UNIVERSIDADE ESTADUAL DE CAMPINAS

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"CATABOLISMO DA LISINA EM SORGO: ISOLAMENTO, PURIFICAÇÃO PARCIAL E CARACTERIZAÇÃO DAS ENZIMAS LOR E SDH E SUA RELAÇÃO COM O ACÚMULO DA LISINA EM SEMENTES".

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RESUMO

A lisina é um aminoácido essencial, sintetizada em plantas através da via metabólica do ácido aspártico. O catabolismo da lisina é desempenhado pela ação de duas enzimas consecutivas, lisina 2-oxoglutarato redutase (LOR) e sacaropina desidrogenase (SDH). A concentração final de lisina solúvel em sementes de cereais é controlada tanto pela taxa de biossíntese quanto de catabolismo, porém, o catabolismo desempenha um papel central neste controle. O entendimento dos aspectos regulatórios das vias metabólicas de biossíntese e degradação da lisina, bem como a manipulação das enzimas relacionadas são importantes para a produção e caracterização de plantas com alta concentração de lisina, principalmente que acumulem lisina nas sementes. Neste trabalho foi isolada, purificada parcialmente e caracterizada a LOR e SDH de sorgo. Estes procedimentos foram particularmente trabalhosos devido a instabilidade e atividades muito baixas das enzimas, embora diversos métodos de purificação e modificações nos tampões tenham sido testados. Um pico principal de atividade para LOR e SDH foi observado e co-purificado após precipitação com sulfato de amônio e cromatografia de troca iônica, sugerindo a existência de um polipeptídeo bifuncional contendo dois domínios, conforme previamente observado para LOR e SDH em outras espécies de plantas estudadas. As

análises da concentração de lisina em sementes sugerem que o sorgo naturalmente contém concentrações de lisina elevadas, quando comparado a outros cereais, o que pode ser explicado pelas baixas atividades de LOR e SDH observadas, de maneira similar a outras plantas que acumulam lisina já estudadas. Devido a estas altas concentrações de lisina, estudos futuros podem ser desenvolvidos para melhor caracterizar o sorgo como uma fonte alimentar rica no conteúdo do aminoácido essencial lisina, entre outros.

ABSTRACT

Lysine is an essential amino acid, synthesized in plants in the aspartic acid metabolic pathway. Lysine catabolism is performed by the action of two consecutive enzymes, lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH). The final soluble lysine concentration in cereal seeds is controlled by both, the synthesis and catabolism rates, with an apparent central role played by the catabolism. The understanding of the regulatory aspects of lysine biosynthesis and catabolism and the manipulation of the enzymes involved in both processes is important for the production and characterization of high-lysine plants. In this work we have isolated, partially purified and characterized LOR and SDH from sorghum. The isolation, purification and even characterization were particularly difficult due to instability and very low activities of the enzymes during purification, although several methods of purification and modifications to the buffer system had been tested. One main peak of LOR and SDH activity was observed and co-purified after ammonium sulphate and anion exchange

chromatography, suggesting the existence of a bifunctional polypeptide containing two domains as previously observed for LOR and SDH of all plant species studied so far. Analysis of lysine concentration in the seeds suggests that sorghum naturally contains much higher lysine concentration than other cereal crops, which may explain the low LOR and SDH activities observed, in a similar manner to other high-lysine plants studied. Due to this high lysine concentration further studies should be carried out in order to better characterize sorghum as a food source of the essential amino acid lysine.

INTRODUÇÃO E OBJETIVO

Plantas, e grande parte das bactérias e fungos, quando supridos por uma fonte adequada de carbono e nitrogênio inorgânico, possuem a capacidade de sintetizar todos os 20 aminoácidos normalmente incorporados em uma proteína, enquanto animais monogástricos podem sintetizar apenas 11 destes aminoácidos. Os nove aminoácidos restantes, os quais são chamados de essenciais, precisam ser suplementado aos animais através de sua dieta.

A síntese de lisina, treonina, metionina e isoleucina, quatro dos aminoácidos essenciais, é iniciada em uma via metabólica complexa e regulada em diversos passos enzimáticos, que tem como precursor o ácido aspártico ou aspartato e conta então com a ação de diversas enzimas que catalisam reações que culminam na síntese desses aminoácidos acima citados (Azevedo *et al.*, 1997).

Como sementes de cereais que constituem a principal fonte de proteínas em plantas são deficientes em lisina e treonina, e os legumes são deficientes em metionina, os estudos dessa via de biossíntese pela conversão do aspartato têm despertado grande interesse devido ao seu potencial para o enriquecimento desses alimentos e melhoria de sua qualidade nutritiva. Estudos bioquímicos e genéticos e mais recentemente moleculares levaram à identificação de alguns mecanismos envolvidos na regulação desta via, mostrando que várias destas enzimas são reguladas por retroinibição pelos aminoácidos como lisina, treonina e metionina que são produtos finais dos diversos ramos da via do ácido aspártico (Rognes *et al.*, 1980) e por interações gênicas (Brennecke *et al.*, 1996).

O catabolismo da lisina em plantas foi primariamente estudado e demonstrado em trigo, milho e cevada, através de experimentos que utilizaram [^{14}C] lisina, com a radioatividade incorporada no ácido α-amino adípico e ácido glutâmico, indicando que este aminoácido é oxidativamente degradado através da sacaropina (Nigam and McConnel, 1963; Lawrence and Grant, 1964; Brandt, 1975; Sodek and Wilson, 1970).

As duas enzimas desta via de degradação foram estudadas em detalhes em animais e microorganismos e recentemente em plantas. A lisina 2-oxoglutarato redutase (LOR, EC 1.5.1.8), (também conhecida como lisina ketoglutarato redutase – LKR) é a primeira enzima desta via, e que condensa lisina e 2-oxoglutarato para formar sacaropina, a qual é então hidrolisada em ácido α-amino adípico e ácido glutâmico em uma reação catalisada pela enzima sacaropina desidrogenase (SDH, EC 1.5.1.9). De acordo com a literatura, a massa molecular das enzimas exibiu alguma variação além de particularidades na modulação e nas características físico-químicas. Em adição, cereais geralmente apresentam enzimas bifuncionais com as atividades de LOR e SDH residindo em um mesmo polipeptídio (Azevedo *et al*., 1997; Azevedo and Lea 2001; Gaziola *et al*., 1997; Lugli *et al*., 2003). Estes aspectos serão melhor discutidos mais adiante nas revisões bibliográficas dos artigos apresentados neste trabalho.

Embora a biossíntese de lisina já foi detalhadamente estudada, com as enzimas chaves como aspartato quinase (AK, EC 2.7.2.4), dihidrodipicolinato sintase (DHDPS, EC 4.1.2.52), homoserina desidrogenase (HSDH, EC 1.1.1.3) entre outras já determinadas (Rognes 1980; Azevedo *et al*., 1992, 1997) e extensivas informações

estejam disponíveis sobre os seus aspectos regulatórios, uma quantidade menor de informações sobre o catabolismo da lisina pode ser encontrada.

Estudos recentes indicam fortemente que o catabolismo de lisina desempenha importante papel no acúmulo de lisina em plantas, sendo que o controle do nível de lisina, particularmente em sementes, pode ser regulado tanto pela sua síntese quanto pelo catabolismo (Arruda *et al.*, 2000). Em milho, estudos iniciais com enzimas envolvidas na degradação da lisina sugeriram que este catabolismo é um dos principais mecanismos que controlam a concentração de lisina solúvel no endosperma (Da Silva and Arruda, 1979; Arruda *et al.*, 1982; Arruda and Da Silva, 1983). Os resultados sugeriram que a concentração de lisina solúvel é controlada principalmente pela taxa de catabolismo, em vez da inibição da sua síntese por retroinibição.

O entendimento do metabolismo da lisina, incluindo a biossíntese e particularmente o catabolismo, pode contribuir para o sucesso na manipulação das enzimas envolvidas e possivelmente, auxiliar em programas que visem à melhoria da qualidade nutritiva em cereais. Particularmente neste caso, o sorgo que ainda não foi estudado neste aspecto, pode ocupar lugar de destaque em programas de melhoramento, em função de sua importância na alimentação humana e principalmente animal, aliada ao fato ser uma espécie resistente a condições ambientais até certo ponto severas, como estresse hídrico e calor.

Dessa forma, baseados nas informações da literatura, nós percebemos a necessidade de elucidar diversos pontos relativos aos padrões de distribuição de proteínas de reserva, aos fatores que determinam a proporção de aminoácidos nas suas diversas classes, particularmente a lisina, e principalmente o papel do

catabolismo na regulação do fluxo metabólico através da via do ácido aspártico. A variabilidade observada nos padrões enzimáticos para LOR e SDH entre as espécies e mutantes estudados, também reforça tal necessidade. Assim, procuramos estudar o catabolismo da lisina em sorgo através da análise destas enzimas (LOR e SDH), já que nenhum estudo com esta abordagem havia sido realizado até então. Nosso objetivo foi o de aumentar o conhecimento básico sobre este metabolismo, visando sua utilização em trabalhos futuros, principalmente a melhoria da qualidade nutritiva deste cereal, bem como de sua melhor utilização em programas de melhoramento e na composição de dietas mais equilibradas.

A seguir, no capítulo 1, brevemente relataremos algumas características da cultura do sorgo e sua utilização apenas em caráter informativo, para justificar a escolha dessa cultura e salientar sua importância. De forma mais detalhada relataremos as informações disponíveis na literatura abordando os diversos aspectos relacionados com este trabalho, destacando a importância do catabolismo para o acúmulo de lisina em sementes. No capítulo 2, apresentaremos os dados mais relevantes de nosso trabalho com o genótipo Massa 03 e também dados obtidos com diversos mutantes para alta concentração de lisina.

CAPÍTULO 1 – REVISÃO DA LITERATURA

Neste capítulo será apresentada a revisão da literatura relativa a importância do sorgo no cenário mundial e nacional, bem como as caracteríticas e importância dos genótipos que apresentam alto acúmulo de lisina. Além disso será apresentado um artigo de revisão sobre o catabolismo da lisina, sendo abordados aspectos regulatórios das enzimas, papeis funcionais e recentes descobertas ao nível bioquímico e molecular, intitulado:

"Lysine Catabolism: flow, metabolic role and regulation".

A Importância do Sorgo e dos Genótipos com Alta Concentração de Lisina

O grão de sorgo [*Sorghum bicolor* (L.) Moench] é a principal base alimentar para milhões de pessoas tanto em países desenvolvidos como em desenvolvimento , como nas regiões semi-áridas da África e Ásia (Weaver *et al.*, 1988).

O sorgo, cultivado para silagem, recebeu considerável atenção na última década, particularmente nos EUA, devido a sua tolerância à seca e extenso período adequado ao plantio (McDonald *et al.*, 1991). No Brasil atualmente ocupa de 10 a 12% da área cultivada para silagem (Rocha *et al.*, 2000).

A produção de silagem é importante na conservação de plantas forrageiras e tem grande importância econômica para a maioria dos países em virtude da produção irregular de forrageiras durante as estações do ano, principalmente no inverno, determinando queda de produtividade dos rebanhos e redução no suporte dos pastos (Brito, 1995; Chaves 1997). Assim, as alternativas e formas de suprir tais necessidades são alvos das pesquisas no campo da nutrição animal (Brito, 1995; Nogueira, 1995).

As culturas de milho e sorgo são as mais utilizadas no processo de ensilagem, por sua facilidade de cultivo, altos rendimentos e qualidade da silagem produzida sem aditivos para estimular a fermentação (Zago, 1999). O sorgo é utilizado devido às suas características agronômicas como a alta produção de forragem, maior tolerância à seca e ao calor, capacidade de explorar maior volume de solo (Brito, 1995). A variabilidade genética para suas características nutricionais, tem permitido um eficiente trabalho de melhoramento, com o desenvolvimento de híbridos de alto valor nutritivo (Zago, 1999).

O sorgo apresenta uma baixa digestibilidade quando comparado a outros cereais principais (Hamaker *et al.*, 1986). Como demonstrado por McLean *et al.* (1981), a digestibilidade de proteínas do sorgo, trigo, milho e de arroz foi de 46%, 81%, 73% e 66%, respectivamente, em crianças. Adicionalmente, esta característica foi independente do conteúdo de tanino (Elkin *et al.*, 1996), o qual é conhecido como redutor da digestibilidade de proteínas de sorgo.

As principais proteínas em grãos de sorgo são as proteínas de reserva prolaminas, denominadas kafirinas, que constituem em cultivares normais aproximadamente 80% das proteínas do endosperma (Hamaker *et al.*, 1995). Estas são classificadas como α-, β-, e γ-kafirinas. As α-kafirinas constituem 80% do total de kafirinas e estão localizadas na luz da região central de corpos protéicos (Shull *et al.*, 1991). As β-, e γ-kafirinas, as quais apresentam grande quantidade de pontes dissulfeto em grãos em desenvolvimento (Oria *et al.*, 1995a), são encontradas principalmente na periferia dos corpos protéicos. Isto sugere que uma barreira enzima-resistente na periferia destes corpos protéicos dificulta o acesso de proteases ao interior onde estão localizadas as proteínas α-kafirinas mais facilmente digeridas (Oria *et al.*, 1995 a, b).

Desde a década de 50, a lisina é relatada como o primeiro aminoácido limitante em sorgo (Shelton *et al.*, 1951) e desde a década de 70, diversos genótipos de sorgo com elevados conteúdos de lisina foram identificados, como os acessos IS11167 e IS 11758, e o gene recessivo *hl* identificado como responsável por esta característica (Singh and Axtell, 1973).

Genótipos de sorgo com conteúdo de lisina elevado, comparados ao sorgo normal mostraram um aumento no conteúdo de não-kafirinas ricas em lisina (Hamaker *et al.*, 1995). Linhagens originadas destes genótipos de sorgo com alto conteúdo de lisina apresentaram digestibilidade de proteínas substancialmente mais altas comparadas a cultivares de sorgo normal e mostraram uma mudança na digestibilidade de α-kafirina com 90-95% de α-kafirinas digeridas em 60 minutos comparadas a 45-60% para cultivares normais (Weaver *et al.*, 1988). Foi demonstrado que mutantes quimicamente induzidos para alto conteúdo de lisina (P7210) apresentaram 25% menos kafirina comparados a um cultivar com baixo conteúdo de lisina (White Martin) e que o gene da α-kafirina é funcional e endospema-específico (Reddy *et al.*, 2001).

