Plant Physiol. (1992) 98, 1139–1147
0032-0889/92/98/1139/09/$01.00/0

Received for publication September 3, 1991
Accepted November 12, 1991

Partial Purification and Characterization of Lysine-Ketoglutarate Reductase in Normal and Opaque-2 Maize Endosperms[1]

Marcia R. Brochetto-Braga[2], Adilson Leite, and Paulo Arruda*

Departamento de Genética e Evolução, Instituto de Biologia (M.R.B.-B., P.A.), and Centro de Biologia Molecular e Engenharia Genética (A.L., P.A.), Universidade Estadual de Campinas, 13081 Campinas, SP, Brazil

ABSTRACT

Lysine-ketoglutarate reductase catalyzes the first step of lysine catabolism in maize (*Zea mays* L.) endosperm. The enzyme condenses L-lysine and α-ketoglutarate into saccharopine using NADPH as cofactor. It is endosperm-specific and has a temporal pattern of activity, increasing with the onset of kernel development, reaching a peak 20 to 25 days after pollination, and thereafter decreasing as the kernel approaches maturity. The enzyme was extracted from the developing maize endosperm and partially purified by ammonium-sulfate precipitation, anion-exchange chromatography on DEAE-cellulose, and affinity chromatography on Blue-Sepharose CL-6B. The preparation obtained from affinity chromatography was enriched 275-fold and had a specific activity of 411 nanomoles per minute per milligram protein. The native and denaturated enzyme is a 140 kilodalton protein as determined by polyacrylamide gel electrophoresis. The enzyme showed specificity for its substrates and was not inhibited by either aminoethyl-cysteine or glutamate. Steady-state product-inhibition studies revealed that saccharopine was a noncompetitive inhibitor with respect to α-ketoglutarate and a competitive inhibitor with respect to lysine. This is suggestive of a rapid equilibrium-ordered binding mechanism with a binding order of lysine, α-ketoglutarate, NADPH. The enzyme activity was investigated in two maize inbred lines with homozygous normal and *opaque-2* endosperms. The pattern of lysine-ketoglutarate reductase activity is coordinated with the rate of zein accumulation during endosperm development. A coordinated regulation of enzyme activity and zein accumulation was observed in the *opaque-2* endosperm as the activity and zein levels were two to three times lower than in the normal endosperm. Enzyme extracted from L1038 normal and *opaque-2* 20 days after pollination was partially purified by DEAE-cellulose chromatography. Both genotypes showed a similar elution pattern with a single activity peak eluted at approximately 0.2 molar KCL. The molecular weight and physical properties of the normal and *opaque-2* enzymes were essentially the same. We suggest that the *Opaque-2* gene, which is a transactivator of the 22 kilodalton zein genes, may be involved in the regulation of the lysine-ketoglutarate reductase gene in maize endosperm. In addition, the decreased reductase activity caused by the *opaque-2* mutation may explain, at least in part, the elevated concentration of lysine found in the *opaque-2* endosperm.

There is little information on lysine catabolism in higher plants. Most of the available data were obtained in studies on the incorporation and metabolism of radiolabeled precursors by plant tissues. Feeding experiments with [^{14}C]lysine demonstrated the incorporation of radioactivity into α-amino adipic acid and glutamic acid in wheat (18) and into saccharopine and diaminopimelic acid in maize and barley (16, 26). In developing endosperm of maize and barley, radiolabeled lysine is incorporated primarily into glutamic acid and proline (4, 26). These findings indicate that lysine is catabolized in plants via the saccharopine pathway.

The first enzymatic evidence for the operation of the saccharopine pathway for lysine catabolism in plants was obtained with the demonstration of LKR[3] activity in immature endosperm of maize (3). LKR (EC 1.5.1.8) condenses lysine and α-ketoglutarate into saccharopine using NADPH as cofactor.

An understanding of the pathways for lysine biosynthesis and degradation in plants has enormous importance because of the limiting concentration of this essential amino acid in major food sources such as cereals. Valuable information can be obtained by the elucidation of the properties of enzymes involved in the biosynthesis and catabolism of lysine and by the use of mutants in which the activities of the enzymes are altered.

Since the discovery of the superior nutritive value of the high lysine maize mutant *opaque-2* (15), there have been many studies on the effects of this mutant gene on protein and amino acid metabolism in maize endosperm. The *opaque-2* gene is located on the short arm of chromosome 7 and its major effect is the reduction of the maize storage protein zein. This is a complex of polypeptides coded by a multigenic family located on chromosomes 4 and 7 (23). The *opaque-2* gene in the homozygous form reduces the zein content of the endosperm by up to 70% (6). The reduction is

[1] This work was supported in part by grants to P.A. from Financiadora de Estudos e Projetos and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). M.R.B.-B. received postgraduate fellowships from Fundação de Amparo a Pesquisa do Estado de São Paulo and CNPq. P.A. and A.L. received research fellowships from CNPq. This research constituted part of the Ph.D. thesis of M.R.B.-B.

[2] Present address: Depto Biologia, Instituto de Biociências, UNESP/Rio Claro, Av. 24A, no 1515, 13500, Rio Claro, SP, Brazil.

[3] Abbreviations: LKR, lysine-ketoglutarate reductase [L-lysine: α-ketoglutarate: NADPH oxidoreductase (*E*-*N*-(glutaryl-2)-lysine forming)]; DAP, days after pollination.

due mainly to a decreased synthesis of the 22 kD α-zeins (12). The *opaque-2* gene was recently cloned (17, 22). The gene encodes a 47 to 48 kD DNA-binding protein (11, 21) that binds to the 22 kD α-zein gene promoter and regulates its transcription (21).

