Quality Protein Maize: A Biochemical Study of Enzymes Involved in Lysine Metabolism

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Quality protein maize (QPM) varieties have been produced by the introduction of opaque-2 modifier genes. Two QPM varieties, BR451 and BR473, a wild type and an opaque-2 variety, have been used to study key enzymes controlling lysine metabolism in the endosperm during development. Aspartate kinase and homoserine dehydrogenase enzymes, which are involved in lysine and threonine biosynthesis, respectively, exhibited identical activity patterns during endosperm development, with a maximum specific activity at 16 days after pollination. The QPM varieties exhibited higher levels of aspartate kinase activity in the endosperm, suggesting an increased rate of lysine biosynthesis when compared to the opaque-2 and wild-type genotypes. Similar results were observed for the lysine ketoglutarate reductase and saccharopine dehydrogenase enzymes, which form a single bifunctional polypeptide involved in endosperm lysine degradation. Both enzyme activities were strongly reduced in the opaque-2 maize variety when compared to the wild-type maize, whereas the QPM varieties exhibited even lower levels of lysine ketoglutarate reductase−saccharopine dehydrogenase activities when compared to the opaque-2 variety. The developmental pattern of enzyme activity showed a different profile when compared to the enzymes involved in lysine biosynthesis, with activity being detected only 12−16 days after pollination (DAP) and maximum activities ∼24 DAP. These results also suggest that the modifier genes have intensified the effect of the opaque-2 mutation on lysine ketoglutarate reductase−saccharopine dehydrogenase. These alterations lead to an increase in soluble lysine in the endosperm of the QPM varieties when compared to the opaque-2 and wild type.

Keywords: *Quality protein maize; aspartate kinase; homoserine dehydrogenase; lysine ketoglutarate reductase−saccharopine dehydrogenase; opaque-2*

INTRODUCTION

Maize has been used worldwide for human and animal consumption, in view of its being an excellent source of carbohydrates. However, one of the main characteristics of this cereal is the low quality of its storage proteins due to the low concentrations of lysine and tryptophan in the main storage protein fraction of the endosperm, zein, which accounts for 50−70% of the storage proteins. Consequently, a diet based on maize must be supplemented with these essential amino acids.

For over 30 years several researchers have worked on the isolation of mutants that might produce higher concentrations of lysine and tryptophan. The opaque-2 mutant was isolated and well characterized (Mertz et al., 1964). However, together with changes in endosperm protein composition and an increase in lysine concentration, some undesirable agronomic traits, such as soft endosperm, higher susceptibility to pathogens, and lower yields compared to normal maize kernels, were associated with the opaque-2 mutant (Vasal, 1994).

The use of modifying genes to alter kernel phenotype has allowed the selection of modified opaque-2 lines that maintain the increased concentrations of lysine and tryptophan in a modified-vitreous endosperm [quality protein maize QPM)]. Since the development of QPM varieties, several studies have been conducted, mainly on the agronomic aspects of the kernel and the plant, such as combining ability for yield and protein quality among QPM hybrids (Pixley and Bjarnason, 1993); kernel hardness and density (Paulis et al., 1993; Moro et al., 1995), protein content (Paulis et al., 1993; Moro et al., 1995); increase in γ-zein storage protein (Moro et al., 1995), lysine content (Paulis et al., 1993; Moro et al., 1995), and nutritional value (Sullivan et al., 1989); and use in the food industry (Martinez et al., 1996). Also, aspects related to the mapping of *opaque-2* modifying loci and their effects on *opaque-2* gene have been studied (Geetha et al., 1991; Lopes et al., 1995). However, the lysine biosynthesis and degradation pathways have not been studied in QPM varieties.

With the development of plant tissue culture techniques, biochemical mutants of various plant species can be induced, selected, and regenerated (Lea et al., 1992). In maize, two mutants resistant to lysine plus threonine growth inhibition, designated ask1-LT19 and ask2-LT20, were isolated and shown to have higher concentrations of soluble threonine when compared to normal

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maize lines (Hibberd and Green, 1982). It has also been shown that the first enzyme of the aspartic acid pathway, aspartate kinase (AK; EC 2.7.2.4), was insensitive to lysine inhibition in the mutants (Dotson et al., 1990). Further biochemical analysis of double-mutant combinations, ask1-Lt19/opaque-2, indicated that the *ask1* gene may be regulated by the *opaque-2* gene at the level of soluble amino acids, total amino acids, storage proteins, and enzyme activity (Azevedo et al., 1990; Brennecke et al., 1996). The genetic analysis carried out showed that the *ask1* gene was linked to the *opaque-2* gene on chromosome 7S (Azevedo et al., 1990). The analysis of a second mutant gene, *ask2*, showed that another lysine-sensitive AK isoenzyme was altered in its inhibition by lysine and the gene was located on chromosome 2L (Dotson et al., 1990; Muehlbauer et al., 1994).

Another group of mutants resistant to *S*-2-aminoethyl-L-cysteine have been selected in some plant species to obtain high-lysine mutants (Bright et al., 1979; Negrutiu et al., 1984; Boyes and Vasil, 1987; Azevedo and Arruda, 1995). For the maize mutants, the increase in soluble lysine was small (Azevedo and Arruda, 1995).