Proteínas de grãos de sorgo mais digestíveis e com alto teor de lisina podem ser um importante benefício para populações submetidas a dietas com conteúdos inadequados de proteínas. Estudos adicionais em andamento, podem aumentar o valor do sorgo como um grão alimentar (Weaver *et al.*, 1988). Porém, o sucesso neste desafio depende de estudos bioquímicos e moleculares do catabolismo e biossíntese de lisina em cereais, com mutantes induzidos e naturais, além da manipulação de enzimas-chaves envolvidas (Azevedo and Lea, 2001; Arruda *et al.*, 2000).

Para um melhor entendimento do metabolismo de lisina, particularmente do seu catabolismo, a seguir serão apresentados e discutidos aspectos relativos a regulação em diferentes espécies, e recentes descobertas ao nível bioquímico e molecular incluídos no artigo de revisão:

Lisine Catabolism: flow, metabolic role and regulation

Lysine catabolism: flow, metabolic role and regulation

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Lysine is an essential amino acid, synthesized in plants in the aspartic acid pathway. The lysine catabolism is performed by the action of two consecutive enzymes, lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH). The steady state of lysine is controlled by both, synthesis and catabolism rates, with the final soluble lysine concentration in cereal seeds a direct result of these processes. In the last 40 years, the enzymes involved in lysine biosynthesis have been purified and characterized from some plant species such as carrot, maize, barley, rice, and coix. Recent reports have revealed that lysine degradation might be related to various physiological processes, for instance growth, development and response to environmental changes and stress. The understanding of the regulatory aspects of the lysine biosynthetic and catabolic pathways and manipulation of related enzymes is important for the production of high-lysine plants.

Key words: amino acids, lysine, metabolism, mutants.

Catabolismo da lisina: fluxo, papel metabólico e regulação: A lisina é um aminoácido essencial, sintetizado na via metabólica do ácido aspártico. O catabolismo da lisina é realizado pela ação das enzimas consecutivas, lisina 2-oxoglutarato redutase (LOR) e sacaropina desidrogenase (SDH). A concentração final de lisina solúvel em plantas, incluindo cereais, é controlada tanto pela taxa de síntese quanto pela de catabolismo. Nos últimos 40 anos, as enzimas envolvidas na biossíntese de lisina foram isoladas, purificadas e caracterizadas em várias espécies vegetais, incluindo cenoura, milho, cevada, arroz e *coix*. Relatos mais recentes revelaram que o catabolismo da lisina pode estar envolvido em vários processos fisiológicos como crescimento, desenvolvimento, resposta a mudanças e estresses ambientais. A manipulação das enzimas dessa via metabólica é imprescindível para obtenção de plantas que acumulem altos níveis de lisina solúvel, bem como um melhor conhecimento desse metabolismo.

Palavras-chave: aminoácidos, lisina, metabolismo, mutantes.

Aspartic acid pathway in plants

Plants, fungi, yeasts and most of the bacteria, usually synthesize all the 20 amino acids incorporated in a protein, while monogastric animals can only synthesize 11 of them. The nine remaining amino acids, which are termed essential, need to be provided by the diet.

The syntheses of the essential amino acids lysine, threonine, methionine and isoleucine are carried out in a complex and strongly regulated metabolic pathway, which has aspartic acid as a precursor with several enzymes being regulated by feedback inhibition (figure 1A). Since cereal seeds constitute the main source of proteins in plants and are deficient in lysine and threonine, extensive studies of this pathway have been carried out, with special attention to the potential improvement of the nutritious quality. Such studies allowed the identification of important regulatory mechanisms, showing that many enzymes are positively or negatively regulated by the end-products of the pathway (feedback) or their analogues (Heremans and Jacobs, 1994; Azevedo et al., 1997; Feller et al., 1999).

Aspartate kinase (AK), the first enzyme of the aspartic acid pathway, has been extracted, partially purified and

well characterized in several higher plants (Azevedo et al., 1992a,b; Azevedo et al., 1997; Dey and Guha-Mukherjee, 1999). A bifunctional polypeptide with both threonine-sensitive AK and homoserine dehydrogenase (HSDH) activities has been observed in some plant species (Azevedo et al., 1992b; Wilson et al., 1991; Texeira et al., 1998). The activity of the enzyme dihydrodipicolinate synthase (DHDPS), which is strongly inhibited by low concentration of lysine, has been shown to be a key regulatory point of the lysine biosynthesis branch of the pathway (Azevedo et al., 1997). Although a great deal of information is available about the lysine biosynthetic enzymes and their regulation, little is know about lysine degradation. Success in obtaining high-lysine plants for human or animal consumption relies on the full understanding of the metabolism of lysine as well as for subsequent genetic manipulation (Azevedo, 2002).

The lysine catabolism in plants

The lysine catabolism (figure 1B) in plants was initially studied in wheat, maize and barley in experiments using ^{14}C-lysine, with the radioactivity being incorporated into α-aminoadipic acid and glutamate, indicating that this amino acid is oxidatively degraded through saccharopine (Brandt, 1975; Sodek and Wilson, 1970).

Recent studies have indicated that the lysine catabolism plays an important role for lysine accumulation in plants and the control of the lysine content, particularly in seeds (Arruda et al., 2000). Initial studies with enzymes involved in the lysine degradation reinforced the main role of catabolism in the control of the soluble lysine concentration in the maize endosperm (Arruda et al., 1982; Arruda and da Silva, 1983). The amount of lysine that was shown to be translocated from other tissues to the developing endosperm, for the synthesis of storage proteins, was 2 to 3-fold higher than what would be necessary (Arruda and da Silva, 1983). Due to excess lysine, an accumulation of lysine in the soluble form would be expected, however this does not occur, since the soluble lysine concentration was maintained at a low level during the development of the endosperm. These low lysine levels could contribute by preventing the inhibition of AK activity and facilitate the subsequent biosynthesis of methionine. These results indicated that the soluble lysine concentration is mainly controlled by the catabolic rate, instead of by the feedback inhibition of its synthesis.

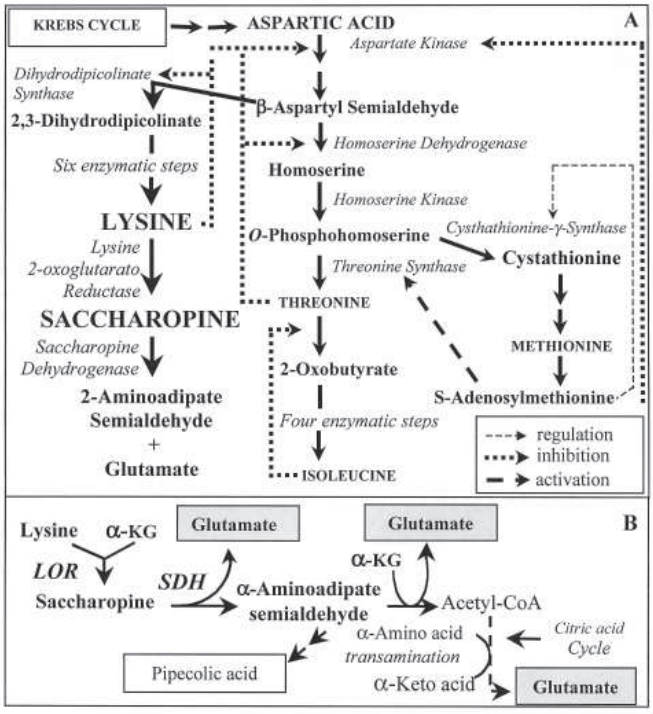

Figure 1. Aspartate metabolic pathway (A). Plant lysine catabolic pathway (B). Modified from Azevedo and Lea (2001).

The purification and characterization of LOR and SDH enzymes

Two main enzymes have been shown to be part of the lysine catabolic pathway in animals, micro-organisms and recently in plants. Lysine 2-oxoglutarate reductase (LOR; EC 1.5.1.8), (also known as lysine α-ketoglutarate reductase, LKR) is the first enzyme of the pathway, which condenses lysine and 2-oxoglutarate to form saccharopine, which is then hydrolyzed to α-amino adipic acid and glutamic acid in a reaction catalyzed by the enzyme saccharopine dehydrogenase (SDH; EC 1.5.1.9). The net result of these two reactions resembles a transaminase reaction in which the α-amino group of lysine is transferred to 2-oxoglutarate to form glutamate (Azevedo et al., 1997; Azevedo and Lea, 2001). Kinetic studies have shown distinct results between maize and rice enzymes, for instance. In maize, saccharopine is a competitive inhibitor of lysine and non-competitive of 2-oxoglutarate, suggesting a mechanism were lysine first interacts with the enzyme, and afterwards with 2-oxoglutarate and NADPH, releasing $NADP^+$ and saccharopine (Brochetto-Braga et al., 1992). The opposite was observed for the rice enzymes (Gaziola et al., 1997). Both enzymes have been

well-characterized in mammals and were shown to be part of one single bifunctional polypeptide (Markovitz and Chuang, 1987). The human bifunctional enzyme is a tetramer with a molecular mass of 460 kDa, with 115 kDa subunits (Fjellstedt and Robinson, 1975a; Markovitz and Chuang, 1987). In fungi and yeast, the structures of LOR and SDH are comprised of monomers of 49 kDa and 73 kDa, encoded by the *Lys1* and *Lys9* genes, respectively (Ramos et al., 1988; Feller et al., 1999). It was only recently that these enzymes have received more attention in plants, being isolated and characterized in such species as maize, rice, soybean, *Phaseolus*, *Arabidopsis*, canola and coix. In maize, rice and coix the bifunctional enzyme LOR-SDH was shown to be endosperm-specific (Gonçalves-Butruille et al., 1996; Gaziola et al., 1997; Lugli et al., 2003; Azevedo and Lea, 2001). In maize (Gonçalves-Butruille et al., 1996), rice (Gaziola et al., 1997), soybean (Miron et al., 2000), *Phaseolus vulgaris* (Cunha-Lima et al., 2002) and coix (Lugli et al., 2002) the activities of LOR and SDH reside in the same bifunctional polypeptide, similar to what has been observed in mammals (Fjellstedt and Robinson, 1975a; Markovitz and Chuang, 1987). Recently, the presence of one additional monofunctional SDH enzyme was demonstrated in *Arabidopsis* (Tang et al., 1997) and canola (Zhu et al., 2000) which is interesting since both have already been reported as having a bifunctional LOR-SDH enzyme (Tang et al., 1997; Zhu et al., 2000). Furthermore, a new monofunctional LOR has now been detected in *Arabidopsis* (Galili et al., 2001) and in cotton (Tang et al., 2002).

The molecular mass of the LOR-SDH enzyme exhibits some variation among plant species (Azevedo and Lea, 2001). In maize, the polypeptide presents a monomeric form of 125 kDa (Gonçalves-Butruille et al., 1996) or 140 kDa (Brochetto-Braga et al., 1982), when determined by SDS-PAGE or native PAGE, respectively, or 260 kDa when determined by gel filtration, in a dimmer structure, with two 117 kDa subunits, which constitutes the native form of the enzyme (Gonçalves-Butruille et al., 1996; Kemper et al., 1999). These subunits could be cleaved by elastase digestion into five bands ranging from 35 kDa to 65 kDa (Kemper et al., 1998). The separation of the five bands during the course of proteolyses could be associated with LOR and SDH activities, and the predominant 65 and 57 kDa bands contained the functional domains of LOR and SDH activities, respectively (Kemper et al., 1998).

In rice, the LOR-SDH protein exhibited a molecular mass of approximately 203 kDa when determined by PAGE and gel filtration, with the presence of multimeric forms, probably dimmeric or tetrameric states 396 kDa (Gaziola et al., 1997). In *Arabidopsis*, a monomeric form of 116 kDa was observed (Tang et al., 1997). In *Phaseolus vulgaris*, the activities of LOR-SDH also reside in a bifunctional protein and depending on the purification procedure, may elute as a monomer of 94 kDa with SDH activity only, or a dimmer of 190 kDa with both enzyme activities (Cunha-Lima et al., 2003). In soybean, monomeric forms of 100 and 123 kDa, and a 256 kDa dimmeric form were identified (Miron et al., 2000).

The regulation of LOR and SDH enzymes in plants

Recent studies with different plant species have demonstrated that lysine may autoregulate its own catabolism *in vivo*, with the enzymes differentially modulated by a intracellular signaling cascade, involving mainly Ca^{2+}, protein phosphorylation-dephosphorylation and ionic strength (Karchi et al., 1995; Miron et al., 1997; Kemper et al., 1998; Gaziola et al., 2000). Karchi et al. (1995) working with tobacco seeds observed that the activity of LOR could be stimulated by exogenous lysine and this stimulatory effect was significantly reduced when the seeds were treated with the Ca^{2+} chelator EGTA, an inhibitory effect that could be overwhelmed with addition of Ca^{2+}. In maize, Ca^{2+} was also shown to modulate LOR activity, whereas SDH activity was not. The Ca^{2+}-dependent LOR activity increase was also tested for inhibition by two structurally different calmodulin inhibitors, which almost completely inhibited the activity of LOR (Kemper et al., 1998). In rice, the results pertaining to the regulation were similar to those observed in maize, for both enzymes (Gaziola et al., 2000). Kemper et al. (1999) reported evidence for a Ca^{2+} effect on the oligomerization state of LOR-SDH from maize. Ca^{2+} stimulated LOR activity through the dimerization of only the LOR domain, and had no effect on the SDH activity. Figure 2A illustrates a model presented by Arruda et al. (2000).

In addition, LOR modulation has been demonstrated in maize with ionic strength, whereas the SDH activity remained unaltered (Kemper et al., 1998). Organic solvents at concentrations that lowered the water activity increased LOR activity (Kemper et al., 1998). In tobacco and

soybean, the LOR activity was modulated with bifunctional polypeptide phosphorylation, but SDH activity was not modulated. The phosphorylation-dephosphorylation with kinase-casein II and alkaline phosphatase respectively, indicated that active LOR is a phosphoprotein with the activity being modulated by the opposite actions of the kinase and phosphatase proteins (Karchi et al., 1995; Miron et al., 1997).

In recent reports, the effects on LOR-SDH activity caused by aminoethyl-l-cysteine (AEC), a lysine analogue, and S-adenosylmethionine (SAM) have also been tested (Gaziola et al., 2000; Lugli et al., 2002). In rice, AEC was shown to be able to substitute for lysine as a substrate for LOR, but less efficiently, whereas SAM did not produce any significant changes (Gaziola et al., 1999; Gaziola et al., 2000). On the other hand, in maize, AEC was not able to substitute for lysine as a LOR substrate (Brochetto-Braga et al., 1992).

