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HOMOSERINE DEHYDROGENASE IN *PISUM SATIVUM* AND *RICINUS COMMUNIS*

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Key Word Index—*Pisum sativum*; Leguminosae; pea; *Ricinus communis*; Euphorbiaceae; castor bean; homoserine dehydrogenase.

Abstract—Homoserine dehydrogenase was extracted from *Ricinus communis* and *Pisum sativum*. The kinetic parameters of the forward and reverse reactions were determined. In the forward reaction only the enzyme from *Ricinus* is inhibited by threonine. The response to K^+ is different for the enzyme from the two sources.

INTRODUCTION

Homoserine dehydrogenase (E.C.1.1.1.3.) is a key enzyme in the biosynthesis of homoserine from aspartate in bacteria, and is subject to complex regulation. In *E. coli* multiple forms of the enzyme exist, each being controlled by different amino acids formed in reactions subsequent to homoserine. In higher plants evidence has been obtained for the same metabolic pathway, and some of the enzymes of the pathway have been isolated. Homoserine dehydrogenase has been previously studied in pea [1] and maize [2]. The maize enzyme resembles the enzyme from microorganisms in the extent and modality of the inhibition by threonine. The aim of the present work was to obtain further information on the regulation of the enzyme in plants. The study of the enzyme from 2 plants sources has been undertaken in view of the difficulties in comparing results from different laboratories, which is due mainly to the extreme sensitivity of the enzyme to slight variation in the experimental conditions. The enzyme from pea (*Pisum*) deserves particular attention because of the unusually high content of the aminoacid homoserine during the germination [3].

RESULTS

(1) *Ratio of the forward and reverse reactions*

Measured in the forward direction of NADPH oxidation, activity of homoserine dehydrogenase from *Ricinus* was twice that of the enzyme from *Pisum* when compared on a fr. wt basis and essentially the same ratio was obtained in terms of the specific activities of the $(NH_4)_2SO_4$ fractions which were 39 and 24 n mol/min/mg protein respectively. The ratio of the forward and reverse reactions, when measured under standard conditions, differs significantly, being *ca* 7 in *Ricinus* and *ca* 3 in *Pisum*. The enzyme from *Neurospora* [4] also has a low ratio of 3, but other homoserine dehydrogenases have a higher ratio. In particular the data of Sasaoka [1] for the enzyme from *Pisum* suggests a ratio between 30 and 60. In order to investigate the presence of contaminating enzymes both preparations were further fractionated by $(NH_4)_2SO_4$ at intervals of 5% saturation. The spread of activity over the fractions was quite broad, but in all fractions containing activity, the ratio between the forward and reverse reaction was constant for both preparations. The enzyme activity was proportional to the protein concn in the range studied, which is below 0·05 ΔA/min for the

Table 1. Kinetic constants of homoserine dehydrogenase from *Ricinus* and *Pisum*

Constant substrate	Varied substrate	*Km* for varied substrate in μM *Ricinus*	*Pisum*
ASA 0·1 mM	NADPH	16	42
ASA 0·1 mM	NADH	37	
NADPH 0·5 mM	ASA	120	51
HS 10 mM	NAD^+	770	1200
HS 10 mM	$NADP^+$	65	36
$NADP^+$ 0·5 mM	HS	860	2500

The reaction mixtures were as described in Experimental. ASA = aspartic semialdehyde; HS = homoserine.

forward reaction and 0·02 ΔA/min for the reverse reaction.

(2) *Stability of enzymes during dialysis*

On dialysis, the enzyme obtained from *Ricinus* was relatively stable in the presence of threonine (1 mM). On dialysing for 16 hr against K-phosphate buffer (0·1 M, pH 7·4), or Tris–HCl (0·1 M, pH 7·4), both buffers containing threonine (1 mM), 90% of the original activity was recovered which was inhibited 75% by threonine (1 mM). Dialysing the enzyme against Tris–HCl buffer (0·1 M, pH 7·4) containing KCl (0·1 M) or in K-phosphate buffer (0·1 M, pH 7·4) a loss of activity of 50% and 30% respectively was observed with a concomitant desensitization towards threonine, whilst inhibition by cysteine was retained. Dialysis against Tris–HCl (0·1 M, pH 7·4) in the absence of KCl reduced the recovery of enzyme activity but sensitivity towards threonine was retained. The enzyme from *Pisum* on the other hand was rather unstable, losing *ca* 60% of its activity in 16 hr regardless of the buffers used. Only in the presence of glycerol (20%) with or without threonine (1 mM) in Tris–HCl buffer (0·1 M, pH 7·4) *ca* 90% of the activity was retained after 16 hr.

