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Purification and Interconversion of Homoserine Dehydrogenase from *Daucus carota* Cell Suspension Cultures[1]

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ABSTRACT

Homoserine dehydrogenase from cell suspension cultures of carrot (*Daucus carota* L.) has been purified to apparent homogeneity by a combination of selective heat denaturation, ion exchange and gel filtration chromatographies, and preparative gel electrophoresis. Carrot homoserine dehydrogenase is composed of subunits of equal molecular weight (85,000 ± 5,000). During purification, the enzyme exists predominantly in two molecular weight forms, 180,000 and 240,000. The enzyme can be reversibly converted from one form to the other, and each has different regulatory properties. When the enzyme is dialyzed in the presence of 5 millimolar threonine, the purified enzyme is converted into its trimeric form (240,000), which is completely inhibited by 5 millimolar threonine and is stimulated 2.6-fold by K^+. When the enzyme is dialyzed in the presence of K^+ and absence of threonine, the purified enzyme is converted into a dimer (180,000), which is not inhibited by threonine and is only stimulated 1.5-fold by K^+. The enzyme also can polymerize under certain conditions to form higher molecular weight aggregates ranging in size up to 720,000, which also are catalytically active. This interconversion of homoserine dehydrogenase conformations may reflect the daily stream of events occurring *in vivo*. When light stimulates protein synthesis, the threonine pool decreases in the chloroplast, while K^+ concentrations increase. The change in threonine and K^+ concentrations shift the homoserine dehydrogenase from the threonine-sensitive to the threonine-insensitive conformation resulting in increased production of threonine, which would meet the demands of protein synthesis. The reverse process would occur in the dark.

The seeds of some crop plants are nutritionally deficient in certain essential amino acids. Barley, for example, is low in available lysine (11), while soybean is low in available methionine (25). Both of these amino acids are derived from aspartate. It may be possible to improve the nutritional value of some crop plants by understanding more about the enzymes and genes involved in amino acid biosynthesis and by modifying those genes to overproduce the desired amino acids.

HSDH[2] (EC1.1.1.3) is the branch point enzyme leading from aspartate to the synthesis of the essential amino acids threonine and methionine. It catalyzes the conversion of β-aspartate semialdehyde to L-homoserine in the presence of the coenzymes NADH or NADPH (3, 17), and is located mainly in the chloroplast (4, 23, 24, 26). In many higher plants (17–19, 22, 26), this enzyme activity is feedback inhibited by the pathway end product, L-threonine, and is stimulated in the presence of potassium ions. HSDH has been extensively studied in crude or partially purified extracts of maize (4, 5, 9, 17), soybean (20, 22), carrot (19, 21), barley (1, 26), and pea (2, 26). Only the enzyme in maize, however, has been subjected to rigorous purification and to some characterization (13, 14, 17, 27). In many plant systems HSDH exists in more than one form. Generally, one form is resistant to feedback inhibition by the pathway end product threonine, while the other forms are highly sensitive to threonine inhibition. In corn, several HSDH isoenzymes have been identified (9, 10, 17, 27). One low mol wt form was threonine insensitive, while the other, larger forms were threonine sensitive. The higher mol wt forms appear to interconvert to different states and are sensitive to *in vitro* environmental factors (9, 10, 13–15). All of these different enzyme states can be inhibited to varying degrees by threonine. There is no evidence indicating that these larger forms in maize deaggregate to form the low mol wt, threonine-insensitive form.

In crude and partially purified extracts of carrot, *Daucus carota*, cell suspension cultures, the HSDH activity is partially inhibited by threonine and is stimulated by K^+ (19, 21). Until now, separate threonine-sensitive and resistant forms have not been demonstrated in carrot.

In this paper we describe the purification to homogeneity and the characterization of distinct forms of HSDH from *D. carota*, demonstrate the reversible conversion of HSDH from a threonine-insensitive form to a threonine-sensitive form, and propose a role for these different enzyme conformations in pathway regulation with respect to threonine and K^+ interactions.

