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Regulation of Lysine Metabolism and Endosperm Protein Synthesis by the *Opaque-5* and *Opaque-7* Maize Mutations

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Two high lysine maize endosperm mutations, *opaque-5* (*o5*) and *opaque-7* (*o7*), were biochemically characterized for endosperm protein synthesis and lysine metabolism in immature seeds. Albumins, globulins, and glutelins, which have a high content of lysine, were shown to be increased in the mutants, whereas zeins, which contain trace concentrations of lysine, were reduced in relation to the wild-type lines B77xB79+ and B37+. These alterations in the storage protein fraction distribution possibly explain the increased concentration of lysine in the two mutants. Using two-dimensional polyacrylamide gel electrophoresis of proteins of mature grains, variable amounts of zein polypeptides were detected and considerable differences were noted between the four lines studied. The analysis of the enzymes involved in lysine metabolism indicated that both mutants have reduced lysine catabolism when compared to their respective wild types, thus allowing more lysine to be available for storage protein synthesis.

KEYWORDS: Aspartate kinase; lysine; lysine 2-oxoglutarate reductase; maize; storage proteins

INTRODUCTION

Amino acid metabolism is a fundamental process for plant growth and development (*1*). Aspartate is the common precursor for the biosynthesis of the essential amino acids lysine, threonine, methionine, and isoleucine (*2*). The aspartate pathway is strongly regulated and has been studied in detail in microorganisms and higher plants (*3*) allowing the identification of the main regulatory steps leading to the synthesis of the amino acid end products (*4*). Cereal seeds constitute a major source of proteins; however, this group of plant species is characterized by a low concentration of lysine in both the soluble and the protein-incorporated forms (*4*).

The enzyme AK (EC 2.7.2.4), which converts aspartic acid into β-aspartyl phosphate, can exist in at least two distinct isoforms, one (or two) sensitive to lysine feedback inhibition and the other sensitive to threonine feedback inhibition, with the latter being a bifunctional polypeptide with the threonine sensitive HSDH isoenzyme (EC 1.1.1.3) (*5*). The AK isoenzymes have been biochemically and molecularly characterized in several plant species (*5*−*12*) and shown to be a major factor in the regulation of the carbon flux through the aspartate pathway (*2*, *3*). HSDH catalyzes the conversion of aspartate semialdehyde to homoserine in the presence of the coenzymes NADH or NADPH and is present in plant species in two isoforms, one resistant and one sensitive to threonine inhibition (*3*). The first enzyme unique to lysine synthesis, dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52), catalyzes the condensation of pyruvate and aspartate semialdehyde into dihydrodipicolinic acid, which is subject to feedback inhibition by micromolar concentrations of lysine, and has also been extensively studied and characterized in plants (*13*, *14*).

Biochemical mutants and transgenic plants have been produced, which exhibit altered concentrations of lysine and other amino acids (*2*, *4*, *15*, *16*). However, the accumulation of lysine was shown to still be limited in the seeds of the cereal crops that had been genetically altered (*4*). The *opaque-2* (*o2*) maize

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mutant is characterized by a higher concentration of lysine in the seeds, which is due to increased concentrations of the amino acid in the soluble form (*17*) and to an altered distribution of the endosperm storage proteins. In the *o2* maize mutant, there is an accumulation of albumins, globulins, and glutelins, which contain higher concentrations of lysine, and a reduction of zeins, which are poor in lysine and account for 50−70% of the endosperm proteins in maize seeds (*4*, *18*). Although the *o2* maize mutant exhibits a more balanced distribution of amino acids when animal nutrition is considered, the higher lysine trait is associated with low yield and a greater susceptibility to pathogens (*17*). An important advance in maize genetics was the identification of seed phenotype modifying genes, which in combination with breeding programs allowed the production of the quality protein maize varieties, which maintain the high lysine and high tryptophan characteristics conditioned by the *o2* mutation but in a modified vitreous endosperm, with favorable agronomic characteristics (*17*, *19*).

More detailed information about the regulation of lysine catabolism was only obtained recently (*4*). Lysine catabolism is controlled by the bifunctional protein LOR−SDH (EC 1.5.1.8 and EC 1.5.1.9, respectively) (*4*). The LOR−SDH bifunctional enzyme has been purified and characterized in some plant species (*12*, *20*−*24*) and shown to be specific to the endosperm tissue in cereal crops (*20*, *21*). The studies involving mutants and transgenic plants provided a better understanding of lysine metabolism and indicated that in order to accumulate lysine in the seed, manipulation of its degradation is also needed (*4*, *25*, *26*).

