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INACTIVE AND ACTIVE ALANINE 2-OXOGLUTARATE AMINE TRANSFERASE IN MAIZE EMBRYO

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Key Word Index—*Zea mays*; Gramineae; maize; germination; alanine degradation; enzyme control.

Abstract—Freshly prepared extracts of maize embryo exhibited a rise of alanine 2-oxoglutarate amine transferase activity when they were treated at temperatures ranging from 35 to 50° before the assay of the enzyme. The same rise of activity was observed when the extracts were incubated at 25° in the presence of alanine. The extracts of maize seeds soaked in the presence of alanine did not show the rise of alanine 2-oxoglutarate amine transferase by heat treatment or incubation with alanine.

INTRODUCTION

The amount of alanine in the proteins of maize endosperm is in excess of that required by the growing embryo. Therefore, a part of alanine is degraded during germination [1, 2]. In previous work [3] we partially purified alanine 2-oxoglutarate amine transferase (AOAT) (EC 2.6.1.2) of maize embryo, which can also use cysteine and serine as substrates. This enzyme seems to be responsible for alanine degradation during the germination of maize, since we were unable to detect alanine dehydrogenase (EC 1.4.1.1) activity in maize, although this common bacterial enzyme has been reported in higher plants [4].

Preliminary work with AOAT (unpublished) suggested the existence of both an active and an inactive (latent) form. The latter became active by moderate heating of the extracts. In this report, we present some results that indicate the activation of the inactive form and relate this with a possible control of the active enzyme level by the amount of alanine to be degraded.

RESULTS

The initial activity of AOAT, measured in the incubation mixture after treatment of crude extracts for 5 min at different temperatures, is represented in Fig. 1 for extracts prepared from maize 'Basto' and from the market. With small quantitative differences, both thermosensitivity curves showed that activity was raised on heating at temperatures in the range of 35–50°. Higher temperature treatments produced a loss of the activity, which disappeared above 60°. The results represented in Fig. 1 were for the initial activity measured by method A (see experimental) but similar results were obtained by measuring the activity with method B. Following this we mostly used extracts from maize of the market, and measured the activity by method A.

As Fig. 2 shows, when the transaminase activity was measured for longer than 6 min, both the preheated and not preheated preparations of the same extract showed the same high activity, which was constant during the time of measurement for the preheated preparation. The un-preheated preparation reached full activity after 8 to 10 min. The time taken to increase to full activity with the un-preheated extracts depended on the amount of extract added to the incubation mixture, being shorter for the higher amount of extract in the range that could be measured before exhausting the NADH in the reaction mixture. The time lag involved in increasing to full activity in the un-preheated extracts was not due to a limited amount of the lactate dehydrogenese (LDH). This activity was measured in separate experiments, Doubling the amount of LDH did not affect the time lag.

For the two forms of AOAT we postulated that the active form was responsible for the initial activity measured with the untreated extract, and that the inactive form accounted for the additional activity

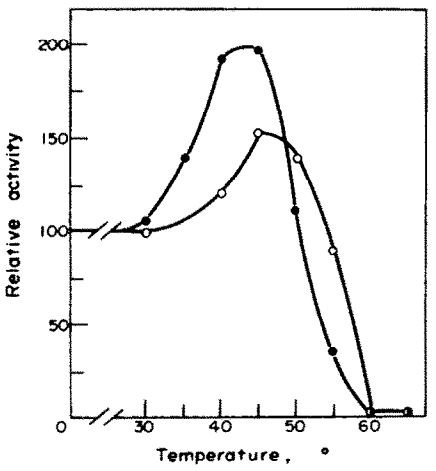

Fig. 1. Alanine 2-oxoglutarate amine transferase activity after heat treatment of extracts of maize 'Basto' (—○—) and that of the market (—●—) at different temperatures. 1 ml fractions of extracts prepared from seeds soaked in water for 3 days, were treated for 5 min at the indicated temperatures. After quick cooling and centrifugation at 5000 *g* for 10 min, activities were measured by method A.