MATERIALS AND METHODS

Cell Cultures

Garden carrot (*Daucus carota* L. cv Danvers) cell suspension cultures were grown by inoculating 0.5 g fresh weight of cells into 250 mL flasks containing 100 mL defined liquid

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[2] Abbreviations: HSDH, homoserine dehydrogenase; DDCA, diethyldithiocarbamic acid.

medium as described previously (19). The cell cultures were periodically checked for microbial contamination by plating cells and medium on Difco nutrient agar (Difco Laboratories, Detroit, MI).[3]

Enzyme Extraction

Chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Cells were vacuum-filtered, resuspended, ruptured, and centrifuged as described previously (19), except that two volumes of cold Tris A buffer (25 mM Tris, 1.0 mM EDTA, 5.0 mM threonine, 20% glycerol [pH 7.6]) plus 1.73 g/l DDCA per gram was used as extraction buffer. The supernatant was fractionated using saturated $(NH_4)_2SO_4$. The pellet resulting from the 33 to 66% fractionation was resuspended and dialyzed overnight against a 100-fold excess of cold Tris A. The volume was adjusted to between one-third and one-half of the g fresh weight of the cells and heated at 55°C in a waterbath for 22 min, while mixing occasionally. The extract was immediately chilled on ice for 5 min. Two volumes of TET buffer (25 mM Tris, 1 mM EDTA, and 2 mM threonine [pH 7.6]) were added, and the preparation was centrifuged at 19,000*g* for 20 min at 4°C. The supernatant was injected onto an HPLC Protein Pak DEAE-5PW column (215 mm × 15 cm) (Millipore, Milford, MA). The flow rate of the column was 4.1 mL per min. The column was washed for 30 min with 150 mM KCl in TET buffer. The enzyme activity was eluted using a linear, 50 min gradient extending from 150 mM to 230 mM KCl. Fractions containing homoserine dehydrogenase of high specific activity were pooled and glycerol was added to 20%. At this point, the pool could be stored overnight at 4°C or frozen. The pool was then concentrated by precipitation with ammonium sulfate to 66%, resuspended, and dialyzed against TET buffer for 18 h, then injected into an HPLC ion exchange Bio-Gel TSK-DEAE-5PW (75 × 7.5 mm) column equilibrated with 25 mM Caps (3-(cyclohexylamino)-1-propanesulfonic acid) buffer (pH 10.0), containing 5 mM threonine. The column was washed for 60 min with 130 mM ammonium sulfate. The enzyme activity was eluted with a gradient from 100 to 200 mM ammonium sulfate. Fractions containing high amounts of activity were pooled, concentrated, and applied (500 μg/gel) to a 9% polyacrylamide slab gel with a 5% stacking gel using a Protean II electrophoresis unit (Bio-Rad, Richmond, CA). The enzyme was subjected to electrophoresis at 4°C with 10 mM Tris-glycine (pH 8.9) as the electrode buffer until the bromphenol blue migrated to 1 cm from the bottom of the gel (about 16 h). End gel lanes were stained specifically for homoserine dehydrogenase activity as previously reported (19). Gels incubated in staining mixture lacking homoserine were used as controls. Areas of the gel containing enzyme activity were excised and electroeluted for 6 h in a Tris borate (pH 8.3) buffer containing 0.3 mM EDTA using an ISCO Electrophoretic Concentrator model 750 (Lincoln, NE) according to manufacturer's instructions.

[3] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of the products or vendors that may be suitable.

Enzyme and Protein Assay

Homoserine dehydrogenase was assayed in the direction of coenzyme oxidation (forward direction) and coenzyme reduction (reverse direction) as described by Matthews and Widholm (19). The assay mixture for the reverse direction contained 0.2 M Tris buffer (pH 9.0), 15 mM homoserine, and either 15 mM NAD or 0.48 mM NADP. KCl (0.1 M) was included in the assay mixture except where otherwise noted. The assay mixture for the forward direction contained 0.2 M potassium-phosphate buffer (pH 7.0), 1.0 mM aspartate semialdehyde, and either 0.5 mM NADPH or 0.3 mM NADH in a total volume of 1 ml. The rate of activity in either direction was linear for at least 5 min and was corrected independently for any change in absorbance noted in the absence of substrate. One unit of homoserine dehydrogenase activity is equal to the amount of enzyme required to produce a change in A_{340} of 0.001/min. Assays lacking homoserine were used as controls. Protein was assayed using the Bio-Rad Protein Assay Kit and microassay procedure.

