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PARTIAL PURIFICATION AND INITIAL STUDIES OF THE TOMATO L-ALANINE:2-OXOGLUTARATE AMINOTRANSFERASE

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SUMMARY

1. The tomato fruit contains two alanine 2-oxoglutarate aminotransferase enzymes (EC 2.6.1.2). The minor one can be extracted by Triton X-100. The major enzyme is soluble. No significant changes are observed in the specific activities of these enzymes during tomato ripening.

2. The soluble enzyme has been purified 660-fold by a procedure involving $(NH_4)_2SO_4$ precipitation, DEAE-cellulose, DEAE-Sephadex and gel filtration on Sephadex G-200. During the purification procedure the enzyme needs protective substances, i.e. glycerol, mercaptoethanol and alanine. The highest specific activity obtained was 120 units/mg of protein.

3. The enzyme reversibly catalyzes the transamination reaction between alanine and 2-oxoglutarate according to the Ping Pong Bi Bi mechanism. The Michaelis constant with respect to alanine is 2.8 mM, to 2-oxoglutarate 0.28 mM, to pyruvate 0.09 mM and to glutamate 2.3 mM.

INTRODUCTION

The properties of aminotransferases in plant tissue have not been characterized extensively because of the presence of inhibiting and denaturing factors (organic acids, phenolic compounds) [1]. It is nevertheless of importance because aminotransferases may play an essential role in fruit ripening [2–5]. Yu et al. [6, 7] have demonstrated the existence of leucine and alanine aminotransferases in tomato.

This article presents a purification method for the L-alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2) from the tomato fruit, and gives a preliminary report of some properties of the partially purified enzyme.

MATERIALS AND METHODS

Tomatoes (*Lycopersicon esculentum*, Heinz 1370 variety) were grown in an open field at the experimental station Puyricard-13 France. The ripening stages have been described by Besford and Hobson [8] and were as follows: Large Green, Mature Green, Green Orange, and Red.

Lactate dehydrogenase was purchased from Sigma (Type 340-10), Glutamate dehydrogenase from Boehingrer, NADH from Calbiochem, DEAE-cellulose from Bio Rad Laboratories, DEAE-Sephadex and Sephadex G-200 from Pharmacia Fine Chemicals and Polyclar AT from General Aniline. Substrates and other chemicals were of analytical reagent grade and purchased from Merck or Fluka.

The glass-ware was treated with 50% HNO_3 to remove any remaining trace of detergent. Only deionized redistilled water was used.

Extraction procedure

Tomatoes were homogenized in 0.2 M potassium phosphate buffer, pH 7.5, containing 10 mM mercaptoethanol, 5 mM EDTA, 20% (v/v) glycerol and 2% (w/v) Polyclar AT. (1 part tissue, 1 part buffer). The homogenate was rapidly brought to pH 7.5 using 1 M KOH and centrifuged at 3500 $\times$ *g* for 20 min. The supernatant was made 10 mM in alanine and cysteine then centrifuged for 30 min at 20 000 $\times$ *g* and used for enzyme purification. In an experiment the pellet which contained the minor enzyme was resuspended in 50 mM phosphate buffer, pH 7.5, containing 10 mM mercaptoethanol, 10 mM alanine, 10 mM cysteine, 20% glycerol. After stirring for 30 min in the presence of 0.5% Triton X-100, the mixture was centrifuged for 30 min at 20 000 $\times$ *g* and the supernatant was analyzed for enzymatic activity and protein.