Other proteins and enzymes are also affected by the *opaque-2* mutation. For example, ribonuclease activity is two- to sixfold higher in *opaque-2* endosperm than in normal endosperm (30). The *opaque-2* endosperm lacks an albumin of 32 kD (b-32) with unknown function whose accumulation pattern is coordinated with zein accumulation in normal endosperms (24). The gene encoding b-32 is transactivated by the *opaque-2* gene product (14). The *opaque-2* mutation also affects lysine catabolism. Feeding experiments with [^{14}C]lysine have demonstrated that the amino acid is degraded to a lesser extent in the *opaque-2* than in normal endosperm (26). Decreased lysine degradation has also been observed in the endosperm of a high lysine barley mutant (4).

In this paper, we report the partial purification and characterization of LKR from immature maize endosperm and the activity of LKR in normal and *opaque-2* endosperms during kernel development. The results are discussed in the context of the properties and regulation of enzyme activity.

MATERIALS AND METHODS

Chemical and Chromatographic Materials

L-Lysine, α-ketoglutaric acid, NADPH, DEAE-cellulose, and other chemicals were purchased from Sigma Chemical Company, Pharmacia Fine Chemicals, E. Merck Darmstadt, and Riedel-de Haen.

Plant Material

Maize (*Zea mays* L.) inbred lines were from the collection of Universidade Estadual de Campinas. The inbred lines L1038 and Cat-100–1, both containing homozygous normal and *opaque-2* counterparts, were obtained by repeated self-pollination of heterozygous plants after at least six backcrosses with a source of *opaque-2* mutant provided by D. V. Glover (Purdue University). Plants were grown in the field, self-pollinated, and harvested from 10 to 50 DAP at 3- or 5-d intervals and stored frozen at −20°C.

Extraction and Quantification of Zein

The endosperms were freeze-dried, ground to a flour, and defatted with acetone. After extraction of soluble proteins with 0.5 M NaCl, zein was extracted at room temperature with 55% isopropanol containing 2% 2-mercaptoethanol according to the method of Paulis *et al.* (20). The nitrogen content of zein extracts was determined by the method of Nkonge and Ballance (19).

LKR Assay

The enzyme was assayed at 30°C in a 1 mL reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.0, 20 mM L-lysine, 10 mM α-ketoglutarate neutralized with KOH, 0.1 mM NADPH, and 0.03 to 0.2 mg protein. Oxidation of NADPH was monitored at 340 nm. One unit of activity was defined as the amount of enzyme required for the oxidation of 1 nmol NADPH min^{-1} at 30°C. The protein concentrations in the enzyme extracts were determined as described by Spector (27).

Preparation of Enzyme Extract

Immature endosperms of inbred line L1038 were hand-dissected from the seeds and homogenized in a Waring blender with buffer A (0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM DTT, 15% glycerol). The homogenate was filtered through four layers of cheesecloth and centrifuged at 22,100*g* for 30 min.

Solid ammonium sulfate was slowly added to the supernatant solution at 4°C to give 35% saturation. The solution was kept at 4°C for 1 h and the precipitate was removed by centrifugation at 22,100*g* for 20 min. The supernatant fluid was brought to 60% saturation with solid ammonium sulfate and, after centrifugation, the pellet was resuspended in a minimal volume of buffer A and desalted by filtration through a Sephadex G-25 column. The desalted fraction was centrifuged at 105,000*g* for 1 h at 4°C.

DEAE-Cellulose Chromatography

The supernatant fraction from ultracentrifugation was diluted to a protein concentration of 3 mg mL^{-1} and applied to a column (18 × 2.5 cm i.d.) of DEAE-cellulose previously equilibrated with buffer A. The column was washed with buffer A until $A_{280} < 0.05$ and then eluted with a linear gradient of 0 to 0.5 M KCl in buffer A at a flow rate of 40 mL h^{-1}. Fractions of 5 mL containing LKR activity were pooled, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 23,700*g* for 20 min at 4°C. The pellet was resuspended in a minimal volume of buffer A and dialyzed overnight against 2.7 L of the same buffer.

Affinity Chromatography

The dialyzed sample was applied to a Blue-Sepharose CL-6B column (8 × 1.5 cm i.d.) equilibrated with buffer A. The column was washed with buffer A and eluted with a 30 mL, 0 to 15 mM NADPH gradient in buffer A at a flow rate of 6.6 mL h^{-1}. Successive 2-mL fractions were collected, dialyzed individually against buffer A, and assayed for LKR activity. The fractions containing enzyme activity were dialyzed against 2 × 2 L 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM DTT.

PAGE

Discontinuous PAGE of native proteins was performed at pH 7.0 and 4°C in 5 to 10% gradient slab gels. Enzyme activity in the gels was detected by a modification of the method of Susor and Rutter (28). After electrophoresis, the gel was washed with 0.1 M potassium phosphate buffer, pH 7.0, and incubated at 30°C for 1.5 h in 0.1 M potassium phosphate buffer (pH 7.0) containing 20 mM L-lysine, 10 mM α-ketoglutarate, and 0.5 mM NADPH. At the end of the incubation period, excess NADPH was removed by soaking

the gel for 10 min in 0.1 M phosphate buffer (pH 7.0). The gel was immediately placed on the surface of a transilluminator with UV light at 260 nm and photographed. In these experiments, the electrophoresed gel was divided into duplicate sections, one of which was used to locate the enzyme activity, as described above. The other section was stained with Coomassie brilliant blue R-250 to locate protein bands.

SDS-PAGE of denatured proteins was performed in 5 to 20% gradient slab gels. The apparent monomeric molecular mass of LKR was estimated by comparison with standard molecular mass markers. Proteins were stained with Coomassie brilliant blue R-250 or a combined Coomassie blue-silver nitrate procedure (7).

Kinetic Studies

Samples of LKR taken from the most active fractions from an independent DEAE-cellulose chromatography experiment were used for kinetic studies. Steady-state product inhibition studies were performed and the results interpreted by a Hanes plot using the ENZFITER program (13).