Analytical Electrophoresis

The purity of the enzyme was monitored by analytical electrophoresis using a Phast System (Phamacia, Uppsala, Sweden) on Phast Gel gradient (10–15%) gels stained for enzyme activity (19) or for protein using silver staining techniques (12). The subunit composition and enzyme purity were analyzed using Phast Gel gradient (10–15%) gels run with SDS buffer strips. Before application to the SDS gels, the enzyme samples were placed in 25% SDS containing 5% (v/v) β-mercaptoethanol, boiled for 10 min, and cooled on ice.

RESULTS

Homoserine dehydrogenase activity in crude and partially purified extracts of carrot cell suspension cultures can be measured readily in the reverse direction by monitoring the reduction of NAD in the presence of homoserine. The NAD-dependent activity of crude preparations is only 46% as active when in the presence of 10 mM threonine as compared to control assays lacking threonine, while the NADP-dependent activity is only 31% as active with 10 mM threonine present in the assay mixture. HSDH activity was purified over 6000-fold from this crude extract (Table I), from a specific activity of 0.03 to a specific activity of over 200. The two purification steps using HPLC greatly increased the specific activity of the preparation, while removing a number of contaminating proteins as monitored on electrophoretic gels (Fig. 1). The enzyme, however, was still not purified to homogeneity at this stage and contained several protein bands which possessed no demonstrable HSDH activity when the gel was stained for HSDH-specific activity. The separation of the major forms of HSDH and contaminating proteins by native gel electrophoresis was sufficient that a band of protein could be excised from the gel representing the major portion of HSDH activity. This single band of activity was electroeluted and further characterized.

Analysis of the purity of the electroeluted enzyme on native polyacrylamide gels indicated that the enzyme could poly-

Table I. *Purification of HSDH*

Sample[a]	Volume	Enzyme Units	Specific Activity[b]	Recovered	Purified
				%	*-fold*
Crude	610	384,024	0.03		
Ammonium sulfate	100	314,900	0.22	82	6.5
Heat denaturation	200	227,800	0.39	72	13
Preparative DEAE	82	141,386	14.6	62	487
Analytical DEAE	10.3	47,576	49.3	60	1,643
Preparative electrophoresis	0.3	13,000	>200	8–33%	>6,000

[a] Data from 10 extractions were averaged. At least 200,000 units of HSDH activity were present after ammonium sulfate precipitation. [b] Expressed as units/g protein.