The product of the *o2* gene is a transcription factor that is specifically expressed in the endosperm (*27*) and has been shown to regulate directly or indirectly some of the storage protein polypeptides and other genes belonging to several metabolic pathways (*18*, *28*−*30*). In the *o2* mutant, LOR−SDH mRNA and protein quantities were markedly reduced, and the expression pattern during grain development was modified (*31*). These large effects suggest that *o2* may play an important role in the developing grain, as a coordinator of the expression of storage protein, and N and carbon metabolism genes.

Considerable information is now available about the *o2* gene and the pleiotropic effects of the mutation; however, very little information is available about lysine metabolism for other opaque mutants that have been classified as high lysine. Recent work by Hunter et al. (*32*) reported an analysis of some of the high lysine mutants. We have also recently reported an extensive study concerning the effects of high lysine opaque and floury maize mutations on lysine metabolism (*33*, *34*). This present report extends our research investigating two opaque mutants, *opaque-5* (*o5*) and *opaque-7* (*o7*), with the aim of obtaining new insights into the regulation of lysine metabolism in maize.

MATERIALS AND METHODS

Maize Mutants. Seeds of the opaque mutants B77xB79*o5* and B37*o7* and their respective wild types, B77xB79+ and B37+, were kindly provided by the Maize Genetics Cooperation Seed Stock Center (Urbana, IL). Plants of all genotypes were grown in the glasshouse under natural daylight at ESALQ-USP, Brazil, with temperatures ranging between 25 and 35 °C, in 4 L pots containing two plants per pot in a commercial potting medium. After preliminary evaluation of enzyme activities during seed development (16, 20, and 24 days after pollination), maize ears were harvested 20 days after pollination (DAP), placed directly into liquid N, and stored at −80 °C until used for enzyme extraction. The experiments were repeated over four summer seasons (1999−2000, 2000−2001, 2001−2002, and 2002−2003), but the results presented in this manuscript were those analyzed during the 2001−2002 summer season.

Preparation of Endosperm Samples. Endosperms were isolated from mature grains previously soaked in water for 30 min, by peeling off the outer tegument and excising the germ. After they were freeze-dried, the endosperms were ground to a powder using a ball mill.

Fractionation of N Constituents. The isolation of endosperm N constituents was undertaken in duplicate as previously described by Landry et al. (*35*) using 100 mg of endosperm meal.

Quantitation of N Constituents. For an accurate quantitation, the NPN and protein content were determined by the ninhydrin assay of α-amino N released after alkaline digestion (3 M NaOH, 130 °C, 45 min) of the trichloroacetic acid (TCA), protein extracts, or acid digestion (6 M HCl, 110 °C, 18 h) of the E_3 extracts and residues, according to the method of Landry and Delhaye (*36*). SAAs were quantitated by ninhydrin without previous digestion of the TCA extracts. The endosperm total N was calculated as the sum of protein from the component fractions.

Amino Acids Analysis. SAAs from mature seeds were extracted and quantitated exactly as described by Azevedo et al. (*33*). Four replicates were analyzed.

Protein Extraction and Two-Dimensional (2D) Gel Electrophoresis of Zein Polypeptides. The procedure for 2D polyacrylamide gel electrophoresis (PAGE) analysis of zein isoforms and spot detection were carried out as described by Consoli and Damerval (*37*).

Statistical Analyses of Zein Isoform Amounts. The intensity of spots stained with colloidal Coomassie blue is generally linearly related to the amount of the polypeptide. Differences in total zein amounts loaded onto the gels were compensated by scaling the raw integrated optical density of every spot *i* in each gel *j* according to the method of Consoli and Damerval (*37*). One way analyses of variance with the genotype as the factor were then performed for each spot on their scaled integrated optical density, and a significant effect was retained at $p < 0.05$. The effect of the different mutations on the amount of zein isoforms was evaluated as compared to the corresponding wild-type background.

Enzymes Partial Purification and Assays. All procedures were carried out at 4 °C unless stated otherwise. Five maize ears were harvested (20 DAP), and the grains were combined and then divided into four sets, which were used for enzyme analysis. The enzymes were extracted and assayed as previously described (*33*, *38*).

Protein Determination. Protein concentrations of the samples were determined as described by Bradford (*39*) using bovine serum albumin as a standard.

