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# COMPARISON OF HOMOSERINE DEHYDROGENASE FROM DIFFERENT PLANT SOURCES

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**Key Word Index**—*Pisum sativum*; Leguminosae; pea; *Ricinus communis*; Euphorbiaceae; castor bean; *Triticum aestivum*; Graminae; wheat; homoserine dehydrogenase; threonine.

**Abstract**—Homoserine dehydrogenase (HSD) was partially purified from castor bean, pea and wheat seedlings. The enzyme from pea had a MW of 75 000 and no sensitivity to threonine when measured in the direction of homoserine formation (forward reaction). The enzyme purified from castor bean had a MW of 290 000–350 000 and exhibited an almost complete inhibition by 1 mM threonine. Furthermore, this enzyme exhibited a polymeric nature as shown by polyacrylamide electrophoresis of the desensitized preparation and by SDS electrophoresis of the native enzyme. In wheat two isoenzymes were separated by gel filtration on Sephadex G 200. The fast-moving fraction (HSD I) was completely inhibited by threonine and exhibited a MW of 280 000, while the slow-moving fraction (HSD II) was insensitive to threonine and had a MW of 75 000. The sensitive enzyme from wheat and castor bean showed an almost absolute requirement for $K^+$. The enzyme from pea and the insensitive form from wheat did not show a requirement for $K^+$. For the wheat enzyme the effect of several amino acids and the main kinetic constants were studied.

## INTRODUCTION

The central role played by homoserine dehydrogenase (HSD) in the metabolism of aspartic acid explains why so much attention has been devoted to this enzyme. An extensive and accurate study on maize HSD, paying particular attention to the ontogenetic aspect, has been reported [1-3]. The structural and regulatory properties of the pea [4] and barley [5] HSD have been described. A recent paper refers to the HSD in developing soybean seedlings [6]. In a previous paper we reported data on the enzyme extracted from pea and castor bean [7]. From these investigations some of the general properties of this enzymatic activity can be assessed, namely the existence of isoenzymes with different sensitivity to the inhibitory end product threonine.

In this study HSD was partially purified from pea, castor bean and wheat seedlings. The main structural properties, the effect of $K^+$, and the effect of several amino acids on wheat enzyme were examined. The main kinetic constants of wheat enzyme were also determined.

## RESULTS

### *Homoserine dehydrogenase from castor bean*

The enzyme was purified by sequential treatment of crude extract with $(NH_4)_2SO_4$, calcium phosphate (hydroxylapatite) and chromatography on Sephadex A-50 (Table 1) followed by molecular sieving on Sephadex G 200. The high sensitivity to threonine inhibition shown by the crude extract was maintained throughout the purification.

This high sensitivity of the enzyme (0.02 mM threonine carried over into the assay mixture with 50 $\mu$l of enzymatic solution containing 1 mM threonine causes 25% inhibition) was taken into account when the activities shown in Table 1 were calculated. The same correction was used in the case of the sensitive activity of wheat (HSD I).

Desensitization to threonine inhibition was observed *in vitro* by dialysing the purified enzyme at 4° in 0.2 M KPi (pH 7.4). Threonine inhibition decreased to 40% after 5 hr and to 10% after 24 hr. The original activity was retained during the treatment. The reverse reaction was also desensitized, and attempts to resensitize the enzyme by threonine addition were unsuccessful. Polyacrylamide electrophoresis of the purified enzyme resulted in a single band. The same analysis, performed on the desensitized enzyme previously concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against a large volume of buffer A, resulted in a single band which migrated faster with respect to the sensitive enzyme. A plot of the relative mobilities in gels at different concentrations by the procedure of Hedrick and Smith [8] revealed that the sensitive and the insensitive enzyme had a slope of 14.0 $\pm$ 1.2 and 7.0 $\pm$ 0.6, respectively, corresponding to the MW of 290 000 $\pm$ 24 800 and 80 000 $\pm$ 6800 as determined from a standard curve determined with several reference proteins. A partially desensitized HSD showed two distinct bands with the same electrophoretic properties described. Gel filtration on Sephadex G 200 indicated a MW of 350 000 $\pm$ 20 000 for the sensitive enzyme, while no activity was recovered after filtration of the desensitized one. Electrophoretic analysis of the sensitive and the desensitized enzyme in the presence of SDS produced a single polypeptide showing a MW of *ca* 80 000 $\pm$ 5000. Occasionally, during the gel filtration step, we obtained a second peak of threonine-insensitive HSD. This peak