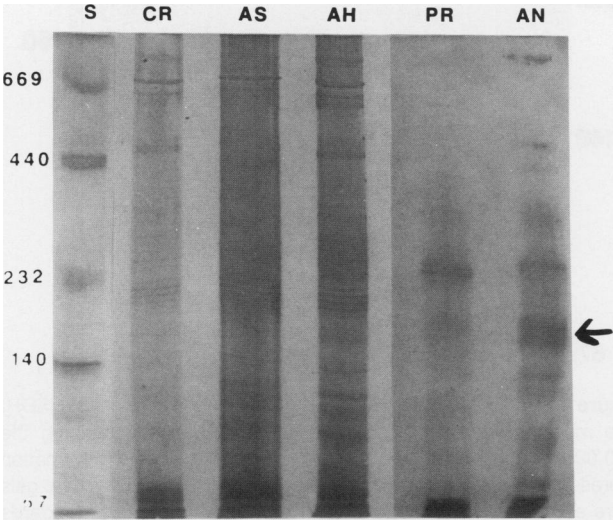

Figure 1. A silver-stained polyacrylamide gel for showing proteins copurifying with HSDH at different purification steps. Mol wt standards (S) were thyroglobulin 669 kD, ferritin 440 kD, catalase 232 kD, lactate dehydrogenase 140 kD, and BSA 67 kD. The lanes representing the original crude extract (Cr), protein after 65% ammonium sulfate precipitation (AS), after heat treatment (AH), after preparative DEAE HPLC column chromatography (PR), and after analytical DEAE HPLC chromatography (AN) contained 0.007, 0.032, 0.042, 1.15, and 4.8 units of HSDH activity, respectively, and each lane contained between 0.6 and 0.9 μg of protein. One μL sample was applied to a Phast (Pharmacia) (10–15%) gradient polyacrylamide gel and subjected to electrophoresis for 305 V h.

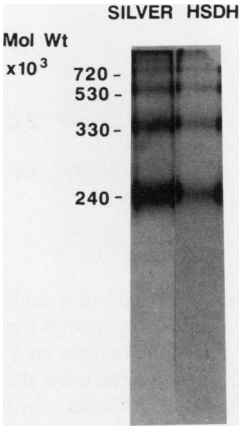

Figure 2. Aggregation of HSDH to form a polymeric ladder on a polyacrylamide gel after elution from a preparative polyacrylamide gel. Each lane contained 0.2 μg protein representing 12 units of HSDH activity. HSDH mol wt ranged from 240 to 720 kD.

merize into several medium to high mol wt forms (Fig. 2). The approximate mol wt of these polymers estimated from polyacrylamide gels were 240,000, 330,000, 530,000, and 720,000. Each of these forms possessed HSDH activity. Some of these forms had not been detected previously and may not exist *in vivo*, but may form only in highly purified fractions of HSDH.

To determine the subunit construction of these forms, the purified enzyme was analyzed on SDS electrophoretic gels (Fig. 3). Only one protein was present on SDS gels when different protein concentrations were used. It had a mol wt of 85,000 ± 5,000. The multiple high mol wt forms appearing on native gels therefore, probably represent trimeric (240,000), tetrameric, hexameric, and octameric (720,000) forms of the enzyme.

During isolation and purification, carrot HSDH activity was visualized on gradient PAGE gels predominantly in two areas. The mol wt coinciding with these two areas as judged by native PAGE were 180,000 and 240,000 (Fig. 4). This would suggest a dimer and a trimer, respectively. These results coincided with those obtained by gel filtration on SW 4000 columns (data not shown).

Efforts were made to shift HSDH from one form to the other by dialyzing aliquots of enzyme against buffers containing 5 mM threonine or 100 or 250 mM KCl in Tris buffer (pH 7.6). The resultant conformation of the enzyme was then determined by PAGE and enzyme specific staining. When the enzyme was dialyzed for 18 h against a Tris buffer containing 5 mM threonine (TT buffer), the 240,000 form (T form) was the only form of HSDH detected by activity staining of polyacrylamide gels. Protein staining of the gel indicated that protein was present at the location of the T form of HSDH, but not at the location of the absent K form. When the enzyme was dialyzed against a Tris buffer containing 250 mM KCl (TK), the 180,000 form (K form) was the only detectable form of HSDH by activity staining of PAGE gels. The enzyme could be redialyzed again to obtain the initial form of the enzyme. Protein staining of the gel indicated a loss of protein in the location of the T form and the concomitant appearance of the protein band at the location

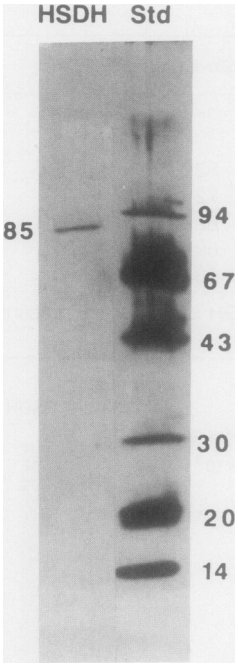

Figure 3. A silver-stained SDS gel of a purified sample of HSDH after electroelution. A 1 μL sample containing 0.2 μg of an 85 kD protein was subjected to electrophoresis on a Phast (10–15%) gradient gel for 90 V h. Mol wt standards were: phosphorylase b, 94 kD; BSA, 67 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20 kD; lactalbumin, 14.4 kD.