Although LOR-SDH from animals, yeast and plants have some different properties, others are common, such as optimum pH, which are neutral for LOR (7.0 to 7.5) and basic for SDH (8.0 to 9.0) (Gonçalves-Butruille et al., 1996; Gaziola et al., 2000).

Regulatory mechanisms controlling lysine metabolism are still not fully understood and some hypothesis have been suggested. Arruda et al. (2000) and Galili et al. (2001), reported alternative hypothesis (figures 2A and 2B), which consider the linkage between LOR and SDH domains that may be responsible for LOR activity modulation through protein intramolecular interactions. If such an *in vivo* mechanism really occurs, theoretically it would be possible to minimize the linkage through the alteration of the ionic strength in enzyme assays *in vitro*. Reinforcing this idea, a low LOR activity was detected in buffers without the addition of NaCl, when compared to the LOR activity levels obtained in buffer containing 100 mM NaCl. In addition, in *Arabidopsis* transformed with a construction, in which the interdomain and SDH domains were deleted, the LOR activity was not affected by salt concentrations (Galili et al., 2001). These results suggest that the interdomain region, as well as the SDH domain, may play a role in an interdomain interaction that affects LOR activity.

Peptides derived from the SDH domain or the interdomain were shown, *in vitro,* to be able to inhibit the activity of peptides derived from the LOR domain (Kemper et al., 1998). This fact suggests the existence of *in vivo* inhibition of the monofunctional LOR by the monofunctional SDH, although this inhibition shows less efficiency than that which occur in the case of the bifunctional LOR. On the other hand, the SDH activity does not appear to be affected by this linkage (Zhu et al., 2000). In a recent report, Zhu et al. (2002) showed that the functional interaction between the LOR and SDH domains is mediated by the linker region and not by specific affinities between these domains.

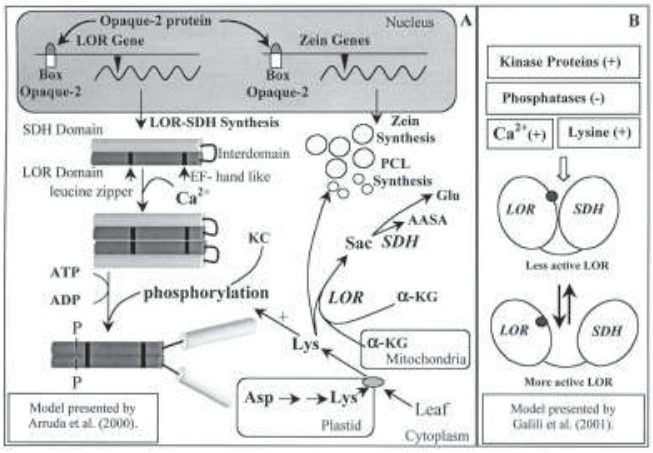

Figure 2. Model of lysine catabolism regulation in the cereal endosperm cells (A). Synthesis in plastids via the aspartate pathway and lysine translocation from vegetative tissues to the endosperm. Part of the lysine is incorporated into proteins containing lysine (PCL), however the largest storage protein fraction is the prolamins, which are deficient in lysine. In maize, the transcriptional activator opaque-2 controls the expression of genes that encode zeins and the bifunctional LOR-SDH enzyme, which is regulated by Ca2+, and is involved in enzyme dimerization, and phosphorylation by casein kinase (KC) in a lysine-dependent manner. As soon as the lysine concentration increases, the activity of LOR increases due to lysine-dependent phosphorylation. The phosphorylation of the LOR domain could inhibit the enzyme, which would be inhibited by the SDH domain and/or interdomain region. The lysine catabolic process leads to an increase in glutamic acid and amino adipic semialdehyde (AASA). Hypothesis suggesting a conformational modulation of LOR-SDH, where the two states may to be found. Calcium, proteins kinases and phosphatases regulate the alteration between the two forms (B).

The metabolic flow through the saccharopine pathway and related implications

In some plant tissues, such as seeds, in which the bifunctional LOR-SDH protein apparently corresponds to the majority of the total LOR activity, the lysine catabolic flow is regulated by LOR modulation via the linkage with SDH. This may contribute to the control of lysine homeostasis through lysine-dependent stimulation of LOR activity (Karchi et al., 1994, 1995).

Dominant induction of the monofunctional LOR and SDH proteins during the abscission process and under stress conditions may maintain a high and temporary catabolic flow, leading to glutamate production. Such a flow would probably be temporary, otherwise it could lead to the depletion of the soluble-lysine pool (Galili et al., 2001). Important information concerning such an aspect has been provided by studies of canola, which demonstrated an increased SDH activity, including the monofunctional isoenzyme, under osmotic stress conditions and followed the increase in LOR activity, when the stress was more severe (Moulin et al., 2000). It has also been suggested that this linkage may influence the metabolic flow, allowing the LOR product, saccharopine, to be sent directly to the catalytic site of SDH (Gonçalves-Butruille et al., 1996). This hypothesis may be questionable, based on the results obtained by Falco et al. (1995), who reported saccharopine accumulation in lysine overproducing transgenic soybean seeds, maintaining some SDH activity.

Considering the regulatory mechanisms described above with the different LOR and SDH K_m values for their substrates (Gonçalves-Butruille et al., 1996; Gaziola et al., 1997; Miron et al., 2000) and the fact that plant LOR-SDH isoenzymes have be located in the cytosol, the SDH domain may act in a physiological non-optimal pH for its activity (Kemper et al., 1999), suggesting that both LOR and SDH activities represent a rate-limiting step in the lysine catabolism (Miron et al., 2000) and that the metabolic flow through the saccharopine pathway is different among plant species and thus subjected to various regulatory points. This observation can be further supported by the work of Falco et al. (1995), who reported saccharopine accumulation in transgenic soybean, whereas canola exhibited accumulation of α-amino adipate semialdehyde, another intermediate of the lysine catabolic pathway. Moreover, the identification of monofunctional SDH enzyme in a limited number of plant species (*Arabidopsis,* canola, and cotton) and the studies of its properties, which are similar to those determined for the bifunctional enzyme, suggests that the presence of a monofunctional SDH may provide an increase in metabolic flow, compensating for the limitation generated by the physiological cellular pH.

The metabolic flow through the degradation pathway may be subjected to diverse regulatory mechanisms that have been studied, with special attention to the possible roles of lysine catabolism in distinct metabolic processes, such as growth, development and response to environmental changes or stress (Arruda et al., 2000; Galili et al., 2001). It may also provide further insights into the role of glutamate, which also originates from lysine catabolism (figures 1 and 3). Glutamate may be utilized as a primary precursor of the metabolite stress-related compounds such as proline, arginine, and γ-aminobutyric acid (GABA) (figure 3), which constitute stress-related signaling (Galili et al., 2001). Reinforcing the different metabolic roles it has recently been demonstrated in plants the existence of animal homologues of glutamate receptors, which appear to regulate different physiological processes (Lam et al., 1998; Brenner et al., 2000). In transgenic *Arabidopsis* plants, the over-expression of these glutamate receptors changed the Ca^{2+} balance, leading to hypersensitivity to ionic stress (Kim et al., 2001).

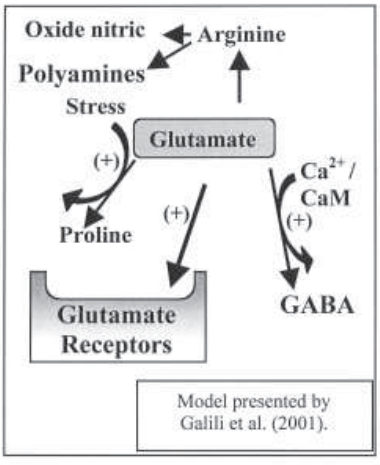

Figure 3. Conversion of glutamate to stress related compounds. The conversion of glutamate to a strong osmolite, proline, by D-pyrroline-5-carboxylate synthase; to GABA via glutamate decarboxylase calcium/calmodulin modulation; to nitric oxide (signaling molecule) via arginine. Glutamate is also a stimulator of glutamate receptors.

The expression and characteristics of LOR and SDH genes

The LOR-SDH gene is abundantly express in floral tissues and seeds in development. The *in situ* mRNA hybridization suggests that the *Arabidopsis* LOR-SDH gene is up-regulated in ovarian tissues, embryos in development and in the outer layers of the endosperm (Tang et al., 1997). Kemper et al. (1999) have demonstrated by *in situ* analysis of SDH activity that the bifunctional enzyme is located in the outer layer of maize developing seeds, whereas in embryos the activity was only slightly detectable, contradicting other studies, which showed an over production of lysine in embryos and subsequent lysine catabolic products (Mazur et al., 1999). These results suggest the possibility of a putative lysine transport mechanism from embryos to the outer layers of the endosperm where lysine is then degraded (Galili et al., 2001). In developing maize seeds, the LOR-SDH gene expression is mediated by the opaque-2 transcription factor, which also controls the expression of genes that encode zein storage proteins (Kemper et al., 1999). cDNA studies of maize (Kemper et al., 1999) and *Arabidopsis* (Tang et al., 1997) have shown the expression of the LOR-SDH bifunctional enzyme. One distinct and short mRNA sequence is translated from the same LOR-SDH gene that encodes the monofunctional enzyme in *Arabidopsis* (Tang et al., 1997). Short maize mRNA sequences have also been observed, however, they do not appear to be translated (Kemper et al., 1999).

Sequencing analysis has revealed that maize and *Arabidopsis* LOR-SDH genes contain the CCAAT and TATA box sequences in the promoter and in an internal region of the same gene, possibly controlling the transcripts of the bifunctional LOR-SDH and the monofunctional SDH (Arruda et al., 2000). In addition, GCN4-like sequences, which are involved in the transcription of genes related to nitrogen metabolism in yeast (Hinnebusch, 1988) and plants (Muller and Kanudsen, 1993), have been found in both the upstream and internal promoters in maize and in the internal promoters in *Arabidopsis*. Furthermore, sites for the linkage of opaque-2 have also be found in the upstream and internal promoters of *Arabidopsis,* but only in the upstream promoter in maize. The absence of this site in the promoter of the maize LOR-SDH gene could be an explanation for the presence of the monofunctional SDH in this plant species (Arruda et al., 2000).

The LOR-SDH gene expression is not restricted to reproductive tissues, since mRNAs have been observed in canola leaves when submitted to osmotic stress (Deleu et al., 1999). Expression analyses of sequences (ESTs) related to the LOR-SDH gene in several plants suggest an abundant expression in the cell division process in various tissues, as well as in cells in the abscission zone and in tissues treated with biotic elicitors (Arruda et al., 2000).

The LOR-SDH locus, as already mentioned, is not restricted to the encoding of the bifunctional LOR-SDH and monofunctional SDH. This locus can also encode for a new monofunctional LOR as in cotton and *Arabidopsis* (Galili et al., 2001). In cotton, monofunctional LOR cDNAs have an identical DNA sequence to the LOR domain, suggesting that this monofunctional LOR is encoded by the some composite locus. The EST database of the abscission zone of cotton contains 1800 sequenced ESTs and presents a relatively high frequency of the monofunctional LOR.

Biochemical mutants and transgenic plants for the production of high lysine plants

Mainly in the last four decades, research groups have focused attention on understanding the biochemical and genetic controls of the aspartate pathway (Azevedo et al., 1997). The data allows researchers to induce and select for lysine and threonine overproducing plants through genetic manipulation of key points of the pathway such as catalysis by the enzymes; AK, HSDH, DHDPS, threonine synthase (TS), LOR and SDH (Heremans and Jacobs, 1994; 1995; Ravanel et al., 1998; Laber et al., 1999; Azevedo and Lea, 2001).

The development of plant tissue culture and *in vitro* regeneration technologies have facilitated the selection of biochemical mutants. Such mutants can be selected in cell cultures treated with mutagenic agents and selected on solid or in liquid medium amended with selective agents, such as amino acids or their analogues. The cells that eventually grow in such conditions may be mutants containing enzymes with altered regulatory characteristics (Azevedo, 2002). A similar system can also be used for embryos of seeds submitted to mutagenesis (Lea et al., 1992). Independent of the procedure utilized, the selected plants need to be genetically evaluated and biochemically characterized, as well as submitted to a complete agronomic analysis (Lea et al., 1992).

Specifically in the case of the aspartate pathway, several mutants were selected in a large number of plant species, with the aim of obtaining cereal plants with the accumulation of lysine in seeds, which exhibited altered enzymes (Azevedo et al., 1997; Molina et al., 2001). Mutants were obtained with isoenzymes of AK that were insensitive to lysine plus threonine feedback inhibition (Bright et al., 1982; Muehlbauer et al., 1994a; Heremans and Jacobs, 1997), which exhibited an overproduction and accumulation of threonine in the leaves and in the seeds but no significant changes in the soluble lysine concentration in the seeds. These results indicated a major role of DHDPS in lysine biosynthesis, since the mutants were still sensitive to lysine feedback inhibition of the DHDPS step of the pathway, therefore driving carbon molecules to threonine biosynthesis (Azevedo and Lea, 2001). Hesse et al. (2002) suggested that after lysine biosynthesis, methionine would be considered the main route for the carbons entering the pathway, instead of threonine biosynthesis. Chiba et al. (1999) showed in *Arabidopsis* that the Cystathionine γ-synthase (CγS) is not feedback-inhibited by end products, but its expression is regulated by methionine at the level of mRNA stability in a process that is activated by methionine or one of its catabolites. This result suggests a central role for CγS in methionine biosynthesis indicating an important flux into the aspartate pathway.

Based on the information provided by the work with the biochemical mutants and on newly developed transformation techniques, a similar strategy has been used to obtain plants that accumulate lysine in the seeds. Transgenic tobacco plants expressing a lysine-insensitive AK from *E. coli* exhibited similar results to those observed for the biochemical mutants, with threonine accumulation, but without changes in the soluble lysine content of the seeds (Shaul and Galili, 1992). Other transgenic plants produced with altered enzyme regulation did not result in accumulation of lysine in seeds (Shaul and Galili, 1993; Falco et al., 1995; BrinchPedersen et al., 1996).