RESULTS

Table 1 provides data concerning the percentage contribution of the main N constituents present in the mature endosperm of the two mutants and their respective wild types. The SAA content for the B37*o7* mutant was reduced by 34% whereas the B77xB79*o5* mutant exhibited a 72% increase in comparison to their respective wild types. For storage proteins, the mutants exhibited similar patterns, with increased concentrations of albumins, globulins, and glutelins, whereas the zeins were reduced when compared to the wild types (**Table 1**), leading to a higher accumulation in the mature endosperm of the nonzein storage protein fractions. The B77xB79*o5* mutant exhibited a slight reduction in the concentration of soluble lysine, whereas the B37*o7* mutant exhibited a 15% higher concentration of soluble lysine when compared to their wild-type counterparts (**Table 1**).

Among the four genotypes studied, 44 zein polypeptides were detected (32 and 41, respectively, in the B37 and B77xB79 backgrounds), classified as α22 kDa zeins, α19 kDa zeins, β14 kDa zeins, γ27 kDa zeins, γ16 kDa zeins, and δ10 kDa zeins, according to their apparent molecular masses in the sodium dodecyl sulfate (SDS) dimension (**Table 2** and **Figure 1**). The sensitivity of the colloidal coomassie blue used to stain the zein in 2D gels was approximately 20 ng of protein. The endosperm of the mutant B37*o7* contained a smaller number of zein

Table 1. Percentage Contribution of N Constituents Present in the Mature Endosperm of the Wild-Type and Opaque Maize Lines[a]

	N constituents								
genotypes	SAA	NPN	A + G	zeins	Glu	nonzeins	P% DM	lysine	% increase
B37+	1.30 (0.14)	2.2 (0.12)	3.3 (0.21)	74.3 (1.1)	20.2 (0.6)	23.5	13.1	0.46 (0.04)	
B37*o7*	0.86 (0.07)	2.1 (0.33)	6.3 (0.39)	65.0 (0.7)	26.6 (0.6)	33.0	11.1	0.53 (0.01)	15
B77xB79+	0.80 (0.07)	2.3 (0.07)	3.6 (0.22)	71.1 (3.4)	23.0 (2.0)	26.6	12.8	0.82 (0.02)	
B77xB79*o5*	1.38 (0.23)	2.0 (0.12)	4.4 (0.24)	66.1 (1.3)	27.5 (1.9)	31.9	14.6	0.78 (0.01)	–5

[a] Data expressed as % (± standard deviation) of endosperm total N. N constituents: SAA; NPN; A + G, albumins + globulins – NPN; nonzeins corresponding to glutelins (Glu) + albumins + globulins; P, endosperm total proteins expressed as % of dry matter. Soluble lysine is expressed as percentage of total SAA pool (± standard deviation) followed by percentage increase in lysine in relation to the wild type.

Table 2. Two-Dimensional PAGE Separation of Zeins from Mature Endosperms[a]

spot	B37		B77xB79		spot	B37		B77xB79	
name	WT	o7	WT	o5	name	WT	o7	WT	o5
γ27Z45	0	0	A	A	α19Z93	0	A	0	A
γ27Z38	0	A	0	0	α19Z9	0	A	A	A
γ27Z28	A	A	A	A	α19Z8	0	0	0	A
γ27Z21	A	A	A	B	α19Z7	A	A	B	A
α22Z94	0	A	0	A	α19Z6	A	A	B	A
α22Z4	A	B	B	A	α19Z5	0	0	A	B
α22Z37	0	0	0	A	α19Z23	A	0	0	A
α22Z31	A	0	A	B	α19Z20	A	B	A	0
α22Z3	B	A	B	A	α19Z19	A	0	0	A
α22Z26	0	0	A	B	α19Z17	A	A	A	A
α22Z2	A	B	A	A	α19Z15	0	0	A	0
α22Z18	A	0	0	A	α19Z122	0	0	0	A
α22Z12	A	0	0	0	α19Z102	A	0	0	A
α22Z118	0	0	0	A	α19Z106	0	0	A	0
α22Z110	0	0	A	0	α19Z10	A	A	A	B
α22Z11	A	A	0	A	γ16Z44	A	0	A	0
α22Z107	0	0	A	0	γ16Z13	A	A	B	A
α22Z105	A	A	A	B	γ16Z100	0	0	A	0
α22Z103	A	B	0	A	β14Z33	A	0	A	B
α22Z1	B	A	A	A	β14Z14/Z104	A	A	A	B
α19Z99	0	A	0	0	δ10Z16	A	A	A	B
α19Z95	0	A	0	A	δ10Z109	A	A	A	B