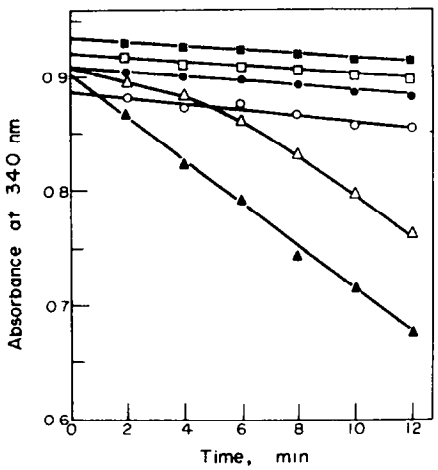

Fig. 2. Absorbance decrease with time due to alanine 2-oxoglutarate amine transferase activity. Incubations for the estimation of the activity by method A were made with 0.1 ml of freshly prepared extracts (—△—) or freshly prepared extracts heated for 5 min at 45° (—▲—), from seeds of maize (market) soaked in water for 3 days. Controls: (—○—) fresh extract without alanine, (—□—) fresh extract without lactate dehydrogenase. (—●—) heated extract without alanine, (—■—) heated extract without lactate dehydrogenase.

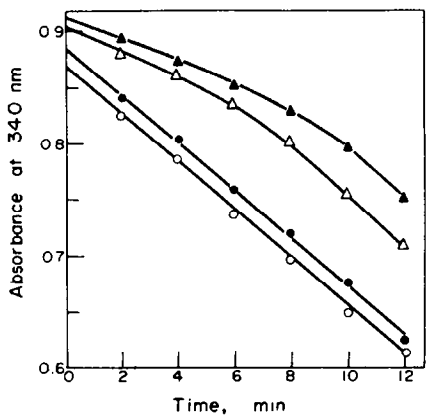

Fig. 3. Absorbance decrease with time due to alanine 2-oxoglutarate amine transferase activity. The incubation mixture for the assay of the activity by method A was preincubated without: 2-oxoglutarate (—○—), NADH (—●—), alanine (—△—), or extract (—▲—), for 15 min before 0 time, when this component was added.

detected after preheating of the extracts for 5 min at 45° or after prolonged incubation of the mixture for the assay of the activity. The preheated enzyme preparations had the same kinetic properties as the un-preheated preparations [3].

Incubation of the extracts for times ranging from 5 to 15 min at 25° did not produce a detectable rise in the initial activity, so it would seem that although the increase in activity is the same for 5 min of heating at 45° as by incubation with the mixture for the estimation of the the activity, the mechanism of raising the activity may be different. It seems that for increasing the activity during measurement, some chemicals of the incubation mixture or of the products of the reaction would be needed. When the activity was measured, after 15 min of preincubation of all the mixture without one of the components, at 25° it was noted that only when alanine is absent in the preincubation mixture, is low initial activity obtained and this rises with time. Fig. 3 shows these findings for the preincubation without 2-oxoglutarate, NADH, alanine or extract. Other assays not indicated in Fig. 3 were preincubation without LDH or pyridoxal phosphate, both of which showed no time lag.

Testing these results *in vivo* in order to determine their significance, we studied the effect of exogenous alanine on the level of the transaminase in maize. Unpublished works with maize 'Basto', showed that alanine added to the water used for soaking the seeds, raised the levels of AOAT. The results with market maize, in terms of specific enzyme activity, are shown for the first 4 days of soaking in Fig. 4. From the first to the third day of soaking in water, a continuous rise of both active and total (active plus inactive) enzyme appears in embroys of maize seeds. During this 3-day period the ratio of active to total enzyme increased from 0 to 0.56. This ratio was 1 on the fourth day, immediately before germination, when, there was a diminution of the total enzyme.

When 10 mM alanine was added to the soaking water, the inactive form of the enzyme was not detected and the active form was still slightly higher than the total enzyme in embryos of seeds soaked in water.