Soluble lysine accumulation was obtained when a lysine-insensitive DHDPS from *Corynebacterium* was expressed in transgenic maize embryos (Falco, 2001). Moreover, the knockout of LOR-SDH by T-DNA insertion resulted in a loss of lysine and its catabolism products, but the combination of these transgenic maize plants resulted in a soluble lysine content in the seeds of about 2- to 3-fold higher than the DHDPS transgenic maize plant (Falco, 2001). Transgenic rice plants have also been obtained in order to improve the nutritional value of the seed, by elevating the lysine concentration (Lee et al., 2001). A constitutive and seed-specific expression of feedback-insensitive maize DHDPS lead to a higher content of soluble lysine in the seeds. The higher rate of lysine biosynthesis obtained with the introduction of the altered DHDPS encoding gene also resulted in an increased rate of lysine catabolism. Even so, the over-expression of the mutant gene of DHDPS in a constitutive manner appears to overcome the lysine catabolism, thus maintaining higher lysine concentrations in the mature seeds (Lee et al., 2001). Azevedo and Lea (2001) in a recent review, suggested that lysine overproduction and accumulation in cereal seeds might be obtained by combining the genetic manipulation of the biosynthesis and lysine degradation mechanisms. Such a suggestion was supported mainly by the fact that the manipulation of enzymes involved in lysine biosynthesis did not produce lysine accumulation in cereal seeds. This could be explained by the fact that vegetables and the maize opaque-2 mutants, which exhibit higher concentration of soluble lysine in the seeds, exhibited a drastic reduction in the lysine catabolic rate in the endosperm, allowing excess lysine to be incorporated into storage proteins, as well as the accumulation in the soluble form (Azevedo and Lea, 2001; Molina et al., 2001). The maize opaque-2 mutant has been extensively studied (Gaziola et al., 1999). This mutation is characterized by an opaque phenotype with a farinaceous endosperm. The high lysine concentration observed in the endosperm is related to an increase in the concentration of soluble lysine and storage proteins with the simultaneous reduction of the prolamin fraction, which has only trace amounts of lysine (Lefèvre et al., 2002). The introduction of the opaque phenotype modifier genes allowed the production of opaque-2 maize lines with good grain productivity, that also exhibit characteristics of high lysine and tryptophan contents, but with a translucent phenotype, which have been denominated as quality protein maize - (QPM) (Vasal, 1994; Gaziola et al., 1999). QPM inbred lines have been included in breeding programs with several hybrid of QPM been produced and agronomically tested that are now commercially available (Gaziola et al., 1999).

Through transcriptome and proteome approaches, the regulatory role of the opaque-2 gene has been confirmed, since a 3' restriction site was shown to be associated with

LOR-SDH mRNA abundance (Lefèvre et al., 2002). The use of such techniques certainly will contribute significantly in the future. Azevedo et al. (1997) suggested that cereal cultivars with high lysine content seeds would probably be available in a short period of time. In a similar manner, Hesse et al. (2002) suggested possible traits to increase methionine synthesis in plants. Seed companies and research institutions have already confirmed such a possibility. Even so, additional studies will still be necessary to completely understand the regulatory aspects of lysine, threonine and methionine metabolism and how these mechanisms can be controlled.

Several informations can be obtained by the investigation of protein concentrations of the opaque and floury maize mutants, and of similar mutants of barley, sorghum and other cereal crops. It is surprising that based on the available information, and to the best of our knowledge, other cereals with high lysine mutants, similar to the opaque-2 mutants of maize, have not been utilized in research programs to study the aspartate metabolic pathway, which could further increase our understanding of lysine metabolism (Azevedo, 2002).

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REFERENCES

Azevedo RA, Arana JL, Arruda P (1990) Biochemical genetics of the interaction of the lysine plus threonine resistant mutant *Ltr*19* with *opaque-2* maize mutant. Plant Sci. 70:81-90.

Azevedo RA, Blackwell RD, Smith RJ, Lea PJ (1992a) Three aspartate kinase isoenzymes from maize. Phytochemistry 31:3725-3730.

Azevedo RA, Smith RJ, Lea PJ (1992b) Aspartate kinase regulation in maize: evidence for co-purification of threonine-sensitive aspartate kinase and homoserine dehydrogenase. Phytochemistry 31:3731-3734.

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46:395-419.

Azevedo RA, Lea PJ (2001) Lysine metabolism in higher plants. Amino Acids 20:261-279.

Azevedo RA (2002) Analysis of the aspartic acid metabolic pathway using mutant genes. Amino Acids 22:217-230.

Arruda P, Sodek L, da Silva WJ. (1982) Lysine-ketoglutarate reductase activity in developing maize endosperm. Plant Physiol. 69: 988-989.

Arruda P, da Silva WJ (1983) Lysine-ketoglutarate reductase-activity in maize-Its possible role in lysine metabolism of developing endosperm. Phytochemistry 22:2687-2689.

Arruda P, Kemper EL, Papes F, Leite A (2000) Regulation of lysine catabolism in higher plants. Trends Plant Sci. 5:324-330.

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.

BrinchPedersen H, Galili G, Knudsen S, Holm PB (1996) Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Mol. Biol. 32:611-620.

Brochetto-Braga MR, Leite A, Arruda P (1992) Partial purification and characterization of lysine-ketoglutarate reductase in normal and opaque-2 maize endosperm. Plant Physiol. 98:1139-1147.

Chiba Y, Ishikawa M, Kijima F, Tyson RH, Kim J, Yamamoto A, Mambara E, Leustek T, Wallsgrove RM, Naito S (1999) Evidence for autoregulation of cystathionine γ-synthase mRNA stability in Arabidopsis. Science 286:1371-1374.

Cunha-Lima ST, Azevedo RA, Santoro LG, Gaziola SA, Lea PJ (2003) Isolation of the bifunctional enzyme lysine 2-oxoglutarate reductase-saccharopine dehydrogenase from *Phaseolus vulgaris*. Amino Acids 24:179-186.

Deleu C, Coustaut M, Niogert MF, Larpher F (1999) Three new osmotic stress-regulated cDNAs identified by differential display polymerase chain reaction in rapeseed leaf discs. Plant Cell Environ. 22:979-988.

Dey M, Guha-Mukherjee S (1999) Phytochrome activation of aspartate kinase in etiolated chickpea (*Cicer arietinum*) seedlings. J. Plant Physiol. 154:454-458.

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Webber P (1995) Transgenic canola and soybean seeds with increased lysine. Biotechnology 13:577-582.

Falco SC (2001) Increasing lysine in corn. Amino Acids 21:57-58.

Feller A, Ramos F, Pierard A, Dubois E (1999) In *Saccharomyces cerevisae*, feedback inhibition of homocitrate synthase isoenzymes by lysine modulates the activation of *LYS* gene expression by Lys14p. Eur. J. Biochem. 261:163-170.

Fjellstedt TA, Robinson JC (1975) Purification and properties of L-lysine-α ketoglutarate reductase from human placenta. Arch. Biochem. Biophys. 168:536-548.

Galili G, Tang G, Zhu X, Gakiere B (2001) Lysine catabolism: a stress and development super-regulated metabolic pathway. Curr. Opin. Plant Biol. 4:261-266.

Gaziola SA, Teixeira CMG, Lugli J, Sodek L, Azevedo RA (1997) The enzymology of lysine catabolism in rice seeds. Isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate/saccharopine dehydrogenase bifunctional polypeptide. Eur. J. Biochem. 247:364-371.

Gaziola SA, Alessi ES, Guimarães PEO, Damerval C, Azevedo RA (1999) Quality protein maize: a biochemical study of enzymes involved in lysine metabolism. J. Agric. Food Chem. 47:1268-1275.

Gaziola SA, Sodek L, Arruda P, Lea PJ, Azevedo RA (2000) Degradation of lysine in rice seeds: Effect of calcium, ionic strength, S-adenosylmethionine and S-2-aminoethyl-L-cysteine on the lysine 2-oxoglutarate reductase-saccharopine dehydrogenase bifunctional enzyme. Physiol. Plant. 110:164-171.

Gonçalves-Butruille M, Szajner P, Torigoi E, Leite A, Arruda P (1996) Purification and characterization of the bifunctional enzyme lysine-ketoglutarate reductase saccharopine dehydrogenase from maize. Plant Physiol. 110:765-771.

Heremans B, Jacobs M (1994) Selection of *Arabidopsis thaliana* (L.) Heynt. Mutants resistant to aspartate-derived amino acids and analogues. Plant Sci. 101:151-162.

Heremans B, Jacobs M (1995) Threonine accumulation in a mutant of Arabidopsis thaliana (L.) Heynth with an altered aspartate kinase. J. Plant Physiol. 146:249-257.

Heremans B, Jacobs M (1997) A mutant of (*Arabidopsis thaliana* L.) Heynth with modified control of aspartate kinase by threonine. Biochem. Genet. 35:139-153.

Hesse H, Kreft O, Maimann S, Zeh M, Willmitzer L, Hofgen R (2001) Approaches towards understanding methionine biosynthesis in higher plants. Amino Acids 20:281-289.

Hinnebusch AG (1988) Mechanism of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cervisiae*. Microbiol. Rev. 52:248-273.

Karchi H, Miron D, Benyaacov S, Galili G (1995) The lysine-dependent stimulation of lysine catabolism in tobacco seed requires calcium and protein-phosphorylation. Plant Cell 7:1963-1970.

Karchi H, Shaul O, Galili G (1994) Lysine synthesis and catabolism are coordinately regulated during tobacco seed development. Proc. Natl Acad. Sci. USA 91:2577-2581.

Kemper EL, Cord-Neto G, Capella AN, Gonçalves-Butruille M, Azevedo RA, Arruda P (1998) Structure and regulation of the bifunctional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase in maize. Eur. J. Biochem. 253:720-729.

Kemper EL, Cord-Neto G, Papes F, Martinez-Moraes KC, Leite A, Arruda P (1999) The role of opaque-2 on the control of lysine degrading activities in developing maize endosperm. Plant Cell 11:1981-1994.

Kim SA, Kwak JM, Jae SK, Wang MH, Nam HG (2001) Overexpression of the *ATGluR2* gene encoding an *Arabidopsis* homologue of mammalian glutamate receptors impairs calcium utilization and sensitivity to ionic stress in transgenic plants. Plant Cell Physiol. 15:74-84.

Laber B, Maurer W, Hanke C, Grafe S, Ehler S, Messerschimidt A, Clausen T (1999) Characterization of recombinant *Arabidopsis thaliana* threonine synthase. Eur. J. Biochem. 263:212-221.

Lam HM, Chui J, Hsieh MH, Meisel L, Oliveira IC, Shin M, Coruzzi G (1998) Glutamate-receptor genes in plants. Nature 396:125-126.

Lea PJ, Blackwell RD, Azevedo RA (1992) Analysis of barley metabolism using mutant genes. In: Shewry PR (ed), Barley: genetics, biochemistry, molecular biology and biotechnology, pp.181–208. CAB International, Wallingford.

Lee SI, Kim HU, Lee YH, Suh SC, Lim YP, Lee HY, Kim HI (2001) Constitutive and seed-specific expression of a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene leads to increased free lysine in rice seeds. Mol. Breed. 8:75-84.

Lefèvre A, Consoli L, Gaziola SA, Pellegrino AP, Azevedo RA, Damerval C (2002) Dissecting the opaque-2 regulatory network using transcriptome and proteome approaches along with enzyme activity measurements. Scientia Agricola 59:407-414.

Lugli J, Campbell A, Gaziola SA, Smith RJ, Lea PJ, Azevedo RA (2002) Enzymes of lysine metabolism from *Coix lacryma-jobi* seeds. Plant Physiol. Biochem. 40:25-32.

Markovitz PJ, Chuang, DT (1987) The bifunctional aminoadipic semialdehyde synthase in lysine degradation. J. Biol. Chem. 262:9353-9358.

Mazur B, Krebbers E, Tingey S (1999) Gene discovery and product development for grain quality traits. Science 285:372-375.

Miron D, Ben-Yaacov S, Karchi H, Galili G (1997) *In vitro* dephosphorylation inhibits the activity of soybean lysine-oxoglutarate reductase in a lysine-regulated manner. Plant J. 12:1453-1458.

Miron D, Ben-Yaacov S, Reches D, Schupper A, Galili G (2000) Purification and characterization of bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase from developing soybean seeds. Plant Physiol. 123:665-663.

Molina SMG, Gaziola SA, Lea PJ, Azevedo RA (2001) Manipulating cereal crops for high lysine accumulation in seeds. Scientia Agricola 58:205-211.

Moulin M, Deleu C, Larher F (2000) L-Lysine catabolism is osmo-regulated at the level of lysine-ketoglutarate reductase and saccharopine dehydrogenase in rapeseed leaf discs. Plant Physiol. Biochem. 38:577-585.

Muehlbauer GJ, Somers DA, Matthews BJ, Gengenbach BG (1994) Molecular genetics of the maize (*Zea mays* L.) aspartate kinase-homoserine dehydrogenase gene family. Plant Physiol. 106:1303-1312.

Muller M, Knudsen S (1993) The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant J. 4:343-355.

Ravanel S, Gakiere B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. Proc. Natl. Acad. Sci. USA 95:7805-7812.

Shaul O, Galili G (1992) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia coli*. Plant Physiol. 100:1157-1163.

Shaul O, Galili G (1993) Concerted regulation of lysine and threonine synthesis in tobacco plants expressing bacterial feedback-insensitive aspartate kinase and dihydro-dipicolinate synthase. Plant Mol. Biol. 23:759-768.

Sodek L, Wilson CM (1970) Incorporation of leucine-C^{14} into protein in the developing of normal and opaque-2 corn. Arch. Biochem. Biophys. 140:29-38.

Tang G, Miron D, Zhu-Shimoni JX, Galili G (1997) Regulation of lysine catabolism through lysine-oxoglutarate reductase and saccharopine dehydrogenase in *Arabidopsis*. Plant Cell 9:1305-1316.

Tang G, Zhu X, Gskiere B, Levanony H, Kahana A, Galili G (2002) The bifunctional *LKR/SDH* locus of plants also encodes a highly active monofunctional lysine-ketoglutarate reductase using a polyadenylation signal located within an intron. Plant Physiol. 130:147-154.

Vasal SK (1994) High quality protein corn. In: Hallawer AR (ed), Specialty Corns, pp.79-120, CRC Press, Boca Raton, FL.

Wilson BJ, Gray AC, Matthews BF (1991) Bifunctional protein in carrot contains both aspartate kinase and homoserine dehydrogenase activities. Plant Physiol. 97:1323-1328.

Zhu X, Tang G, Galili G (2000) Characterization of the two saccharopine dehydrogenase isozymes of lysine catabolism encoded by the single composite *AtLKR-SDH* locus of *Arabidopsis*. Plant Physiol. 124:1363-1372.

Zhu XH, Tang GL, Galili G (2002) The activity of the Arabidopsis bifunctional lysine-ketoglutarate reductase/ saccharopine dehydrogenase enzyme of lysine catabolism is regulated by functional interaction between its two enzyme domains. J. Biol. Chem. 277:49655-49661.

