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Activatory Action of Trypsin on Epidermis Dopa-oxidase *

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Trypsin activated the Dopa-oxidase enzyme of frog epidermis, while carboxypeptidase «a» achieved only 50 % of this activation. The enzyme can be activated by passing it through a column of insoluble trypsin coupled to Sepharose. Some properties of inactive and active dopa-oxidase are compared: *a*) Apparent molecular weight and Stokes radius of active enzyme are higher than those of the inactive one. *b*) The entropy change for denaturation of inactive enzyme is about 108 cal $\times$ mol^{-1} $\times$ °K^{-1}; while the value is only —3.6 cal $\times$ mol^{-1} $\times$ °K^{-1} for the active enzyme. It is hypothesized that the activatory process consists of a tryptic rupture accompanied by a spatial unfolding of the enzyme molecule.

Monophenol monooxygenase (5) (E.C. 1.14.18.1) is a copper protein which has two different catalytic activities, usually denominated tyrosinase and dopa oxidase. According to their actions they transform tyrosine into dopa or dopa into dopaquinone. In both cases atmospheric oxygen is the oxidant. The enzyme is the only clearly identified one which participates in melanogenesis and seems to appear in an inactive form in some tissues (3, 4, 13).

Some time ago, it was found that in frog epidermis, both in the ventral (unpigmented) and dorsal (pigmented) zones, the enzyme was present in an inactive form but was convertible into an active one by means of trypsin (10). In the epidermis of other animals, including humans, the properties of this enzymatic system are not well known, either because of its low concentration or that the enzyme is present in an inactivated or inhibited form, whose activation has not yet been elucidated. The possibility exists that the proteolytic activation of the enzyme has a certain physiological role on some ocassions and the existence of proteolytic enzymes in the skin is known (2). It is therefore interesting to study the characteristics of the enzyme from frog epi-

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dermis, since it is present in a high concentration and is easily activated.

Our investigation is a study of the nature of this activatory process to determine if the trypsin action is of the classical proteolytic type and if other conformational factors that can play an important role also exist.

Materials and Methods

The frogs *(Rana esculenta ridibunda)* were obtained from local suppliers from November to March. After this date specific activities were drastically diminished. Epidermis was separated from dermis after incubation with a 2 M NaBr solution during 24 hours at 0-4° C. After washing several times with bidistilled water, a negative bromide reaction was reached and epidermis was lyophilized and kept at 0-4° C until used.

Enzyme preparation. 1 g lyophilized epidermis was ground in a mortar for 10 minutes with purified sand-sea and 20 ml of bidistilled water. After centrifugation at 15,000 × g for 20 minutes, supernatant was discarded and the sediment was treated again in mortar with 20 ml 0.1 M sodium phosphate *p*H 7.0 and then centrifuged at 18,000 × g for 30 minutes. Supernatant was centrifuged at 105,000 × g for 2 hours. In this way a solution of the enzyme was obtained. A saturated solution of $(NH_4)_2SO_4$ (neutralized at *p*H 7.0 with OHNa) was added until a saturation degree of 40 % was reached, and the solution was kept overnight. After centrifugation at 15,000 × g for 30 minutes, sediment was removed and saturated solution of $(NH_4)_2SO_4$ was added to the supernatant until a 55 % was reached, and the solution was kept overnight. The solution was kept for at least 8 hours after which the precipitate was recovered and 5 ml 0.1 M sodium phosphate buffer *p*H 7.0 added. This preparation was used as the source of the enzyme after the adequate dilution with the above buffer. All operations were performed at 0-4° C.

Measurement of dopa oxidase activity. The measurement medium, with a total volume of 2.6 ml in 0.1 M sodium phosphate buffer *p*H 7.0, contained 15 μmoles of L-dopa. At 0 time, 1 ml of activated enzyme solution was added and formation of dopachrome was spectrophotometrical checked at 475 nm. Since the absorbance increment was not proportional to the time, the tangents of the curves were drawn at 0 time.