RESULTS

Preparation and Purification of LKR

For enzyme purification, a crude extract was prepared by homogenizing 3000 20-DAP endosperms in buffer A. The sequential steps of enzyme purification are shown in Table I. For the routine monitoring of enzyme activity, except for crude extract, the fractions obtained in each step were desalted through Sephadex G-25 columns. Over 95% of the LKR activity units was recovered in the 35 to 60% ammonium sulfate fraction. This preparation was enriched 3.5-fold in relation to the crude extract. The enzyme activity recovered in the 35 to 60% ammonium sulfate fraction was stable for up to 2 weeks when stored at 0 to 4°C. This fraction was clarified, desalted, and small amounts of precipitated protein were sedimented by centrifugation at 105,000*g* for 1 h. This increased the specific activity by 45% in relation to the 35 to 60% ammonium sulfate fraction. An increased percentage of recovery of total activity was observed after ammonium sulfate fractionation and ultracentrifugation (Table I). This could be explained by the fact that crude extract was assayed without desalting given underestimated values. After ultracentrifugation, the desalted preparation was loaded onto a DEAE-cellulose column and eluted with a KCl gradient in buffer A (Fig. 1). A large amount of protein lacking LKR activity did not bind to the column. A single peak of activity eluted between 0.2 and 0.3 M KCl. The specific activity of the most active fraction of this preparation was enhanced 97-fold in relation to the crude extract (Table I). The purest preparation was obtained after binding the most active fractions from DEAE-cellulose chromatography onto a Blue Sepharose CL-6B column, which was then eluted with a 0 to 15 mM NADPH gradient (Fig. 2). The addition of different combined concentrations of L-lysine and α-ketoglutarate together with NADPH did not improve the elution of enzyme from the affinity column (data not shown). The total recovered enzyme activity after affinity chromatography was 13%, corresponding to a specific activity of 411 nmol min^{-1} mg^{-1} protein. The purified fraction was enriched 275-fold (Table I).

Apparent Molecular Mass

The preparations obtained at each step of enzyme purification were subjected to SDS-PAGE. A band of 140 kD was enriched after the purification steps and became the predominant band in the affinity chromatography fraction (Fig. 3, lane 5). Minor contaminant bands of lower molecular mass were present after the final step of enzyme purification. To verify that the predominant protein in the affinity chromatography preparation represented the LKR enzyme, this fraction was subjected to nondenaturating PAGE. Part of the gel was stained for protein and the remainder was used to locate enzyme activity. The predominant band of 140 kD present in the affinity chromatography preparation (Fig. 4, lane 1) corresponded to the LKR activity detected in the duplicate gel (Fig. 4, lane 2).

Substrate Specificity

The substrate specificity of LKR has been tested previously by adding several amino acids and ketoacids such as L-ornithine, L-glutamine, L-asparagine, D-lysine, diaminopimelic acid, pyruvic acid, and oxalocetate to the incubation

Table I. *Purification of Lysine-Ketoglutarate Reductase from Immature Maize Endosperms Harvested at 20 DAP*

Purification Step	Volume	Total Protein	Total Activity	Specific Activity	Recovery
	mL	*mg/mL*	*units*[a]	*units/mg*	%
Crude extract	1158	3.7	6514	1.5	100
35–60% $(NH_4)_2SO_4$ precipitate	76	20.2	8023	5.2	123
Ultracentrifugation at 105,000*g*	76	15.4	8919	7.6	137
DEAE-cellulose chromatography	3	5.8	2534	146.0	39
Blue-Sepharose chromatography	8	0.3	822	411.0	13

[a] nmol NADPH min^{-1} at 30°C.

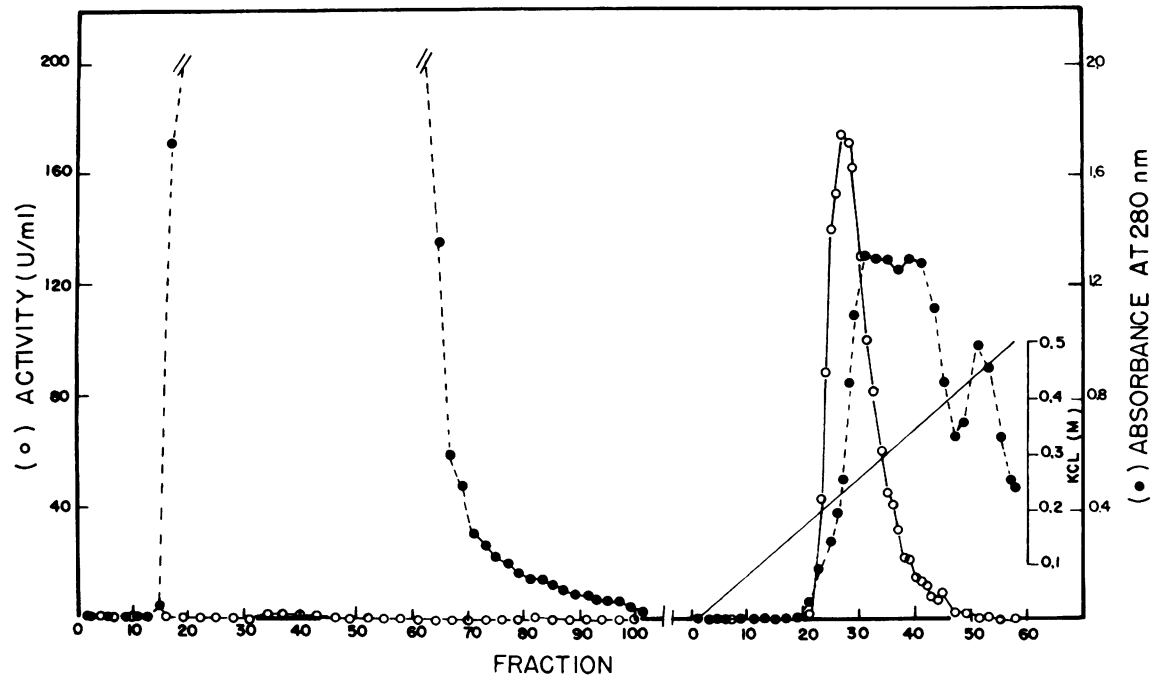

Figure 1. Elution pattern of LKR activity and protein from the DEAE-cellulose column. (○) Enzyme activity; (●) protein measured by absorbance at 280 nm; (—) KCl concentration gradient.