Table 1. Purification steps of homoserine dehydrogenase in castor bean, pea and wheat

| Step | Volume (ml) | Protein (mg) | Tot. act. (units*) | Sp. act. units/mg) | Purification (fold) | Recovery | % inhibition by threonine |
|---|---|---|---|---|---|---|---|
| Castor bean | | | | | | | |
| Crude extract | 375 | 2250 | 124 | 0.055 | 1 | 100 | 87 |
| $(NH_4)_2SO_4$ fraction | 55 | 1040 | 139 | 0.13 | 2.3 | 111 | 86 |
| Hydroxylapatite | 150 | 195 | 66 | 0.34 | 6.2 | 53 | 89 |
| DEAE Sephadex A-50 | 40 | 4.4 | 30 | 6.8 | 124.0 | 24 | 90 |
| Pea | | | | | | | |
| Crude extract | 220 | 880 | 26 | 0.03 | 1 | 100 | 12 |
| $(NH_4)_2SO_4$ fraction | 23 | 640 | 25 | 0.04 | 1.3 | 96 | 5 |
| Hydroxylapatite | 15 | 33 | 14 | 0.24 | 8.0 | 54 | 8 |
| DEAE Sephadex A-50 | 11 | 4 | 11 | 2.8 | 91.0 | 42 | 10 |
| Wheat | | | | | | | |
| Crude extract | 30 | 172 | 3.39 | 0.02 | 1 | 100 | 54 |
| $(NH_4)_2SO_4$ fraction | 3.0 | 8.2 | 2.71 | 0.03 | 1.5 | 80 | 58 |
| Sephadex G-200 HSDH I | 30 | 6.1 | 1.02 | 0.17 | 8.5 | 30 | 100 |
| HSDH II | 28. | 4.4 | 0.85 | 0.19 | 9.5 | 25 | 0 |

Procedure as described in Experimental.

* One unit of homoserine dehydrogenase catalyses the oxidation 1 μmol of NADPH per min in standard condition.

represented *ca* 10–15% of total activity. When submitted to electrophoretic analysis in polyacrylamide 7.5% this fraction had the same electrophoretic mobility as the desensitized enzyme. Furthermore, gel filtration on the Sephadex G 200 column used for MW determination, indicated a MW of 80 000 ± 5000.

*Homoserine dehydrogenase from pea*

The HSD was purified through the same steps used for castor bean enzyme and remained almost completely insensitive to threonine throughout purification (Table 1). However, threonine was a powerful inhibitor of the reverse reaction, confirming data previously obtained [7]. By filtration on a Sephadex G 200 column, previously standardized with reference proteins, a MW of 75 000 ± 5000 was obtained.

In view of the fact that two isoenzymes of HSD has been reported in etiolated pea seedlings, var. Pillert Fenomen [4], the activity of the enzyme in extracts of the seedlings was followed from the beginning of germination but the same lack of sensitivity was obtained. MES–KOH (0.1 M, pH 6.5) and the extraction buffer reported in ref. [4] [KPi 50 mM (pH 7.2), 1 mM EDTA, 1 mM threonine, 14 mM mercaptoethanol and 15% by vol. glycerol] were also used, but we were unable to produce a sensitive activity (< 15% inhibition).

*Homoserine dehydrogenase from wheat*

The HSD was partially purified by $(NH_4)_2SO_4$ precipitation and gel filtration (Table 1). Gel filtration on Sephadex G 200 resulted in two symmetrical peaks of activity. When allowance was made for the overlapping region, the first peak (HSDI) was almost completely inhibited by threonine 1 mM while the second peak (HSD II) was completely insensitive to threonine. All fractions showing respectively only HSD I activity and HSD II activity were pooled separately, concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against a large volume of buffer A. In order to determine the MW these two preparations were filtered through a Sephadex G 200 column previously standardized with reference proteins (see Experimental). A MW of 280 000 ± 10 000 was obtained for HSD I and 75 000 ± 5000 for HSD II. Desensitization to threonine was observed during seedling development. HSD obtained from 10-day-old seedlings was 80–90% insensitive to threonine, while the 6-day-old seedlings usually collected for the experiments reported here were 60% insensitive to threonine.

