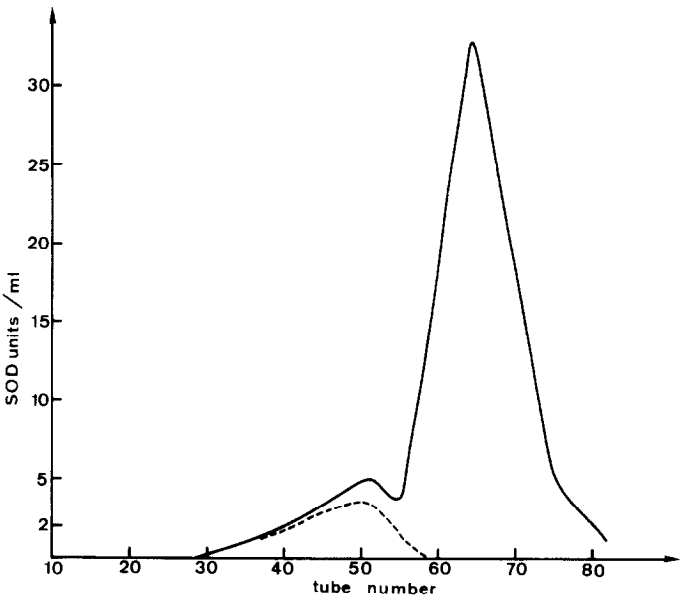

Fig. 2 Separation of Cu - SOD and Mn - SOD in an extract of adult flies on Sephadex G-100 (1. 6 x 90 cm). Solid line-total SOD activity, broken line - SOD activity resistant to KCN.

Like bovine erythrocuprein the enzyme is heat resistant at least up to 55° C, and both the major and minor components may be considered as typical cytoplasmic copper-zinc superoxide dismutases.

A third SOD is also present but differs from the two others in that the molecular weight (approximately 90 000) corresponds to a mitochondrial manganese SOD. This enzyme, present in much smaller amounts, is readily separated from the erythrocuprein type SODs by gel filtration on Sephadex 100 (Fig. 2). Migration on acrylamide gels electrophoresis is slower (R_F 0. 10) than for the copper containing SODs, in accord with the larger molecular weigh the isoelectric point of 5. 7 being intermediate between those of the other two enzymes. The activity is not inhibited by cyanide nor is it destroyed on incubation with $CHCl_3$/EtOH and thus resembles human mitochondrial SOD rather than the manganese enzyme from chicken liver.

TABLE I

SUPEROXIDE DISMUTASE ACTIVITIES PER GM

	Total	Cu-SOD	Mn-SOD	% Mn-SOD / Total SOD
Eggs	1181	1120	61	5.2
Larva	1243	1192	51	4.1
Pupa (2 days)	1338	1287	51	3.8
Pupa (9 days)	1388	1330	58	4.2
Adult fly	1762	1527	235	13.3

Activities are expressed as luminol units (10), using inhibition of the chemiluminescence of luminol in the system xanthine oxidase - O_2 - hypoxanthine as method of estimation.

It was of interest to determine the changes in activity of SOD during development of the fly not only with respect to total activity per gram of material, but also the relative amounts of copper enzyme (inhibited by cyanide) corresponding to cytoplasmic SOD and manganese enzyme (no inhibition) corresponding to mitochondrial SOD. Attempts to estimate these activities in crude extracts gave completely aberrant results owing to the presence of large quantities of lipids and lipoproteins which consume $O_2^{\cdot-}$. The different superoxide dismutases were therefore partially separated (and purified from lipid material) by centrifugation on sucrose gradients, followed by estimation of total activity as well as cyanide resistant activity. All four stages -eggs, larva, pupa (at 2 days and at 9 days, the latter corresponding to the latest time before emergence of the fly) and adults were examined by this approach. In the case of the adult fly, filtration on Sephadex 100 was also used for partial purification, and gave identical results. The values obtained are shown in Table I.

It can be seen that until emergence of the fly, very little difference occurs in superoxide dismutase activities present in the different stages of development. The adult fly shows an increase in total activity, half of this

being due to a four fold increase in the mitochondrial enzyme. It may be noted that the large increase in mitochondria, mentioned earlier, develops mainly in the thorax, whereas in this work the entire insect was used. Separation of the thorax from the rest of the insect would presumably give rise to an even greater apparent increase.

Thus final stages of development of the fruit fly Ceratitis capitata are accompanied by a large increase in number of mitochondria which results in a similar increment of mitochondrial superoxide dismutase (400 %) and a proportionately much lower increase in cytoplasmic SOD (15 %). No significant changes are observed in the three stages, eggs, larva and pupa prededing emergence of the adult fly, in contrast with marked changes in lipid metabolism occuring at the apolysis stage between larva and pupae (3).

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