(3) *Effect of coenzymes and substrates*

The enzymes from both sources were active in both forward and back reactions with NADH and NADPH, or with NAD^+ and $NADP^+$ respectively. Simple Michaelis–Menten kinetics were obtained, except for the NADH linked activity with the *Pisum* enzyme in which the *Km* could not be determined because of the low affinity of the enzyme for NADH. For homoserine oxidation, the values for V_{max} with NAD^+ or $NADP^+$ were almost the same with preparations from both plants, even though the *Km* for the 2 coenzymes differ considerably. In the case of the enzyme from *Ricinus* the V_{max} with NADPH was about three times that observed in the presence of NADH. A summary of kinetic constants is presented in Table 1.

(4) *Effect of amino acids*

(a) The *Ricinus* enzyme was affected in both directions by many amino acids and especially by threonine and cysteine. The inhibition by threonine reaches 75% at a concentration less than 1 mM. The same degree of inhibition is obtained by cysteine but at 3 mM. The concentrations needed for 50% inhibition were: threonine, 0·1 mM; cysteine, 1 mM; serine, 2 mM; homoserine, 3 to 4 mM. Little effect is shown by aspartic acid, methionine and isoleucine. The effect of the aminoacids on the reverse reaction is shown in Table 2.

(b) For the *Pisum* enzyme in the forward reaction, only cysteine significantly affects the activity. Cysteine affects only the intercept of the double reciprocal plot of initial velocity vs aspartic semialdehyde (ASA), with no effect on the slope, showing the so-called "uncompetitive inhibition" with a Ki = 0·33 mM. Using NADPH as varied substrate the double reciprocal plot was affected both on the

Table 2. Effect of various aminoacids on the $NADP^+$ linked reaction measured in presence and absence of KCl

	+KCl		−KCl		+KCl		−KCl	
	ΔA_{340} $min^{-1} \times 10^3$	% Inhibition	ΔA_{340} $min^{-1} \times 10^3$	% Inhibition	ΔA_{340} $min^{-1} \times 10^3$	% Inhibition	ΔA_{340} $min^{-1} \times 10^3$	% Inhibition
Control	24·0		6·0		20·0		13·7	
Threonine 10^{-3} M	6·9	71	4·9	17	13·2	34	2·0	85
Cysteine 10^{-3} M	4·0	83	0·3	95	3·8	81	1·7	87
Serine 10^{-3} M	22·1	8	3·6	40	17·5	12	10·0	20
Aspartate 10^{-3} M	23·0	4	4·3	28	19·0	5	11·0	20

The reaction mixtures were as described in Experimental.

slope and intercept, indicating a mixed type inhibition, with a $Ki = 0{\cdot}37$ mM. Threonine, serine and aspartic acid at 1 mM produce a very slight inhibition (*ca* 10%). In the reverse reaction the enzyme is inhibited by threonine, cysteine and aspartic acid (Table 2). KCl increases the inhibition produced by threonine whilst the reverse is true for other amino acids tested.

(5) *Effect of KCl*

The enzymes respond differently towards KCl. From the few experiments on dialysis, the enzyme from *Ricinus* seems to be inactivated and desensitized towards threonine in the presence of KCl if threonine is absent. The enzyme from *Ricinus* showed no activity when assayed in the forward reaction in MES–Tris buffer (0·1 M, pH 6·5) in the absence of KCl.

The activity can be restored to the same level obtained with the standard K-phosphate buffer by adding KCl to a final concentration at 0·2 M. On the other hand the *Pisum* enzyme shows a reduction of only 20% of its activity when measured in MES–Tris buffer in the absence of KCl. In the reverse reaction the presence of KCl in the assay mixture greatly enhances the activity of both enzymes. In fact when measured with homoserine (5 mM) and $NADP^+$ (0·5 mM) in the presence of KCl (0·1 M) the activity of the *Ricinus* enzyme increases 2·6 fold, whilst activity of the *Pisum* enzyme increases 3·3 fold.