One unit of enzyme is the quantity which transforms 1 μmol of L-dopa per minute at 20° C; for dopachrome $\epsilon = 3.7 \times 10^4$. The enzyme was always previously diluted in such a way that less than 0.03 units were present in the reaction medium. Above this figure, there was not proportionally between the measurement and the amount of the enzyme in the medium.

Activation of the enzyme in bath. To 1.0 ml of 0.1 M sodium phosphate buffer containing 0.1 ml of enzyme solution, 10 μl of trypsin solution (1 mg/ml) was added and the mixture incubated for 5 minutes at 37° C, after which the enzyme was totally activated. In other cases, pancreatin or carboxypeptidase «a» was used instead of trypsin.

Activation of the enzyme using a column with insoluble trypsin. Trypsin was coupled to CNBr-activated Sepharose 4B in the following way: 1 g of gel swollen overnight with 20 ml of 10^{-3} M HCl solution was poured into a Pharmacia K 9/15 column. The gel was washed with 200 ml of acid solution at a speed of 80 ml/hour and with the same rate, 50 ml of 0.1 M $NaHCO_3$ buffer solution containing 0.5 M NaCl, was added. A solution of 10 mg of trypsin in 5 ml of the above coupling

buffer was then passed through the column and was recirculated during 24 hours at a rate of 5 ml/hour, after which the column was washed with another 200 ml of $NaHCO_3$-NaCl buffer. Next, 100 ml of 1 M ethanolamine at *p*H 8.0 were added at the same velocity, since ethanolamine is known to react with any remaning active group. Finally three washing cycles were used to remove non-covalent adsorbed protein. Each cycle consisted of a wash with 25 ml 0.1 M acetate buffer *p*H 4.0 containing 1 M NaCl, followed by a wash with 25 ml 0.1 M borate buffer, *p*H 8.0, containing 1 M NaCl.

The yield of coupled trypsin was 90,8 % as calculated by measuring the absorbance at 280 nm before and after the coupling process.

The activation of dopa oxidase was performed in the following way: the trypsin-Sepharose column was washed with 50 ml 0.1 M sodium phosphate buffer *p*H 7.0 and then 1 ml of enzyme solution was passed through it at a rate of 5 ml/hour. Activities of several fractions were checked. If the speed of the enzyme circulation was increased to 100 ml/hour, only 65 % of activation was noted. All operations were performed at 0-4° C. The enzyme after passing through the column was not further activated by addition of a trypsin solution and incubation at 37° C.

Gel filtration. A Pharmacia K 26/70 column was employed and manufacturer's instruction were followed for preparing and using gels of Sephadex G-100, Sephadex G-300 Superfine and Sepharose 6B. The following standards were used (Molecular weights and Stokes' radii in nm in brackets): 1) cyt c (13,500; 1.64); 2) trypsin (23,000; 1.94); 3) chymotrypsinogen (25,000; 2.25); 4) ovalbumin 45,000; 2.90); 5) bovine serum albumin (67,000; 3.61); 6) yeast alcohol dehydrogenase (83,000; 4.6); 7) collagenase (109,000; 4.9); 8) catalase (240,000; 5.22); 9) γ-globulin (153,000; 5.22); 10) ferritin (540,000).

Trypsin activity. Trypsin activity was determined by using the colorimetric method at 405 nm with benzoylarginine-p-nitronilide as substrate (6). One unit is the amount of enzyme which converts 1 μmole of substrate per minute at 25° C.

Protein determination. Protein were evaluated colorimetrically (9) or spectrophotometrically (8).

Results

The activatory effect of trypsin can also be obtained, at least in part, by using carboxypeptidase «a» (table I). The absence of trypsin activity was checked in the solution of carboxypeptidase «a». However, the addition of both trypsin and carboxypeptidase «a» did not increase the activation produced by trypsin alone. This activatory action of carboxypeptidase «a» is in contrast to the results reported by BARISAS and MCGUIRE (1) using and enzyme extracted from *Rana pipiens pipiens*. Pancreatine had the same behaviour as trypsin.

