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THE CHARACTERISTICS OF ASPARTATE TRANSAMINASE ENZYME IN TOBACCO TISSUE CULTURES

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Abstract

The optimal temperature of the activity of the aspartate transaminase enzyme obtained from tobacco tissue cultures is 53°C at 8.5 pH. The activation entalpy of the reaction is 33.5 KJ, Km = $1.15.10^{-5}$M on aspartate. The reaction does not require pyridoxal phosphate. Ca^{2+}, Sr^{2+}, Ba^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Pb^{2+}, Mn^{2+} do not influence the enzyme activity.

Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{+}, semicarbazide hydroxylamine, parachlor-mercurybenzoate inhibit the reaction. There is correlation between the inhibitory effect and the electron structure of the metal ions. Aspartate transcminase activity is measurable in every culture, while in the case TRP substrate transamination is only detectable in the so-called habituated cultures.

Key words: Aspartate transaminase, tryptophan-transaminase, tobacco callus, ion effect, enzyme inhibition, *Nicotiana tabacum* cv. Xanthi.

Abbreviations: ASP = aspartate, DNFH = dinitrophenylhydrazine, GLU = glutamic acid, GOT = glutamic acid-oxaloacetic acid-transaminase, IAA = -indole-3-acetic acid, PCMB = parachlor-mercurybenzoate, TRP=tryptophan.

Introduction

The aspartate transaminase (SMITH and WILLIAM, 1951) is one of the mostly studied enzymes among the plant-transaminases. It participates in the metabolism of amino acids (FOWDEN, 1967), takes part in the pathway of the glyoxylic acid (TOLLBERT and YAMAZAKI, 1969), the C_4-reaction pathway, and the intercellular transport of the metabolites (HATCH, 1971), It catalyzes the transformation of tryptophan in to indole-3-pyruvic acid from which indole-3-acetic acid is formed (GORDON, 1961; LARSEN, 1967; SCHNEIDER et al., 1972; HERKLOSS and LIBBERT, 1976). Certain metal ions influence the activity of the enzyme (HAPPOLD and TURNER, 1957; NADKARNI and KAMALA, 1962; PATWARDHAN, 1960; VERJEE and EVERED, 1969). Latter authors studied the influence of metal ions on the enzyme in different plants. The obtained results are contradictory in many cases. In our opinion the contradictions cannot be fully explained by the variations in species. Examination of certain substances and physiological processes in plant segments is very complicated due to the differentiation of the cells, since the tissues of various structures may react differently from the meristematic cells. Morphologically and physiologically the cell- and tissue cultures are homogeneous systems. Their cells are dedifferentiated. The keeping under sterile conditions of the tissue cultures is also easier and more controllable, therefore, it seemed reasonable to study a few basic characteristics of this important enzyme in tobacco callus culture. Since the role of the enzyme in the auxin synthesis also belonged to our range of interest, in one series habituated callus cultures capable of auxin synthesis were used, and normal callus cultures not growing without auxin were used in the other experiments. As the enzyme transforms aspartate with a rate of nearly two ordes

higher than it does in the case of tryptophan (FOREST and WIGHTMAN, 1972) it seemed reasonable to study certain features of the aspartate transaminase, enzyme.

Materials and methods

Cultivation of tobacco callus

Nicotiana tabacum cv. Xanthi pith parenchyma callus cultures were used in our experiments, grown on Murashige-Skoog media (MURASHIGE and SKOOG, 1962, SKOOG and LINSMAIER, 1965). The habituated cultures were grown auxin-free media, while the heterotrophic calluses were cultivated on media containing 3 mg/l IAA and 0.1 mg/l 2.4-D. The media contained 0.04 mg/l kinetin in every case. The cultures were grown in Conviron growth chambers at 25°C under light of 1.23 mWatt/cm².