More recently, the development of plant transformation and molecular biology techniques have given rise to a better understanding of the regulation of amino acid biosynthesis and degradation and have allowed the development of a new strategy to obtain lysine- and threonine-overproducing plants. Genes encoding for key enzymes of the aspartic acid pathway from plants or *Escherichia coli*, which have been altered or are less sensitive to the inhibition by lysine and threonine, can be transferred and combined in other plants to obtain accumulation of lysine and threonine (Shaul and Galili, 1992; 1993; Azevedo et al., 1997; Muhitch, 1997).

Lysine is synthesized in the aspartic acid metabolic pathway in plants (Azevedo et al., 1997). Biochemical mutants selected in vitro that accumulate threonine and lysine, such as ask1-LT19 and ask2-LT20, have key regulatory enzymes altered in their regulation leading to the accumulation of the amino acids of the pathway (Heremans and Jacobs, 1995). The enzyme AK has been characterized and studied in detail in maize and in several other plant species (Dotson et al., 1989; Azevedo et al., 1992a, 1997; Heremans and Jacobs, 1997; Teixeira et al., 1998). Three AK isoenzymes have been identified, two lysine sensitive and one threonine sensitive. The threonine-sensitive AK isoenzyme is part of a bifunctional protein involving the threonine-sensitive homoserine dehydrogenase isoenzyme (HSDH; EC 1.1.1.3) (Azevedo et al., 1992b; Muehlbauer et al., 1994). These two enzymes, AK and HSDH, are involved in lysine and threonine biosynthesis. Dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52) is another enzyme involved directly in the biosynthesis of lysine (Frisch et al., 1991). DHDPS activity is feedback inhibited by lysine in all plants studied so far (Azevedo et al., 1997).

Two other enzymes, lysine ketoglutarate reductase (LKR or LOR; EC 1.5.1.8) and saccharopine dehydrogenase (SDH; EC 1.5.1.9), are involved in lysine degradation in maize (Gonçalves-Butruille et al., 1996; Kemper et al., 1998) and rice (Gaziola et al., 1996, 1997). Both enzyme activities have been shown to be part of a single bifunctional polypetide in maize (Gonçalves-Butruille et al., 1996) and rice (Gaziola et al., 1997) and to be specific to the endosperm (Brochetto-Braga et al., 1992; Gonçalves-Butruille et al., 1996; Gaziola et al., 1997). LKR activity has been shown to be stimulated by calcium and lysine (Kemper et al., 1998).

The effect of the opaque-2 modifying genes on the regulation of the enzymes controlling the biosynthesis and degradation of lysine has not been determined. The focus of this study was the biochemical analysis for AK, HSDH, LKR, and SDH, extracted from the developing endosperm in wild-type, opaque-2, and QPM maize varieties.

MATERIALS AND METHODS

Plant Material. The maize inbred line R1, the QPM varieties BR451 and BR473 (Embrapa, Sete Lagoas), and the Maya opaque-2 (donated by Instituto Agronômico de Campinas, Brazil) were used for the extraction and partial purification of the AK, HSDH, LKR, and SDH enzymes.

Growth of Plant Material. Plants for all genotypes were grown in the field and self-pollinated. Maize ears at 8, 12, 16, 20, 24, 28, and 32 days after pollination (DAP) were harvested directly into liquid nitrogen and stored at −70 °C until used in the extraction procedure. The experiment was repeated in two summer seasons (1996−1997 and 1997−1998). Five maize ears for each DAP stage selected were harvested, combined, and mixed. The endosperms were isolated and used for enzyme and soluble amino acid extractions. Embryos were not excised from the 8 DAP maize samples.

AK Extraction and Partial Purification. All procedures were carried out at 4 °C unless stated otherwise. Frozen endosperms were extracted in 5 volumes of buffer A [50 mM Tris-HCl, 200 mM KCl, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM EDTA, 1 mM DTT, 2 mM L-lysine, 2 mM L-threonine, 10% (v/v) glycerol, and 5% (w/v) insoluble polyvinylpyrolidone, pH 7.4]. The extract was filtered through three layers of miracloth and centrifuged at 16000*g* for 30 min to completely remove the cell debris from the extract. Solid ammonium sulfate was slowly added to 30% saturation by gently stirring for at least 30 min. The suspension was centrifuged at 16000*g* for 30 min and the supernatant subjected to a second ammonium sulfate precipitation at 60% saturation for 30 min with continuous stirring. Precipitated protein was recovered by centrifugation at 16000*g* for 30 min and the protein pellets were dissolved in a small volume of buffer B [25 mM Tris-HCl, 1 mM DTT, 0.1 mM L-lysine, 0.1 mM L-threonine, and 10% (v/v) glycerol, pH 7.4]. The sample was loaded onto a Sephadex G50 column (2.5 × 20 cm) equilibrated with 5 column volumes of buffer B and run under gravity. The desalted sample was collected and assayed for AK activity.