CAPÍTULO 2

Neste capítulo será apresentado um manuscrito a ser submetido à publicação na revista **"Journal of Agricultural and Food Chemistry"**. Este artigo trata dos procedimentos de isolamento purificação e caracterização das enzimas envolvidas no catabolismo da lisina, bem como da caracterização do genótipo de sorgo Massa 03 no presente trabalho, sendo abordados fatores que podem favorecer um maior acúmulo de lisina em sementes. **A metologia detalhada encontra-se na seção de Anexos**.

Partial Purification and Characterization of Lysine Catabolism Enzymes From Sorghum Seeds and Massa 03-Genotype Characterization for High Lysine Content

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Running headline: lysine metabolism in sorghum seeds.

Abbreviations list: AK, aspartate kinase; DHDPS, dihydrodipicolinate synthase; HSDH, homoserine dehydrogenase; LOR, lysine 2-oxoglutarate; OPA, o-phthaldialdehyde; SDH saccharopine dehydrogenase; TS, threonine synthase;

Keyword index: lysine, amino acids, high-lysine cereal, nutrition quality, mutants.

Abstract

Lysine is an essential amino acid synthesized in plants in the aspartic acid pathway. The lysine catabolism is performed by the action of two consecutive enzymes, lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9). The final soluble lysine concentration in cereal seeds is controlled by both, synthesis and catabolism rates. The production and characterization of high-lysine plants species depend on the knowledge about the regulatory aspects of lysine metabolism and manipulation of the key enzymes. We for the first time isolated, partially purified and characterized LOR and SDH from developing sorghum seeds, which exhibited very low levels of activity, which may explain, at least in part, the higher content of total lysine observed in the seeds. These enzymes showed to be endosperm specific and were very unstable during the isolation and purification procedures. LOR and SDH exhibited some distinct properties when compared to the enzymes isolated from other plant species, particularly elution molarity from anion-exchange chromatography and the presence of multimeric forms with distinct molecular masses.

Introduction

Cereal protein is a major component of the total protein consumption in most countries. An increasing number of cereal crops has also been used to feed animals in most developed countries. Sorghum grain is the main staple food for people living in semiarid regions of Africa and Asia and it is also a major feed grain, used both in developed and developing countries (*1*). With the identification of the high lysine mutant gene (*hl*) (*2*), new perspectives for improving the nutritional quality of sorghum grain have opened.

Amino acids are important nitrogen containing compounds (*3*). Plants usually synthesize all 20 amino acids incorporated in a protein (*4*). Monogastric animals can only synthesize 11 of them, and the remaining essential amino acids must therefore be provided in the diet. Since cereal seeds constitute the main source of plant protein and are characterized by a deficiency in lysine, tryptophan and threonine, detailed studies of the aspartate metabolic pathway in which lysine and threonine are synthesized, have been carried out in order to better understand the metabolism of these essential amino acids, providing information for the genetic manipulation of plants in order to improve the nutritional value of cereal seeds in particular (*5*, *6*).

Although the regulation of amino acid metabolism in higher plants may be analogous to that in microorganisms (in which metabolic engineering of amino acids has been successfully achieved), the multicellular nature of higher plants introduces

additional levels of complexity that render metabolic fluxes much more difficult to predict and engineer (*7*).

The syntheses of the essential amino acids lysine, threonine, methionine and isoleucine are carried out in a complex and strongly regulated metabolic pathway, which has aspartic acid as a precursor with several enzymes being regulated by feedback inhibition (*5*).

Several research groups have focused their attention on understanding the biochemical and genetic controls of the aspartate pathway, inducing and selecting for lysine and threonine overproducing plants through genetic manipulation of the key steps catalyzed by the enzymes aspartate kinase (AK, EC 2.7.2.4), homoserine dehydrogenase (HSDH, EC 1.1.1.3), dihydrodipicolinate synthase (DHDPS, EC 4.1.2.52), threonine synthase (TS; EC 4.2.3.1), lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9) (*8-12*). Mutants containing an AK insensitive to lysine plus threonine feedback inhibition and transgenic tobacco plants expressing a lysine-insensitive AK from *Escherichia coli* exhibited overproduction and accumulation of threonine, but had no significant changes in the soluble lysine concentration in the seeds (*13-15*). Other transgenic plants with altered enzyme regulation did not result in significant lysine accumulation in the seeds (*16*, *17*). Azevedo et al. (*18-20*) showed that increased lysine content in some maize mutants could be attributed to reduced lysine catabolism by reduced endosperm LOR and SDH activities, reduced prolamin and increased non-zein proteins contents and alterations in the regulation of enzymes involved in lysine biosynthesis.

Although extensive information is available about the biosynthetic enzymes such as AK, DHDPS, HSDH, until recently very little about lysine degradation was known (*21*).

Recent studies have indicated that lysine catabolism plays an important role in lysine accumulation in plants and the control of the lysine content, particularly in seeds (*22*, *23*). Previous reports indicated that the soluble lysine concentration is mainly controlled by the rate of catabolism, instead of the feedback inhibition of its synthesis (*24*).

The lysine catabolic pathway in plants is constituted by LOR, which condenses lysine and 2-oxoglutarate to form saccharopine, which is then hydrolyzed to α-amino adipic acid and glutamic acid in a reaction catalyzed by the enzyme SDH (*23*). Monofunctional LOR and SDH and bifunctional LOR-SDH polypeptides have been observed in plants (*25-27*). In maize (*25*), rice (*28*) and coix (*29*) the bifunctional enzyme LOR-SDH was shown to be endosperm-specific.

Lysine was also shown to be able to regulate its own catabolism *in vivo*, with the enzymes differentially modulated by an intracellular signaling cascade, involving mainly Ca^{2+}, protein phosphorylation-dephosphorylation and ionic strength (*30-32*).

Regulatory mechanisms controlling lysine metabolism are still not fully understood and some hypotheses have been suggested. Arruda et al. (*22*) and Galili et al. (*33*) reported alternative hypothesis, which consider the linkage between LOR and SDH domains responsible for LOR activity modulation through protein intramolecular interactions. Zhu et al. (*34*) showed that the functional interaction is mediated by the

linker region and not by specific affinities between these domains. The regulatory mechanisms described above and in other studies (*35*, *36*), suggest that both LOR and SDH activities represent a rate-limiting step in the lysine catabolism, and the metabolic flow through the saccharopine pathway is different among plant species and thus subjected to various regulatory points.

Azevedo and Lea (*6*) suggested that lysine overproduction and accumulation in cereal seeds might be obtained by combining the genetic manipulation of the biosynthesis and lysine degradation mechanisms. This could be explained by the fact that vegetables and the maize opaque-2 mutant, which contain higher concentration of soluble lysine in the seeds, exhibit a drastic reduction in the lysine catabolic rate in the endosperm, thus allowing excess lysine to be incorporated into storage proteins, as well as increasing the accumulation in the soluble form (*23*, *37*). The authors further suggested that cereal varieties with high lysine content in the seeds would probably be available in a short period of time, a fact already confirmed by several seed companies and research institutions (*6*). Even so, additional studies will be necessary to completely understand the regulatory aspects of lysine, threonine and methionine metabolism and how these mechanisms can be controlled (*5*).

Further information may be obtained by investigating in more detail the expression and synthesis of storage proteins in high-lysine mutants, such as the opaque and floury maize mutants (*18-20*). It is surprising that based on the available information, and to the best of our knowledge, other high-lysine cereal mutants, similar to the opaque-2 mutant of maize, have not been used in research programs to study the

aspartate metabolic pathway, which could further increase our understanding of lysine metabolism.

Success in obtaining high-lysine plants for human or animal consumption relies on the full understanding of the metabolism of lysine as well as for subsequent genetic manipulation (*21*, *23*). We report the first detailed study about lysine catabolism in sorghum with the isolation and partial characterization of LOR and SDH from developing seeds.

Material and Methods

Plant Material

Sorghum [*Sorghum bicolor* (L.) Moench (Massa 03 – Dow Agroscience)] was used for extraction of the enzymes and amino acids. Immature seeds were harvested at three distinct stages of development: 93, 97 and 100-101 days – stage 1, 2 (milk stage) and 3, respectively. Plants were grown in the field station of the Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, in the summer season of 2001. The developing seeds were harvested directly into liquid nitrogen and stored at -80 ºC for further analysis. Other sorghum lines (accessions IS 11758, IS 11167, IS 16210, IS 10477, IS 22204, IS 5603) used for Infrared analysis were kindly donated (except the Massa 03) by the International Crop Research Institute for Semi Arid and Tropics – ICRISAT - India. These mature seeds were previously stored in a chamber maintained at 15 ºC and 45% humidity for a period of at least three days before analysis.

Enzymes extraction

All procedures were carried out at 4 ºC. Immature seeds were used for enzyme extraction. LOR and SDH were extracted from frozen seeds in five volumes of buffer as described by Gaziola et al. (*28*) with some modifications [15% glycerol, 10% PVPP

(w/v)]. The extracts were filtered through several layers of gauze and centrifuged at 15000*g* for 30 min to completely remove the cellular debris.

Ammonium sulphate precipitation and enzymes partial purification

The supernatant was submitted to sequential precipitation exactly as described by Lugli et al. (*29*). The supernatant was divided in two fractions and precipitated by slowly adding solid ammonium sulphate and gently stirring for 30 min to give precipitation sequences of 0-20%, 20-40%, 40-60%, 60-80% and 0-30%, 30-50%, 50-70% ammonium sulphate saturation. After each step, the suspension was centrifuged at 15000*g* for 30 min and the precipitated protein dissolved in a small volume of buffer A (100 mM Tris-HCl pH 7.4, containing 1 mM DTT, 1 mM EDTA and 10% (v/v) glycerol). The dissolved pellets were then loaded onto Sephadex G25 columns (10 mL total volume) equilibrated in buffers A and run under gravity. The desalted samples were assayed for LOR and SDH activities.

LOR and SDH partial purification.

The enzymes were extracted as described above and partially purified with 30-70% ammonium sulphate saturation. After desalting the resuspended protein pellets on Sephadex G25 columns as described above, the extracts were used in all experiments and for further purification steps. Rice and maize LOR and SDH enzymes were used as controls of enzyme activity and were partially purified with 25-45% and 0-

60% ammonium sulphate saturation as described by Gaziola *et al.* (*28*) and Azevedo et al. (*18*), respectively.

An anion exchange chromatography step was performed for the sorghum enzymes. Both step-wise and linear gradient types of elution were tested. The desalted samples were applied to a DEAE-Sephacel column (2.5 x 15 cm; flow rate 1 $mL.min^{-1}$) equilibrated in buffer A. The column was washed with buffer A (the unbound fraction was stored at -20 °C for further analysis) and a step-wise elution (0-100, 100-200, 200-300, 300-400, 400-500 mM KCl) was carried out. A final wash with 1 M KCl was also performed and the fractions stored for further analysis for enzyme activity. Similarly, another sample was eluted using a linear gradient of 0-250 mM KCl, from the same column and same running conditions. The linear gradient eluted fractions containing LOR-SDH activity were combined and applied to a gel filtration Superose 12HR 10/30 column, connected to an Akta Purifer System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was equilibrated and protein elution was carried out in buffer A (flow rate of 0.5 $mL.min^{-1}$) and fractions (0.5 mL) were immediately assayed for LOR activity.

Enzyme assays

Enzyme activities were assayed as described previously by Azevedo et al. (*18*), with a minor alteration (100 μL of extract were added to the reaction mixture). LOR/SDH activities were expressed as nmol $NADPH/NAD^+$ oxidized/reduced. min^{-1}. mg $protein^{-1}$.

SDH activity staining

SDH activity staining was performed using non-denaturing PAGE gels as described by Gaziola *et al.* (1997). Maize SDH enzyme sample partially purified was also applied to the gels and used as control of SDH activity and for enzyme comparison.

Protein determination

The protein concentration of all samples was determined by the Bradford method (*38*) using bovine serum albumin (BSA) as a standard.

Amino acid extraction and analyses

Soluble amino acids were extracted from immature sorghum seeds essentially as described by Bieleski and Turner (*39*). Immature seeds were weighted, extracted in five volumes of Methanol:Chloroform:Water (12:5:3, v/v/v) and then centrifuged at 2500*g*. The pellet was submitted to hydrolysis in 6M HCl for 22 h at 110 ºC before amino acid analysis. The samples were centrifuged and the supernatant stored at 4 to 8 ºC. Soluble and total amino acids samples were quantified as described by Yemm and Coking (*40*), and separated by reverse-phase HPLC as described by Gaziola et al. (*37*), using the OPA derivatization method. A Spherisorb ODS-2 C18 column was eluted with 0.8 mL min^{-1} flux to form a linear gradient with solutions of 65% methanol and phosphate buffer, pH 7.5 (50 mM sodium acetate, 50 mM disodium phosphate, 1.5 mL acetic acid, 20 mL

tetrahydrofuran and 20 mL methanol in 1 L water). A standard solution containing all amino acids was derivatized with OPA and utilized for column profile and elution sequence identification. Each amino acid analysis was performed with three replicates using independent preparations.

Infrared analyses

The infrared absorbance transmission analyses were performed in a Feed & Food 1255 Infratec Analyzer (Foss Tecator AB, Hönas, Sweden). Twenty three mature seeds of each sorghum line were analyzed in each step of the programmed analysis and the results were expressed as means. Three replicates were analyzed for the mutants and normal genotypes (mutants: IS 11758, IS 11167, IS 16210, IS 10477, IS 22204, IS 5603; wild-type: Massa 03).

Results and Discussion

Isolation, partial purification and characterization

Lysine metabolism is particularly important in cereal crops due to the low concentration of this amino acid in both, soluble form and incorporated into seed storage proteins. Although most of the key enzymes controlling the metabolism of lysine, threonine and methionine have been isolated and characterized in cereal and legume plant species, in sorghum the enzymes have never been studied. We have isolated and studied the two key enzymes that control lysine catabolism in higher plants from sorghum seeds. Some of the chemical and physical properties reported for other plant species have also been observed in sorghum, although some of them were specific to the sorghum enzymes. For instance, the optimum pH (**Figure 1**) for LOR (7.5) and SDH (higher than 8.5), were similar to those already reported for other species, such as maize (*25*) and rice (*28*), a characteristic which appears to vary very little in higher plants.