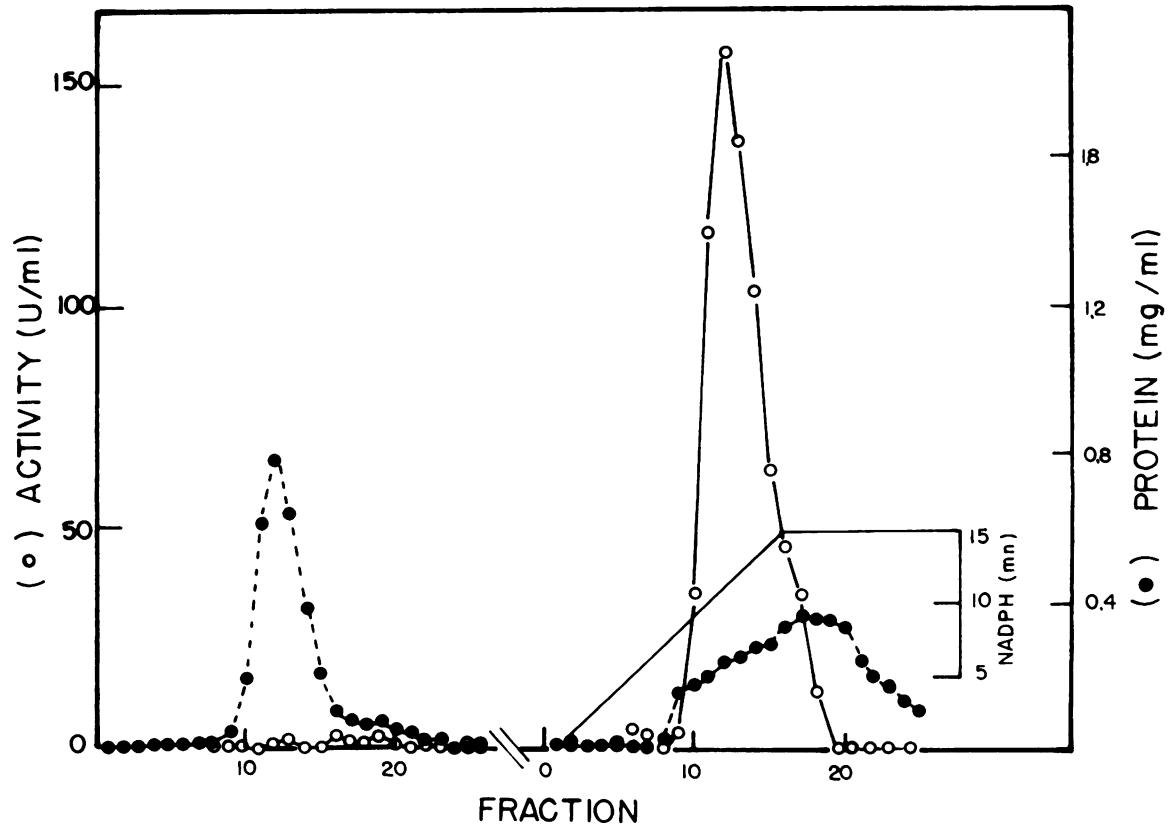

Figure 2. Elution pattern of LKR activity and protein from Blue-Sepharose CL-6B column. (○) Enzyme activity; (●) protein measured by the Coomassie blue method; (—) NADPH concentration gradient.

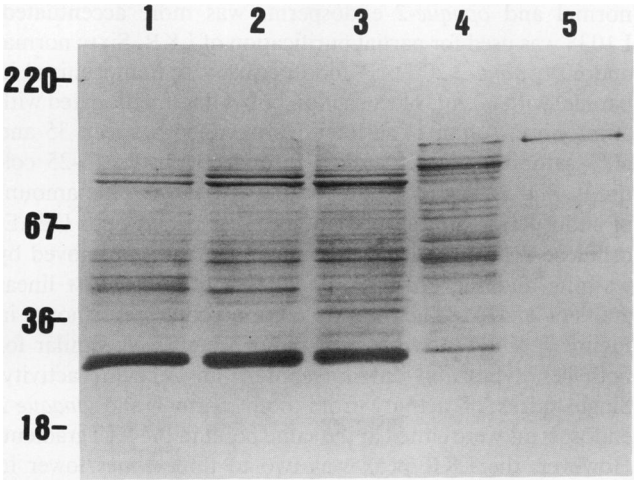

Figure 3. SDS-PAGE of enzyme purification steps. The denaturated proteins were subjected to electrophoresis through a 5 to 20% gradient slab gel. The numbers at the left indicate the following standard proteins: ferritin, 220 kD; albumin, 67 kD; lactate dehydrogenase, 36 kD; ferritin subunit, 18.5 kD. Lane 1, crude extract; lane 2, 35 to 60% ammonium sulfate precipitate; lane 3, ultracentrifugation at 105,000*g*; lane 4, DEAE-cellulose column chromatography; lane 5, Blue-Sepharose CL-6B chromatography. Lanes were loaded with 1 to 10 μg of protein.

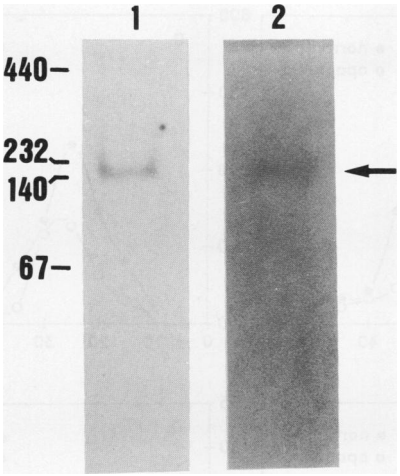

Figure 4. PAGE under nondenaturating conditions of the enzyme preparation obtained after chromatography on Blue-Sepharose CL-6B. The numbers at the left indicate the following standard proteins: ferritin, 440 kD; catalase, 232 kD; lactate dehydrogenase, 140 kD; albumin, 67 kD. Lane 1, protein stained with Coomassie blue-silver; lane 2, duplicate gel assayed for LKR activity. The arrow indicate the band of LKR activity.