HSDH Extraction and Partial Purification. All procedures were carried out at 4 °C unless stated otherwise. Frozen endosperms were extracted in 5 volumes of buffer C [50 mM Tris-HCl, 200 mM KCl, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, 3 mM DTT, 5 mM l-threonine, 10% (v/v) glycerol, and 5% (w/v) insoluble polyvinylpyrolidone, pH 7.5]. The extract was filtered through three layers of miracloth and centrifuged at 16000*g* for 30 min to completely remove the cell debris from the extract. Solid ammonium sulfate was slowly added to 30% saturation by gently stirring for at least 30 min. The suspension was centrifuged at 16000*g* for 30 min and the supernatant subjected to a second ammonium sulfate precipitation at 60% saturation for 30 min with continuous stirring. Precipitated protein was recovered by centrifugation at 16000*g* for 30 min, and the protein pellets were dissolved in a small volume of buffer D [25 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.1 mM L-threonine, and 10% (v/v) glycerol, pH 7.5]. The sample was loaded onto a Sephadex G50 column (2.5 × 20 cm) equilibrated with 5 column volumes of buffer D and run under gravity. The desalted sample was collected and assayed for HSDH activity.

LKR and SDH Extraction and Partial Purification. All procedures were carried out at 4 °C unless stated otherwise. Frozen endosperms were extracted in 5 volumes of buffer E [100 mM potassium phosphate, 50 mM KCl, 1 mM EDTA, 1

mM DL-dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 10% (w/v) glycerol, and 5% (w/v) insoluble polyvinylpyrrolidone, pH 7.0]. The homogenate was first filtered through three layers of miracloth and then centrifuged at 15000*g* for 30 min to remove cell debris. The supernatant was adjusted to 30% ammonium sulfate saturation by gently stirring for at least 30 min. The suspension was centrifuged at 15000*g* for 30 min and the supernatant subjected to a second ammonium sulfate precipitation at 55% saturation for 30 min with continuous stirring. After centrifugation at 15000*g* for 30 min, the sedimented proteins were dissolved in 10 mL of buffer E (minus phenylmethylsulfonyl fluoride and insoluble polyvinylpyrolidone). The sample was then loaded onto a Sephadex G25 column (2.6 × 20 cm) previously equilibrated with buffer F [100 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol, pH 7.4] and run under gravity. The desalted sample was collected and assayed for LKR and SDH activities.

Assays for AK, HSDH, LKR, and SDH. AK activity was assayed routinely in a final volume of 500 μL as described by Brennecke et al. (1996). The assay mixture comprised 100 μL of Tris-HCl (100 mM), pH 7.4, containing 1 mM DTT and 20% (v/v) glycerol, 100 μL of aspartic acid (500 mM), pH 7.4, 50 μL of magnesium sulfate (125 mM), 50 μL of ATP (200 mM), pH 7.4, 50 μL of hydroxylamine (4 M), pH 7.4, 50 μL of H_2O, and 100 μL of enzyme extract. The assay was started by the addition of 50 μL of ATP and incubated at 35 °C for 30 min. The assay was terminated by the addition of 500 μL of $FeCl_3$ reagent [670 mM $FeCl_3$, 370 mM HCl, and 20% (w/v) TCA]. After centrifugation for 10 min at 20000*g* to remove precipitated protein, the absorbance of the supernatant was measured at 505 nm. Controls containing lysine and threonine were normally included to ensure that the activity measured was due to AK and to identify isoenzymes sensitive to lysine and threonine. Four replicates were carried out for each assay.

HSDH activity was assayed routinely spectrophotometrically at 340 nm in a final volume of 1.0 mL at 30 °C as described by Azevedo et al. (1992b). The assay mixture contained 800 μL of Tris-HCl buffer (100 mM), pH 9.0, containing 150 mM KCl, 1 mM DTT, and 0.5 mM EDTA, 100 μL of DL-homoserine (200 mM), 100 μL of NADP (4.8 mM), and 100 μL of enzyme. The rate of activity was linear for at least 3 min and was corrected for any change in absorbance noted in the absence of the substrate (homoserine). The effect of threonine on the HSDH activity was determined by the addition (10 μL of a 1 M solution) of the amino acid to the assay mixture. Four replicates were carried out for each assay.

LKR activity was routinely assayed spectrophotometrically in a 1.0 mL cuvette at 30 °C by following the change in absorbance at 340 nm over a 15 min period, with appropriate adjustments for a lysine-free blank. The assay mixture contained 100 mM Tris-HCl, pH 7.4, 20 mM L-lysine, 10 mM α-ketoglutarate neutralized with KOH, 0.14 mM NADPH, and enzyme sample in a 0.9 mL assay volume.

SDH activity was routinely measured spectrophometrically in a 1.0 mL cuvette by following the rate of substrate-dependent reduction of NAD^+ to NADH monitored at 340 nm at 30 °C over a 15 min period, with appropriate adjustments for a saccharopine-free blank. The reaction mixture contained 100 mM Tris-HCl, pH 8.4, 2 mM saccharopine, 2 mM NAD^+, and enzyme sample in a 0.9 mL assay volume. Activities were expressed as nanomoles of NADPH/NAD^+ oxidized/reduced per minute per milliliter.

