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Demonstration of Tyrosinase Isozymes in *Celerio euphorbiae* Pupae by Serological Methods

Wykazanie obecności izozymów tyrozynazy owadziej z *Celerio euphorbiae* w oparciu o metody serologiczne

Выявление присутствия изозимов тирозиназы насекомых из *Celerio euphorbiae* серологическим методом

INTRODUCTION

Many authors have reported on heterogeneity of tyrosinase (o-diphenol : O_2 oxidoreductase, EC 1.10, 3.1) isolated from various materials. The works of Burnett (3, 12) and Pomerantz (8) give evidence of the presence of tyrosinase isozymes in animal material whereas those of Smith and Krueger (11), Bouchilloux et al. (2), Robb and Mapson (9), and Jolley (5) point to similar properties of this enzyme in plants. Those authors have frequently described their preparations as "multicomponent in nature".

This paper is an attempt to elucidate the character of tyrosinase isolated from *Celerio euphorbiae* pupae by the method of Trojanowski and Dernałowicz (13). By means of chromatography on brushite gel ($CaHPO_4 \cdot H_2O$) it was possible to separate tyrosinase into 5 active fractions which differed in their activity towards the substrates: catechol, DL-DOPA (DL-3,4-dihydroxyphenylalanine), L-tyrosine and p-cresol. The occurrence of tyrosinase isozymes in *Celerio euphorbiae* is dealt with by means of immunological methods: double diffusion in agar gel and immunoelectrophoresis. These methods, particularly immunoelectrophoresis, can be used successfully in that kind of studies because of their high sensitivity and selectivity (6).

MATERIAL AND METHODS

Material: Pupae of the moth (*C. euphorbiae*) were used in the investigations. The caterpillars of this moth appear at the beginning of July and they live till the end of August. After their pupation they were stored and kept at +4°C.

Determination of protein and enzyme activity. The above mentioned determination was carried out by the methods described in the paper of Trojanowski et al. (13). The spectrophotometric and Folin-Ciocolteu methods were used for estimation of protein concentration and the manometric method of Warburg for estimation of enzymatic activity.

Determination of polysaccharides. The content of polysaccharides was determined by a modified method of Dżułyńska et al. (4). The sample containing 1 mg of lyophilized enzyme * was dissolved in 1 ml of 0.9% NaCl with addition of 5 ml of anthrone reagent prepared after Wenke (20 ml of dist. water, 80 ml of conc. H_2SO_4, 80 mg of pulverized anthrone). After being mixed the sample was incubated in water bath for 5 minutes and then cooled. After 10 minutes the extinction at 570 mμ was read.

Double diffusion in agar gel according to Ouchterlony (7). The following composition of gel was prepared: 1.2% of Difco agar in 0.005 phosphate buffer *pH* 7 with addition 0.85% NaCl and 0.001% merthiolate. The gel was poured on Petri dishes in which wells were punched. The distance between the serum wells and those for the antigen was 1 cm. After depositing the serum and antigens, the dishes were incubated in a humid chamber at 25°C for 5 days, whereupon the final reading was done.

Immunoelectrophoresis (according to Scheidegger — 10). On each glassplate (76 × 26 mm) 4.5 ml of 1% Difco-Noble agar in barbital buffer (barbitone sodium 5×10^{-2} M, barbital 2.82×10^{-2} M) *pH* 8.2 were distributed. Wells for serum or antigen were punched in the gel and after filling them up, electrophoresis was carried out using electric current of 0.8 mA for 1 cm of width; the voltage was fixed at 180 V. The time of electrophoresis was 4.5 hr. After the distribution small grooves were cut in the gel and after filling them up with antiserum they were left in a humid chamber at 25°C.

Complement-fixation reaction. For exact characterization of serological properties of the antigen used for immunization and antibody level in antiserum, complement fixation was done. Standarized estimation was carried out in the Department of Microbiology at the Medical Academy, Lublin.