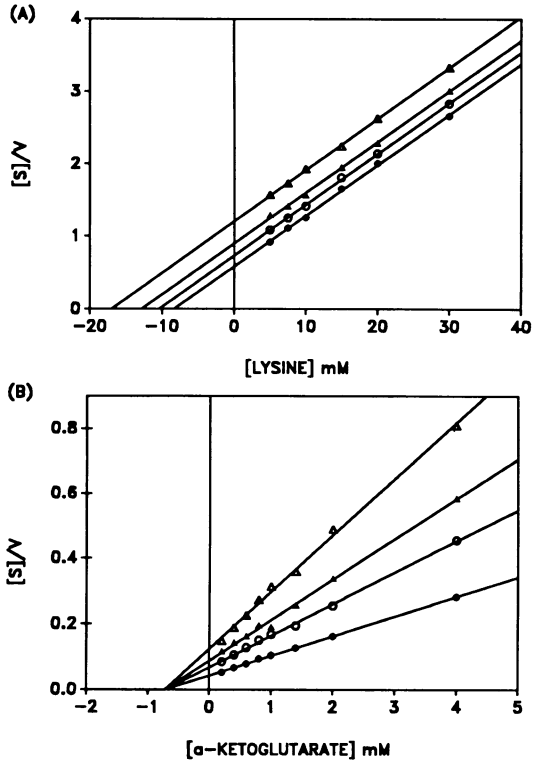

Figure 5. Inhibition of lysine-ketoglutarate reductase activity by saccharopine. A, Lysine concentration was varied from 0 to 30 mM. Saccharopine concentrations were 0 (●), 0.1 (○), 0.2 (▲), and 0.4 (△) mM. B, α-Ketoglutarate concentrations were varied from 0 to 4 mM. Saccharopine concentrations were 0 (●), 0.1 (○), 0.2 (▲), 0.4 (△) mM. In A and B, NADPH was at 0.1 mM.

mixture. None of these compounds was able to substitute for L-lysine or α-ketoglutarate (2, 3). The enzyme was active with NADPH, but not with NADH (3). In addition, the capacity of *S*-2-aminoethyl-L-cysteine and L-glutamate to substitute for substrates and/or inhibit LKR activity was tested. Neither compound could substitute for L-lysine or α-ketoglutarate or inhibit LKR activity (data not shown).

Kinetic Analysis

Steady-state product-inhibition analyses revealed that saccharopine was a competitive inhibitor with respect to lysine (Fig. 5A), with a K_i value of 0.14 mM as estimated by the replot of the data from Figure 5A (saccharopine concentration *versus* K_m). Saccharopine was a noncompetitive inhibitor with respect to α-ketoglutarate (Fig. 5B), with a K_i value of 0.21 mM as estimated by a replot of the data from Figure 5B (saccharopine concentration *versus* 1/kkat). It was not possible to determine the kinetic properties of LKR with respect to NADPH under the experimental conditions used.

Coordinate Regulation of Zein Synthesis and LKR Activity by the *opaque-2* Mutation

Zein begins to accumulate in both normal and *opaque-2* endosperms around 10 DAP. After a short lag phase up to 12

to 15 DAP, zein increases exponentially, reaching its maximum rate of accumulation at 25 DAP (Fig. 6). It is important to note that zein accumulation reaches its maximum rate at the time of maximum LKR activity (see below). As shown in Figure 6, the *opaque-2* mutation reduces zein accumulation in both inbred lines, but the reduction is higher in L1038 (Fig. 6A) than in Cat-100–1 (Fig. 6B). The reduction in zein synthesis affected primarily the accumulation of the 22 kD α-zein in both inbred lines (data not shown). These results demonstrate that the *opaque-2* mutation in our inbred lines exhibits the same characteristic alterations in zein synthesis that have been reported by other investigators (12).

LKR assays were carried out with endosperms obtained from the same ears as those used for the zein studies. Crude extracts, prepared in parallel from 20 normal and *opaque-2* endosperms sampled at each developmental stage, were fractionated with ammonium sulfate, desalted through Sephadex G-25 columns, and assayed for LKR activity. The enzyme activity was characterized by a similar developmental pattern in all materials analyzed, increasing in immature kernels, reaching a peak at an intermediate stage of development and decreasing as the kernels approached maturity (Fig. 6). However, the total activity was twofold higher in L1038 (Fig. 6C) than in Cat-100–1 (Fig. 6D). In general, this difference between inbred lines was also observed for zein accumulation and it may reflect differences in the genetic background of the inbred lines. Great differences in endosperm RNase activity have been observed in different maize inbred lines and hybrids (29). The LKR activity of *opaque-2* endosperms was two to three times lower than that of normal endosperms. As was observed for zein synthesis, the effect of the *opaque-2* mutation on LKR activity was more marked in L1038 (Fig. 6C) than in the Cat-100–1 inbred line (Fig. 6D).

Because the enzyme activity of normal endosperm was higher in the L1038 inbred line and the difference between normal and *opaque-2* endosperms was more accentuated, L1038 was used for partial purification of LKR. Sixty normal and 60 *opaque-2* 20-DAP endosperms were homogenized in parallel with 60 mL of extraction buffer and fractionated with ammonium sulfate. The fractions obtained between 35 and 60% saturation were desalted through Sephadex G-25 columns, and 22 mg of protein, representing the same amount of endosperm from each genotype, was loaded onto DEAE-cellulose columns. The unbound proteins were removed by washing and the enzyme eluted with a 0 to 0.5 M linear gradient of KCl. The enzyme elution profiles are shown in Figure 7. The amount of unbound protein was similar for both genotypes and did not contain any enzyme activity. Single peaks of activity from both normal and *opaque-2* endosperms were eluted at the same point in the KCl gradient. However, the LKR peak was two to three times lower in *opaque-2* than in normal endosperm. These partially purified fractions were used for a comparison of enzyme properties of normal and *opaque-2* endosperms. Both enzymes possessed similar physical and electrophoretic properties (data not shown).