Protein Determination. Protein concentrations of the samples were determined as described by Bradford (1976) using bovine serum albumin as a standard.

Amino Acid Analysis. For the quantification of soluble amino acids in the developing endosperms, the samples were analyzed as *o*-phthaldialdehyde (OPA) derivatives by analytical high-performance liquid chromatography (HPLC) as described by Marur et al. (1994). The amino acids were separated on a Superpac (Pharmacia) ODS-2 column. The elution of the amino acids was performed with a linear gradient of 20−100% B (methanol 65%) in buffer A (50 mM NaOAc, 50 mM Na_2HPO_4, 1.5 mL of HAc, 20 mL of tetrahydrofuran, and 20 mL of methanol, pH 7.2) in 50 min at a 1 mL/min flow rate. Because the lysine derivative is rapidly degraded, a second analysis using a 15 min total gradient time was performed.

RESULTS AND DISCUSSION

QPM varieties and hybrids have been produced and are now available for the market in several countries. The undesirable kernel characteristics, which are a common feature in the opaque-2 maize grains, have been considerably reduced in QPM varieties due to the introduction of opaque-2 modifier genes.

Enzymes such as AK and HSDH have been isolated and studied in several plant species (Azevedo et al., 1997). AK, which regulates the biosynthesis of lysine in plants, has been studied previously in maize tissues, including the endosperm (Henke and Wahnbaeck-Spencer, 1979; Brennecke et al., 1996). For HSDH, however, very little is known about its regulation in the maize endosperm.

AK showed the same activity pattern during the development of the maize endosperm, irrespective of genotype (Figure 1). The highest level of AK activity was observed at 16 DAP, falling to nearly zero at 28 DAP. The result is similar to those of previous studies (Henke and Wahnbaeck-Spencer, 1979; Brennecke et al., 1996), in which a higher rate of metabolism was observed ∼15−25 DAP, followed by a continuous reduction of the rate of metabolic activity. At the 15−25 DAP stage, amino acids are being synthesized to be used in storage protein synthesis. Although AK activity in all genotypes exhibited the same activity pattern during endosperm development, various levels of AK activity were observed among the genotypes analyzed (Figure 1). The wild-type endosperm (Figure 1A) exhibited a 2-fold higher specific AK activity than the opaque-2 endosperm (Figure 1B). Both QPM varieties exhibited higher levels of AK activity than opaque-2, with a 3-fold increase for QPM BR451 (Figure 1C) and a 5-fold increase for QPM BR473 (Figure 1D) for the 16 DAP endosperms. These increases in AK activity in QPM genotypes do not seem to be directly related to a specific AK isoenzyme, because the patterns of AK inhibition by lysine, threonine, and both amino acids together did not show any significant variation. The only exception was the QPM BR451 variety, in which threonine did not inhibit AK activity until 24 DAP (Figure 1C), which suggests that the increase in total AK activity might be due to a reduced sensitivity of AK to threonine or that the lysine-sensitive AK isoenzymes are strongly predominant in this genotype. However, threonine was able to inhibit part of the AK activity in QPM BR473 (Figure 1D), suggesting that a threonine-sensitive AK isoenzyme is present in this genotype. Furthermore, the AK activity that is inhibited by lysine was shown to be predominant in all genotypes tested, which confirms that the AK isoenzymes of maize sensitive to lysine inhibition are present in the endosperm and also agrees with previous results shown for maize (Muehlbauer et al., 1994). It has been shown that maize contains at least two distinct lysine-sensitive AK isoenzymes (Dotson et al., 1989; Azevedo et al., 1992a); however, we did not purify the enzymes to a stage in which the isoenzymes could be separated. Therefore, the results obtained for lysine inhibition comprise total lysine-sensitive AK activity. Although both lysine-sensitive AKs were analyzed together, the results did not show any major variation for lysine inhibition.

The results obtained for HSDH activity were similar to those exhibited by AK activity. The peak of HSDH