In order to activate totally the enzyme, a tryptic activity of 3 units/ml in the medium of activation was necessary. An excess of trypsin did not inactivated the dopa-oxidase activity (figure 1), at least

Table I. *Activatory effects of some enzymes on the DOPA-oxidase activity.*
The added proteolytic agents were incubated for 5 minutes at 37° C before the measurement of DOPA-oxidase activity was made. When trypsin + carboxypeptidase «a» were used two incubations were carried out.

Addition	Concentration (mg/ml)	Dopa-oxidase activity (units)
None	—	0.000
Trypsinogen	5	0.000
Pancreatin	50	0.016
Trypsin	5	0.016
Carboxypeptidase «a»	5	0.008
Trypsin+ carboxypeptidase «a»	5	0.016

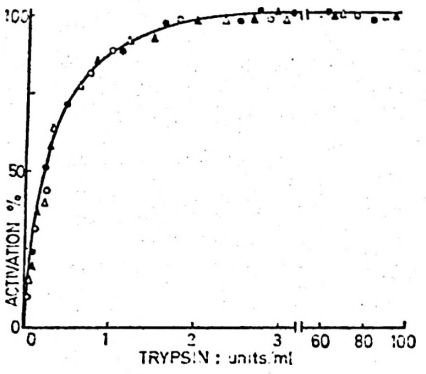

Fig. 1. *Activatory effect of trypsin on DOPA-oxidase activity.*
Samples of the enzyme incubated for 5 minutes at 37° C with different solutions of trypsin. ●—● Sigma, type III (565 mUnits/mg); O—O Merck, 2,000 E/g (12 mUnits/mg); ▲—▲ Difco, 1:250 (68 mUnits/mg); △—△ Merck, pancreatin (5 mUnits/mg).

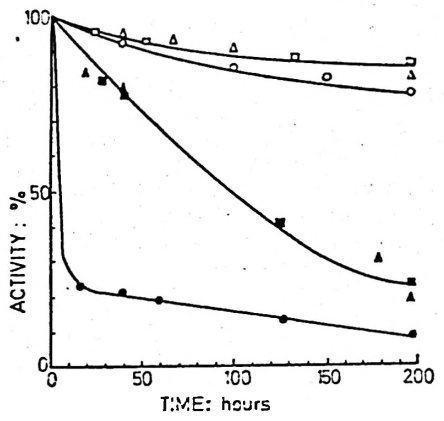

Fig. 2. *Unstability of DOPA-oxidase produced by the presence of trypsin.*
Three different types of enzyme were used: *a*) native, non-activated enzyme; *b*) activated enzyme by passing it through insoluble trypsin coupled to Sepharose; therefore, the solution does not contain trypsin; *c*) activated enzyme by incubation with trypsin; the solution contains trypsin. △—△ Native enzyme, at 0° C. □—□ Active enzyme, without trypsin, at 0° C. O—O Active enzyme, with trypsin, at 0° C. ▲—▲ Native enzyme, at 30° C. ■—■ Active enzyme, without trypsin, at 30° C. ●—● Active enzyme, with trypsin, at 30° C.

when activities measurements were performed immediately after the activation. However, the stability of the enzyme was affected by the presence of trypsin after a long period of time; it was therefore preferible to activate the enzyme by passing it through a column of insoluble trypsin coupled to Sepharose 6B. In this way properties of the enzyme were not affected by the action of trypsin. In figure 2 it is patent that inactive or active enzyme in the absence of trypsin is very stable, but in the presence of trypsin it can lose activity quickly.

