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Protective Effect of Pyridoxal-5-Phosphate on Glutamate Decarboxylase from Lupin Seeds

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The effect of cofactor on enzyme activities of crude extract and purified enzyme from *Lupinus albus* seeds with respect to heat inactivation is studied. Pyridoxal-5-phosphate (PLP) protects the enzyme activity with a maximum of 67×10^{-5} M in the case of crude extract, and 135×10^{-5} M when purified enzyme is used. The incubation with PLP of the enzyme samples increases the enzyme reaction velocities and decreases the energy of activation calculated from the Arrhenius graph. The formation of a enzyme-PLP complex, which is activated when enzyme and cofactor are incubated together is suggested.

Many papers dealing with amino acid metabolism enzymes, and in particular, with amino acid decarboxylases and aminotransferases have referred to the protective effect of pyridoxal-5-phosphate (PLP), a coenzyme in these reactions, on enzyme inactivation by heating or by various inhibitors. MARDASHEV (14) has shown, in experiments with decarboxylases of arginine and lisine, that PLP restores the activity inhibited by cysteamine. NOVOGRODKY and MEISTER (7) have suggested, after their studies on β-aspartic acid decarboxylase, that PLP, besides its action as a coenzyme, could have another effect on PLP-dependent enzymes, contributing to maintain the structural integrity of protein conformation which disappeared in the case of denaturing. SHUKUYA and SCHWERT (8), MALIUS (3), and BLETHEN *et al.* (1), use this protective effect in several steps for purifying PLP-dependent enzymes.

Materials and Methods

Glutamate decarboxylase (L-glutamate-1-carboxy-lyase E.C.4.1.1.15) was purified from *Lupinus albus* seeds using MAYOR'S method (5) with some modifications. The change of pH was carried out only once (this step is repeated twice in the original procedure). Before the ammonium sulphate step, 7×10^{-4} M of PLP were added to supernatant and the mixture was heated for 10 min. at 50°.

Glutamate decarboxylase activity was measured manometrically at 37° in the Warburg device (mod. Braum SG-35). The experimental conditions were: 30 μmoles of sodium glutamate, 0.4 μmoles of

Table I. *Effect of increasing concentration of PLP on heat inactivation of glutamate decarboxylase. Crude extract.*

Crude extract 1/6 w/v in citrate buffer M/15 pH 6.2 was mixed PLP and heated as indicated. The samples were centrifuged and 1 ml portions of supernatant fluids were assayed following the standard conditions: 30 μmoles of sodium glutamate, 0.4 μmoles of PLP and 200 μmoles of sodium phosphate buffer pH 5.7 in a final volume of 2 ml. Activity was measured with Warburg manometric assay, as μliters of CO_2 released in 10 minutes. Protein was determined by the procedure of LOWRY *et al.* (2). The data were expressed as percent of activity.

PLP added $M \times 10^{-5}$	Enzyme activities			Remaining protein			Specific activities		
	50º	55º	60º	50º	55º	60º	50º	55º	60º
--	34	15	7	80	80	80	42	14	9
3.4	--	80	--	--	77	--	--	104	--
6.8	--	83	--	--	77	--	--	108	--
13.5	91	95	73	87	82	84	105	115	87
20.2	--	96	--	--	80	--	--	120	--
27.5	97	95	82	81	84	82	120	113	100
67.5	96	--	86	82	--	84	118	--	102
135.0	97	--	87	86	--	85	113	--	101
270.0	84	--	78	84	--	89	100	--	87
675.0	80	--	58	96	--	95	83	--	61
1.350.0	32	--	18	96	--	96	33	--	19

PLP, and 200 μmoles of sodium phosphate buffer (pH 5.7), in a final volume of 2 ml. Under these conditions, a constant volume of gas is released with respect to time during the first 12-16 minutes. The enzyme activity of the preparations is defined as the number of μliters of CO_2 released in 10 minutes. Protein content was determined by the Folin-Ciocalteu method of LOWRY *et al.* (2).