Enzyme assays

The alanine aminotransferase was assayed by following the pyruvate formation by the rate of NADH oxidation catalyzed by lactate dehydrogenase [9]. In the same way, the production of the 2-oxoglutarate was followed by the rate of NADH oxidation coupled with glutamate dehydrogenase [10, 11]. Each test, in a final volume of 3 ml, contained 150 μmoles Tris–HCl, pH 7.25 at 37 °C, 0.3 μmoles NADH, 50 μl lactate dehydrogenase or 5 μl glutamate dehydrogenase, 150 μmoles L-alanine or 60 μmoles L-glutamate, 15 μmoles NH_4Cl in the test of the reverse reaction and the enzyme extract. After a 10-min incubation at 37 °C, the reaction was started by the addition of 15 μmoles of 2-oxoglutarate or 3 μmoles of pyruvate and followed during 5 min at 340 nm with an Unicam SP 800 spectrophotometer. One enzyme unit is defined as the amount required to transform 1 μmole of substrate per min under the described conditions. Unless otherwise specified, the units refer to pyruvate formation. The specific activity is the number of units per mg of protein.

The amino acid specificity was assayed (a) by a coupled assay system involving malic dehydrogenase and NADH in the case of aspartate, (b) by determining the 2,4-dinitrophenylhydrazone formation in the case of leucine [12], (c) by the alkali-catalyzed conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde for tyrosine [13]. Glycine, threonine, methionine, arginine, phenylalanine and tryptophan were assayed by the glutamate formation [14] in comparison to the alanine substrate.

The 2-oxoacid specificity was assayed by pyruvate formation for glyoxylate and 2-oxo-3-methylvalerate. In the case of glyoxylate, the interference with lactic dehydrogenase was prevented by the presence of Tris buffer which forms a Schiff's base with glyoxylate [15].

Other methods

Proteins were precipitated with trichloroacetic acid [16] and quantitatively

analyzed according to the method of Lowry et al. [17] using bovin serum albumin as a standard.

The enzymatic solutions were concentrated by ultra-filtration in an Amicon cell equipped with a Diaflo membrane PM 30.

The apparent molecular weight was determined by gel chromatography according the method of Laurent and Killander [18]. A column of Sephadex G-200 (1.5 cm × 90 cm) was equilibrated with a 50 mM Tris–HCl, pH 7, containing 10 mM mercaptoethanol, 0.01 mM pyridoxal phosphate and 5% glycerol, and calibrated with human γ-globulin, bovin serum albumin, ovalbumin and α-chymotrypsin.

The p*I* was determined by electrofocusing [19] on a 110-ml LKB 8100 column, with pH 3–10 Ampholines LKB.

Acrylamide–agarose gel electrophoresis [20] was carried out with Tris–borate buffer, pH 9.2 (ref. 21). A current of 10 V/cm was applied for 3 h at 4 °C. Proteins were stained with Coomassie blue [20] and the enzymatic activity was revelated according to the method of Boyde and Latner [22].

RESULTS

Change in the alanine aminotransferase activity throughout the ripening process

Total aminotransferase activity and total protein content were estimated at several stages of the ripening process. It appears that some activity (minor enzyme) is localized in the sediment and requires Triton X-100 for solubilization. The total enzymatic activity as well as the protein content decreases during ripening, so that changes in the specific activities are not significant (Fig. 1). Furthermore the minor enzyme was found to have an almost constant activity during tomato development, corresponding to 20–25% of the activity in the supernatant.