When the aproximate molecular weights of inactive and active dopa-oxidase were calculated using Sephadex G-100 and Sephadex G-200 Superfine gels, the results were not satisfactory because of the high molecular weight of the enzyme in the first case. With Sephadex G-200 Superfine, elution volumen of both inactive and active enzyme were very close, but molecular weight of the active enzyme was always, paradoxically, superior to that of the inactive one. Using Sepharose 6B gel, with a great resolution towards proteins with higher molecular weights, the apparent molecular weights were 115,000 for the inactive enzyme and 210,000 for the active one (fig. 3). This difference may be due not to a real increase of the molecular weight during activation, but to a change of the spatial conformation producing an active enzyme more unfolded than the inactive and, therefore, with a lower elution volume. The existence of two apparently different molecular weights corresponding to the active and inactive enzymes is in opposition to the data from BARISAS and MCGUIRE (1) who claimed a molecular weight of 200,000 for both the active and proenzyme.

In figure 3 the relation between $\sqrt[3]{K_D}$ and Stokes radius is also shown. For the inactive enzyme Stokes radius was estimated to be 4.2 nm and more than 5 nm for the active enzyme. In this case, Stokes

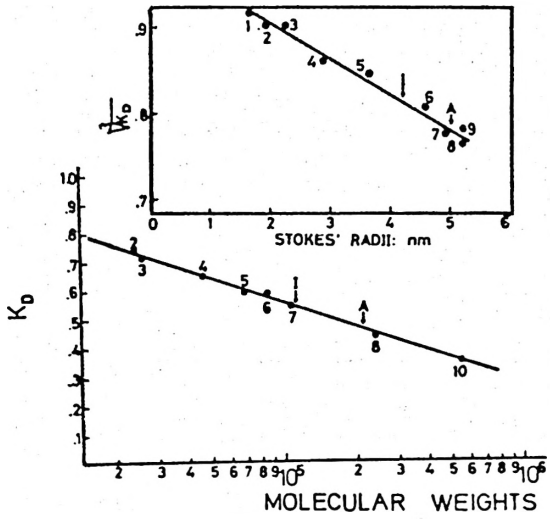

Fig. 3. *Determination of approximate molecular weights and Stokes radii.*
Experimental data are expressed in terms of K_D, which is defined as $K_D = \frac{V_e - V_o}{V_i - V_o}$, where V_e is the elution volume of the protein; V_o is the void volume (determined by dextran blue 2,000) and V_i the internal volume of the gel. K_D values were plotted either versus molecular weights of versus Stokes radii according to PORATH (12): $K_D = A - B.\ a$, in which A and B are constants and a is the Stokes radius. The figures in the graph correspond to the standard indicated in Material and Methods. «A» is active enzyme after activation with insoluble trypsin. «I» is inactive enzyme, without activation.

radiues is very close to that of catalase and γ-globulin (5.22 nm). Howerer these two proteins, with the same Stokes radius. differ 80,000 in their molecular weights. Therefore, an accurate calculation of the molecular weights by the usual calibration curve in that zone could not be made. In any case, the inactive enzyme has a higher value for its Stokes radius than the active one.

In denaturation studies, solutions of inactive or active enzyme (activated with the insoluble trypsin) were placed in thermostatic baths and the residual activities were measured at different times after activation of the inactive enzyme by addition of a solution of trypsin. The results, (fig. 4), showed that up to 70° C, the classical thermal denaturation did not take