In one attempt to separate active and inactive forms of the enzyme, extracts of maize soaked for 3 days in water were filtered on a column of Sephadex G-100, and fractions collected to assay the activity of AOAT immediately, or after 5 min of treatment of the fractions at 45°. In current experiments, both the active form of the enzyme and the supposed precursor of the additional activity obtained after heat treatment, elute together. This indicates a similar MW (*ca* 100000). In spite of the rise of activity after heat treatment of the fractions the activity measured in fresh fractions after column treatment rose little or undetectably during the time of reaction, indicating an almost complete loss of some factor presumably required for the increase of activity detected at 25°.

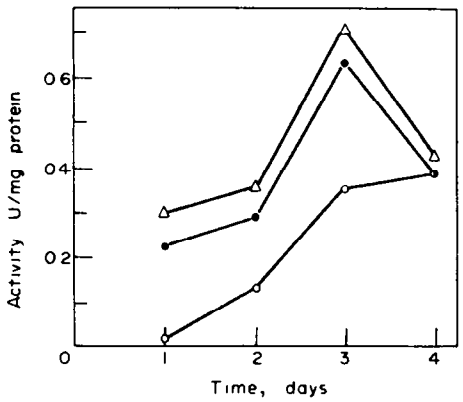

Fig. 4. Effect of alanine on the levels of alanine 2-oxoglutarate amine transferase of maize embryo. Activity was measured by method A in extracts from seeds (market) soaked for the indicated times. Initial activities (—○—), and maximal activities at the end of the incubation (—●—) of the extracts soaked in water. Initial or maximal activities (—△—) of seeds soaked in water with 10 mM alanine.

DISCUSSION

During the germination of seeds specific metabolic processes take place which are different from those of the adult plant. The germinating seed is, consequently, increasingly used in the study of enzyme controls [5–9].

It seems obvious that the degradation of alanine during the germination of maize is catalyzed by the enzyme AOAT, which must perform this function of alanine degradation only during the first stages of germination. In this regard the kinetic properties of the enzyme [3] clearly favoured its operation in the sense of converting alanine to pyruvate. Therefore, one sees the possibility of a control of the level of the enzyme by the amount of available alanine, which would determine the amount of active enzyme according to the excess of substrate to be degraded.

The results of increase of activity shown in Figs 1 and 2 cannot be attributed to the scarcity of LDH of the assay mixture, since this was always in excess. Furthermore, the temperature of the assay mixture was constant and did not change more than 2° during measurement. It seems clear that both results are indicative of a genuine increase of the activity AOAT by either of the two treatments, and due also to the absence of an alanine effect on the coupling enzyme or on other activities of the extracts or commercial LDH, and by the fact that dialyzed extracts also show the effects of increase of activity.

The results shown in Fig. 3 also emphasize the existence of a specific process of increase of the activity, stimulated by alanine.

The fact that there is a greater amount of the active enzyme in seeds soaked in the presence of alanine, also indicates a positive control of the level of AOAT by alanine. This control must operate principally by favouring the active form of the enzyme, because in seeds soaked with alanine there is, apparently, no inactive form of the enzyme. Since during the first day of soaking in water there was inactive but no active form of the enzyme, the results indicate a conversion of an essentially inactive protein into an active enzyme by alanine, and not of the transition of a less to a more active enzyme. The quick rise of both active and total enzyme after alanine addition (Fig. 4) agrees with the almost parallel increases of AOAT and the reported increases of free alanine during germination of maize [10], both being substantial at the second day.

The results of gel filtration and the fact that the initially active enzyme has the same kinetic properties as the full active preparations, suggest that there is only one active form of the enzyme with the same MW as the inactive proenzyme. The mechanism of the conversion of the inactive into the active enzyme is not yet known. The results of gel filtration suggest the existence of a cofactor for the more physiological and alanine activated transition at 25°. The possibility of the participation of inhibitory substances whose elimination would be responsible for the transition of the inactive into the the active enzyme cannot be ruled out.

Enzyme level regulation by transitions between active and inactive forms is, of course, a well-known mechanism [11–13]. In the case of AOAT it seems an appropriate mechanism for the control of alanine degradation in the embryo, on order to utilize the nitrogen of alanine in excess.