Ammonium sulphate partially purified extracts of roots, stems and leaves from seven days-old seedlings and immature seeds of sorghum were prepared and the activities of LOR and SDH were measured and compared to rice seed extracts prepared in the same way, which was used as a control, for tissue specificity analysis. LOR and SDH activities could only be detected in immature seeds (Data not shown), confirming previous data obtained from others cereal seeds like maize (*25*), rice (*28*) and coix (*29*),

in which the activities of LOR and SDH have also only been detected in the developing seed. Such specificity appears to be a characteristic of cereal crops, since legume crops have shown different results. For instance, in *Phaseolus vulgaris* LOR and/or SDH activities were detected in roots, pods, leaves, cotyledons and hypocotyls, but below the limit of detection in seeds (*41*, *42*). The result obtained in sorghum further confirms that lysine degradation via the saccharopine is higher in cereal seeds when compared to legume seeds. Immature sorghum seeds were used for further partial purification and characterization.

Three stages of seed development were tested and LOR and SDH activities were mainly recovered in the intermediary stage of maturation (milk stage) in a similar way to rice (*28*) and maize (*37*) and with 40-70% ammonium sulphate saturation (**Figure 2A**). The ammonium sulphate saturation range to isolate the enzymes have been shown to vary among the plant species studied so far, being 25-50% for rice (*28*) and 35-60% for maize (*43*), indicating that the majority of the enzyme activities can be generally isolated in the range of 40-50% ammonium sulphate saturation, concentration which also precipitates the highest amounts of total protein in sorghum seeds (**Figure 2B**).

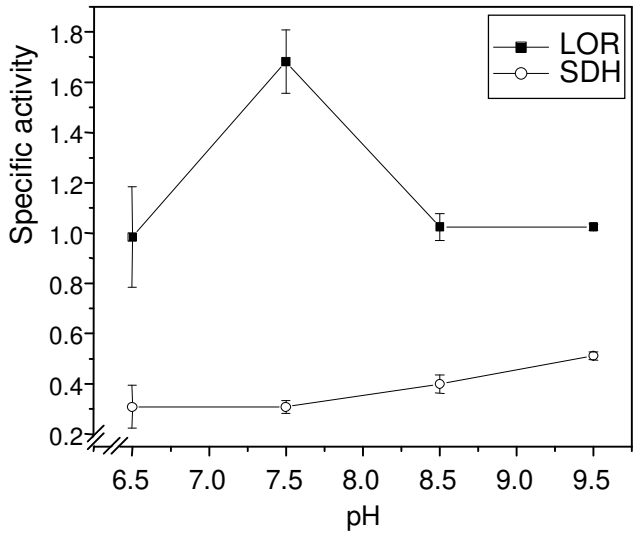

Figure 1. Determination of optimum pH for sorghum LOR and SDH activities. An ammonium sulphate partially purified LOR and SDH sample was used in the enzymatic assays. Enzyme activities are expressed in nmol NADPH/oxidized. min $^{-1}$. mg $^{-1}$ of protein for LOR and in nmol NAD/reduced. min $^{-1}$. mg $^{-1}$ of protein for SDH ± SE.

Table 1. Purification of LOR and SDH enzymes from developing sorghum seeds. (A) mg for total protein; (B) nmol of NADPH/oxidized.min^{-1}. mg $^{-1}$ of protein for LOR and NAD/reduced .min^{-1}.mg $^{-1}$ of protein for SDH.

Step	Total protein (A)	Total activity		Specific activity (B)		Fold Purification		Yield %	
		LOR	SDH	LOR	SDH	LOR	SDH	LOR	SDH
Crude extract	2260	-	-	-	-	-	-	-	-
Sephadex G-25	1838	1201	548	0,65	0.30	-	-	-	-
DEAE Sephacel	6.56	100	21	15.25	3.2	23.5	9.1	8	2.83

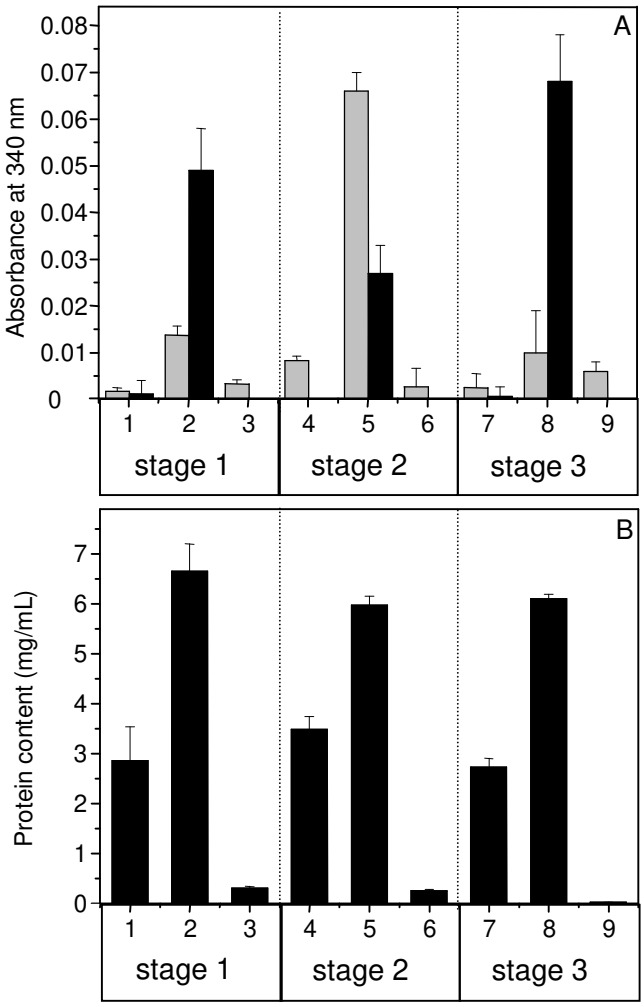

Figure 2. Identification of ammonium sulphate preciptation range using the absorbance at 340 nm (A) of LOR (gray box) and SDH (black box) reaction mixture assay and protein content (B) from extracts partially purified from the three seed developmental stages [1, 2 and 3 (early, intermediary and later, respectively] at different ammonium sulphate saturation ranges (1, 4 and 7 – 0 to 40%; 2, 5 and 8 – 40 to 70%; 3, 6 and 9 – 70 to 80%).

Curiously, LOR and SDH activities recovered from sorghum seeds were shown to be very low when compared to any of the other plant species analyzed so far in the literature. Furthermore, it has been shown that the enzymes activities in cereal seeds are normally much higher when compared with other plant species (*5*, *6*). However, rice seeds, which have been shown to contain higher lysine concentration in the mature seed when compared to other cereal crops, also exhibited reduced LOR and SDH activities (*23; 27*).

Several protein separation methods have been tested in order to produce a high purification of LOR and SDH enzymes from sorghum stage 2 immature seeds (data not shown), however, purification of the enzymes had to be limited to ammonium sulphate precipitation and an anion exchange chromatography step (**Table 1**) due to the higher instability after the ammonium sulphate precipitation step, even after several variations to the buffer systems had been tested, including the addition of several different protease inhibitors, normally leading to significant or even complete loss of both enzyme activities. Although some high purification of these enzymes have been obtained for the majority of the plant species studied so far, for a few, including cereal crops such as coix, enzyme activity has been shown to be extremely unstable (*29*). The purification of LOR and SDH from *Phaseolus vulgaris* has also been difficult for similar reasons, although LOR activity was considerably more sensitive than SDH activity to *in vitro* manipulation (*41*, *42*) even though some positive results were obtained with the addition of BSA, which can act against lipids and fatty acids impairing the proteins from complexion, and with higher EDTA concentrations suggesting that a change in the phosphorylated state of the enzymes occurred or that both enzymes activities are

dependent upon ions that were chelated by EDTA (*42*). Phosphatase inhibitors such as vanadate and sodium molibdate have also shown distinct effects on LOR and SDH activity further confirming the occurrence of different states of phosphorylation of these enzymes, but with LOR being more strongly modulated than SDH activity in plants (*41*, *42*).

Initially, a step-wise elution from a DEAE-Sephacel column was attempted with both enzymes being eluted in the 100 mM KCl step, while the other four concentrations of KCl did not elute any LOR or SDH activities (**Figure 3A**). Moreover, no residual LOR and SDH activity was left bound to the column, which was checked by washing with 1 M KCl or in the unbound fraction. When the samples were eluted from the same column with a 0-250 mM KCl linear gradient (**Figure 3B**), only one peak of LOR and SDH activity was eluted by approximately 80 mM KCl, confirming the data obtained in the step-wise elution (**Figure 3A**). The KCl concentration used for the elution of sorghum LOR-SDH was in a completely different range, being much lower than the concentrations previously reported for the elution of these enzymes from other plant species. For instance, the maize enzymes were co-eluted with 200 mM KCl (*25*), whereas in rice a 160 mM KCl was used for the elution of LOR and SDH (*28*).

The enzyme LOR/SDH ratio of activity obtained for sorghum was approximately 5, whereas in rice and maize LOR/SDH ratios of 1.2 and 4, respectively, were observed (*25*, *28*). These results suggest differential levels of stability or activity of LOR and SDH among these species. LOR and SDH specific activity (nmol NADPH oxidized and NAD reduced .min $^{-1}$.mL^{-1}.mg^{-1} protein, respectively) levels after ammonium sulphate (A.S.) or polyethylene glycol (PEG) precipitation and DEAE-Sephacel was also variable. In

maize genotypes the values observed from various studies were 5.2 (A.S.) and 146 (DEAE-Sephacel) for LOR (*41*); 19 and 8.6 (PEG), 160 and 40 (DEAE-Sephacel) (*22*), 36 and 24 (PEG) (*26*) for LOR and SDH respectively. In rice Gaziola *et al.* (*23*) reported low activity for LOR and SDH enzymes [7.74 and 6.16 (AS); 11.77 and 9.7 (DEAE-Sephacel)]. In sorghum the lowest values were observed [0.65 and 0.3 (AS); 15.25 and 3.2 (DEAE-Sephacel)] for LOR and SDH respectively.

Although LOR and SDH activities were only tested after ammonium sulphate and anion exchange chromatography steps, both enzymes co-purified as has been observed for other plant species [see (*6*) for a review], indicating that in sorghum, LOR and SDH may also be present as a bifunctional polypeptide, which agrees with the fact that a bifunctional LOR-SDH protein appears to be a common feature in plants (*6*, *21*). In this study, no evidence was obtained for the presence of monofunctional enzymes, although monofunctional LOR and SDH enzymes have also been recently reported in some plant species and shown to be differentially expressed and modulated (*22*, *27*, *33*).

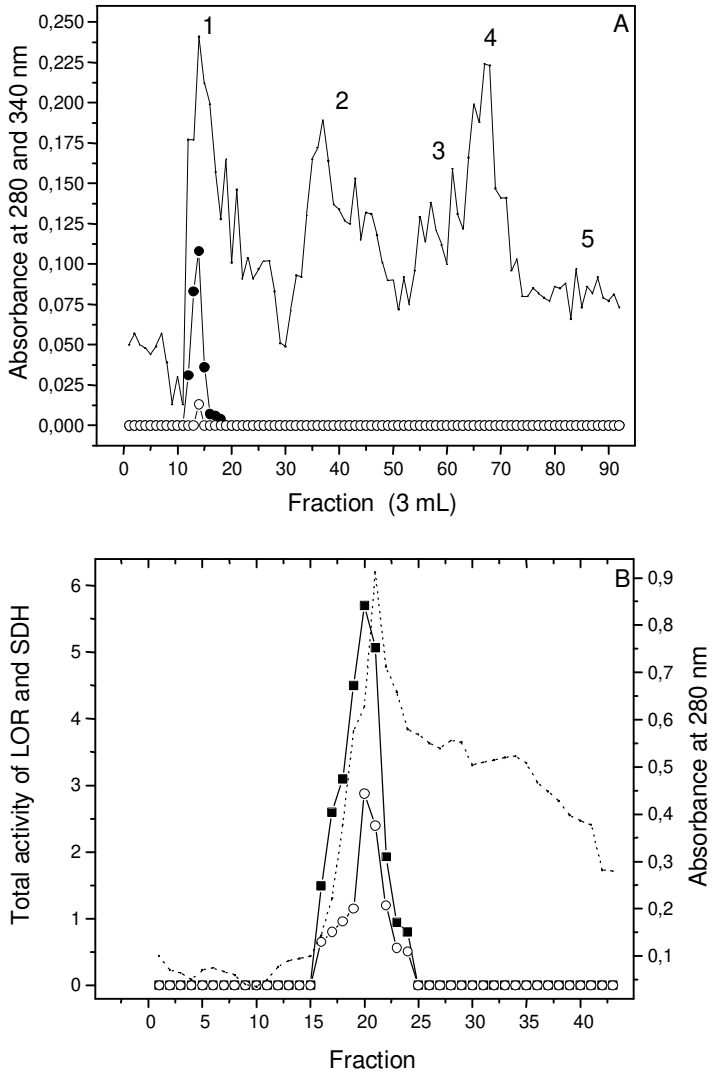

Figure 3. Elution profile of LOR and SDH enzymes from a DEAE-Sepacel anion exchange chromatography column. In (A), step-wise elution system. KCl concentrations: (1- 100 mM; 2- 200 mM; 3- 300 mM; 4- 400 mM; 5- 500 mM). In (B), linear gradient system (0 to 250 mM KCl). (⋯⋯) protein absorbance at 280 nm; (–■–) LOR activity; (–O–) SDH activity.

The differential metabolic flux through this catabolic branch of the aspartate pathway has been correlated to environmental changes and stress and plays a central role in the accumulation of lysine in the seeds by probably affecting LOR and SDH expression and activities (*44*). This could also be valid for sorghum and may probably interfere with these enzymes and others of the pathway such as AK, HSDH and DHDPS. However, it is very difficult to go any further with aspects related to regulation or the presence of other polypeptides since the aspartate pathway as a whole is completely unknown in sorghum, therefore, the existence of other LOR and SDH monofunctional enzymes cannot be ruled out based on the data obtained in this research. Although very high losses of LOR and SDH activities were verified after the anion exchange chromatography step, some samples, which maintained relatively high LOR and SDH activities, have been applied to a gel filtration Superose 12HR 10/30 (Pharmacia) column to estimated the molecular masses of LOR and SDH. The elution profile and enzyme assays revealed four peaks of LOR activity, but not SDH, with apparent molecular masses of approximately 270 kDa, 205 kDa, 157 kDa and 76 kDa in elution order (**Figure 4**). The lack of SDH activity could suggest that the peaks represent monofunctional LOR enzymes, however, the combined activity of all fractions suggest a further loss in activity, which could have more dramatically affected SDH activity. On the other hand, Kemper et al. (*31*) separated LOR and SDH polypeptides by elastase digestion with the proteolysis carried out not affecting SDH activity, while LOR showed inactivation. Furthermore, the SDH-containing polypeptides inhibited the enzymatic activity of the LOR-containing polypeptides (*31*). In rice developing seeds, up to five multimeric forms of SDH activity bands ranging up to 202 kDa were observed in

distinct experiments involving non-denaturing PAGE (*32*). In maize, the bifunctional native enzyme (dimeric form) was estimated at 260 kDa, but monomers of 125 kDa were also observed, which maintained enzyme activity (*43*).