*Kinetic constants, effect of amino acids, effect of* $K^+$

The main kinetic constants for pea and castor bean enzyme were reported previously [7]. The kinetic constants of wheat enzyme were determined on the preparation used for the MW determination. The data are the average of 3 determinations ± s. d.

HSD I was effective both with NADPH and NADH as substrates. The apparent $K_m$ (NADPH) measured in the presence of 0.1 mM aspartate semialdehyde (ASA) was 43 ± 6 μM and $K_m$ (NADH) measured in the same conditions was 31 ± 5 μM. The $K_m$ (ASA) measured in the presence of 0.5 mM NADPH was 240 ± 15 μM. HSD II was effective only in the presence of NADPH, showing a $K_m$ (NADPH) of 32 ± 5 μM in the presence of 0.1 mM ASA and a $K_m$ (ASA) of 130 ± 10 μM in presence of 0.5 mM NADPH. The ratio between the forward and the reverse reaction measured respectively at pH 7.4 and 9.0 in standard conditions in the presence of NADP(H) was 25 for HSD I and 7 for HSD II. HSD I showed 30% stimulation changing the pH of the assay medium from 6.5 to 7.4 in the forward reaction, while no effect in this range was shown by HSD II. Furthermore, HSD II was completely insensitive to threonine when assayed in the reverse reaction in standard conditions.

The effect of several amino acids related to the aspartate pathway on pea and castor bean enzyme has been reported previously [7]. At 10 mM concentration the effectiveness as inhibitors on wheat HSD I was (± s. d.): cysteine (100% ± 1%) = threonine (100% ± 3%) > aspartic acid (40% ± 7%) > serine (27% ± 4%) > homoserine (20% ± 5%). Inhibition on HSD II was shown by cysteine (90% ± 5%), aspartic acid (50% ± 6%), serine (30% ± 3%) and homoserine (23% ± 4%).

The effect of $K^+$ previously reported for pea and castor bean [7] has been investigated in the partially purified enzymes. Since the enzymes are purified and stored in the

Table 2. Effect of $K^+$ on the activity of homoserine dehydrogenase in castor bean, pea and wheat, measured in different buffers in the forward reaction in presence of NADPH

| Buffer | % of activity in standard conditions | | | | |
|---|---|---|---|---|---|
| | Wheat | | Castor bean | | Pea |
| | HSD I | HSD II | HSD native | HSD desensitized | |
| KPi 0.1 M (pH 6.5) (standard conditions) | 100 | 100 | 100 | 100 | 100 |
| KPi 0.1 M (pH 6.5) + 1 mM thr. | 0 | 103 ± 5 | 10 ± 2 | 90 ± 2 | 90 ± 3 |
| MES–Tris 0.1 M (pH 6.5) | 5 ± 1 | 62 ± 4 | 15 ± 3 | 95 ± 5 | 98 ± 1 |
| MES–Tris 0.1 M (pH 6.5) + $K^+$ 0.1 M (chloride) | 14 ± 5 | 98 ± 3 | 102 ± 1 | — | — |
| MES–Tris 0.1 M (pH 6.5) + $K^+$ 0.1 M (phosphate) | 87 ± 6 | | 104 ± 6 | | — |
| LKB-ampholine (pH 6.5) | 0 | | 12 ± 3 | | — |
| LKB-ampholine (pH 6.5) + $K^+$ 0.1 M (chloride) | 39 ± 5 | | 96 ± 2 | — | — |
| LKB-ampholine (pH 6.5) + $K^+$ 0.1 M (phosphate) | 101 ± 2 | | 102 ± 4 | — | — |

The LKB-ampholine buffering solution was prepared adding 5 ml of LKB ampholine, pH 3.5–10 (dry content 40%, w/v) to 95 ml of distilled water. Activities are expressed as per cent of that observed in standard conditions and the results are the average of three determinations. The error is presented as s.d.

presence of $K^+$, a certain amount of the cation is carried over with the enzyme into the reaction mixture. In most cases 10 $\mu$l of enzyme preparation containing 0.13 M $K^+$ was used, which brought the $K^+$ concentration in the $K^+$-free buffers to 0.5 mM.