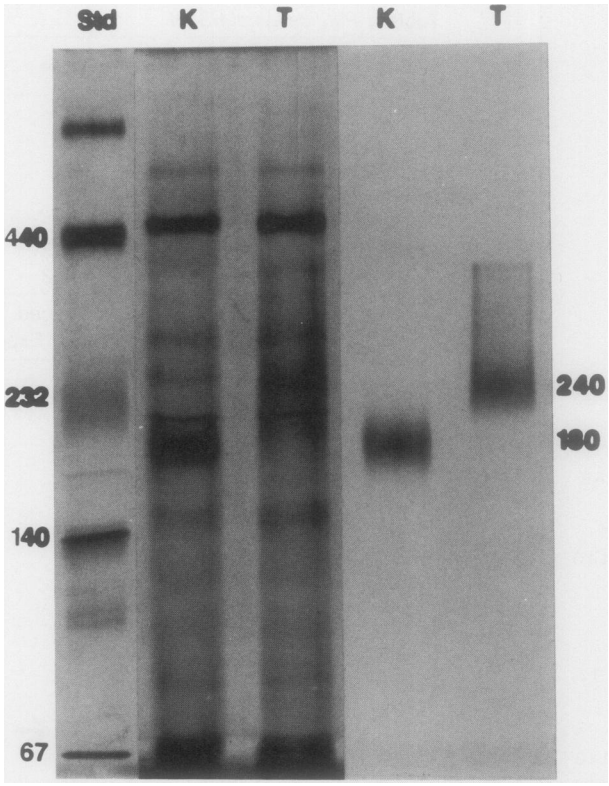

Figure 4. Polyacrylamide gel containing the K and T forms of HSDH. The mol wt standards are as in Figure 1. Lanes containing the 180,000 D (K) conformation and the 240,000 D (T) conformation represented 0.2 μg protein and 1.3 units of HSDH activity. The gels were silver stained or stained for HSDH activity. Mol wt standards were as in Figure 1.

of the K form. Incubation of HSDH in TT or TK buffer had little effect on total activity; however, the regulatory properties of the enzyme were markedly buffer-dependent (Table II). The T form of the enzyme is highly sensitive to inhibition by threonine and can be completely inhibited by 10 mM threonine. This is true regardless of whether the enzyme activity is assayed in the forward (coenzyme oxidation) or reverse (coenzyme reduction) direction. In contrast, the K form is not inhibited by threonine.

The T form of HSDH was stimulated about 2.6-fold by K^+, while the K form of the enzyme was stimulated about 1.6-fold. Continued exposure of the enzyme to K appears to induce a conformational change which is slow.

The differential sensitivity to inhibition by threonine by the K and T forms of the enzyme can be demonstrated visually by staining polyacrylamide gels for HSDH activity in the presence and absence of threonine (data not shown). The T form of HSDH does not stain in the gel in the presence of threonine, while the K form stains to the same degree with or without threonine.

In actuality, more than one form of HSDH was present in the K conformation area of the gel. These forms could be seen by increasing the time of electrophoresis and by staining the gel for HSDH activity for a shorter period of time, so that the activity bands did not diffuse together (Fig. 5). Three or four very close bands of HSDH activity could be distinguished on the gel. In a further effort to determine if these conformations could be altered or if new forms could be found, the enzyme was dialyzed in buffers containing combinations of several different effectors and these preparations were subjected to gel electrophoresis. Glycerol (20% v/v) had a mild effect on the balance of T and K forms by shifting the equilibrium toward the slowest migrating K form (Fig. 5).