Extracts from different seed and seedling tissues from normal and *opaque-2* genotypes were prepared using the same methodology that was utilized in the endosperm studies. No LKR activity was detected in extracts from immature embryos, shoots, or roots (data not shown).

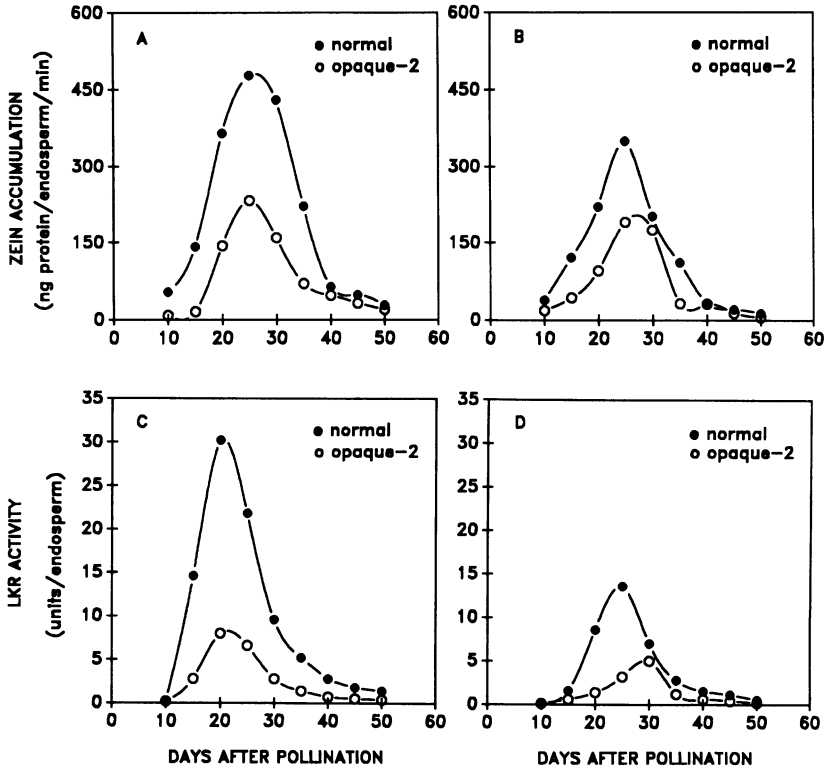

Figure 6. Zein accumulation and lysine-ketoglutarate reductase activity in normal (●) and *opaque-2* (○) endosperms. Zein was extracted from freeze-dried immature L1038 (A) and Cat-100–1 (B) inbred line endosperms with 55% isopropanol containing 2% 2-mercaptoethanol. Immature L1038 (C) and Cat-100–1 (D) endosperms sampled during kernel development were homogenized with extraction buffer and fractionated with ammonium sulfate. The fractions obtained between 35 and 60% saturation were desalted through Sephadex G-25 columns and assayed for enzyme activity. Equal amounts of endosperm were used for each genotype at each stage of kernel development. Each point represents duplicate assays.

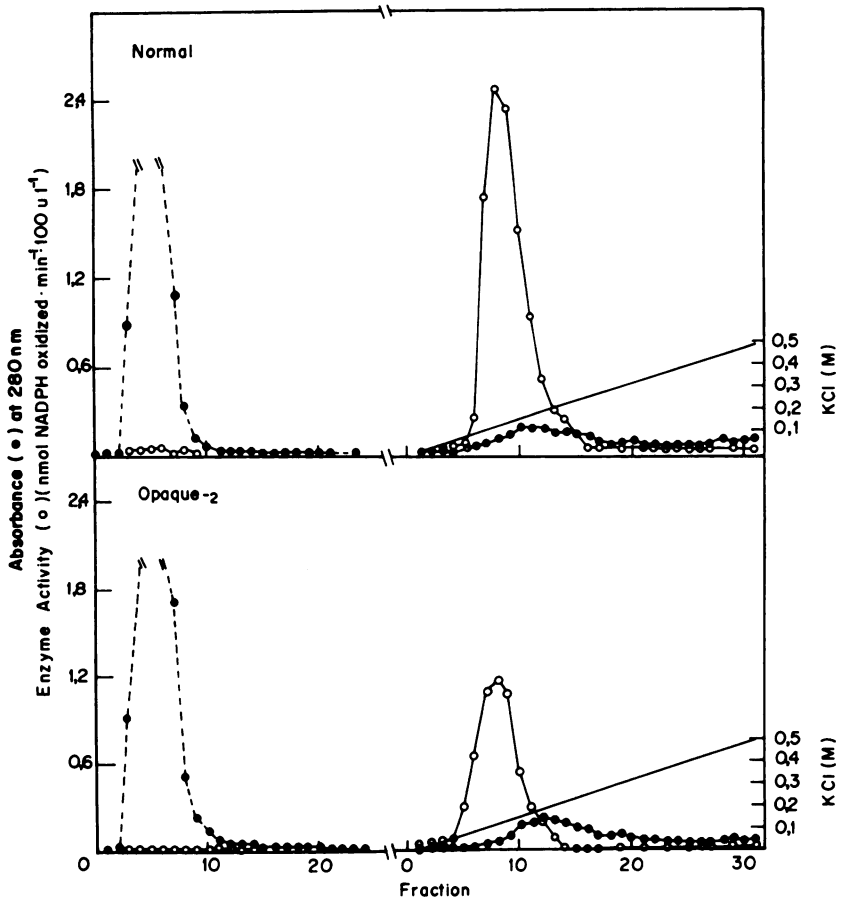

Figure 7. Elution pattern of lysine-ketoglutarate reductase activity from DEAE-cellulose columns with normal and *opaque-2* endosperm extracts from L1038 maize inbred line sampled at 20 DAP. (○) LKR activity; (●) protein measured by absorbance at 280 nm; (—) KCl concentration gradient.