Results

OPTIMUM CONCENTRATION OF PLP FOR PROTECTIVE EFFECT AGAINST HEATING. Both crude extract and purified enzyme were mixed with PLP to obtain concentrations between 3.4×10^{-5} M and 1.3×10^{-2} M. The mixtures were heated for 8 minutes at the temperatures shown in tables I and II. After cooling in an ice-cold water bath, the samples were centrifuged at 4° and 10,000 × g. The enzyme activities and protein content were measured in the supernatant fluids under the above mentioned conditions. The results in tables I and II (given in percents)

Table II. *Effect of increasing concentrations of PLP on heat inactivation of glutamate decarboxylase. Purified enzyme.*

The conditions were as per the experiments of Table I except that purified enzyme was used, and was heated for 10 minutes at 55°.

PLP added $M \times 10^{-5}$	Enzyme activity	Remaining protein	Specific activity
0	35	99	35
13.5	40	82	49
27.0	52	80	65
67.5	76	88	85
135.0	87	91	96
270.0	93	98	95
675.0	59	92	64
1.350.0	50	99	50

show the variations from the normal value under the standard conditions, for a crude extract or purified enzyme, sample neiher heated nor mixed with PLP. According to these results, the optimum PLP concentration to protect against heat inactivation of the enzyme is about 67×10^{-5} M for crude extract and 135×10^{-5} M for the purified enzyme.

EFFECT OF TEMPERATURE AND TIME ON THE ENZYME ACTIVITY OF THE CRUDE EXTRACT, PROTECTED WITH PLP. Figure 1 shows the percents of initial decarboxylizing enzyme reaction velocities in crude extract heated at 55° for 10, 20, 30, and 40 minutes in the presence or absence of PLP. It is quite clear that the PLP protection in the crude extract markedly decreases the continuous effect of heat inactivation.

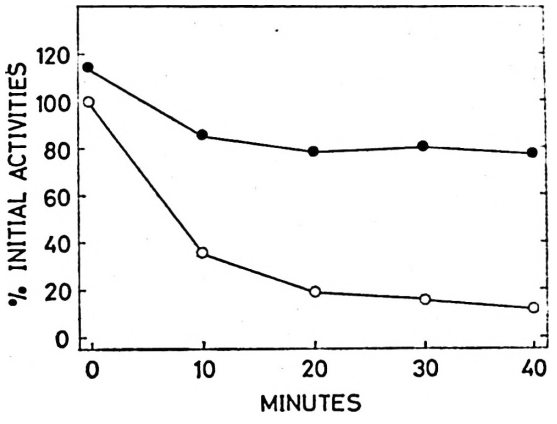

Fig. 1. *Protective effect of PLP on heat inactivation of glutamate decarboxylase activities at 55°.*
Crude extract 1/6 (w/v) in citrate buffer M/15 pH 6.2 added PLP 7×10^{-7} M (●) and without addition (○), were incubated in a constant temperature water bath at 55° C, at the indicated times 2 ml aliquots were removed and carried out to and ice-cold water bath. The samples were centrifuged and 1 ml portions of supernatant fluids were assayed following the standard conditions: 30 μmoles of sodium glutamate, 0.4 μmoles of PLP, and 200 μmoles of sodium phosphate buffer pH 5.7, in a final volume of 2 ml. Activities were measured with Warburg manometric assay as μliters of CO_2 released in 10 minutes at 37° C.

The data in figure 2 indicate the changes in initial enzyme reaction velocities in the extract with and without optimum PLP concentrations, and heated at 30, 40, 50, 60, and 70°, for 8 minutes. After cooling in an ice-cold water bath and centrifuging the initial reaction velocities were tested, using the supernatant fluids under the standard conditions. This figure shows that the maximum temperature protection comes at about 50°; and that, in the interval from 30° to 50°, heating increases the activity up to 140 % with respect to the unprotected, unheated extract.