Extraction of transaminase enzyme

The 3 weeks old tobacco calluses were homogenized in mortar at 0—5 °C in 0.05 M Tris-HCl buffer (MATHERON and MOORE, 1973). The EDTA concentration of the buffer was 0.01 M, that of mercaptoethanol was 0.001 M. The fresh weight and buffer ratio of the callus was 1:3. The homogenate was centrifuged in a cooling centrifuge for 30 min. at 1000 g and 5 °C. The supernatant was saturated with ammonium sulfate up to 35%. After standing overnight centrifugation according to the above described procedure was carried out once more. This time the supernatant was saturated with ammonium sulfate up to 80%. After standing overnight and a repeated centrifugation the precipitate was resolved in 0.05 M Tris-HCl buffer (pH8.5) it was dialysed against 0.05 M Tris-HCl buffer in a refrigerator (5 °C) for one day. The protein extract was centrifuged as described above; concentrated with Aquacide II, centrifuged again and the supernatant was used for our studies.

Measuring of aspartate transaminase enzyme activity

The determination of enzyme activity were carried out with the method of REITMAN and FRANKEL cf. Sós (1974). The composition of GOT substrate was 1 mM α-ketoglutaric acid + 0.2 M L-aspartic acid in 0.05 M Tris-HCl buffer (pH 8.5). One ml of GOT substrate was preincubated at 37 °C for 5 min, then mixed with 200 µl protein extract (1.7 mg/ml protein). After 20 min. 1 ml of dinitrophenylhydrazine after further 20 min. 10 ml of NaOH was added. The absorption of the solution was measured with spectrophotometer at 500 nm after 5 min. Instead of the enzyme, a mixture containing 0.2 ml H_2O was used as comparative solution.

Measuring of tryptophan transaminase enzyme activity

Applying the method of LIU (1978) several disturbing circumstances occurred (GAÁL and KÖVES, 1981) during the course of the activity measurements by spectrophotometry, therefore the TRP transaminase activity was followed by the quantitative measuring of GLU end-product. The incubation mixture was composed of 0.01 M EDTA, 0.03 M TRP, 0.0002 M pyridoxal phosphate, 0.855 g enzyme protein in 10 ml borate buffer (pH 8.5). The mixture was taken into two halves and icubated at 45 °C for 15 min. Then 0.5 ml buffer was added to one of the mixtures, and 0.5 ml 0.06 Mμ-ketoglutaric acid-containing buffer to other. After 1 h the reaction was stopped with 3 drops of cc. HCl solved in 2 ml of ethanol. The solution was saturated at low temperature, centrifuged and the supernatant was evaporated in a vacuum, then dissolved in 1 ml buffer. GLU was determinded with the help of an AAA 881 Mikrotechna (product of Czehoslovakia) automatic amino acid analyser. Na-citrate (pH 3.25; 4.25; 5.28) was used as buffer. The samples were evaporated to dryness and dissolved in 1 ml buffer.

Determination of protein

Protein was determined by the method of LOWRY et al. (1951).

Results

pH-DEPENDENCY OF THE ENZYME ACTIVITY

The activity of the enzyme was measured between pH 6—11. The changes in enzyme acitivity in the function of pH are described by a maximum curve. The point of the maximum is at pH 8.5 (Fig. 3).

DEPENDENCE OF ENZYME ACITIVITY ON THE TEMPERATURE

The temperature-dependency of the aspartate transaminase enzyme acitivity was studied between 19—62 °C. The activity-maximum was at 53 °C (Fig. 1). Though activity was highest at this temperature, the rate of the reaction was only constant for 30 min. Therefore, the measuring were made at 37 °C, where the rate of substrate-transformation was lower, however, it showed no changes for several hours. The activation entalpy of the reaction was 35.5 KJ, obtained by the graphic presentation of the linear form of the Arrhenius equation (Fig.2).