DISCUSSION

It has been demonstrated in this and in an earlier publication (2) that LKR is very active in developing maize endosperm. The maize enzyme exhibits specificity for lysine and α-ketoglutarate, NADPH is required as a cofactor, and saccharopine is the product of the reaction (2, 3).

The apparent molecular mass of maize LKR, as determined by denaturating and nondenaturating PAGE, is approximately 140 kD (Figs. 3, 4). The similarity of molecular mass estimations obtained by gel electrophoresis under denaturating and nondenaturating conditions suggests that the holoenzyme is a monomer.

The enzyme binding mechanism and order of substrate addition, examined by product inhibition kinetics, showed competitive inhibition by saccharopine with respect to lysine and noncompetitive inhibition toward α-ketoglutarate. Comparing with the results of product inhibition kinetics obtained for LKR purified from human placenta (9), the kinetics observed for maize LKR suggest a rapid equilibrium-ordered binding mechanism in which lysine binds first, followed by α-ketoglutarate and NADPH, leading to the release of $NADP^+$ and saccharopine. For the human enzyme, a similar ordered mechanism was suggested, except that α-ketoglutarate binds first followed by lysine (9).

This is the first report of LKR being highly purified from a plant source, so the only information available for comparative purposes are data pertaining to the mammalian enzyme (9). The solubility properties differ as the human enzyme is recovered between 26.5 and 32.5% ammonium sulfate (9), whereas the maize enzyme is precipitated between 35 and 60% saturation (Table I). The two enzymes differ markedly with respect to molecular mass, as the human enzyme, purified from placenta (9), is multimeric with a native molecular mass of 480 kD, whereas the maize enzyme is apparently composed of a single polypeptide chain with an apparent molecular mass of 140 kD (Figs. 3, 4). The maize enzyme also differs from the human enzyme with respect to its kinetic properties; maize LKR is not inhibited by *S*-2-aminoethyl-L-cysteine, nor does this lysine analog substitute for lysine as a substrate as it does with the human enzyme (9). The competitive pattern obtained for lysine and the noncompetitive pattern obtained for α-ketoglutarate contrast with those observed with LKR purified from human placenta, in which saccharopine was a competitive inhibitor with respect to α-ketoglutarate and a noncompetitive inhibitor with respect to lysine (9).

It is noteworthy that the pattern of LKR activity is related to the rate of zein accumulation and total nitrogen input in maize endosperm (Fig. 6) (2). This could be of physiological importance for the following reasons. First, zein is devoid of lysine (6) and, because zein is the most abundant protein accumulated in the endosperm, the demand for lysine during

endosperm development would appear to be very low. Second, although lysine is synthesized in the developing endosperm (25), substantial amounts of this amino acid (approximately 5%), believed to be produced by hydrolysis of soluble proteins accumulated in the leaves before anthesis, are translocated to the endosperm during development (1). In such circumstances one would expect an excess of lysine to accumulate in the developing endosperm. This is not the case, however, as total lysine is approximately 1.5% (2) and the free lysine concentration in the endosperm is maintained at low levels throughout development (2). The free lysine should be maintained at a low concentration because this amino acid is a feedback inhibitor of enzymes involved in early steps of the aspartate pathway (8, 10). The inhibition of aspartate kinase (8), the first enzyme in the pathway, results in a lack of intermediary substrates for the synthesis of methionine. Because only small amounts of methionine are translocated to the kernel (1), this could limit protein synthesis and have adverse effects on the development of the endosperm. The high activity of LKR during endosperm development may prevent the accumulation of free lysine, and if this is the case it would be expected that both zein synthesis and lysine catabolism operate under the same regulatory system. We suggest that the genes coding for LKR and zeins could be under the control of the same regulators.

There is also evidence to suggest that LKR could be under the control of *opaque-2,* a gene that transactivates the expression of the 22 kD α-zein genes in maize endosperm. Indications that lysine catabolism is higher in normal than in *opaque-2* endosperm were obtained by feeding [^{14}C]lysine to developing ears segregating for normal and *opaque-2* endosperm (26). Further evidence was obtained by analyzing the lysine concentration in the vascular sap of a segregating ear containing normal and sugary-*opaque-2* endosperm. The lysine concentration in vascular sap was double that observed in normal, but similar to that found in sugary-*opaque-2,* endosperm (5). In the current study, two maize inbred lines containing homozygous normal and *opaque-2* versions were used to investigate whether the *opaque-2* mutation affects the activity of LKR. The *opaque-2* inbred lines exhibited the characteristic reduction in zein accumulation (Fig. 6), due mainly to a repression in the synthesis of the 22 kD zein class. The activity of LKR was greatly reduced in the *opaque-2* endosperm at all stages of development, but this effect was accentuated in the L1038 inbred line, which also showed a more marked reduction in zein accumulation (Fig. 6). The results demonstrate that the *opaque-2* mutation affects zein synthesis and LKR activity in a similar developmental pattern. The partial purification on DEAE-cellulose (Fig. 7) of enzyme extracted from normal and *opaque-2* endosperms suggested that the opaque-2 mutation may affect the synthesis of LKR rather than its structure. The recent cloning of the *Opaque-2* gene has demonstrated that *Opaque-2* codes for a DNA binding protein that transactivates the 22 kD zein and b-32 genes (14, 17, 21, 22). It is possible that the same *trans*-acting factor is involved in the expression of the LKR gene. Furthermore, low LKR activity in the *opaque-2* endosperm may be responsible for the reduced lysine catabolism in the mutant endosperm and thus contribute, at least in part, to the elevated concentration of lysine in *opaque-2* compared with normal endosperm.