The different response to $K^+$ is shown in Table 2, where the activity in different buffers is reported as per cent of activity in standard phosphate buffer. The LKB ampholyte system (ampholine), which is composed of aliphatic polyamino-polycarboxyl acids, was used as monovalent cation free buffer. The 3.5–10 pH system at 2% w/v concentration was found suitable. Some of the monovalent cations were also added to the reaction mixture in the form of phosphate, because wheat HSD I shows maximum activity in phosphate buffer (its maximum activity is not restored when $K^+$ in the form of chloride is added to the $K^+$-free buffer). On the contrary, the cation effect on castor bean enzyme was the same irrespective of whether chloride or phosphate was used.

The sensitive activity of wheat (HSD I) and the activity of castor bean showed an almost absolute requirement for the monovalent cation. The desensitized form of castor bean enzyme and pea enzyme maintained the original activity in the absence of $K^+$ in MES–Tris buffer while wheat insensitive enzyme (HSD II) had a significant reduction of activity.

In order to determine the cation specificity, 0.1 M of each of the various cations were added to the ampholyte buffer. The results reported in Table 3 indicate that $K^+$ is the most effective activating cation, followed by $Na^+$ and $NH_4^+$. $Li^+$ and $Cs^+$, added in the chloride form only to castor bean enzyme, show little activation.

Table 3. Effect of various cations on the activation of native HSD from castor bean and HSD I from wheat

| Cations | | % of activity in standard conditions | |
|---|---|---|---|
| | | Castor bean HSD native | Wheat HSD I |
| $K^+$ (phosphate) | 100 mM | 102 ± 4 | 101 ± 2 |
| | 40 mM | 71 ± 3 | 77 ± 2 |
| | 20 mM | 48 ± 4 | 51 ± 4 |
| | 10 mM | 33 ± 2 | 30 ± 1 |
| $Na^+$ (phosphate) | 100 mM | 77 ± 2 | 56 ± 3 |
| $NH_4^+$ (phosphate) | 100 mM | 67 ± 3 | 27 ± 2 |
| $Li^+$ (chloride) | 100 mM | 28 ± 3 | |
| $Cs^+$ (chloride) | 100 mM | 14 ± 4 | - |

The LKB ampholine system was used as monovalent cation-free buffer (see Table 2). The molarity is referred to the cation concentration. Activity is expressed as per cent of that observed in standard conditions and the results are the average of three determinations. The error is presented as s.d.

## DISCUSSION

The data presented here are consistent with the general picture obtained in previous studies [1-7]. Even though we failed to find a threonine-sensitive form of the enzyme in pea cv Quimper Glory, evidence has been produced for the existence of this molecular form in the *Pisum* genus [4]. On the contrary, in castor bean we usually observed only the sensitive form. Occasionlly we obtained an insensitive form in a small amount, mostly in more recent experiments in which we used old seeds. Furthermore, the presence of this form was not related to the age of the seedlings. The presence in pea plants of an enzymatic activity almost completely insensitive to threonine was attributed by us to the presence of an unusually large amount of homoserine [7]. The reason for the presence of the highly controlled activity in castor bean remains to be explained. The hypothesis that can be made is that the supply of oxaloacetate derived from the glyoxylate cycle, particularly active during germination of oily seeds, would cause an overflow of carbon through the aspartate pathway in the absence of control. However, HSD activity almost completely sensitive to threonine inhibition has been also found in leaves of field-grown plants (data not shown).

The pea enzyme appears to be rather peculiar in showing inhibition by threonine in the reverse reaction (unidirectional inhibition). Both HSD II from wheat and the desensitized form of castor bean are insensitive to threonine in the reverse reaction. This characteristic of the pea enzyme further favours the reduction of aspartate semialdehyde to homoserine, which in pea seedlings plays an important role in the mobilization of nitrogen and carbon reserves.