Table II. *Comparison of HSDH Forms*

HSDH (about 15 units/assay) was assayed in the presence (+K) and absence (−K) of 200 mL KCl. In some assays threonine was present at 10 mM. The enzyme was shifted to the K form by overnight dialysis in the presence of 250 mM KCl and to the T form by dialysis in the presence of 5 mM threonine. Recoveries of enzyme activity ranged from 70 to 95%. At least 1000 units of enzyme activity were dialyzed in each experiment.

	Assay Supplement	Enzyme Form	
		T	K
NAD-dependent activity			
% inhibition by Thr	+K	100	0
	−K	100	0
K stimulation		2.57×	1.55×
NADP-dependent activity			
% Inhibition by Thr	+K	75	0
	−K	100	0
K stimulation		1.00×	2.40×
NAD/NADP	+K	1.81	2.40
	−K	0.65	1.96

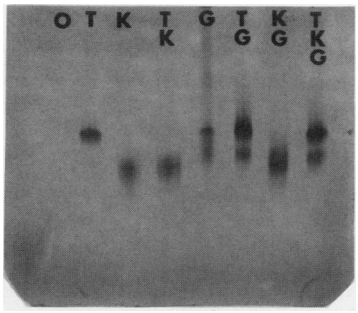

Figure 5. Multiple bands of the K conformation of HSDH and the effect of glycerol. No activity was present when HSDH was dialyzed overnight against 25 mM Tris (pH 7.6) with no additions (0). The K form was composed of 3 or 4 bands of activity (K). Other lanes contain the threonine conformation (T), or are preparations dialyzed overnight in the presence of glycerol and threonine (TG), KCl and glycerol (KG), or threonine, KCl, and glycerol (TKG). The lanes of the 10 to 15% gradient polyacrylamide gel contained 1 μL samples with 1.3 units of HSDH activity and 0.2 μg protein.

The addition of 10 mM dithiothreitol to the dialysis buffer with and without threonine, glycerol or KCl, or combinations thereof had no detectable effect on the enzyme conformation, nor did the pH from 5.3 to 9.5 (data not shown).

DISCUSSION

HSDH from carrot cell suspension cultures could be interconverted between threonine-sensitive and threonine-insensitive forms *in vitro*. This is in distinct contrast to the results obtained with HSDH extracted from maize cell suspension cultures (27). While the threonine-sensitive HSDH of maize cell suspension cultures (HSDH II) does not appear to interconvert between threonine-sensitive and insensitive forms, Krishnaswamy and Bryan (14) have demonstrated that the maize seedling enzyme can exist as a homodimer or a homotetramer with subunits of 86,000 mol wt, and that the relative amounts of these two forms are influenced by threonine. These forms are both inhibited by threonine.

The presence of an interconverting threonine-sensitive and threonine-insensitive form of HSDH in *Daucus carota* differs not only from results obtained from examinations of maize (3, 4, 9, 10, 27), but also with those of soybean (20, 22) and barley (1). Each of these species possesses separate threonine-sensitive and insensitive enzyme forms, which are probably different gene products. HSDH II is composed of two subunits with mol wt of 89,000 and 93,000 (27). The threonine-insensitive HSDH from maize cell suspension cultures (HSDH I) is a 70,000 mol wt dimer consisting of two 38,000 subunits (27). Maize cell suspension culture HSDH isozymes I and II could not be interconverted *in vitro*. The subunit mol wt of the maize seedling HSDH II (86,000) is similar to that of carrot cell suspension culture HSDH (85,000).

The threonine-resistant form of carrot HSDH (the K conformation) has a mol wt of 180,000. Presumably, it may be a homodimer, composed of two 85,000 subunits. The threonine-sensitive form (K-conformation), with a native mol wt of 240,000, is probably a homotrimer. Higher mol wt forms of carrot HSDH can exist *in vitro*, creating a mol wt ladder with increments of 85,000. Interestingly, HSDH from *Rhodospirillum rubrum* (subunit = 48,000), can also aggregate to form a threonine-sensitive form in the presence of threonine (6–8, 16). As the pH of the incubation buffer is decreased from 7.1 to 6.3, the amount of aggregation of *R. rubrum* HSDH increases from 20 to 80% (7). Increasing the threonine concentration results in an increase in polymerization of the enzyme, and at 20 mM threonine, almost all of the enzyme exists as polymeric species. Catalytically active aggregates ranging from 300,000 to 2×10^6 can be detected by PAGE and by gel filtration (6). In contrast to *R. rubrum* HSDH, the aggregation of carrot HSDH was not dependent upon pH being between pH 5.8 and pH 6.8 (data not shown). Also, while threonine favored the appearance of the trimeric (240,000) form of the enzyme, it did not appear to cause the formation of higher mol wt forms of the enzyme.