ACKNOWLEDGMENT

We would like to thank Dr Alan Crozier (Glasgow University, UK) for the critical reading of the manuscript.

LITERATURE CITED

1. **Arruda P, Silva W** (1979) Amino acid composition of vascular sap of maize ear peduncle. Phytochemistry **18:** 409–410
2. **Arruda P, Da Silva WJ** (1983) Lysine-ketoglutarate reductase activity in maize; its possible role in the metabolism of developing endosperm. Phytochemistry **22:** 2687–2689
3. **Arruda P, Sodek L, Da Silva WJ** (1982) Lysine-ketoglutarate reductase activity in developing maize endosperm. Plant Physiol **69:** 988–989
4. **Brandt AB** (1975) In vivo incorporation of lysine-C^{14} into the endosperm proteins of wild type and high lysine barley. FEBS Lett **52:** 288–291
5. **Da Silva WJ, Arruda P** (1979) Evidence for the genetic control of lysine catabolism in maize endosperm. Phytochemistry **18:** 1803–1805
6. **Dalby A, Tsai CY** (1975) Comparison of lysine and zein and non-zein protein contents in immature and mature maize mutants. Crop Sci **15:** 513–515
7. **De Moreno MR, Smith JF, Smith RV** (1985) Silver staining of proteins in polyacrylamide gels: increased sensitivity through a combined Coomassie blue-silver stain procedure. Anal Biochem **151:** 466–470
8. **Dotson SB, Somers DA, Gengenbach BG** (1990) Kinetic studies of lysine-sensitive aspartate kinase purified from maize suspension cultures. Plant Physiol **93:** 98–104
9. **Fjellstedt TA, Robinson JC** (1975) Purification and properties of L-lysine-alpha-ketoglutarate reductase from human placenta. Arch Biochem Biophys **168:** 536–548
10. **Frisch DA, Gengenbach BG, Tommey AM, Sellner JM, Somers DA, Myers DE** (1991) Isolation and characterization of dihydrodipicolinate synthase from maize. Plant Physiol **96:** 444–452
11. **Hartings H, Maddaloni M, Lazzaroni N, DiFonzo N, Motto M, Salamini F, Thompson R** (1989) The *O2* gene which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. EMBO J **8:** 2795–2801
12. **Kodrzycki R, Boston RS, Larkins BA** (1989) The *opaque-2* mutation of maize differentially reduces zein gene transcription. Plant Cell **1:** 105–114
13. **Leatherbarrow RJ** (1987) Enzfiter. A non-linear regression data analysis program for the IBMPC. Biosoft, Cambridge, UK
14. **Lohmer S, Maddaloni M, Motto M, Di Fonzo N, Hartings H, Salamini F, Thompson RD** (1991) The maize regulatory locus *Opaque-2* encodes a DNA-binding protein which activates the transcription of the b-32 gene. EMBO J **10:** 617–624
15. **Mertz ET, Bates LS, Nelson OE** (1964) Mutant gene that changes protein composition and increase lysine content of maize endosperm. Science **145:** 279–280
16. **Moller BL** (1976) Lysine catabolism in barley (*Hordeum vulgare* L). Plant Physiol **57:** 687–692
17. **Motto M, Maddaloni M, Ponziani M, Brembilla M, Marotta R, Di Fonzo N, Soave C, Thompson R, Salamini F** (1988) Molecular cloning of the *o2-m5* allele of *Zea mays* using transposon marking. Mol Gen Gen **212:** 488–494
18. **Nigan SN, McConnell WB** (1963) Studies on wheat plants using carbon^{14} compounds. XIX. Observations on the metabolism of lysine C^{14}. Can J Biochem Physiol **41:** 1367–1371
19. **Nkonge C, Ballance GM** (1982) A sensitive colorimetric procedure for nitrogen determination in micro-Kjeldahl digests. J Agric Food Chem **30:** 416–420
20. **Paulis JW, Wall JS, Sanderson J** (1978) Origin of high methionine content in *sugary-1* corn endosperm. Cereal Chem **55:** 705–712

21. **Schmidt RJ, Burr FA, Aukerman MJ, Burr B** (1990) Maize regulatory gene *opaque-2* encodes a protein with a "leucine-zipper" motif that binds to zein DNA. Proc Natl Acad Sci USA **87:** 46–50
22. **Schmidt RJ, Burr FA, Burr B** (1987) Transposon tagging and molecular analysis of the maize regulatory locus *opaque-2*. Science **238:** 960–963
23. **Soave C, Suman N, Viotti A, Salamini F** (1978) Linkage relationship between regulatory and structural gene loci involved in zein synthesis in maize. Theor Appl Genet **52:** 263–267
24. **Soave C, Tardini L, Di Fonzo N, Salamini F** (1981) Zein level in maize endosperm depends on a protein under control of the *opaque-2* and *opaque-6* loci. Cell **27:** 403–410
25. **Sodek L** (1976) Biosynthesis of lysine and other amino acids in the developing maize endosperm. Phytochemistry **15:** 1903–1907
26. **Sodek L, Wilson CM** (1970) Incorporation of leucine-C^{14} and lysine-C^{14} into protein in the developing endosperm of normal and *opaque-2* corn. Arch Biochem Biophys **140:** 29–38
27. **Spector T** (1978) Refinement of the Coomassie blue method of protein quantitation. Anal Biochem **86:** 142–146
28. **Susor WA, Rutter WJ** (1971) Method for the detection of pyruvate kinase, aldolase and other pyridine nucleotide linked enzyme activities after electrophoresis. Anal Biochem **43:** 147–155
29. **Wilson CM** (1980) Plant nucleases VI. Genetic and developmental variability in ribonuclease activity in inbred and hybrid corn endosperms. Plant Physiol **66:** 119–125
30. **Wilson CM, Alexander DE** (1967) Ribunuclease activity in normal and *opaque-2* mutant endosperm of maize. Science **155:** 1575–1576