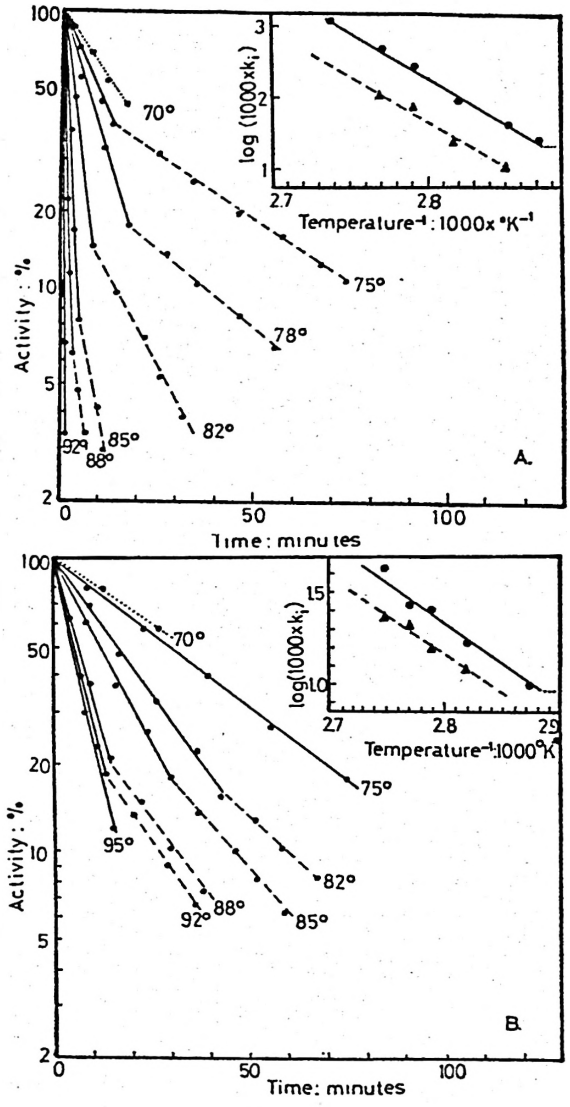

Fig. 4. *Thermal denaturation of DOPA-oxidase. Activation energy for denaturation.*
k_i values were deduced for each of the two zones at each temperature (—— 1st zone; - - - - 2nd zone) and log k_i was plotted against temperature^{-1}. A. Inactive, native enzyme. B. Active enzyme after activation with insoluble trypsin.

Table II. *Thermodynamic characterIstics of the denaturation of DOPA-oxidase.*

Enzyme	Temperature (° C)	Velocity constant k_i ($10^5 \times seg^{-1}$)	Activation energy: E_a (kcal × mol^{-1})	ΔH (kcal × mol^{-1})	ΔG (kcal × mol^{-1})	ΔS (cal × mol^{-1} × °K^{-1})	Q_{10} ($k_{88°}/k_{78°}$)
Inactive	75	450	60.80	60.10	22.04	108	10.5
	88	7.160		60.08	20.69	109	
Actived	75	161	22.00	21.30	22.58	—3.6	3.0
	88	638		21.28	22.45	—3.5	

place. In that range of temperature, the inactivation kinetics were of the first order, but the velocity constants of inactivation did not increase when temperature was rising, but always reamined as $k_i = 0.02$ minutes^{-1} in the range 40-70°C. In contrast, over 75° C denaturation kinetics were also of the first order, but the velocity constant or denaturation, k_i had higher temperatures and different behaviours were obtained by using inactive or active enzymes. From the values of the slopes of the graphs obtained by plotting log k_i against temperature^{-1}, activation energies for the denaturation process were calculated to be 60,800 cal × mol^{-1} for the inactive enzyme and 22,000 cal × mol^{-1} for the active one.

At two determined temperatures, 75° and 88° C, enthalpy (ΔH), free energy (ΔG) and entropy (ΔS) changes were calculated from the expression: $\Delta G = RT \ln \frac{K}{h} - \ln k_i$; $\Delta H = Ea\text{-}RT$; T. $\Delta S = \Delta H\text{-}\Delta G$, in which K = Boltzmann constant; h = Planck constant; T = °K. Temperature coefficient $Q_{10} = K_{88°}/K_{78°}$. In table II, these thermodynamyc characterstics are shown. In figure 4 two different straigh lines at each temperature of denaturation can sometimes be seen. Values of table II refer to the first zone because activation energies were similar in both zones.

It is noteworthy that entropy change had a positive and relatively high value in the case of the inactive enzyme. On the other hand, when the enzyme had previously been activated by trypsin, the entropy change was near 0. Likewise, Q_{10} was higher for the inactivate enzyme than for the active one.

Kinetics data from activated enzyme were not affected if a solution of trypsin was added to the medium during activity measurement. In this way, it was concluded that the addition of trypsin in order to measure residual activities of the inactive enzyme had no effect other than its activatory action.