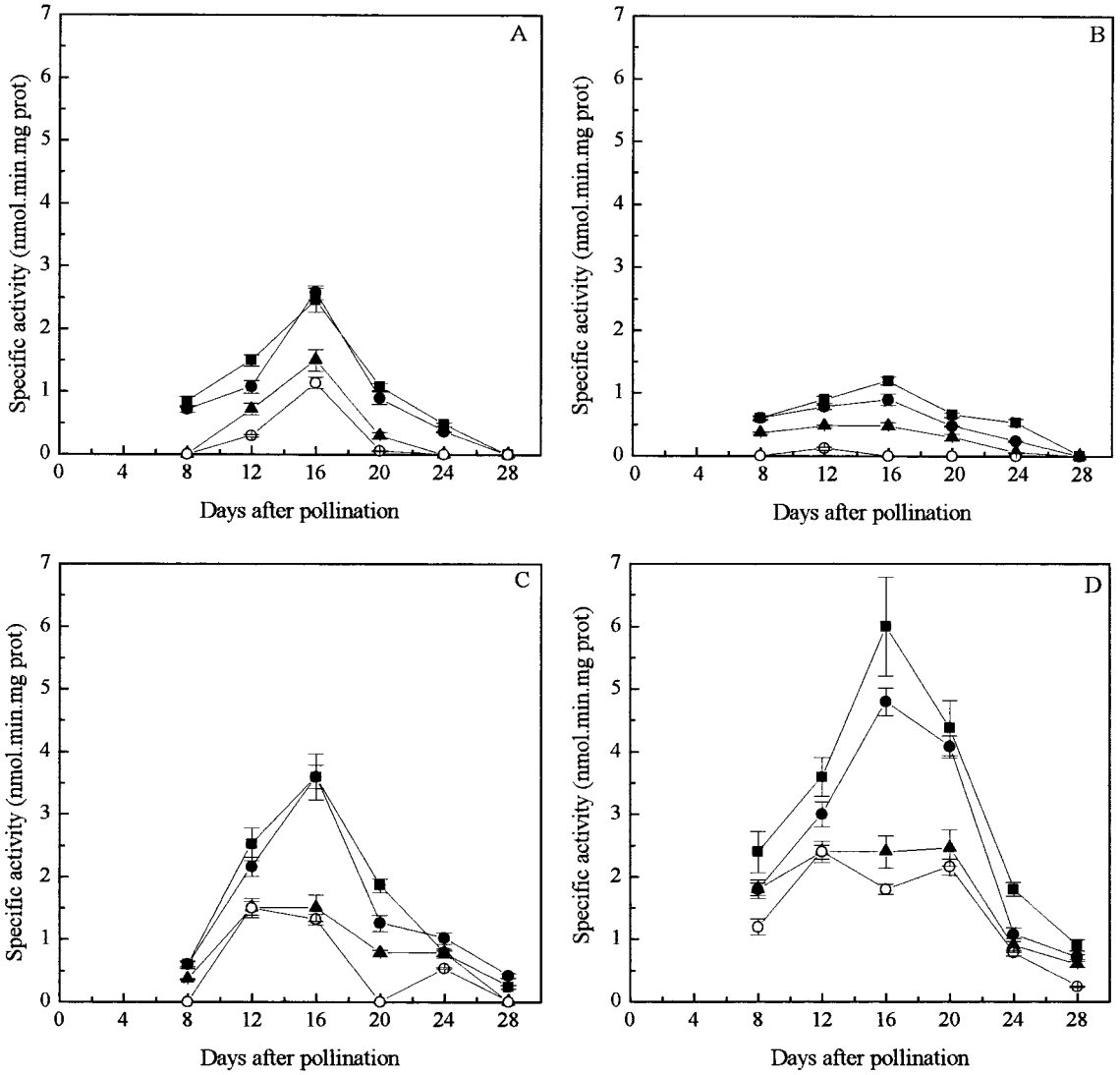

Figure 1. Aspartate kinase activity during the development of maize endosperms: (A) inbred line R1, (B) Maya opaque-2, (C) QPM BR451, and (D) QPM BR473; (■) aspartate kinase control, (▲) plus 10 mM lysine, (●) plus 10 mM threonine, and (○) plus 10 mM lysine + threonine.

activity during endosperm development was observed at 16 DAP for all genotypes tested (Figure 2) and coincided with the AK activity profile of endosperm development (Figure 1). Somewhat surprising were the low levels of total HSDH activity observed in maize endosperms. HSDH activity in most other plant tissues has been shown to be higher than AK activity (Bryan, 1990).

HSDH is normally present in higher plants and microorganisms as two distinct isoenzymes: one threonine resistant (HSDH-R) and the other threonine sensitive (HSDH-S). The addition of threonine to the assay mixture permits the determination of which HSDH isoenzyme was active during each stage of endosperm development. The inhibition caused by threonine varied little during the stages of development analyzed in this study, confirming that variations in total HSDH cannot be explained by differential activity of the HSDH isoenzymes (Figure 2). Both HSDH-R and HSDH-S isoenzymes are present at all stages of development tested. The inhibition of HSDH by threonine was stronger in opaque-2 and QPM varieties, suggesting that HSDH-S is predominant in opaque-2 genotypes (Figure 2). This result for HSDH is of special interest because it has been suggested in the literature that the HSDH-S isoenzyme is involved in amino acid biosynthesis, whereas the physiological function of the HSDH-R isoenzyme is not clear (Bryan, 1990). The increase in the inhibition of HSDH by threonine may be a result of a direct increase of the HSDH-S isoenzyme activity, which could regulate the distribution of carbon between the threonine and lysine branches of the pathway, confirming the metabolic role of the HSDH-S in amino acid biosynthesis in plants. This hypothesis suggests that the DHDPS enzyme should also be considered in future studies, because DHDPS is known to play a key role in the regulation lysine biosynthesis (Bryan, 1990; Azevedo et al., 1997). In contrast to that observed for AK activity, the QPM BR473 produced the lowest level of HSDH among all genotypes (Figure 2D), whereas for the wild-type, opaque-2, and QPM BR451 genotypes, the levels of total HSDH activity were approximately the same, suggesting some variation among the stages of development among the genotypes (Figure 2). On the basis of these results, it is difficult to suggest that one particular HSDH or AK isoenzyme is directly affected by the introduction of the opaque-2 modifier genes in QPM varieties. However, it is clear that the total AK activity is enhanced in QPM varieties (Figure 1C,D) when compared to the opaque-2 Maya variety (Figure