SDH activity staining in non-denaturing PAGE revealed the presence of only one SDH activity band (**Figure 5**). The band exhibited a differential mobility when compared to the maize extract used as a control, which exhibited two SDH bands of activity, suggesting a different molecular mass for the enzyme, which was smaller than the maize predominant SDH band enzyme. It is possible that other isoenzymes are present in sorghum as discussed earlier, however, the low activity did not allow better characterization of the band, which could have confirmed such a possibility. Furthermore, activity staining for LOR, which could have confirmed the nature of the sorghum band, monofunctional or bifunctional, has already proven to be very difficult in all other species even when exhibiting much higher LOR activities (*6*). Several enzyme preparations were used for enzyme activity staining, but only the DEAE-Sephacel concentrated sample allowed the detection of some SDH activity in the gels. In other plant species like rice (*28*, *32*), coix (*29*) and maize (*20*, *25*), partially purified extracts with ammonium sulphate allowed the detection of enzyme activity in the gels. By activity staining, up to 5 bands of SDH have been identified in rice seeds (*23*). In maize, Azevedo et al. (*20*) reported the presence of two bands of SDH activity, in contrast to one major SDH band observed previously for maize in a different study (*25*) and coix (*29*). Gaziola *et al.* (*28*) suggest that the formation of multimeric forms in rice is a result of possible interaction of different isoforms present in the rice variety used. The non-

denaturing PAGE is a powerful technique and with better enzyme preparations should be used in future studies to analyze the nature of LOR and SDH sorghum enzymes.

The amount of information on LOR and SDH enzymes currently available in the literature is still relatively small when compared to other enzymes of the aspartate metabolic pathway. Even so, recent reports have added a considerable amount of new information. It seems likely, that although LOR and SDH are part of a single polypeptide, the LOR domain or the monofunctional LOR enzyme differs in several properties, related mainly to modulation as well as regulation, between plant species. Nevertheless, SDH appears to exhibit similar patterns of structure and regulation among different plant species (*23*).

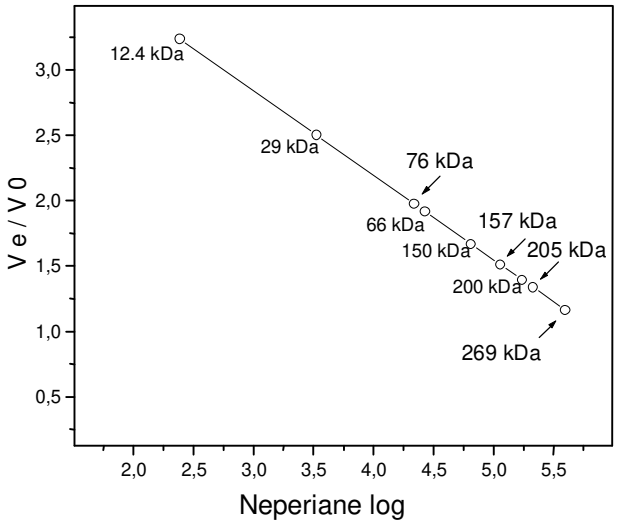

Figure 4. Estimation of the molecular mass of the sorghum LOR by a fractionation of partially purified LOR preparation on a gel filtration-Superose 10/30 HR column. Calibration curve made using standard molecular mass protein markers: Catalase (200 kDa), Alchool dehydrogenase (150 kDa), Bovine serum albumine (66 kDa); Carbonic anhydrase (29 kDa) and cytochrome *c* (12,4 kDa). The void volume was determined using the dextran blue (2000 kDa).

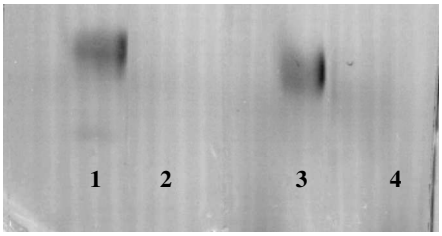

Figure 5 SDH activity staining PAGE. lanes: (1) SDH activity from maize; (2) substrate free revelation for maize extract; (3) SDH activity from sorghum; (4) substrate free revelation for sorghum extract. The maize and sorghum extracts are partiallty purified byt ammonium sulphate and anion exchange chromatography (DEAE sephacel) steps respectively.

Genotipe characterization

The amino acids contents of seeds were determined by colorimetric procedures (**Figure 6**). The data indicate a variation in the accumulation trend of both, soluble and protein amino acids, with the soluble amino acids exhibiting a more reduced variation, whereas the protein amino acids continuously increase with the seed development, indicating the deposition of amino acids into storage proteins during the seed maturation process.

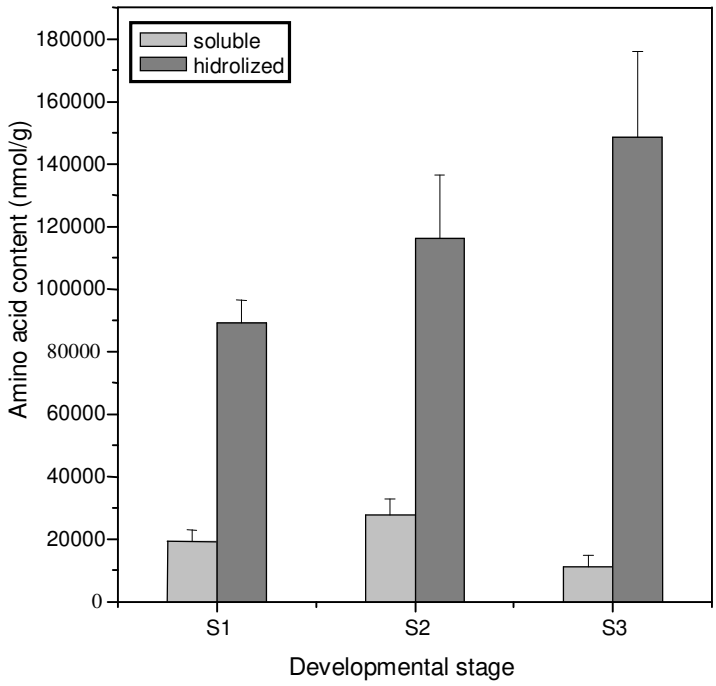

Figure 6. Total soluble and Protein amino acid content from developing seeds at different imature stages. S1 - early development stage (93 days post emergency); S2 – intermediary stage (97 days post emergency – milk stage); S3 – later development stage (101 days post emergency).

In relation to lysine concentration, both soluble and protein lysine were shown to be in high concentrations when compared to the values reported for other cereal crops (**Figure 7**). The stage 2 of seed development exhibited the highest concentration of lysine and in general for all other amino acids as well (soluble and protein). The absolute lysine concentration was shown to be almost 2-fold higher in the protein when compared to the soluble concentration. In high-lysine rice mutants the total soluble amino acids pool was 75% when compared to the wild-type, but soluble lysine, methionine, phenylalanine and proline were considerably lower in the mutant seed (*45*). It was also suggested that later in the development an enhanced lysine accumulation occurred, suggesting an extension of the temporal sequence of events during which lysine-rich proteins are being synthesized and packaged (*45*). These amino acids are probably incorporated rapidly into specific classes of storage proteins. In addition, these results also suggest a higher rate of synthesis of these proteins as well as a reduction in the rate of catabolic activity in the mutants than in the wild-type. In barley, Munck (*46*) reported that the lysine-rich fraction (albumins and globulins) is synthesized at a faster rate during early stages of seed formation, whereas lysine-poor storage proteins (such as the prolamins) dominate later stages of protein synthesis. Glutelins, which exhibit intermediate lysine content, increase linearly with time during seed development. Thus, if a similar mechanism it assumed to occur in sorghum seeds, the low activities of LOR and SDH may be favorable to lysine incorporation into lysine-rich proteins.

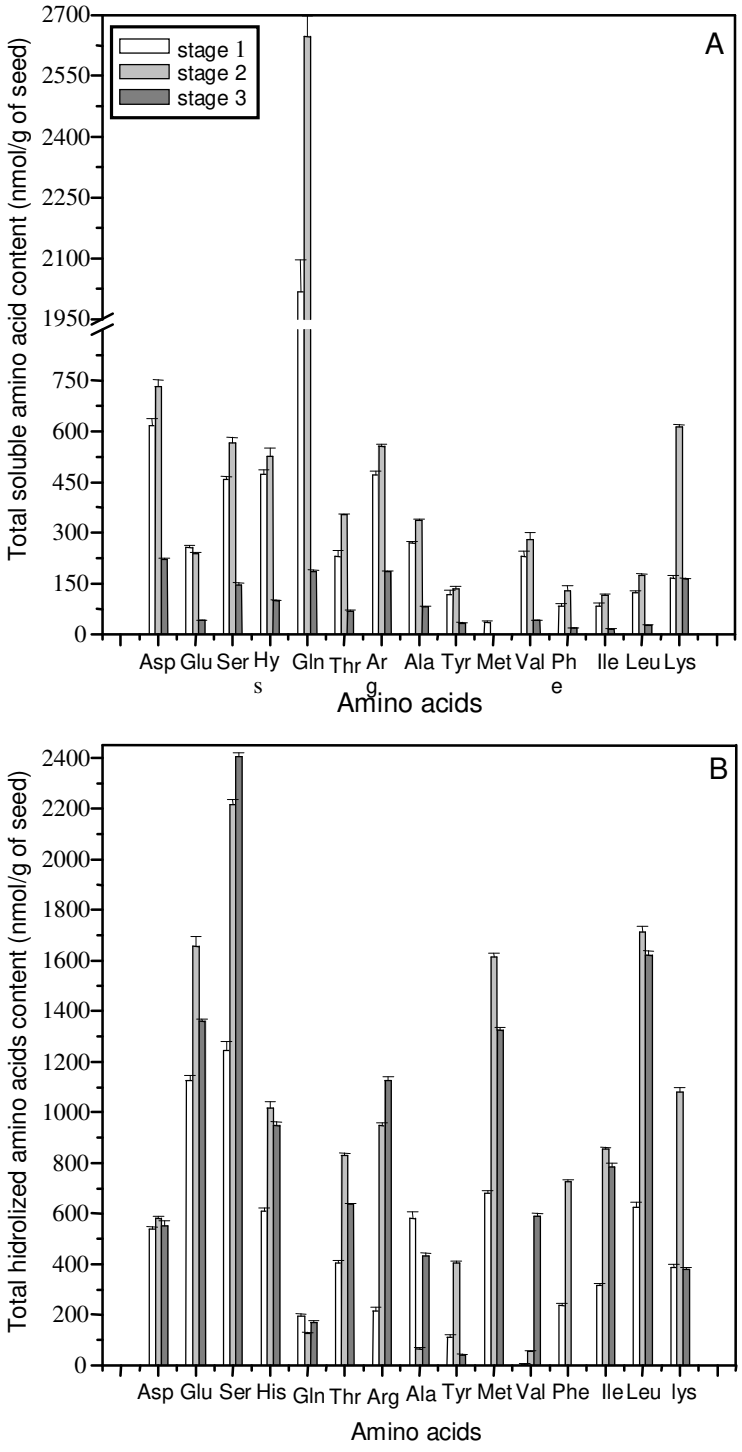

Figure 7. Content of recovered soluble (A) and hidrolized proteins amino acids (B) by HPLC from imature seeds at three developmental stages.

The relative concentrations (**Table 2**) of soluble aspartic acid (precursor of lysine biosynthesis), threonine, isoleucine, methionine and lysine during the later seed development stages, suggest that carbon molecules may be being driven to the lysine branch of the aspartate pathway. This is particularly important since it indicates that there may be an altered regulation of the key enzymes controlling the distinct branches of the pathway, which can be confirmed in the future by a complete analysis of all enzymes, including AK, HSDH, DHDPS and TS, in both, wild-type and high-lysine sorghum mutants.

As a preliminary test, an infrared absorbance transmission analysis was carried out using mature seeds of several high-lysine sorghum mutants, which exhibited relatively similar lysine concentration to the Massa 03 line used in this study (**Figure 8**). The combined results of amino acid analysis for Massa 03 (**Figure 7**) and the infrared (**Figure 8**) clearly indicates that the commercial genotype Massa 03 used for the isolation of LOR and SDH has a high lysine concentration, since the concentrations is in the same range as the high-lysine mutants analyzed. This could explain the very reduced LOR and SDH activities, which characterized this sorghum line during isolation and purification, in a similar manner to the maize high-lysine mutants, such as the opaque-2, which exhibits higher lysine content due to a drastic reduction in lysine catabolism and altered distribution of storage protein (*18-20*). To confirm such a possibility, LOR and SDH activities were determined during sorghum development and in rice seeds (milk stage, which accounts for the highest LOR and SDH activities) (**Figure 9**). The results indicate that both sorghum enzymes exhibited very low activities when compared to the activity in rice immature seeds. Gaziola *et al.* (*28*) isolated and

purified LOR and SDH from rice developing seeds and showed that the activities are reduced when compared to other cereal crops analyzed, which could partially explain the naturally higher lysine concentration normally observed in rice when compared to other cereal crops. In a similar manner, maize opaque-2 mutant and derived quality protein maize (QPM) varieties, also exhibited drastic reductions in lysine degradation when compared to wild-type maize lines also leading to higher lysine concentration in the seed (*37*, *43*). Several other high-lysine maize mutants also exhibited reduced LOR and SDH activities (*18-20*). When legume plant species, which normally present elevated lysine concentrations, is concerned, LOR and SDH activities were shown to be very low and even not detectable in some plant tissues, indicating that the high-lysine concentrations was also at least in part explained by the reduced rate of lysine catabolism (*41*). Therefore, the results obtained in this research confirm all these previous reports suggesting that a reduction in the rate of lysine catabolism is essential for lysine accumulation in seeds. They also emphasize that the genetic manipulation of crops for the high-lysine trait should consider the degradation pathway, which appears to be more important than lysine synthesis, especially for the seeds as previously suggested (*23*).