DiCamelli and Bryan (9) reported that other cations stimulate HSDH activity, but to a lesser extent. The order of HSDH activity activation was $K^+ > Na^+ > NH_4^+ > Li^+ > Rb^+$. We have seen a shift of HSDH to the K conformation in the presence of $(NH_4)_2SO_2$ as well as in the presence of K^+.

HSDH from carrot cell suspension cultures was purified over 6000-fold to apparent homogeneity using ion exchange chromatography and preparative electrophoresis. Previously, HSDH has been purified to homogeneity from maize seedlings (14) and maize cell suspension cultures (27). The HSDH from maize seedlings had to be purified over 5000-fold to obtain homogeneity, while HSDH forms I and II from maize cell suspension cultures were purified 1800- and over 5000-fold, respectively. HSDH from maize cell suspension culture was purified using affinity columns, including Octyl Sepharose, Blue Sepharose, and ADP-agarose (26). Krishnaswamy and Bryan (14) used ADP-agarose and immunoaffinity chromatography to purify HSDH from maize seedlings, but their attempts to use Blue Sepharose and Octyl Sepharose were unsuccessful. We also found these latter two column matrices unsuitable for purification of HSDH from carrot suspension culture cells (our unpublished data).

Interconversion of HSDH in carrot among threonine-inhibitable and threonine-uninhibitable conformations may provide a mechanism for controlling threonine production *in vivo*. As threonine levels decrease in the carrot cell, the equilibrium among the enzyme forms may shift to increase the amount of the K conformation which is not inhibited by threonine. This would allow increased threonine production. When the threonine levels are increased beyond the needs of the cell, the equilibrium of the enzyme forms then may shift toward the T form which is strongly inhibited by threonine, thus decreasing threonine production. If this mechanism of threonine regulation does occur *in vivo* in carrot, then it appears to be different from that found in corn and soybean, which have at least two molecularly distinct forms of HSDH which do not interconvert.

LITERATURE CITED

1. **Aarnes H** (1977) A lysine-sensitive aspartate kinase and two molecular forms of homoserine dehydrogenase from barley seedlings. Plant Sci Lett **9**: 137–145
2. **Aarnes H, Rognes SE** (1974) Threonine-sensitive aspartate ki-

nase and homoserine dehydrogenase from *Pisum sativum*. Phytochemistry **13:** 2713–2724