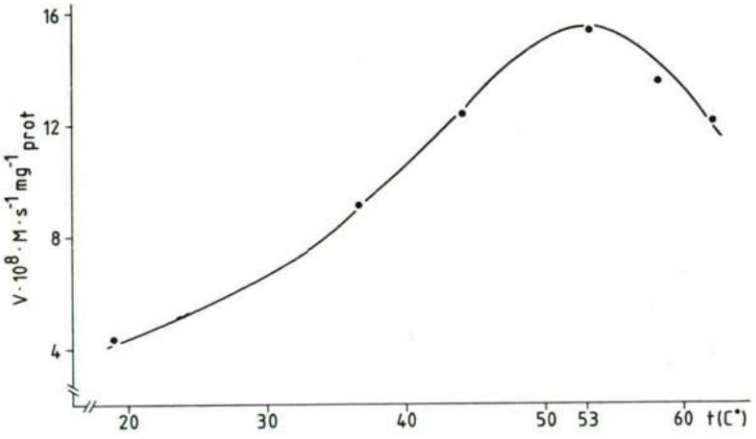

Fig. 1. Temperature-dependency of the transamination reaction. Ordinate: enzyme activity. Abscissa: temperature. Composition of the reaction mixture is described in „Materials and Methods"

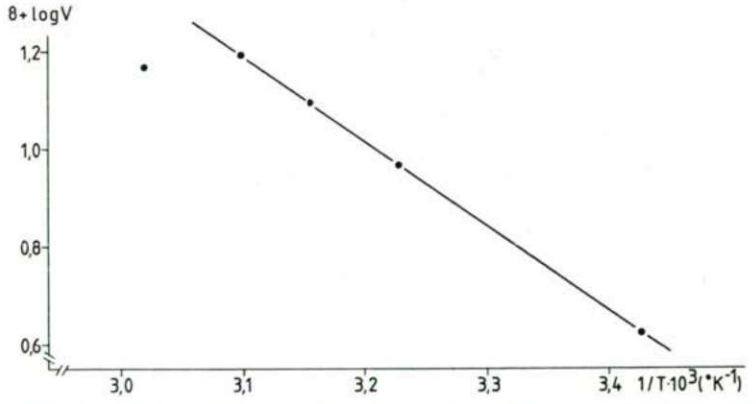

Fig. 2. Determination of the activation entalpy from the linear form of the Arrhenius equation. The ordinate shows the acitivity, which is: M $s^{-1}mg^{-1}$ protein.

ASP AND TRP TRANSAMINASE ENZYME ACITIVITIES

The acitivity of aspartate transaminase at 37 °C and pH 8.5 was $9.10^{-8}M.s^{-1}$ mg^{-1} protein, while that of TRP transaminase enzyme was $0.58.10^{-8}M.s^{-1}.mg^{-1}$ protein. According to our measurings the activity of the enzyme obtained from the tobacco callus was not stimulated by pyridoxal phosphate (Table 3).

Table 1. Changes in the specific acitivity of the aspartate transaminase enzyme and the rate of inhibition in presence of various metal ions.

	Initial concentration of ion (μM)	Concentration of the formed pyruvate 10^{-4}M	Inhibition %
control	0·00	3.83	0.00
Hg^{2+}	1.66	3.83	0.00
	12.50	3.22	26.00
	50.00	1.98	49.00
	100.00	1.66	57.00
	200.00	0.78	80.00
Cd^{2+}	1.66	3.83	0.00
	12.50	2.88	25.00
	50.00	1.74	55.00
	100.00	1.05	73.00
	200.00	0.52	87.00
Zn^{2+}	1.66	3.57	7.00
	12.50	3.57	7.00
	50.00	2.61	32.00
	100.00	2.18	44.00
	200.00	1.39	64.00
Cu^{2+}	1.66	3.66	5.00
	12.50	3.66	5.00
	50.00	3.50	7.00
	100.00	2.87	26.00
	200.00	2.26	31.00
	400.00	0.87	88.00
control	0.00	4.35	0.00
Ag^{+}	1.66	4.01	6.00
	3.13	3.92	11.00
	6.25	3.66	17.00
	12.50	2.45	44.00
	25.00	2.17	51.00

The composition of the reaction mixture is described in "Materials and Methods".

KINETICS OF THE TRANSAMINASE REACTION

The linear form of the Lineveawer-Burk function was used for the determination of the kinetic parameters (Fig. 4). In the case of aspartic acid substrate a value of $K_m = 1.11.10^{-5}$M; in the case of α-ketoglutaric acid $K_m = 5.8.10^{-5}$M value was received.