[a] Fourty-four zein isoforms were revealed by 2D PAGE. Every isoform number is prefixed by the name of the zein class. For a given background, statistical analyses were performed to test for significant differences in isoform amounts, and genotypes sharing the same letter did not differ significantly (A indicates an amount significantly greater than B). The same letter in different backgrounds does not indicate an equivalent amount. Numerical values of standardized spot volumes are available on request.

isoforms detected on the 2D gels as compared to the wild type (B37+), indicating a decrease in both zein amount and diversity. In the B77xB79 background, 14 zein polypeptides appeared and eight disappeared specifically in the *o5* mutant. This proportion of qualitative changes is about twice the one observed in the B37 background (22 vs 13) and could be due to the heterozygous nature of B77xB79. In contrast to the *o7* mutation that reduced the diversity in zein polypeptides, the *o5* mutation resulted in a greater diversity of the 2D zein polypeptide pattern as compared to the wild type. For each background, the effect of the mutation on the amount of every isoform was tested using analyses of variance on standardized spot volumes. Both mutations had large effects, since more than 60% of the isoforms differed in amount as compared to their wild types. The mutations *o5* and *o7* had little effect on the γ27 kDa isoforms, whereas large effects of both mutations were observed on the α-zein isoforms. The *o7* mutation decreased the relative amount of about half of the isoforms in the α22 kDa zein class, while *o5* increased the relative amount of more than half of the isoforms of both α-zein classes, even resulting in the appearance of six α22 kDa and seven α19 kDa isoforms not detected in the wild type. The *o5* mutation also markedly decreased the relative amounts of the low molecular mass zeins (β14 kDa and δ10 kDa classes), while *o7* had little effect on these isoforms.

The activities of the enzymes involved in lysine metabolism have been studied in maize endosperm, exhibiting a peak of activity between 16 and 24 DAP depending on the enzyme assayed (*17*, *33*, *34*). In this study, the enzymes AK, HSDH, LOR, and SDH were extracted from the developing seeds (20 DAP) of the wild types and mutants. The activity of AK varied considerably. The introduction of the mutations *o7* and *o5* resulted in significant decreases in AK activity when compared to their wild-type counterparts (**Table 3**). The inhibition of the enzyme activity by lysine also varied, exhibiting a lower inhibitory effect in the mutant lines. The amino acid threonine also exhibited an inhibitory effect on AK activity, which was particularly high in the *o5* mutant (53.6% inhibition), whereas the combination of both amino acids produced greater inhibitory effects than any of the amino acids added separately (**Table 3**).

HSDH was shown to be reduced in the *o5* mutant (7.9%) and increased in the *o7* mutant (34%), when compared to their wild-type counterparts (**Table 3**). When threonine was added to the assay mixture, inhibition of HSDH activity was observed in the wild types and respective mutants, varying from 7.7 to 13.8% (**Table 3**).

Table 3 shows the activity of the enzymes LOR and SDH in 20 DAP endosperms, both involved in lysine degradation. The B37*o7* mutant exhibited slightly reduced LOR and SDH activities (11 and 23%) when compared to the B37+ wild type, with a slight variation in the LOR/SDH ratio, which was close to 1.0. For the B77xB79*o5* mutant, a drastic reduction in the LOR activity was detected (8.9-fold) in comparison to the B77xB79+ wild type, while SDH activity was only reduced by 1.8-fold. The LOR/SDH ratio for the wild type and mutant varied greatly and was different from that obtained with the two B37 lines (**Table 3**). The activity of SDH was also determined following native PAGE (**Figure 2**). Two SDH isoenzyme bands of different intensity were observed following PAGE for all genotypes; the second more electronegative band was always lower in activity. The SDH activity based on band intensity was shown to be slightly reduced in both mutants.