Preparation of antigens and immunization of animals. Preparation of tyrosinase and its partial purification was carried out after Trojanowski et al. (13). In the lyophilized sample showing tyrosinase activity, protein, polysaccharides and tyrosinase activity were determined. In 10 mg of the liophilizate there was 3.12 mg of protein (estimated by Folin's method) and 0.24 mg of polysaccharides. The enzymatic activity of lyophilizate was expressed in units Qo_2 and

* For the estimation of polysaccharides the homogenate of pupae was prepared with buffered 0.9% NaCl instead of 0.25 M saccharose solution.

it amounted to 190 towards catechol and 104 towards p-cresol. In order to produce the antiserum 340 mg of the lyophilizate were dissolved in 30 ml of 0.9% NaCl and dialized. After the dialysis the sample was filtrated through Schott G-5 filter. Three rabbits were immunized intravenously by introducing the antigen according to Adams' method (1). Each animal received totally 63 mg of protein in 18 injections (3 series of 6 injections per week). The animals were bled after 10 days following the last injection.

A part of the lyophilizate of the crude tyrosinase preparation was subdued to chromatographic separation on brushite gel to separate it into 5 active fractions (13) and into inactive protein fraction which left the column first. After determining the protein content, those fractions were examined in serological reactions.

RESULTS

Characteristics of antiserum. The examined antisera produced the precipitates in the tube with 7.8 μg of protein. The study of immunological properties of the antigen and of the level of antibodies in the sera was determined by complement fixation reaction (Table 1 and 2).

Table 1. Complement-fixation test

Antigen	Serum dilution:												
	serum I						serum II						control
	1/10	1/20	1/40	1/80	1/160	1/320	1/10	1/20	1/40	1/80	1/160	1/320	
Lyophilizate solution (mg protein/ml 0.9 % NaCl)	++++	+++	+++	++	–	–	++++	+++	++	–	–	–	–

Table 2. Immunological properties of the antigen in complement-fixation test

Sera	Antigen dilution:				
	1/2	1/4	1/8	1/16	control
serum I dil. 1/80	++++	++	—	—	—
serum II dil. 1/40	+++	++	+	—	—

Reaction of double diffusion in agar gel. 0.15 ml of antisera were introduced into the middle well in the gel on Petri dishes. The surrounding wells (4 wells in each rosette) were filled either with lyophilizate solution (3.2 mg per 1 ml 0.9% NaCl which equalled to

the concentration of 1 mg protein) and its dilution, or with fractions passed through chromatographic column. Fractions showing activity and that of inactive protein were deposited on plates either directly after passing through the column or they were condensed by means of Sephadex G-25 till protein concentration of 1 mg/ml was reached. The content of protein in effluent after passing through the column in the individual fractions was the following: fr. B (inactive protein) — 12%, I fr. — 19%, II fr. — 22.9%, III fr. — 37%, IV fr. — 8.5%, V — 0.6%. Also commercial sample of mushroom tyrosinase was used as antigen at concentrations: 70, 35, 7, 3.5, 1 and 0.7 mg/ml of 0.9% NaCl. The above experiments were also carried out with various dilutions of antisera in which the best results were obtained. The results obtained by the double-diffusion method in agar gel are shown in Table 3 and are partially illustrated in Fig. 1.

Immunoelectrophoresis. The same set of antigens was used in immunoelectrophoresis. The antigen was subdued to electrophoresis in one series of experiments and the antiserum in another series.