INFLUENCE OF CATIONS ON THE ASPARTATE TRANSAMINASE ENZYME ACTIVITY

For studies on the ion effect the 1 ml GOT substrate was preincubated with 0.5 ml solution containing various ions, and then 0.2 ml enzyme solution was added. The

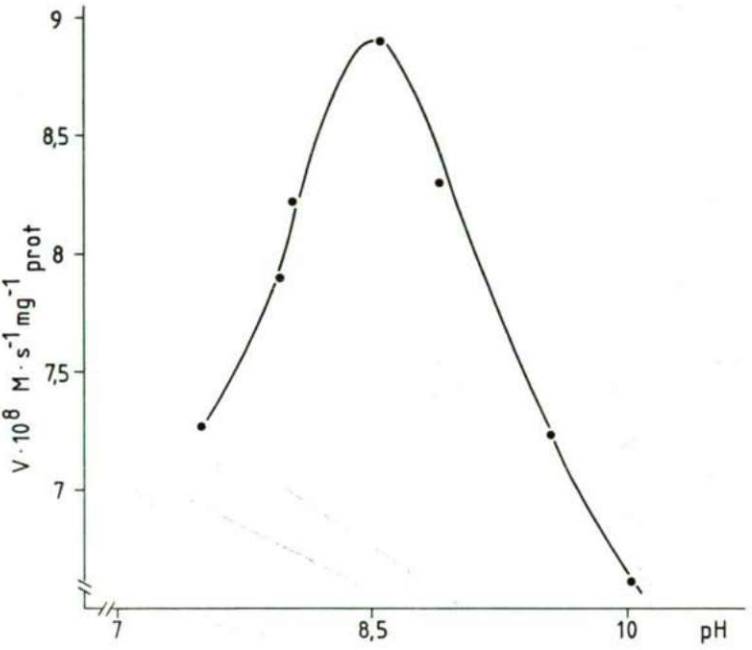

Fig. 3. pH-decencency of the transamination reaction.

Table 2. Influence of PCMB, hydroxylamine and semicarbazide on the aspartate transaminase enzyme activity.

PCMB μM	Spec. act. 10^{-1}M s^{-1} mg^{-1} protein	Hydroxylamine and semicarbazide μM	Spec. activity	
			hydroxylamine	semicarbazide
			μM $s^{-1}mg^{-1}$ protein	
0.0	8.6	0.0	8.2	8.2
2.0	8.2	100.0	6.4	5.1
4.0	7.8	200.0	5.7	3.6
6.0	2.7	300.0	5.3	2.3
8.0	1.6	400.0	5.0	1.3

The composition of the reaction mixture is described in „Materials and Methods"

reaction was followed for 30 min. The effect of 13 ions was studied in the experiments: Ca^{2+}, Sr^{2+}, Ba^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{+} (Fig. 5).

From the listed ions the last five decreased the rate of aspartic acid transamination. Table 1 shows the initial concentrations of the ions, with the inhibition pertaining to them.

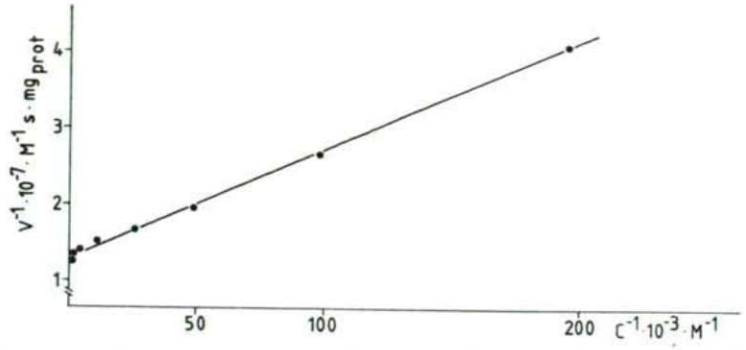

Fig. 4. Changes in activity according to Lineveawer-Burk. The reciprocity of the activity is plotted against the reciprocity of the aspartic acid initial concentrations.