DISCUSSION

The aspartic acid pathway, which leads to the synthesis of lysine, threonine, and methionine, has been studied in detail in order to better understand the regulation of this highly complex pathway (*2*). To accumulate lysine in the seeds, recent reports have shown that manipulation of the catabolism of lysine may be perhaps more important than manipulation of its synthesis (*4*). The analysis of the high lysine *o2* maize mutant confirmed that the higher lysine concentration in this mutant was partially due to a drastic reduction in the rate of lysine breakdown in the endosperm (*17*, *40*), which in association with the altered

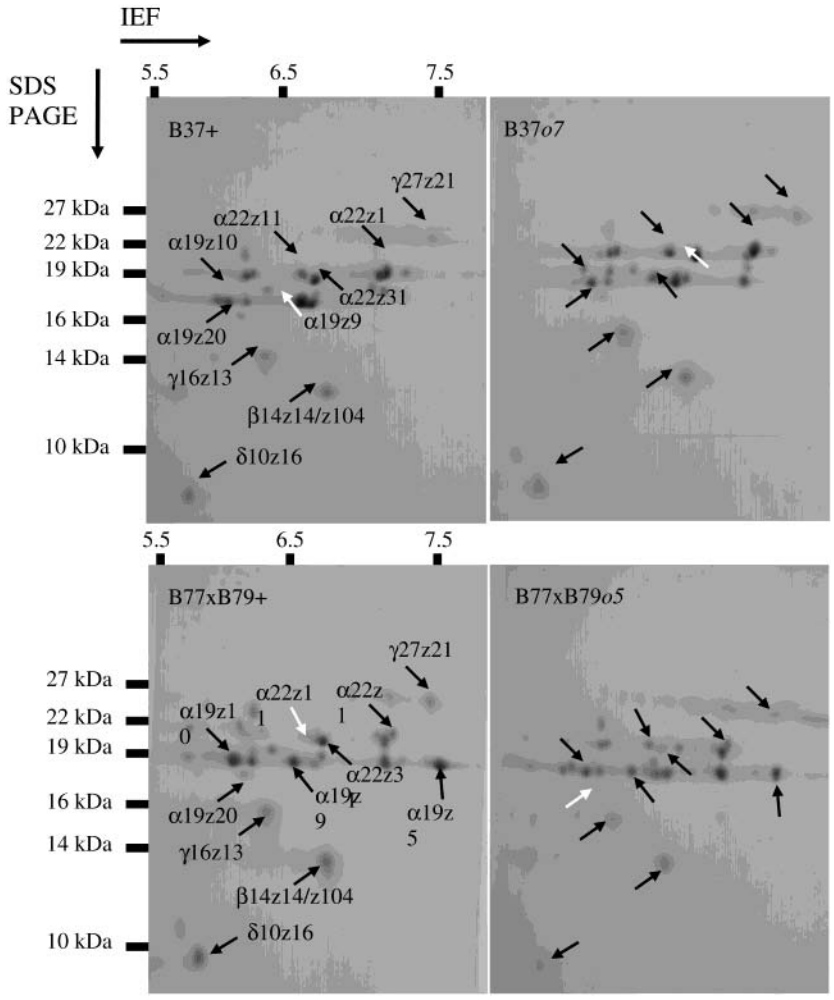

Figure 1. Two-dimensional PAGE separation of zein proteins from the mature endosperm of B37 wild type, B37*o7* mutant, B77xB79 wild type, and B77xB79*o5* mutant. The black arrows point to a numbered individual isoform on each gel, whereas a white arrow indicates an isoform that is deficient in the specific genotype.

Table 3. Specific Activity of AK (nmol min^{-1} mg^{-1} Protein), HSDH (nmol min^{-1} mg^{-1} Protein), LOR (nmol NADPH Oxidized min^{-1} mg^{-1} Protein), and SDH (nmol NAD^+ Reduced min^{-1} mg^{-1} Protein) in 20 DAP Maize Endosperms[a]

	genotypes			
enzyme	B37+	B37*o7*	B77xB79+	B77xB79*o5*
AK				
control (SD)	25.030 (0.793)	9.033 (0.411)	7.708 (0.213)	1.901 (0.090)
% inhibition by L	50.6	35.0	59.6	16.4
% inhibition by T	23.0	26.0	4.9	53.6
% inhibition by LT	78.9	53.6	74.7	70.0
HSDH				
control (SD)	20.3 (0.61)	27.3 (1.00)	21.5 (0.97)	19.8 (0.43)
% inhibition by T	13.8	7.7	12.1	9.1
LOR (SD)	2.926 (0.151)	2.610 (0.111)	3.397 (0.197)	0.380 (0.039)
SDH (SD)	3.237 (0.193)	2.500 (0.099)	2.410 (0.080)	1.300 (0.111)
LOR/SDH ratio	0.90	1.04	1.41	0.29

[a] Control: specific activity with no inhibitor present. Standard deviation (± SD) values were all below 5% for the lysine (L), threonine (T), and lysine plus threonine (LT) treatments. Five millimolar amino acids L, T, and LT was used.