Threonine behaves differently in the presence and absence of KCl. Thus in the case of the *Pisum* enzyme threonine is an effective inhibitor in the presence of high concn of KCl, the value of inhibition increasing from 20 to about 70% in presence of KCl 0·1 M, whilst in the case of the *Ricinus* enzyme the inhibition produced by threonine is drastically reduced from 80 to 10% in presence of KCl 0·2 M. A kinetic analysis of the data suggests the possibility of competition between threonine and KCl. The effect of KCl on the inhibition from the other amino acids tested is rather similar, resulting in a decreased inhibition. In view of the fact that kinetic interactions between the activating cation and the inhibitor are different, the effect of KCl on the kinetic parameters of the reverse reaction were investigated. The results are similar in both cases, the *Km* for homoserine being significantly decreased in the presence of KCl, whilst the *Km* for $NADP^+$ remains unaltered.

For the *Pisum* enzyme in the absence of KCl the *Km* for homoserine is 10 mM and in the presence of KCl (0·1 M) it is 2·5 mM. For the *Ricinus* enzyme the *Km* for homoserine in the presence of KCl (0·1 M) was 0·86 mM and in the absence of KCl the *Km* was 2·5 mM. It must be pointed out that in the reverse reaction we are dealing with a cation effect, because adding 250 μmol of Tris–HCl to the standard buffer neither the stimulation of the catalytic activity nor the threonine inhibition is significantly affected.

DISCUSSION

The critical position of homoserine dehydrogenase in the metabolism of aspartate has led to many investigations on the catalytic and regulatory properties of this enzyme. In general threonine inhibits the formation of homoserine. Homoserine dehydrogenase from *Ricinus* follows this general pattern but the enzyme from *Pisum* shows little inhibition by threonine. The role of threonine as an allosteric regulator has been demonstrated in the bicephalic aspartokinase–homoserine dehydrogenase from *E. coli* K 12 [5] and from *R. rubrum* [6]. On the basis of kinetic and desensitization experiments a specific regulatory site has been also suggested for the enzyme from *Zea mays* (2) and *P. fluorescens* [8].

(*a*) *Homoserine dehydrogenase from* Pisum

The observation that the enzyme from *Pisum* is not inhibited by threonine in the forward reaction could account for the unusually high content of homoserine in germinating peas. It should be noted that threonine is a powerful inhibitor of the back reaction and thus the kinetics of the *Pisum* enzyme resembles the unidirectional inhibition reported for glutamate dehydrogenase from *Blastocladiella* (9). Unidirectional inhibition is difficult to explain because of the principle of microreversibility. Further detailed studies are necessary to ascertain the possible existence of homoserine dehydrogenase isoenzymes differing in their responses to threonine. In some microorganisms [7,11,12] isoenzymes have been observed differing in their responses to threonine. Homoserine in pea seedlings plays a special rôle. The hypothesis

currently favoured suggests that this aminoacid is implicated in the transport of carbon and nitrogen [13,14]. It is apparent that this role can be better fulfilled in the absence of threonine inhibition. In the reverse reaction the inhibition is apparent only in the presence of KCl. It is difficult to ascribe a physiological role to the effect of KCl on the oxidation of homoserine, if as Grant and Voelkert claim [10] the reaction is not readily reversible. On the other hand, Goas [15] demonstrated the formation of labelled aspartate after feeding decotyledonized seedlings with homoserine.

The effect of cysteine in the forward reaction deserves comment. The kinetics show uncompetitive inhibition with respect to ASA. Cysteine in higher plants is involved in the metabolism of homoserine by a reaction in which methionine is formed by a transulfuration reaction with "activated" homoserine [16]. Consequently cysteine might play a role in the metabolic control of homoserine dehydrogenase. A general mechanism with the formation of a ternary complex between cysteine, the enzyme and the coenzyme has been proposed to explain the effect of cysteine on maize homoserine dehydrogenase [2]. It is difficult to say, however, that cysteine inhibition occurs *in vivo*. Measurable amounts of cysteine or cystine could not be detected in germinating peas. Very small amount of cystine, mostly in cotyledons were found by Lawrence and Grant [17] who analyzed different part of the seedlings. These results suggest the absence of control of the formation of homoserine from ASA in *Pisum*.