3. **Bryan JK** (1980) The synthesis of the aspartate family and the branched chain amino acids. *In* BJ Miflin, ed, The Biochemistry of Plants: A Comprehensive Treatise, Vol 5. Academic Press, New York, pp 403–452
4. **Bryan JK, Lissik EA, Matthews BF** (1977) Changes in enzyme regulation during growth of maize. III Intracellular location of homoserine dehydrogenase in chloroplasts. Plant Physiol **59:** 673–679
5. **Bryan JK, Lochner NR** (1981) Quantitative estimates of the distribution of homoserine dehydrogenase isozymes in maize tissues. Plant Physiol **68:** 1400–1405
6. **Datta P** (1970) Homoserine dehydrogenase of *Rhodospirillum rubrum*. II Purification and characterization of the enzyme. J Biol Chem **245:** 5779–5787
7. **Datta P, Epstein CC** (1973) Homoserine dehydrogenase of *Rhodospirillum rubrum*. Enzyme polymerization in the presence and absence of threonine. Biochemistry **12:** 3888–3892
8. **Datta P, Gest H, Segal HL** (1964) Effects of feedback modifiers on the state of aggregation of homoserine dehydrogenase of *Rhodospirillum rubrum*. Biochemistry **51:** 125–130
9. **DiCamelli CA, Bryan JK** (1975) Changes in enzyme regulation during growth in maize. II. Relationships among multiple molecular forms of homoserine dehydrogenase. Plant Physiol **55:** 999–1005
10. **DiCamelli CA, Bryan JK** (1980) Comparison of sensitive and desensitized forms of maize homoserine dehydrogenase. Plant Physiol **65:** 176–183
11. **Fuller MF, Mennie I, Crofts RMJ** (1979) Amino acid supplementation of barley for the growing pig No. 2, optimal additions of lysine and threonine for growth. Br J Nutr **4:** 333–340
12. **Heukeshoven J, Dernick R** (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6:** 103–112
13. **Krishnaswamy S, Bryan JK** (1983) Ligand-induced interconversions of maize homoserine dehydrogenase among different states. Arch Biochem Biophys **222:** 449–463
14. **Krishnaswamy S, Bryan JK** (1983) Characterization of ligand-induced states of maize homoserine dehydrogenase. Arch Biochem Biophys **227:** 210–224
15. **Krishnaswamy S, Bryan JK** (1986) Use of monoclonal antibodies for the purification and characterization of the threonine-sensitive isozyme of maize homoserine dehydrogenase. Arch Biochem Biophys **246:** 250–262
16. **Mankovitz R, Segal HL** (1969) Dissociation-produced loss of regulatory control of homoserine dehydrogenase of *Rhodospirillum rubrum*. II Some properties of the regulatable and nonregulatable forms. Biochemistry **8:** 3765–3767
17. **Matthews BF, Gurman AW, Bryan JK** (1975) Changes in enzyme regulation during growth of maize. I Progressive desensitization of homoserine dehydrogenase during seedling growth. Plant Physiol **55:** 991–998
18. **Matthews BF, Reardon EM, Turano FJ, Wilson BJ** (1988) Amino acid biosynthesis in plants: approaching an understanding at the molecular level. Plant Molec Biol Reporter **6:** 137–154
19. **Matthews BF, Widholm JM** (1978) Regulation of lysine and threonine synthesis in carrot cell suspension cultures and whole carrot roots. Planta **141:** 315–321
20. **Matthews BF, Widholm JM** (1979) Enzyme expression in soybean cotyledon, callus, and cell suspension culture. Can J Bot **57:** 299–304
21. **Matthews BF, Widholm JM** (1979) Expression of aspartokinase, dihydrodipicolinic acid synthase and homoserine dehydrogenase during growth of carrot cell suspension cultures on lysine- and threonine-supplemented media. Z Naturforsch **34c:** 1177–1185
22. **Matthews BF, Widholm JM** (1979) Regulation of homoserine dehydrogenase in developing organs of soybean seedlings. Phytochemistry **18:** 395–400
23. **Mills WR, Lea PJ, Miflin BJ** (1980) Photosynthetic formation of the aspartate family of amino acids in isolated chloroplasts. Plant Physiol **65:** 1166–1172
24. **Mills WR, Wilson KG** (1978) Amino acid biosynthesis in isolated pea chloroplasts: Metabolism of labeled aspartate and sulfate. FEBS Lett **92:** 129–132
25. **Russell LE, Cromwell GL, Stahley TS** (1983) Tryptophan, threonine, isoleucine, methionine supplementation of twelve percent protein, lysine supplemented corn-soybean meal diet for growing pigs. J Animal Sci **56:** 1115–1123
26. **Sainis JK, Mayne RG, Wallsgrove RM, Lea PJ, Miflin BJ** (1981) Localisation and characterisation of homoserine dehydrogenase isolated from barley and pea leaves. Planta **152:** 491–496
27. **Walter TJ, Connelly JA, Gengenbach BG, Wold F** (1979) Isolation and characterization of two homoserine dehydrogenases from maize suspension cultures. J Biol Chem **254:** 1349–1355