Wheat enzyme shows the presence of two molecular forms in similar amounts in the youngest seedlings. The sensitivity to threonine inhibition decreases during seedling aging, due to the increase of the level of HSD II relative to HSD I (data not shown). This has been confirmed in field-grown wheat both in the grain and in the flag leaf [9]. Desensitization of HSD to threonine inhibition during growth has been also reported for maize and soybean seedlings [3, 6]. In these two plants, however, this effect was due to a decreased sensitivity to $K^+$ stimulation of molecular forms in which $K^+$ stimulation was strictly associated with threonine inhibition. Forms insensitive to threonine and to $K^+$ stimulation, showing lower MW, appeared to decline in their relative amount during growth. Furthermore, the ontogenetic pattern appeared rather complex, the sensitive activity being constituted by charge isomers.

In maize seedlings, desensitization to threonine with aging was related to the increase of both threonine and isoleucine with respect to lysine during growth [6]. The same variation in amino acid composition is commonly observed in wheat grains during maturation and has been also observed in the flag leaf by us [9]. In barley seedlings this progressive desensitization of HSD activity with aging has not been noted [5]. Though the general characters of barley and wheat HSD are similar, several differences are worth noting. In barley both molecular forms utilize NADPH and NADH, revealing higher activity in the presence of NADPH [5], while the insensitive form of the wheat enzyme shows no measurable activity in the presence of NADH in the standard enzyme assay. The significance of this behaviour is unknown. Furthermore, the sensitive form of wheat (HSD I) has a MW which is consistent with it being the tetrameric form of HSD II, while a dimeric form results from gel filtration for the sensitive form of barley enzyme [5]. In maize seedlings a monomeric structure for the threonine-insensitive enzyme and a dimeric structure for the threonine-sensitive enzyme have been suggested [3]. It is worth noting, however, that the interconversion of the insensitive form into the sensitive one has never been observed.

The effect of monovalent cations on enzyme catalyzed reactions is widely reported in literature. The mechanism of activation mostly involves a conformational transition or a direct effect on the orientation of the substrate. It is difficult to ascribe a regulatory role to such effects, in view of the small fluctuation of cation concentration in most cells, even though the effective intracellular cation concentration is not yet well defined. The average concentration of $K^+$ is relatively steady during growth, but it can fall when the supply from the roots is low [10]. In our conditions higher $K^+$ amounts would be expected during early growth. The increase of the monomeric form, insensitive to $K^+$ stimulation, would overcome this decline in $K^+$ concentration. We have reported previously the effect of $K^+$ on the reverse reaction of HSD from pea and castor bean [7]. We found a stimulation of the activity of both enzymes in the presence of KCl, while threonine inhibition was stimulated in pea enzyme and reduced in castor bean enzyme in a competitive manner. On the contrary, in maize and soybean seedlings activation by monovalent cation and threonine inhibition measured in the reverse reaction were strictly related [3, 6]. The reason for this divergence is still to be explained. From our results, $K^+$ stimulation of the enzyme in the forward reaction seems to be related to the oligomeric form of the enzyme, being much reduced in the insensitive form of wheat and in pea and almost absent in the desensitized form of castor bean. On the contrary the requirement for the monovalent cation appears almost absolute for the sensitive form of wheat and castor bean.

Even though it is difficult to evaluate the physiological implication from *in vitro* studies of single enzymes, the response of this important regulatory enzyme to $K^+$ concentration could be of interest for the nutritional requirements of cereals in connection with the application of fertilizer.

## EXPERIMENTAL

Pea (*Pisum sativum* cv Quimper Glory), castor bean (*Ricinus communis*) and wheat (*Triticum aestivum* cv Marzotto) were grown in a growth chamber at 27° in moist vermiculite with a photoperiod of 12 hr. Wheat and pea plants were collected 6 days after planting and castor bean after 10 days. The expts reported were performed several times, giving substantially identical results. Leaves were used for the expts.