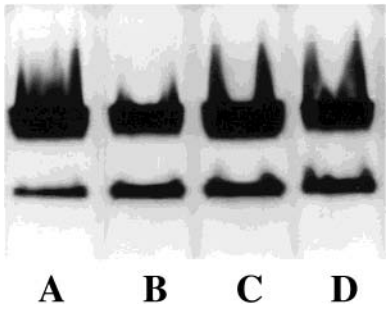

Figure 2. Activity of SDH following the separation of the proteins of 20 DAP maize endosperm by native PAGE: (**A**) B37+ wild type, (**B**) B37*o7*, (**C**) B77xB79+, and (**D**) B77xB79*o5* maize lines.

distribution of the storage proteins resulted in a high lysine concentration in the seed. Various other endosperm mutants classified as high lysine are available, but little is known about lysine metabolism in their seeds (*33*). Here, we have reported the characterization of two of these mutants, *o5* and *o7*. Instead of transferring the mutations to one common inbred line, we have analyzed each one by always comparing the results against the respective wild-type counterpart. The B77xB79*o5* mutant exhibited a yellow leaf phenotype as previously described (*32*), and this continued into the late stages of development.

The increased lysine concentration reported in the literature for the *o2* mutant can be due to an altered distribution of the endosperm proteins and to the soluble lysine accumulated in the endosperms (*15*). The B37*o7* mutant concentration of total SAA was shown to be reduced but exhibited an increased relative concentration of soluble lysine when compared to the wild type. Moreover, the nonzein storage proteins, which are

rich in lysine, were shown to be increased when compared to the wild type, which on balance indicates that the B37*o7* mutant exhibits an increased total lysine concentration in the endosperm. Although the B77xB79*o5* mutant exhibited a decreased relative concentration of soluble lysine but an increased concentration of total SAA, the high lysine trait reported for this mutant is likely to be due to the increased proportion of nonzein storage proteins as observed for the B37*o7* mutant. Furthermore, Hunter et al. (*32*) reported a 1.4-fold increase in soluble lysine for the *o5* mutation in a different genetic background (W64A).

Hunter et al. (*32*) used one-dimensional SDS−PAGE to compare qualitative and quantitative differences in zein patterns among a range of opaque mutants (including *o5* but not *o7*), and except for *o2*, little effect of the mutations was observed. The analysis was refined by immunoblotting with specific antisera, which demonstrated alterations in the amount of distinct isoforms of zeins. Using 2D PAGE, we have been able to observe complex patterns of alterations in the mutants B77xB79*o5* and B37*o7*, as compared to their respective wild types. The various isoforms are not due to artifacts during isolation and/or fractionation but to genetic differences in charge and amino acid content of the individual proteins (*41*). The introduction of the mutations had a major effect on the α-class of zein isoforms. The proportion of α22 kDa zein isoforms that was present in different amounts in the mutant as compared to the wild type was similar in *o5* and *o7*, but the *o5* mutation appeared to have a greater effect on the α19 kDa isoforms. Previous studies have indicated that the *o7* mutation can cause a reduction in the α19 kDa zein (*42*). We have shown here that the global reduction in zeins is complex at the isoform level, since some of the isoforms decreased in amount while other ones increased. In fact, a given mutation can increase or decrease the relative amount of different isoforms belonging to the same class of zein, indicating very specific effects (*33*). The *o5* mutation also resulted in a decreased amount of all low molecular mass *β*14 and *δ*10 kDa isoforms of zein.