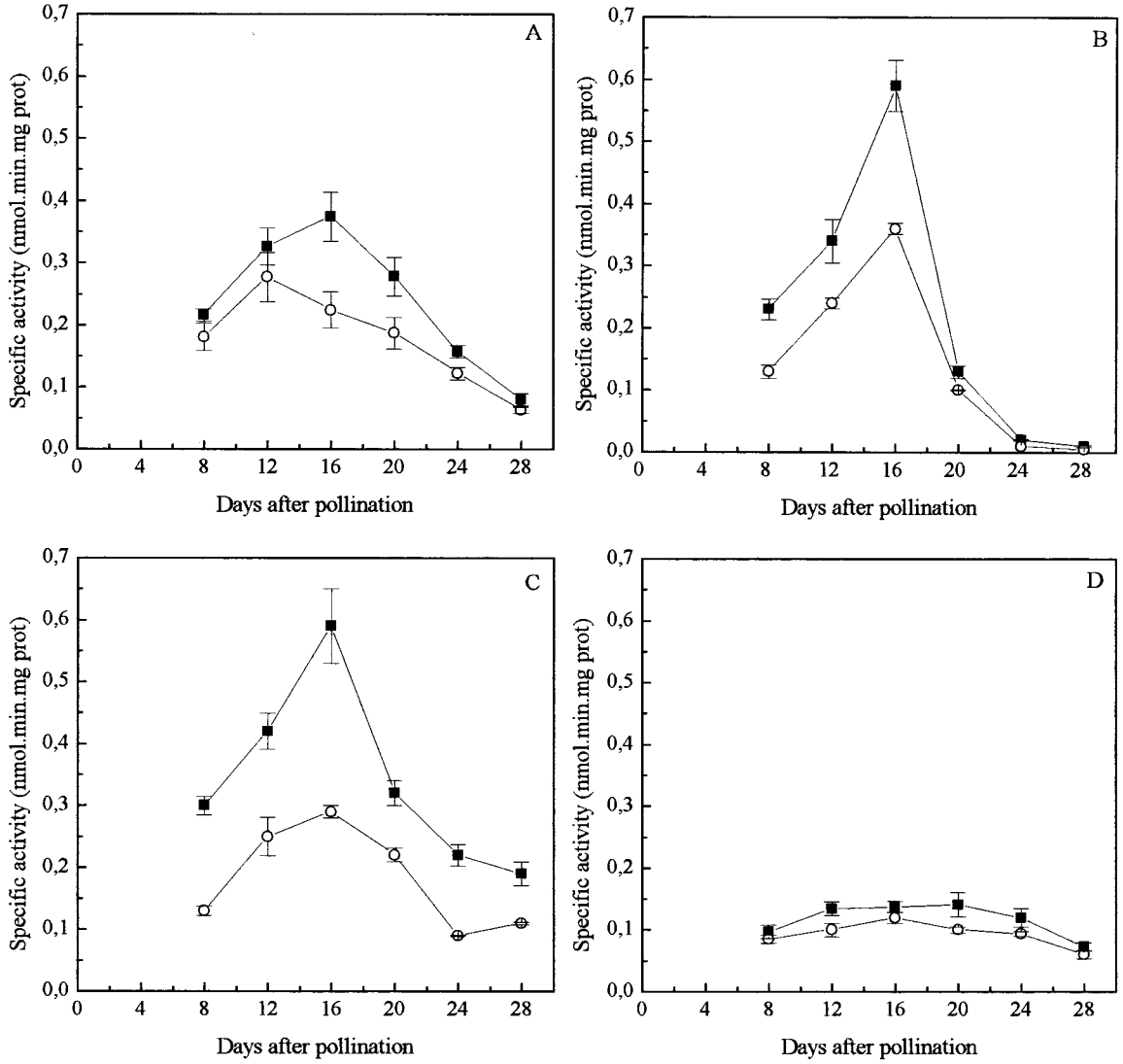

Figure 2. Homoserine dehydrogenase activity during the development of maize endosperms: (A) inbred line R1, (B) Maya opaque-2, (C) QPM BR451, and (D) QPM BR473; (■) homoserine dehydrogenase control and (○) plus 10 mM threonine.

1B), suggesting that an increase in the number of carbon molecules entering the pathway leading to lysine and threonine biosynthesis is taking place. Moreover, the total HSDH activity is enhanced in opaque-2 and QPM BR451 genotypes when compared to the wild type, which further support an increase in the activity of enzymes controlling lysine and threonine biosynthesis. It has also been shown previously that the introduction of the *Ltr*1* gene, which encodes an altered lysine-sensitive AK, into the opaque-2 maize mutant leads to an intensified effect of the opaque-2 mutation on amino acid synthesis, storage protein synthesis (Azevedo et al., 1990), and AK activity (Brennecke et al., 1996).