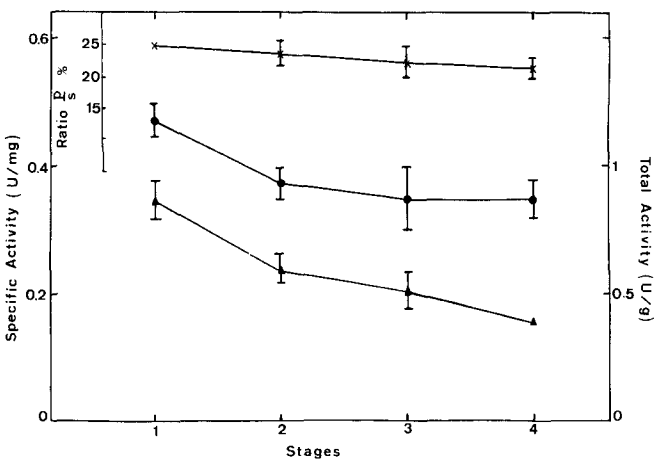

Fig. 1. Effect of ripening on the alanine aminotransferase activity. Four stages were investigated: 1, Large Green; 2, Green Mature; 3, Green Orange; 4, Red. Tomatoes were extracted as described under Materials and Methods. Protein and enzyme activity were assayed in both the soluble and particulate extracts. Cross bars indicate the range of values obtained on three or four samples. Total alanine aminotransferase activity extracted per g of fresh tissue (▲—▲), specific activity of supernatant (●—●), ratio of particulate (P) and soluble (S) activities expressed in % (×—×).

Purification of the soluble alanine aminotransferase

The whole procedure was performed at 4 °C. 1500 g of tomatoes (green orange stage) were extracted as described under Materials and Methods. The supernatant at 3500 × *g* containing 10 mM alanine and cysteine was centrifuged at 60 000 × *g* for 1 h and the resulting supernatant was used for futher purification.

An equal volume of neutralized saturated $(NH_4)_2SO_4$ solution was added to the crude extract, the pH being maintained at 7.5. The solution was allowed to stand for 90 min and the precipitate was discarded. The supernatant was brought to 65% saturation (by the addition of saturated solution) and gently stirred overnight. The precipitate was isolated by centrifugation, dissolved in 10 mM phosphate buffer, pH 7.5, containing 10 mM mercaptoethanol, 10 mM alanine, 5 mM cysteine, 5% glycerol (Buffer A) and dialyzed for 24 h against this buffer. The dialysate was applied on a DEAE-cellulose column (2 cm × 20 cm) equilibrated with 10 mM phosphate buffer pH 7.5, containing 1 mM mercaptoethanol and 5% glycerol. After washing, the column, was developed by a 700-ml linear gradient elution from 0.01 to 0.2 M potassium phosphate. The fractions containing the activity were pooled together and concentrated into a small volume which was dialyzed against 20 mM Tris–HCl, pH 7, containing 10 mM mercaptoethanol, 10 mM alanine and 5% glycerol (Buffer B) and applied on a DEAE-Sephadex column (1.6 cm × 12 cm) equilibrated with 20 mM Tris–HCl, pH 7, containing 1 mM mercaptoethanol and 5% glycerol. The column was washed with Buffer B and developed with a 300-ml linear gradient elution from 0.1 to 0.35 M KCl in Buffer B. The chromatographic profile is shown in Fig. 2. All fractions containing activity were pooled together, concentrated and layered onto the surface of a Sephadex G-200 column which was developed as described in the molecular weight determination. After two consecutive gel filtrations under the same conditions the purified enzyme was kept at −20 °C in a very small volume of 50 mM

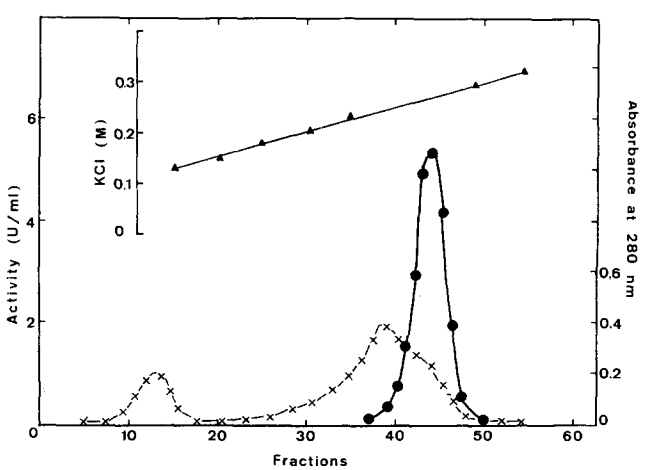

Fig. 2. Alanine aminotransferase purification on DEAE-Sephadex. The enzyme solution (about 5 ml containing 8.2 mg of proteins and 123 enzyme units) was applied on a DEAE-Sephadex column (1.6 cm × 12 cm) equilibrated with Buffer B. The elution was performed as indicated in the text at a flow rate of 25 ml/h. 5-ml fractions were collected and analyzed: protein (×—×), alanine aminotransferase activity (●—●), KCl concentration (▲—▲).