CHANGES IN THE ENERGY OF ACTIVATION. In the following experiments, we study the effect of temperature on crude extract and the purified enzyme, in one case with PLP added and 10 minutes of heating at 55°, and in the other case without PLP and unheated. In each case the samples were centrifuged and the enzyme activities measured in the supernatant

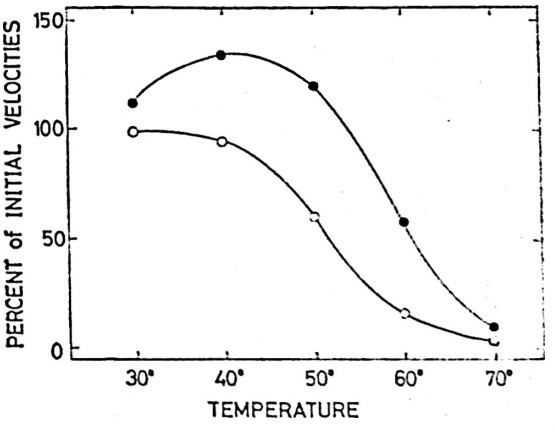

Fig. 2. *Protective effect of PLP on heat inactivation of glutamate decarboxylase activities at different temperatures.*
The same conditions were followed as described in the legend for Figure 1 except that the samples were incubated at the indicated temperature for 10 minutes.

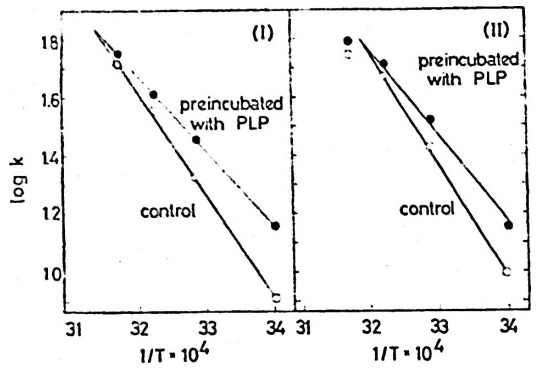

Fig. 3. *Arrhenius energy of activation.* The reaction mixture contained: 30 μmoles of sodium glutamate, 0.4 μmoles of PLP, 200 μmoles of sodium phosphate buffer pH 5.7 and crude extract (I) or purified enzyme (II). Black signs refer to experiments with the samples added with PLP 7×10^{-4} M and heated previosly at 55° C for 10 minutes. Open signs refer to experiments with the samples without any previous treatment.

fluids. The reaction was allowed to take place in the Warburg device after incubation for 15 minutes at 21, 32, 37, 42, 45, and 49°. All mixtures were preincubated at the reaction temperatures in order to achieve thermal equilibrium. The apparent Arrhenius energy of activation was determined from plottings of log K versus 1/T (fig. 3) between 21° and 42° in which interval the graph is linear. The apparent energy of activation determined from this graph was approximately 15.600 cal. per mol in the case of crude extract and 13.000 cal. per mol, when crude extract mixed with PLP and heated at 55° for 10 minutes was used. The apparent energy of activation for the purified enzyme was 16.500 cal. per mol and 14.500 cal. per mol for purified enzyme mixed with PLP and heated for 10 minutes at 55°.

Discussion

The data presented in Table I indicate that there are some levels of PLP concentrations which provide a maximum protective effect against heating: 67×10^{-5} M for crude extract, and 135×10^{-5} M for purified enzyme. This concentration will increase in spite of the fact that the enzyme denaturing decreases progresively, as is shown by the higher values for the remaining proteins; however the drop in enzyme activities may be due to the increase in ionic stregth, specifically in Na^+ ions to which this enzyme is very sensitive and whose inhibitory action cannot be compensated for by increasing of the cofactor (experiments in progress).

Our findings concerning the increase of enzyme activity observed in experiments with crude extract protected with PLP and heated according to figure 2, led us to think that the binding in the enzyme-cofactor complex become stronger when both enzyme and cofactor are incubated at 40°. This complex would be the responsible for the higher values in the initial enzyme reaction velocities. The Arrhenius plot in figure 3 shows the energies of activation. In both cases (crude extract and purified enzyme), lower values were obtained when the samples were incubated with PLP. These results accord with activation in enzyme-cofactor complex formation.

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