The enzymes LKR and SDH, which are involved in the catabolism of lysine, have been isolated from maize endosperm (Gonçalves-Butruille et al., 1996). It has been shown that LKR activity is 3−5-fold lower in the opaque-2 endosperm when compared to the wild-type endosperm (Brochetto-Braga et al., 1992). Other recent studies have demonstrated that the accumulation of lysine is also controlled by the rate of its catabolism (Falco et al., 1995; Gaziola et al., 1997). Karchi et al. (1994) reported that lysine induces the activity of LKR in tobacco. The increased AK and HSDH activities in QPM varieties suggest that an increased rate of lysine biosynthesis is taking place; however, lysine accumulation does not necessarily happen, because it may be incorporated into storage proteins and the excess may be subsequently degraded by enzymes of lysine catabolism such as LKR and SDH.

To verify some of these possibilities, LKR and SDH were also extracted and measured during the development of the endosperm of all genotypes. The developmental patterns for LKR and SDH were identical (Figure 3). Peaks of LKR and SDH activities were observed at 24 DAP. The LKR and SDH activity profile clearly show that the catabolism of lysine did not coincide with its biosynthesis. LKR and SDH activities could be detected only after 16 DAP, indicating that a high rate of lysine biosynthesis at the early stages of endosperm development was followed by a high rate of lysine degradation at later stages. The identical activity patterns for LKR and SDH were expected because both are involved in lysine catabolism but principally because both activities are part of a single bifunctional polypeptide (Gonçalves-Butruille et al., 1996). Also, similar activity ratios would be expected for LKR and SDH. As expected, LKR activity showed a reduced activity in the opaque-2 genotype (Figure 3B) when compared to the wild type (Figure 3A), confirming the results of previous studies (Brochetto-Braga et al., 1992). In QPM varieties, the reduction in LKR and SDH activities observed in