TABLE I

PURIFICATION OF TOMATO ALANINE AMINOTRANSFERASE

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Crude extract	1650	300	0.18	1	100
50–65% $(NH_4)_2SO_4$	390	245	0.63	3.5	82
DEAE-cellulose	30	180	6	33	60
DEAE-Sephadex	8.2	123	15	83	41
First Sephadex G-200	2	96	48	270	32
Second Sephadex G-200	0.66	80	120	660	27

Tris–HCl, pH 7.4, containing 10 mM mercaptoethanol, 10 mM alanine and 20% glycerol.

The results of a typical purification are shown in Table I. The alanine aminotransferase was 660-fold purified from crude extract with a yield of 27%; the best specific activity obtained was 120 units/mg of protein.

Electrophoresis

In polyacrylamide agarose gel, the enzymatic activity appears as a broad and diffuse band migrating towards the anode. From this experiment, one can estimate the degree of purity of the preparation to be between 35 and 45%.

Stability

Glycerol, alanine and mercaptoethanol were effective in protecting the enzyme and so were always present throughout the purification process. 2-Oxoglutarate did not exhibit a better protective effect than alanine. In the crude extract alanine aminotransferase was entirely stable for several months at −20 °C and was unaffected by repeated freezing and thawing. Likewise, the purified enzyme exhibited 80% of residual activity after 6 months storage at −20 °C.

For the study of stability as a function of pH, the enzyme was incubated at 4 °C for 24 h in Tris–HCl (pH 7.4); phosphate (pH 6.5) and citrate (pH 5.5, 5 and 4.5) buffers which all contained 10 mM alanine 10 mM mercaptoethanol and 5% glycerol. A strong loss of activity occurred under pH 6.5.

The enzyme was quickly inactivated by heating at 50 °C. Pyridoxal phosphate and serum albumin did not prevent denaturation, whereas 0.1 M alanine gave a slight protective effect.

Molecular weight and pI

The apparent molecular weight is about 100 000 ± 5000. The alanine aminotransferase gives a single peak by electrofocusing with a maximum at pH 4.5 ± 0.1.

Kinetic studies

None of the following aminoacids and 2-oxoacids were substrates for the enzyme: aspartate, glycine, threonine, leucine, methionine, arginine, phenylalanine, tyrosine, tryptophan, glyoxylate and 2-oxo-3-methylvalerate.

The alanine aminotransferase reversibly catalyzes the transamination reaction

but at different rates: the maximum rate of the forward reaction (V_f) is four times higher than the maximum rate of the reverse reaction (V_r). The addition of pyridoxal phosphate does not increase the activity, a finding which suggests that the holoenzyme structure was preserved.

The effect of pH on the alanine aminotransferase activity was determined in a Tris–HCl medium of 0.040 ionic strength. The activity decreases rapidly under pH 7 and over pH 7.5. The pH optimum was in the range 7.25 to 7.45.

Double-reciprocal plots of the initial rates versus the first substrate concentration for different concentrations of the second substrate, gave a family of parallel straight lines. The plots of the intercept values from the ordinate or abscissa axis, versus the inverse of the concentrations of the second substrate gives straight lines which permit an estimation of the Michaelis constants for each substrate. All these experimental results imply the formation of a binary enzyme–substrate complex and agree with a Ping Pong Bi Bi type reaction mechanism. According to this mechanism it was found experimentally that the product of the forward reaction, L-glutamate, acts as a competitive inhibitor with respect to alanine (Fig. 3).