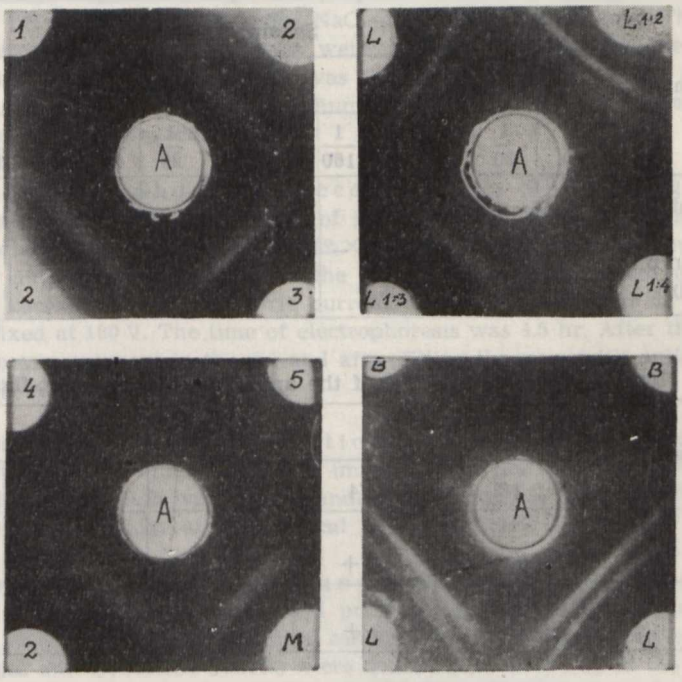

Fig. 1. Reaction of double diffusion in agar gel of the lyophilizate and fractions from brushite column against specific antiserum; A — antiserum, B — inactive protein, 1, 2, 3 — fractions of tyrosinase, L — lyophilizate solution (3.2 mg/ml 0.9% NaCl), 1 : 2, 1 : 3, 1 : 4 — dilutions of lyophilizate solution, M — mixture of active fractions of tyrosinase

Table 3. Double diffusion test

Antigens	Number of precipitation lines	
	full serum	diluted serum 1:2
Lyophilizate full	2	2
dil. 1:2	2	2
dil. 1:3	2	1
dil. 1:4	1	1
dil. 1:8	1	1
Fractions after the column		
B	0	—
I	1	—
II	2	—
III	2	—
IV	0	—
V	0	—
Fractions after the column (condensed)		
B	0	0
I	1	1
II	2	2
III	2	2
IV	1	0
V	0	0
Fraction mixture after condensation	2	2
Mushroom tyrosinase		
70 mg/ml	1	—
35 mg/ml	1	—
7 mg/ml	1	—
3.5 mg/ml	0	—
1 mg/ml	0	—
0.7 mg/ml	0	—

Precipitation results were similar to those obtained in precipitation by double diffusion in agar gel (Fig. 2). When the antigen was separated electrophoretically the results were more differentiated. For the lyophilizate 3 lines were obtained which suggested the existence of three separate protein fractions. The presence of three precipitation lines was also found for fraction III, but fraction II pointed to the presence of two precipitation lines (Fig. 3 b, c). It was found that the fraction B is not capable of forming precipitates with antiserum. For fraction I and IV single precipitation lines were formed, of which that for fraction IV was

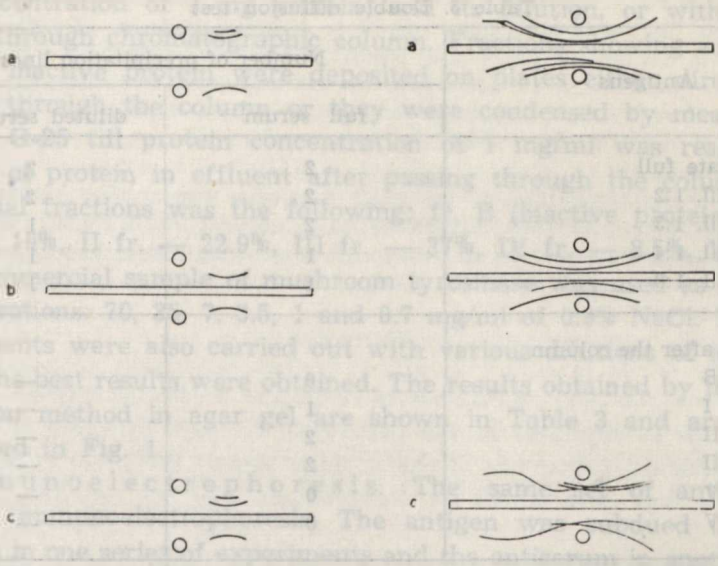

Fig. 2. Scheme of the immunoelectrophoretic pattern of the antiserum against the lyophilizate solution (3.2 mg/ml 0.9% NaCl) and fractions from brushite column, a — against lyophilizate solution, b — against II fraction, c — against III fraction. At the top — full serum, at the bottom — diluted serum 1 : 2.