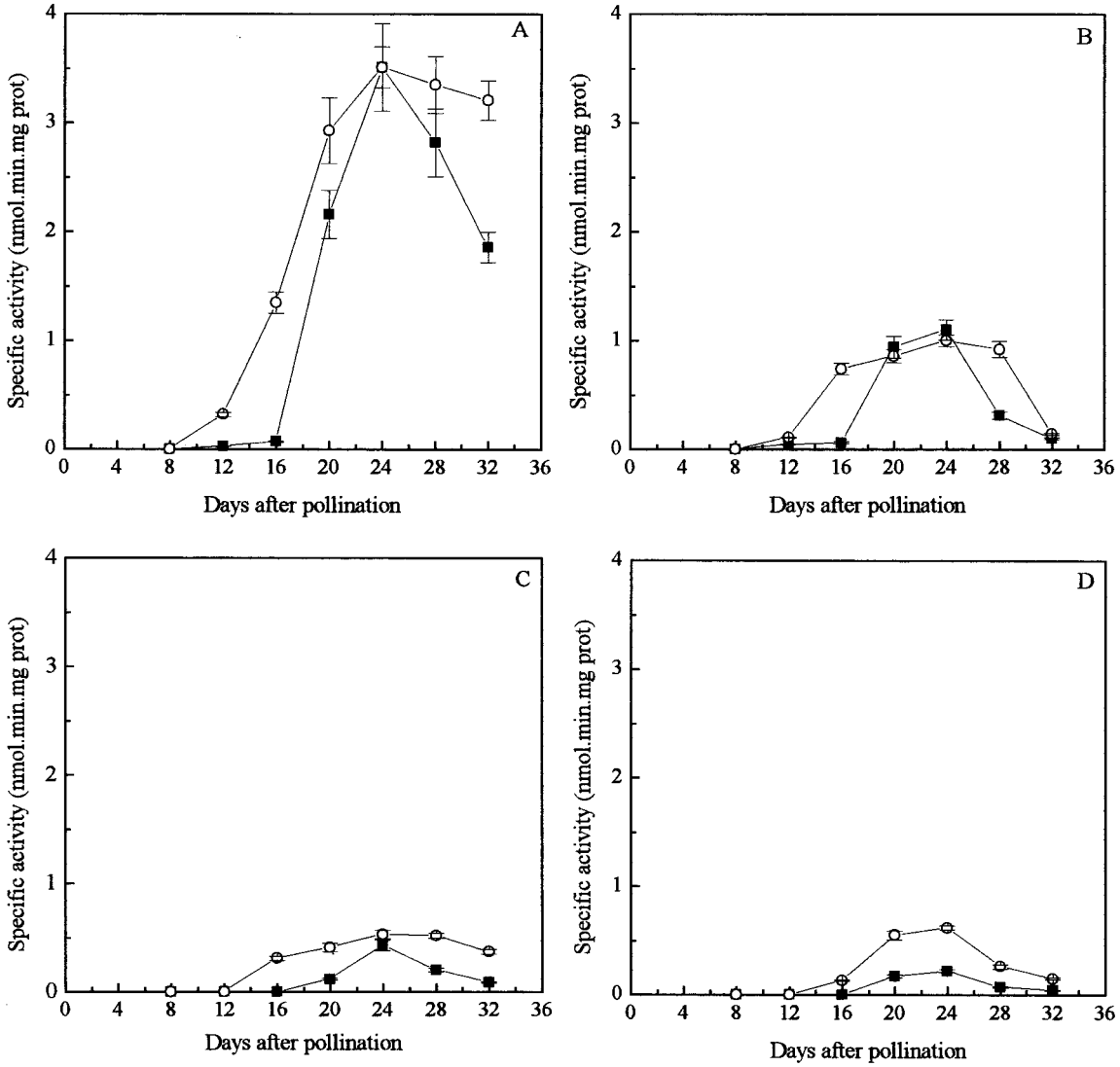

Figure 3. Lysine ketoglutarate reductase (○) and saccharopine dehydrogenase (■) activities during the development of maize endosperms: (A) inbred line R1, (B) Maya opaque-2, (C) QPM BR451, and (D) QPM BR473.

Table 1. Soluble Lysine Absolute Contents in Developing Endosperms of Maize[a]

	8 DAP	12 DAP	16 DAP	20 DAP	24 DAP	28 DAP
R1 (wild type)	0.24 ± 0.01	0.15 ± 0.01	0.37 ± 0.03	0.19 ± 0.02	0.17 ± 0.01	0.15 ± 0.02
opaque-2	0.35 ± 0.02	0.63 ± 0.07	0.73 ± 0.05	0.61 ± 0.02	0.31 ± 0.05	0.32 ± 0.03
QPM BR451	0.38 ± 0.05	0.57 ± 0.07	0.52 ± 0.01	0.53 ± 0.04	0.65 ± 0.04	0.42 ± 0.03
QPM BR473	0.46 ± 0.04	0.66 ± 0.03	0.71 ± 0.02	0.73 ± 0.03	0.32 ± 0.04	0.32 ± 0.03

[a] Each value represents the mean of four replicates (in μmol g^{-1} dry wt. ± SD).

Table 2. Soluble Lysine Relative Contents in Developing Endosperms of Maize[a]

	8 DAP	12 DAP	16 DAP	20 DAP	24 DAP	28 DAP
R1 (wild type)	0.43 ± 0.02	0.34 ± 0.01	0.35 ± 0.05	0.88 ± 0.05	0.73 ± 0.04	1.01 ± 0.08
opaque-2	0.38 ± 0.02	0.51 ± 0.02	0.59 ± 0.02	0.51 ± 0.03	0.90 ± 0.06	1.02 ± 0.05
QPM BR451	0.35 ± 0.01	0.66 ± 0.08	1.14 ± 0.07	1.62 ± 0.13	0.92 ± 0.02	1.20 ± 0.07
QPM BR473	0.31 ± 0.03	0.46 ± 0.03	0.66 ± 0.06	0.76 ± 0.02	0.94 ± 0.05	1.30 ± 0.09

[a] Each value represents the mean of four replicates (in percent of total ± SD).

opaque-2 were even greater (Figure 3C,D). SDH had not been studied in opaque-2 genotypes previously, but the results were identical to those described for LKR (Figure 3). The results suggest that the introduction of modifier genes enhanced the effect of opaque-2 on LKR and SDH enzymes by reducing both enzyme activities even further, leading to a higher availability of lysine. As observed for AK, it is likely that the *opaque-2* gene also regulates the activities of LKR and SDH as it does for several proteins during endosperm development (Damerval and de Vienne, 1993; Damerval and Le Guilloux, 1998).

The analysis of soluble amino acids during endosperm development in this study showed higher levels of soluble lysine in the QPM varieties and opaque-2, when compared to the wild type (Table 1). Among QPM varieties and the opaque-2 Maya variety, the absolute level of lysine did not vary significantly, suggesting that

although the degradation of lysine had been reduced in QPM, lysine did not accumulate in the soluble form, possibly because it was incorporated into proteins. The increases in absolute levels of soluble lysine in opaque-2 and QPM varieties were not accompanied by increases in their relative values (Table 2). The relative values remained similar for all genotypes and varied among the stages of endosperm development. These results are similar to those of a previous paper in which, although the soluble lysine concentration increased in the opaque-2 maize endosperm when compared to the wild type, its relative value was reduced due to an overall increase in the soluble fraction of amino acids in the opaque-2 mutant (Azevedo et al., 1990). The same result has also been observed for the double-mutant combination opaque-2/*Ltr*1* (Azevedo et al., 1990). The analysis of all other amino acids in the soluble fraction did not indicate any significant changes attributable to the modifier genes introduced in the QPM varieties.

In summary, this work suggests that the modifier genes used to produce QPM varieties have induced increases in activity of enzymes that participate in lysine biosynthesis and a reduction in the activity of enzymes involved in lysine catabolism, leading to a higher absolute level of soluble lysine. Further studies involving regulatory aspects of the *opaque-2* gene should consider this group of modifier genes and the genes encoding key enzymes, such as AK, HSDH, and LKR−SDH, controlling lysine metabolism.

ABBREVIATIONS USED

AK, aspartate kinase; HSDH, homoserine dehydrogenase; LKR, lysine ketoglutarate reductase; QPM, quality protein maize; SDH, saccharopine dehydrogenase.

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LITERATURE CITED

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