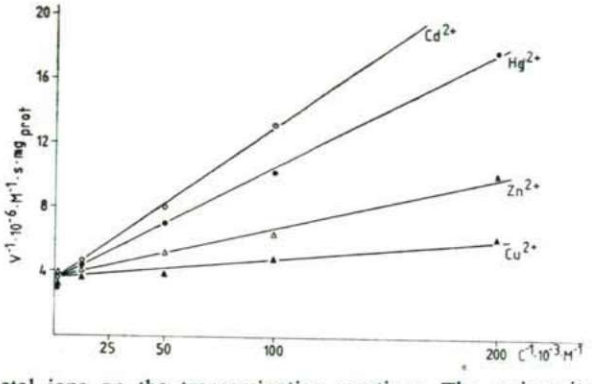

Fig. 5. Effects of metal ions on the transamination reactions. The reciprocity of the activity is plotted against the initial concentrations of metal ions.

Table 3. The influence of pyridoxal phosphate with different initial concentrations on aspartate transaminase acitivity.

Pyridoxal phosphate μM	Absorption
0.00	0.161
4.00	0.140
6.25	0.150
12.50	1.160

Discussion

It can be seen from the results that the rate of TRP transamination is lower by two orders than that of aspartate transformation. This is in accordance with the expectations since through the transamination of TRP IAA develops with low intensity (SCHNEIDER et al., 1972) both in the tissues and in vitro. The TRP transaminase enzyme activity could not be measured in the auxin heterotrophic culture, which is in coordination with the rather low IAA-level if this culture, also known by us (KÖVES et al., 1981). On the contrary, the aspartate transaminase enzyme was observed to be equally active in both cultures.

Our earlier gas-chromatographic determinations indicate that the auxin heterotrophic callus also contains IAA — although in lower amount — as does the autotrophic (habituated) culture (KÖVES et al., 1981). Therefore, it is assumed that in the heterotrophic culture IAA is not formed by indolepyruvic acid pathway. The possible alternatives are: the synthesis starting with TRP-decarboxylase or the forming of IAA in a non-enzymatic way. LIU et al. (1978), however, did not find TRP decarboxylase activity in Nicotiana calluses, and could not demonstrate tryptamine as an intermedier. Others, e. g. PHELPS and SEQUERIRA (1968) demonstrated IAA-synthesis throug h tryptamine in the cell-free extract of tobacco terminal buds; and SIMTH (1977) found tryptamine in Nicotiana leaves. According to SATOH and ESHASHI (1982) TRP does not only transform into IAA on the effect of a decarboxylase in cocklebour seeds, but doing so it also increases the production of ethylene.

LIU et al. (1978) measured the TRP transaminase activity in tumorous and nontumorous calluses, and experienced that in the tumorous tissue culture pyridoxal phosphate did not influence the enzyme activity, while the enzyme from the mutants showed pyridoxal phosphate-dependency. The enzyme activity of the nontumorous tobacco calluses studied by us did not increase in the presence of pyridoxal phosphate even when the enzyme extract was previously filtered on Sephadex G-25.

According to FOWDEN (1965), as well as MATHERON and MOORE (1973) semicarbazide and hydroxylamine react with the aldehyde groups, and inhibit the pyridoxal phosphate-dependent reactions, while the PCMB is a sulfhydryl reagent. According to our measurings these compounds also inhibit enzyme activity in the presence of pyridoxal phosphate (Table 2). Since there is also inhibition despite the co-enzyme bound to the apo-enzyme, it is our assumption that the compounds in question effect another active centre of the enzyme.

From the 13 ions studied by us only the Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, and Ag^{+} influenced the enzyme activity and acted as inhibitors. These ions are sulfhydryl reagents, therefore it is probable that they exert their inhibitory effect by blocking the SH-group of the enzyme. Their common characteristic is that on their external electron shell the electron arrangement is $s^2p^6d^{10}$, form which the Cu^{2+} froms and exception, nevertheless, it also fulfils this condition in a reduced state.

The electron arrangement on the external shell of the ions, the diameters of ions of sheath of solvents the compactness of the ions largely influence the interrelationship with the active centers of the enzyme. The complete or partial conformity of these features may produce similar effect on the enzyme activity. Taking them into consideration, the explanation of the contradictions found in the literature, as well as the proposal of experiments may both become easier.

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