Lysine metabolism has been investigated in maize (*2*) including the *o2* mutation (*17*), which was until recently the only source of information in the literature as far as lysine metabolism is concerned. We have used two distinct genetic backgrounds, B37 and B77xB79; however, the effect of the mutations was always analyzed against the wild-type counterpart, which should limit differences due to developmental age. The results observed for AK provide evidence that there is a wide variation in terms of total AK activity depending on the genetic background used, with a 3.2-fold higher activity in the B37+ background. The introduction of both mutants resulted, independent of the genetic background, in a strong reduction of total AK activity, in the order of 64% for the B37*o7* and 75% for the B77xB79*o5* mutants (**Table 3**). The presence of the *o7* mutation caused a similar but not identical reduction in both lysine and threonine sensitive forms of AK. The reduction in inhibition by lysine agrees with the values previously observed for the *o2* mutant (*17*, *33*, *43*, *44*). However, the effect of the *o5* mutation was more intense and suggested that this mutation may be directly affecting the lysine sensitive and threonine sensitive isoenzymes, in a different manner. The lysine sensitive activity in the B77xB79*o5* mutant was reduced by 93%, while the threonine sensitive activity was increased by 170%, when compared to the wild type (**Table 3**). Although changes in total HSDH activity were detected, there was little evidence of an effect of either mutation on the activity of the threonine sensitive form of HSDH (**Table 3**). Although HSDH and DHDPS share the same substrate (aspartate semialdehyde), which could eventually be a key point in determining the flux of carbon through the pathway, leading to threonine or lysine biosynthesis, it has been suggested that HSDH does not have a regulatory role in the biosynthesis of lysine (*2*).

The enzymes of lysine catabolism, LOR and SDH, were also analyzed in all four genotypes and exhibited significant alteration in activity depending on the mutant (**Table 3**). LOR and SDH were initially identified as one bifunctional enzyme containing both enzyme domains (*4*, *25*, *26*), and later, monofunctional LOR and SDH enzymes were identified (*26*). The results reported in the literature generally indicated that SDH activity is more stable than LOR activity (*4*). Although the SDH activity staining did not exactly provide the same results that were observed in the spectrophotometer assay, it allowed the identification of two clearly separated isoenzymes, indicating that the SDH activity was due to the presence of two bands **(Figure 2)**. The predominant band is likely to be the bifunctional polypeptide, while the second band of faster mobility, which appeared in all genotypes, is likely to be the monofunctional SDH such as observed in *Arabidopsis* (*45*). However, the level of the activity of this minor band cannot explain the variation in SDH activity observed between the genotypes studied. For the B77xB79*o5* mutant, the predominant band appears to be reduced when compared to the wild type but not the minor band, suggesting that the *o5* mutation may have an effect on the bifunctional LOR−SDH. The *o7* mutation did not cause a major effect on LOR and SDH activities; however, the introduction of the *o5* mutation in the B77xB79 maize inbred line resulted in a 90% reduction in LOR activity, when compared to the wild type, in a manner similar to that previously observed for the *o2* (*17*, *31*, *33*), *floury 1*, and *floury 2* mutations (*33*). There was, however, only a 46% reduction of SDH activity in the mutant, clearly indicating a main effect of the *o5* gene on the LOR domain of the bifunctional LOR/SDH protein, leading to a completely altered ratio of LOR/SDH activity in relation to the wild type (**Table 3**). This result further suggests, as observed for AK, that the *o5* mutation is directly affecting lysine metabolism in a similar way to that observed for the *o2* mutation, particularly when LOR activity is concerned. It has been shown in previous studies that LOR has an essential role in the regulation of lysine catabolism, since this enzyme is modulated by Ca^{2+}, ionic strength, and protein phosphorylation/dephosphorylation in several plant species (*22*, *37*, *46*); however, such modulation effects do not appear to influence SDH activity. The pleiotropic regulation is also supported by the effect of the mutations on the storage proteins analyzed by 2D PAGE.

The increases in the concentration of soluble lysine, nonzein storage proteins, and the effect on specific zein isoforms, associated to the effect of lysine metabolic enzymes, are likely to be due to the direct effect of the *o5* mutation, although it is not as intense as that previously observed for the *o2* mutation (*17*, *32*). As already mentioned, several other genes in the same phenotypic class as the *o2* gene may also strongly influence lysine metabolism and storage protein synthesis and accumulation in maize. Recently, the effect of other opaque and floury mutations has indicated that the catabolism of lysine catalyzed by the enzyme LOR and possibly the biosynthetic enzymes AK and to a lesser extent HSDH may be under the regulation of opaque and floury genes (*33*). Therefore, a new range of studies, including the analysis of other enzymes of the aspartate pathway, particularly DHDPS, must be carried out to determine the precise biochemical and molecular regulation caused by such mutations.

ABBREVIATIONS USED

AK, aspartate kinase; HSDH, homoserine dehydrogenase; LOR, lysine 2-oxoglutarate reductase; N, nitrogen; NPN, nonprotein nitrogen; SDH, saccharopine dehydrogenase; SAA, soluble amino acids.

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