When the inactive enzyme was heated either for 5 minutes or 60 minutes at temperatures of 37, 45, 50, 60, 70, 75, and 85° C, in neither case was the enzyme activated with this thermal treatment alone. Addition of trypsin solution or the use of the insoluble trypsin column were necesary in all these cases for determination of the residual activities.

Discussion

The possible existence of an *in vivo* control of the activation process of the enzyme is indicated by the fact that dopa-oxidase concentration is the same in the dorsal and pigmented zone as in the ventral and unpigmented zone of frog epidermis. In the literature about plant polyphenolase or dopa-oxidase from melanome, the existence of natural activatory

or inhibitory substances has been described (7, 11, 14), but in frog epidermis, trypsin does not seem to act by eliminating an inhibitor, because it has been impossible to separate this hypothetical inhibitor from the enzyme through the use of techniques, such as centrifugation, differential thermal treatments, differential ammonium sulphate precipitations, dialysis, sieving filtration and chromatographic and electrophoretic operations used in current research on this enzyme. It is more logical to think, that trypsin acts on the enzyme molecule, and can produce a more or less limited proteolysis and a conformational change in the molecule. One of these two actions — or both simultaneously — would be responsible for the activation.

The fact that carboxypeptidase «a», can achieve a certain degree of activation indicates that proteolytic activity of trypsin is limited and that possibly the enzyme is not broken into several fragments. Furthermore, molecular weight is not diminished in an appreciable form after the trypsin actuation. On the contrary, all the existent data seem to show that the activation process is performed simultaneously with the unfolding of the enzyme molecule. Therefore, two main effects are produced: (a) an increment in the enzyme molecule size, as can be deduced from gel filtration; (b) the ordered state of the native molecule loses this order during the unfolding which accompanies the activation. Effectively, thermal denaturation of the inactive native enzyme has a relatively high positive change of entropy, which means that a change from an ordered to a less ordered state has taken place. On the other hand, the same thermal treatments on the active enzyme determine a change of entropy near 0, which indicates that during denaturation of the active enzyme no great change in the spatial order of the molecule has been effected. It can be hypothesized that during the activatory action of trypsin, a positive change of entropy takes place and the enzyme is unfolded as is also shown by a higher Stokes radius.

Our study on banana phenolase (unpublished results) has indicated a similar situation for thermal denaturation; the process takes place only at temperatures above 72° C, and the entropy change is 190 cal $\times$ mol^{-1} $\times$ °K^{-1}. In melanoma tyrosinase (3) one of the forms of the enzyme has at 55°C an entropy change for its denaturation of 136 cal $\times$ mol^{-1} $\times$ °K^{-1}.

It is noteworthy that the velocity constants for thermal inactivation in the case of the inactive enzyme are higher that those of the active enzyme, that is to say, at one predetermined temperature the native form of the enzyme needs less time for its denaturation than the active form, which possibly adquires a less-energized state (more stable) after its activation. The activatory process of the enzyme not only consisted of its unfolding since thermal treatments by themselves did not activate the enzyme but also needed for its conversion some kind of proteolytic action on its molecule.

Resumen

La tripsina activa el enzima DOPA-oxidasa de epidermis de rana, responsable de las primeras etapas de la melanogénesis. Carboxipeptidasa «a» sólo consigue producir un 50 % de la activación. La activación del enzima se pudo conseguir pasándolo a través de una columna de tripsina insoluble ligada a Sepharose. De la comparación del enzima inactivo y activo se deduce que: *a*) el peso molecular aparente y el radio de Stokes del enzima activo son mayores que los del inactivo; *b*) el cambio de entropía para la desnaturalización térmica del enzima activo es 108 cal $\times$ mol^{-1} $\times$ °K^{-1}, mientras que para el enzima activado es sólo —3,6 cal $\times$ mol^{-1} $\times$ °K^{-1}, por todo lo cual se sugiere que el proceso de activación debe consistir en una ruptura tríptica acompañada de un importante desplegamiento espacial de la molécula del enzima, que pasaría a un estado más desordenado.

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