(*b*) *Homoserine dehydrogenase from* Ricinus

The enzyme extracted from *Ricinus* shares features with most of the homoserine dehydrogenases studied so far, in particular with the maize enzyme. It is inhibited in both directions by threonine and several other aminoacids. It should be noted that our enzyme is significantly less sensitive to aspartate inhibition in the forward reaction compared with the results of Bryan from maize [2]. The maize enzyme can be desensitized to threonine inhibition in the presence of K-phosphate with a loss of 20% of its activity while retaining cysteine inhibition. We observed the same trend upon storage in the extraction buffer at 4°, and after passage through a Sephadex G 200 column previously equilibrated with the extraction buffer. The presence of threonine throughout the purification protects the catalytic and control properties of the enzyme. This is consistent with threonine acting at a specific control site and is frequently observed in allosteric enzymes. The effect of KCl on the catalytic activity of the *Ricinus* enzyme as well on *Pisum* enzyme appears similar, lowering the *Km* for homoserine. In *Ricinus* KCl reduces the inhibition produced by threonine and the kinetics are consistent with competition between threonine and KCl. Similar behaviour has been reported for the enzyme from *P. fluorescens* [8]. In this case the authors suggest that threonine is an allosteric inhibitor and KCl induces conformational changes. In *Pisum*, however, we observed uncompetitive kinetics which suggest that the sites for threonine and KCl may be different.

(*c*) *Comparison between the two enzymes*

The effect of KCl and threonine on the back reaction of the two enzymes is shown in the first 2 lines of Table 2. KCl appears to interact with the effector threonine in different ways. In the case of the *Pisum* enzyme threonine acts as a potent inhibitor in the presence of KCl, whereas in the *Ricinus* enzyme threonine produces little inhibition in the presence of KCl. The effect of KCl on the inhibition from the other amino acids tested appears to be similar, resulting in both cases in a decreased inhibition. In the forward reaction K-phosphate can replace KCl and it is likely that the cation, and not the anion, is responsible for the effect. The two enzymes differ in their response to K^+, the *Ricinus* enzyme showing an absolute K^+ requirement for homoserine formation. The 2 enzymes differ also in their response to threonine, which appears ineffective with the *Pisum* enzyme. Such behaviour could play a physiological role, allowing the continuous synthesis of homoserine, which in *Pisum* is utilized for carbon and nitrogen transport.

EXPERIMENTAL

Seeds of peas (*Pisum sativum* cv. Quimper Glory) and castor bean (*Ricinus communis*) were grown at 27°. The leaves were collected 15 days after germination.

Extraction of homoserine dehydrogenase from the leaves. Leaves (100 g) were ground in a cold mortar with glass powder (12 g) and Tris–HCl buffer (150 ml, 0·1 M pH 9) containing EDTA (1 mM) and 2-mercaptoethanol (1 mM). After filtration through 4 layers of cheesecloth, the extract was centrifuged at

20000 *g* for 30 min. The supernatant was treated with $(NH_4)_2SO_4$ and the ppt between 35% and 55% satn collected by centn. This fraction containing most of the activity, was dissolved in 10 ml of Tris–HCl buffer (0·1 M pH 7·4) and precipitated again with $(NH_4)_2SO_4$ at 60% satn. The suspn was stored at 4° and the enzymes prepared from *Pisum* and *Ricinus* were stable for several weeks. Before use, the suspension was centrifuged and the ppt dissolved in the Tris–HCl buffer (0·1 M pH 7·4), then passed through a column of Sephadex G 25 equilibrated with the same buffer.

Preparation of aspartic semialdehyde (ASA). The ASA was prepared according to the method of ref. [18]. The concn of ASA was determined enzymatically with an excess of NADPH using the enzyme obtained from *Pisum*. The soln of ASA, analysed with an aminoacid analyser was shown to contain about 0·5 mol of aspartic acid mol^{-1} of ASA. This concn of aspartate did not interfere with the kinetic studies of the enzyme.

Protein determination. Proteins were determined by the method of ref. [19].

Determination of homoserine dehydrogenase activity. The enzymatic activity was measured in a spectrophotometer equipped with a recorder and a temp-controlled cell compartment. For the forward reaction (ASA + NADPH → HOMOSERINE + $NADP^+$) the standard mixture contained: KPi (pH 6·5, 460 μmol); EDTA (2·5 μmol); 2 mercaptoethanol (2·5 μmol); NADPH (0·13 μmol); ASA (0·25 μmol) and enzyme in a final vol of 2·5 ml. In the reverse reaction the standard mixture was: Tris–HCl buffer (pH 9,230 μmol); EDTA (2·5 μmol), 2-mercaptoethanol (2·5 μmol); KCl (250 μmol); $NADP^+$ (1·3 μmol); L-homoserine (25 μmol) and enzyme in a final vol of 2·5 ml.

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