EXPERIMENTAL

Maize (*Zea mays*) strain 'Basto' (obtained from the Spanish Agric. Ministry) or bought on the market, was soaked in H_2O at 25°, in sterile conditions, in Petri dishes. In some of the cases indicated below, the H_2O contained 10 mM alanine pH 7. At different times of soaking, embryos of some 40 seeds were removed by cutting and ground in a mortar. Lipids were extracted from the paste with 10 vol. of *n*-hexane for 15 min, the paste was then suspended in 3 vol. of extraction buffer containing: 50 mM KPi, 5 mM mercaptoethanol and 1 mM EDTA-Na_2 pH 7. The suspension was sonicated for 1 min at amplitude 8 and centrifuged at 10000 *g* for 30 min; the supernatant being the crude extract. All these operations were carried out in a cold room. The activity AOAT was measured at 25° by decrease in *A* 340 nm, by coupling with NADH oxidation, following two methods. In method A, the transaminase reaction between alanine and 2-oxogluratate was coupled with lactate dehydrogenase (EC 1.1.1.27) in order to measure the production of pyruvate by its reduction with NADH, in a system containing a final vol. of 3 ml (in μmol): Tris–HCl pH 8 (150), alanine (40), potassium 2-oxoglutarate pH 8 (40), NADH (0.4), pyridoxal phosphate (0.2), 5 units of lactate dehydrogenase and appropriate amounts of transaminase preparations. In method B, the transaminase reaction between pyruvate and glutamate was coupled with glutamate dehydrogenase (EC 1.4.1.4) to measure the production of 2-oxoglutarate by its reduction with NADH in a system containing a final vol. of 3 ml (in μmol): Tris–HCl pH 8 (150), $(NH_4)_2SO_4$ (150), potassium pyruvate pH 8 (40), potassium glutamate pH 8 (40), NADH (0.4), pyridoxal phosphate (0.2), 5 units of glutamate dehydrogenase and appropriate amounts of transaminase preparations. The controls without the amino acids or the auxiliary enzymes showed similar low activities, which were subtracted in both methods. The incubation mixtures, without the 2-oxoacids did not show detectable activity. Before the addition of the last component of the reaction mixture (usually 0.1 to 0.2 ml of transaminase preparations) the mixture was preheated at 25° for 5 min. Sephadex G-100 filtrations were made in the cold room in columns (1.7 × 17 cm) previously equilibrated with extraction medium. Protein was measured according to ref. [14]. A unit of enzyme activity is defined as the amount that transforms one μmol of substrate in one min. Sp. act. refers to mg of protein. The auxiliary enzymes and coenzymes used were from Sigma.

REFERENCES

1. Dupuy, J., Boutin, J and Dupaigne, G. (1971) *Compt. Rend.* **272**, 2548.
2. Sodek, L. and Wilson, C. M. (1973) *Biochim. Biophys. Acta* **304**, 353.
3. Martin, M., Garcia de la Banda, I. and Sabater, B. (1976) *Rev. Espan. Fisiol.* **32**, 131.
4. Bryan, J. K. (1976) *Plant Biochemistry* (Bonner, J. and Varner, J. E. eds) 3rd ed., 525–560. Academic Press, New York.
5. Oaks, A. (1965) *Plant Physiol.* **40**, 149.
6. Kadam, S. S., Singh, J. and Mehta, S. L. (1973) *Phytochemistry* **12**, 1221.
7. Kanamori, T. and Matsumoto, H. (1974) *Plant Sci. Letters* **3**, 431.
8. Matthews, B. F., Gurman, W. and Bryan, J. K. (1975) *Plant Physiol.* **55**, 991.
9. Deng, Q.-I. and Ives, D. H. (1976) *Biochim. Biophys. Acta* **377**, 84.
10. Margaris, N. S. and Thanos, C. A. (1974) *Phytochemistry* **13**, 1645.
11. Holzer, H. and Duntze, W. (1971) *Ann. Rev. Biochem.* **40**, 345.
12. Sabater, B. (1973) *FEBS Letters* **29**, 63.
13. Fischer, M. and Schwartz, D. (1974) *Biochim. Biophys. Acta* **364**, 200.
14. Lowry O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.