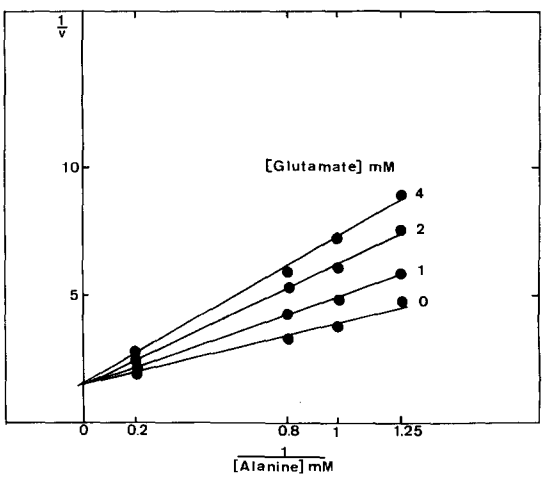

Fig. 3. Inhibition by glutamate of the forward reaction. Lineweaver–Burk representation with alanine as variable substrate, at a fixed concentration of 2-oxoglutarate: 0.5 mM, for fixed L-glutamate concentrations.

The K_m evaluated for each substrate are: alanine (K_a):2.8 mM; 2-oxoglutarate (K_o):0.28 mM; glutamate (K_g):2.3 mM; pyruvate (K_p):0.09 mM.

The equilibrium constant, calculated from the Haldane relation [23]:

$$K_{eq} = \left[\frac{V_f}{V_r}\right]^2 \frac{K_p \cdot K_g}{K_a \cdot K_o}$$

is equal to about 4, favoring pyruvate formation.

DISCUSSION

Two forms of alanine aminotransferase enzyme are present in the tomato. The major one is soluble; the minor one sedimenting at 20 000 × g remains particulate.

The ratio of these two forms, soluble versus particulate, remains nearly constant during ripening and is found between 4 and 5.

Though the successive stages of ripening are sometimes hardly recognized [8], the total enzymatic activity decreases as total protein content during tomato development. This result agrees with that obtained by Yu and Spencer [6, 7]. This work confirms the participation of alanine aminotransferase during the tomato ripening phase, though it is not possible to evaluate the incidence of such a reaction in the biogenesis of volatile compounds in the aroma.

Partial purification of the soluble enzyme has been achieved. On the basis of an electrophoretic experiment its degree of purity was estimated to be between 35–45%. This purification needs protective agents and neutral pH conditions. Some physical properties and some enzymatic properties of this partially purified preparation have been tested. Our results can be compared to those obtained with animal enzymes as, to our knowledge, no detailed study in fruit alanine aminotransferase has been reported yet.

The tomato alanine aminotransferase seems more labile than the animal ones: in addition its lability is below pH 6.5, this enzyme is quickly inactivated at 50 °C, while the pig heart and rat liver enzymes remain unaffected at 60 °C (refs 11 and 21). The molecular weight was estimated to be around 100 000 in agreement with the values found for pig heart (100 000) (ref. 11) and rat liver (114 000) (ref. 21) enzymes.

Like all other known alanine aminotransferases, this enzyme reversibly catalyzes a transamination reaction according to the Ping Pong Bi Bi mechanism [10, 24]. Furthermore, the K_m for 2-oxoacids are very much smaller than those for amino acids, as is the case for other alanine aminotransferases [24, 25]. Yet, the tomato enzyme exhibits a high affinity towards its substrates: the different K_m values are 5- to 10-fold smaller than the K_m values of the rat liver enzyme [25].

The calculated equilibrium constant is equal to about 4. Bulos and Handler [10] give a value of 2.2 for the pig heart enzyme, but for the rat liver enzyme the authors find an equilibrium constant of 1.6 (ref. 26) (calculated according to the Haldane relation) for the formation of alanine. Thus it seems that, in vitro, the tomato alanine aminotranferase preferentially performs transamination between alanine and 2-oxoglutarate. It is interesting to note that Yu et al. [27] found the glutamic acid concentration in the tomato is at least 10 times that of alanine.

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