Fig. 3. Scheme of the immunoelectrophoretic pattern of the lyophilizate and active fractions of tyrosinase from brushite column against the specific antiserum; a — ieph. of lyophilizate, b — ieph. of II fraction, c — ieph. of III fraction

faintly visible despite condensation with Sephadex G-25. Difficulties with condensation of fraction V (protein precipitated of the solution during condensation) caused that in condensed solution there was obtained only 100 μg/ml of protein which appeared insufficient to develop a specific reaction.

DISCUSSION

As it was already mentioned at the beginning of this paper many authors suggest the occurrence of tyrosinase isozymes both in plants and animals. In the light of the results presented it seems that in case of tyrosinase from insects the occurrence of several forms of active tyrosinase should be taken into account. This has been demonstrated in this work chiefly by the method of immunoelectrophoresis. Its application gave results pointing to the presence of three separate protein components in the preparation examined. The same results were obtained regardless of the fraction used. A single precipitation line for fraction IV was obtained only after condensation, for fraction V protein concentra-

tion was too low to obtain the precipitation effect. The comparison of the results obtained by the two immunological methods applied makes it possible to find a considerably smaller sensitivity of the double diffusion method in agar gel as compared with immunoelectrophoresis in examination of the active fractions of enzyme. It appears, however, that chromatography on the brushite gel does not permit, in the case of tyrosinase from *C. euphorbiae*, an exact separation and isolation of the individual forms of the active enzyme. This has been indicated by double and triple lines obtained for fractions II and III in immunoelectrophoresis. Fractions of active tyrosinase leaving the column seem to be a mixture of tyrosinase particles of various electrophoretic mobility.

The choice of the subject of this work is justified also by earlier results of Adams' work (1), who used nonspecific precipitation in floculation test to demonstrate immunogenic reaction of mushroom tyrosinase. Adams used a partially purified preparation of tyrosinase isolated from *Psaliota campestris* as antigen. He found that sera obtained after immunization of animals with this antigen did not precipitate tyrosinase isolated from another mushroom *Lactarius piperatus*. The results of his work do not coincide with experimental data obtained by such authors as Nishimura (6) and Kirk, Campbell, Seaston (quoted after Adams).

It appears therefore that the immunological response to mushroom tyrosinase of antiserum obtained by immunization of animals with insect tyrosinase deserves attention. A considerable concentration of mushroom tyrosinase as it was used in the present case suggests a smaller relationship of this antigen to the obtained antisera.

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STRESZCZENIE

Heterogenność tyrozynazy owadziej w poczwarkach *Celerio euphorbiae* badano przy pomocy metod serologicznych. Zastosowanie metody podwójnej dyfuzji w żelu agarowym pozwoliło stwierdzić w badanym materiale występowanie dwu frakcji tyrozynazy; przy pomocy immunoelektroforezy wykazano obecność trzech odrębnych serologicznie frakcji tego enzymu. Dodatnia reakcja pomiędzy specyficzną surowicą, uzyskaną wobec tyrozynazy z poczwarek, a tyrozynazą grzybową wskazuje na brak swoistości gatunkowej tyrozynazy w odczynach serologicznych.

РЕЗЮМЕ

Гетерогенность тирозиназы насекомых из *Celerio euphorbiae* исследовали серологическим методом. При применении метода двойной диффузии в агаровом желе установлено присутствие в исследуемом материале двух фракций тирозиназы; при иммуноэлектрофоретических исследованиях обнаружили присутствие трех серологически особых фракций этого энзима. Положительная реакция между специфической противосывороткой, полученной в присутствии тирозиназы из личинок, и грибной тирозиназой указывает на недостаток своеобразных признаков тирозиназы в сероreакциях.

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