*Extraction and purification of enzyme.* All purification steps were carried out at 0–4°. The leaves were homogenized with 0.1 M Tris HCl (pH 9), 0.1 M KCl, 1 mM EDTA, 1 mM threonine, 14 mM mercaptoethanol and 20% glycerol. The v/w ratio was 1.5. The extract was filtered through cheesecloth and centrifuged at 30 000 *g* for 30 min. The supernate was fractionated with $(NH_4)_2SO_4$, the ppt. between 35 and 55% satn was collected by centrifugation and dissolved in a small vol. of buffer A [0.1 M KPi (pH 7.4) containing 1 mM EDTA, 1 mM threonine, 1.4 mM mercaptoethanol and 20% glycerol]. Wheat extract was applied directly to a 2.5 × 100 cm Sephadex G-200 column equilibrated with buffer A at a flow rate of 5 ml/hr with the aid of a

peristaltic pump. Fractions of 5 ml were collected. Two symmetrical HSD peaks, partially overlapping, were obtained. All fractions of the first peak showing the highest (>95%) inhibition by threonine 1 mM (HSD I) and all fractions of second peak showing complete insensitivity to threonine (HSD II) were pooled separately, conc by $(NH_4)_2SO_4$ pptn and dialysed against a large vol. of buffer A. This prepn was used for the expts. The following steps were common to pea and castor bean extract. The extract was dialysed overnight against 500-fold excess of 20 mM KPi (pH 7.4) with the same addition as in buffer A (buffer B). The dialysed soln was stirred for 10 min in the presence of hydroxylapatite equilibrated with 10 mM KPi buffer (pH 7.4). The gel containing the adsorbed HSD enzyme was collected by centrifugation and resuspended in 50 mM KPi (pH 7.4) with the same addition as in buffer A. After stirring for 10 min the suspension was centrifuged and the supernate, containing HSD, was pptd with $(NH_4)_2SO_4$ at 60% satn. The ppt. was collected by centrifugation, dissolved in a small vol. of buffer B and dialysed overnight against a large vol. of the same buffer. The dialysed enzyme was then adsorbed on a 5 × 20 cm DEAE-Sephadex (A-50) column and eluted with a linear gradient of KPi buffer (pH 7.4) from 0.02 to 0.5 M (total vol. 2.). The active fractions present as a single peak were pooled and the proteins pptd with $(NH_4)_2SO_4$ at 60% satn were dissolved in a small vol. of buffer A. 1.3 ml of this soln was applied to a 1.2 × 100 cm Sephadex G 200 column equilibrated with buffer A and was eluted with the same buffer at 2.5 ml/hr. Fractions of 2.5 ml were collected. The eluted enzyme was pptd with $(NH_4)_2SO_4$ at 60% satn, centrifuged, dissolved in a small vol. of buffer A and dialysed against a large vol. of the same buffer. This prepn was used for the expts.

*Disc gel electrophoresis and MW determinations.* Disc gel electrophoresis was carried out on 4, 5, 6, 7 and 7.5% acrylamide gels according to ref. [11]. 50 μl of enzymatic soln were used. HSD was located by incubation of the gels as described in ref. [4] with the addition of 150 mM KCl. The MW of the enzymes was determined by comparing their relative mobilities with reference standards according to ref. [8]. The following standards were used: catalase (240 000), lactate dehydrogenase (140 000), BSA (65 000) and ribonuclease (13 000). The expts were run in triplicate. MW of the enzymes was also determined by gel filtration on a 1.2 × 100 cm Sephadex G 200 column, void vol. 40 ml, equilibrated with buffer A. Fractions of 2.5 ml were collected at 2.5 ml/hr with the aid of a peristaltic pump. The elution vol. of the enzymes was compared with the same standards as above. The expts were run at 4° in triplicate and results are given ± s.d. SDS electrophoresis was run according to ref. [12]. Catalase (60 000) and lactate dehydrogenase (36 000) polypeptide chains were used as markers.

*Enzyme assay and protein determination.* Enzyme assay, ASA prepn and protein determination were performed as previously described [7]. The HSD activity was measured in the direction of ASA reduction (forward reaction) unless otherwise stated.

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