On going research in our laboratory with several sorghum high-lysine mutants have already indicated that dramatic alterations in the storage protein fractions take place and may also account for the high lysine concentration observed in sorghum (A. Vendemiatti and R.R. Ferreira, *unpublished data*). The storage protein kafirin (sorghum prolamime) concentration was determined for Massa 03 sorghum and also for a high-lysine mutant IS 11758 (accession of ICRISAT). The data showed reduced values for IS

11758 (12%), which are compatible with literature and lower values for Massa 03 (4%) (A. Vendemiatti and R.R. Ferreira, *unpublished data*). Sastry et al. (*47*) reported that the yields of kafirins from IS 4404 (wild type) was higher (14.5%) than that of IS 11758. High-lysine cultivars of sorghum can contain 25% less kafirin and an increased alcohol insoluble reduced glutelin without affecting the total protein content (*48*). This reduced prolamins fraction contributed to higher lysine content, since prolamins are characterized for the trace levels of lysine.

Table 2. The aspartate/amino acid ratios for sorghum variety Massa 03.

	Amino acid	Means SE
Stage 1	Thr	0.366 ± 0.04
	Met	0.056 ± 0.006
	Ile	0.126 ± 0.015
	Lys	0.267 ± 0.023
Stage 2	Thr	0.476 ± 0.011
	Met	----------
	Ile	0.150 ± 0.010
	Lys	0.835 ± 0.015
Stage 3	Thr	0.311 ± 0.024
	Met	----------
	Ile	0.074 ± 0.002
	Lys	0.739 ± 0.008

*Means and standard errors of the means (SE) are from three analyses.

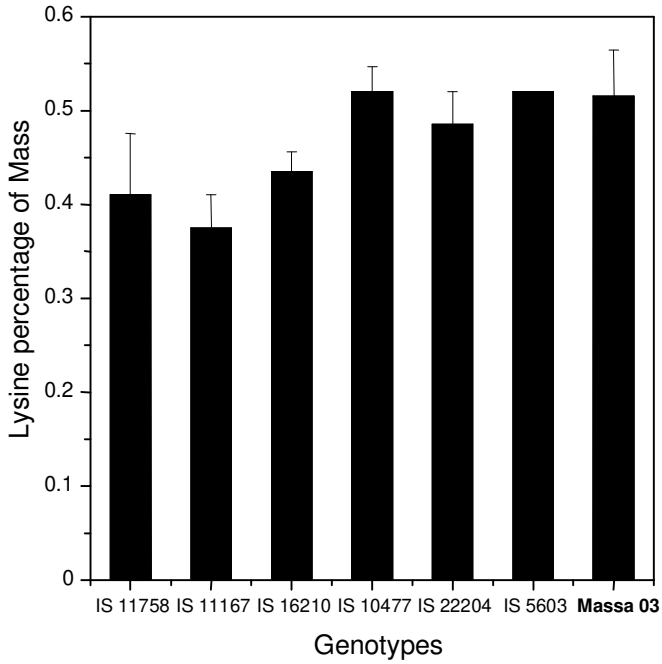

Figure 8. Lysine percentage analysis of several mutants (ICRISAT accessions) and a commercial hybrid Massa 03 – Dow Agroscience determined by infrared absorbance transmission. The results are expressed on % of dry seed mass.

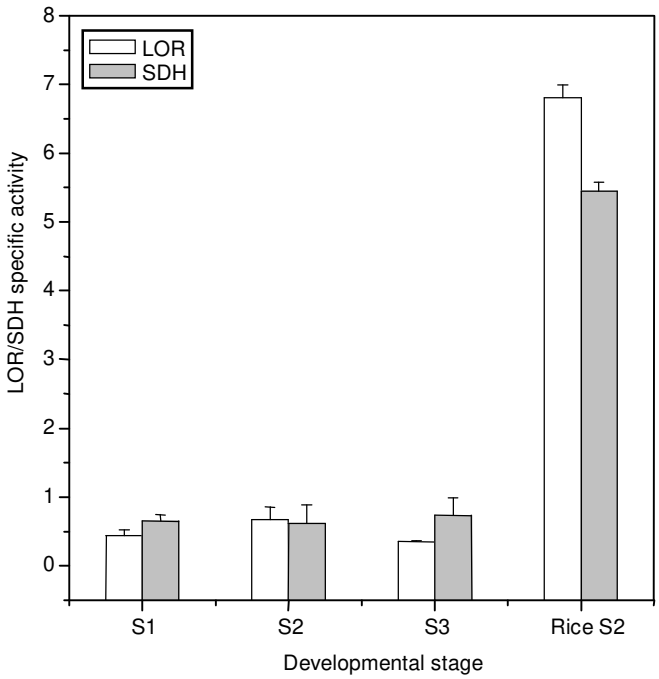

Figure 9. LOR and SDH activities in sorghum seeds of developmental stages and in developing rice seeds at a similar stage (milk stage). The activities were expressed in nmol NADPH oxidized. mg of protein^{-1} .min $^{-1}$for LOR and nmol NAD reduced. mg of protein^{-1} .min $^{-1}$ for SDH.

There is a good nutritional potential for this high-lysine sorghum hybrid (Massa 03), particularly due to the high lysine content observed in the seeds. This characteristic may be interesting for the nutritional balance in the human diet, mainly in the developing countries, where an animal protein based diet is not possible for most of the population. It would also be an important protein source for animal feed. Furthermore, apart from the high yields that can be obtained with sorghum when compared to other cereal crops, the storage protein and amino acid content variability should be considered in

further breeding programs to contribute to a better understanding of amino acid and storage protein metabolism.

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References

(1) Weaver, A. C.; Hamaker, B. R.; Axtell, J. D. Discovery of grain sorghum germ plasm with high uncooked and cooked in vitro protein digestibilities. *Cereal Chem.* **1998**, *75*, 665-670.

(2) Singh, R.; Axtell, J. D. High lysine mutant gene (*hl*) that improves protein quality and biological value of grain sorghum. *Crop Sci*. **1973**, *13*, 535-539.

(3) Medici, L. O.; Azevedo, R. A.; Smith, R. J.; Lea, P. J. The influence of nitrogen supply on antioxidant enzymes in plant roots. *Funct. Plant Biol.* **2004**, *31*, 1-9.

(4) Lea, P. J.; Azevedo, R. A. Primary products: amino acids. In *Encyclopaedia of Applied Plant Sciences*; Thomas, B., Murphy, D. J., Murray, B. G., Eds.; Elsevier/Academic Press: New York, **2003**; pp. 871-883.

(5) Azevedo, R. A.; Arruda, P.; Turner, W. L.; Lea, P. J. The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. *Phytochemistry* **1997**, *46*, 395-419.

(6) Azevedo, R. A.; Lea, P. J. Lysine metabolism in higher plants. *Amino Acids* **2001,** *20*, 261-279.

(7) Galili, G.; Hofgen, R. Metabolic engineering of amino acids and storage proteins in plants. *Metabolic Engineering* **2002**, *4*, 3-11.

(8) Heremans, B.; Jacobs, M. Threonine accumulation in a mutant of *Arabidopsis thaliana* (L.) Heynt. with an altered aspartate kinase. *J. Plant Physiol.* **1995**, *146*, 249-257.

(9) Ravanel, S.; Gakiere, B.; Job, D.; Douce, R. The specific features of methionine biosynthesis and metabolism in plants. Proc. Natl. Acad. Sci. USA **1998**, *95*, 7805-7812.

(10) Laber, B.; Maurer, W.; Hanke, C.; Grafe, S.; Ehler, S.; Messerschimidt, A.; Clausen, T. Characterization of recombinant *Arabidopsis thaliana* threonine synthase. *Eur. J. Biochem.* **1999**, *263*, 212-221.

(11) Falco, S. C. Increasing lysine in corn. *Amino Acids* **2001**, *21*, 57-58.

(12) Lee, S. I.; Kim, H. U.; Lee, Y. H.; Suh, S. C.; Lim, Y. P.; Lee, H. Y.; Kim, H. I. Constitutive and seed-specific expression of a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene leads to increased free lysine in rice seeds. *Mol. Breed.*, **2001**, *8*, 75-84.

(13) Bright, S. W. J.; Kueh, J. S. H.; Franklin, J.; Rognes, S. E.; Miflin, B. J. Two genes for threonine accumulation in barley-seeds. *Nature* **1982**, *299*, 278-279.

(14) Heremans, B.; Jacobs, M. A mutant of (*Arabidopsis thaliana* L.) Heynh. with modified control of aspartate kinase by threonine. *Biochem. Genet.* **1997**, *35*, 139-153.

(15) Shaul, O.; Galili, G. Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia coli*. *Plant Physiol.* **1992**, *100*, 1157-1163.

(16) Shaul, O.; Galili, G. Concerted regulation of lysine and threonine synthesis in tobacco plants expressing bacterial feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. *Plant Mol. Biol.* **1993**, *23*, 759-768.

(17) Brinch-Pedersen, H.; Galili, G.; Knudsen, S.; Holm, P. B. Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. *Plant Mol. Biol.* **1996**, *32*, 611-620.

(18) Azevedo, R. A.; Damerval, C.; Landry, J.; Lea, P. J.; Bellato, C. M.; Meinhardt, L. W.; Le Guilloux, M.; Delhaye, S.; Toro, A. A.; Gaziola, S. A.; Berdejo, D. B. A. Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. *Eur. J. Biochem.* **2003**, *270*, 4898-4908.

(19) Azevedo, R. A.; Damerval, C.; Lea, P. J.; Landry, J.; Bellato, C. M.; Meinhardt, L. W.; LeGuilloux, M.; Delhaye, S.; Toro, A. A.; Gaziola, S. A.; Varisi, V. A.; Gratão, P. L. Endosperm protein synthesis and lysine metabolism in distinct opaque maize seed mutants. *Funct. Plant Biol.* **2004**, *31*, 339-348.

(20) Azevedo, R. A.; Lea, P. J.; Damerval, C.; Landry, J.; Bellato, C. M.; Meinhardt, L. W.; LeGuilloux, M.; Delhaye, S.; Varisi, V. A.; Gaziola, S. A.; Gratão, P. L.; Toro,

A. A. Regulation of lysine metabolism and endosperm protein synthesis by the *opaque-5* and *opaque-7* maize mutations. *J. Agric. Food Chem.* **2004**, in press.

(21) Fornazier, R. F.; Azevedo, R. A.; Ferreira, R. R.; Varisi, V. A. Lysine catabolism: flow, metabolic role and regulation. *Braz. J. Plant Physiol.* **2003**, *15*, 9-18.

(22) Arruda, P.; Kemper, E. L.; Papes, F.; Leite, A. Regulation of lysine catabolism in higher plants. *Trends Plant Sci.* **2000**, *5*, 324-330.

(23) Azevedo, R. A. Analysis of the aspartic acid metabolic pathway using mutant genes. *Amino Acids* **2002**, *22*, 217-230.

(24) Arruda, P.; Sodek, L.; Silva, W. J. Lysine-ketoglutarate reductase activity in developing maize endosperm. *Plant Physiol.* **1982**, *69*, 988-989.

(25) Gonçalves-Butruille, M.; Szajner, P.; Torigoi, E.; Leite, A.; Arruda, P. Purification and characterization of the bifunctional enzyme lysine-ketoglutarate reductase saccharopine dehydrogenase from maize. *Plant Physiol.* **1996**, *110*, 765-771.

(26) Miron, D.; Ben-Yaacov, S.; Reches, D.; Schupper, A.; Galili, G. Purification and characterization of bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase from developing soybean seeds. *Plant Physiol.* **2000**, *123*, 665-663.

(27) Tang, G.; Zhu, X.; Gakiere, B.; Levanony, H.; Kahana, A.; Galili, G. The bifunctional *LKR/SDH* locus of plants also encodes a highly active monofunctional lysine-ketoglutarate reductase using a polyadenylation signal located within an intron. *Plant Physiol.* **2002**, *130*, 147-154.

(28) Gaziola, S. A.; Teixeira, C. M. G.; Lugli, J.; Sodek, L.; Azevedo, R. A. The enzymology of lysine catabolism in rice seeds. Isolation, characterization, and

regulatory properties of a lysine 2-oxoglutarate/saccharopine dehydrogenase bifunctional polypeptide. *Eur. J. Biochem.* **1997**, *247*, 364-371.

(29) Lugli, J.; Campbell, A.; Gaziola, S. A.; Smith, R. J.; Lea, P. J.; Azevedo, R. A. Enzymes of lysine metabolism from *Coix lacryma-jobi* seeds. *Plant Physiol. Biochem*. **2002**, *40*, 25-32.

(30) Miron, D.; Ben-Yaacov, S.; Karchi, H.; Galili, G. *In vitro* dephosphorylation inhibits the activity of soybean lysine-oxoglutarate reductase in a lysine-regulated manner. *Plant J.* **1997**, *12*, 1453-1458.

(31) Kemper, E. L.; Cord-Neto, G.; Capella, A. N.; Gonçalves-Butruille, M.; Azevedo, R. A.; Arruda, P. Structure and regulation of the bifunctional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase in maize. *Eur. J. Biochem.* **1998**, *253*, 720-729.

(32) Gaziola, S. A.; Sodek, L.; Arruda, P.; Lea, P. J.; Azevedo, R. A. Degradation of lysine in rice seeds, Effect of calcium, ionic strength, S-adenosylmethionine and S-2-aminoethyl-L-cysteine on the lysine 2-oxoglutarate reductase-saccharopine dehydrogenase bifunctional enzyme. *Physiol. Plant.* **2000**, *110*, 164-171.

(33) Galili, G.; Tang, G.; Zhu, X.; Gakiere, B. Lysine catabolism: a stress and development super-regulated metabolic pathway. *Curr. Opin. Plant Biol.* **2001**, *4*, 261-266.

(34) Zhu, X. H.; Tang, G. L.; Galili, G. The activity of the Arabidopsis bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase enzyme of lysine catabolism is regulated by functional interaction between its two enzyme domains. *J. Biol. Chem.* **2002**, *277*, 49655-49661.

(35) Kemper, E. L.; Cord-Neto, G.; Papes, F.; Martinez-Moraes, K. C.; Leite, A.; Arruda, P. The role of opaque-2 on the control of lysine degrading activities in developing maize endosperm. *Plant Cell* **1999**, *11*, 1981-1994.

(36) Moulin, M.; Deleu, C.; Larher, F. L-Lysine catabolism is osmo-regulated at the level of lysine-ketoglutarate reductase and saccharopine dehydrogenase in rapeseed leaf discs. *Plant Physiol. Biochem.* **2000**, *38*, 577-585.

(37) Gaziola, S. A.; Alessi, E. S.; Guimarães, P. E. O.; Damerval, C.; Azevedo, R. A. Quality protein maize: a biochemical study of enzymes involved in lysine metabolism. *J. Agric. Food Chem.* **1999**, *47*, 1268-1275.

(38) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.

(39) Bieleski, R. L.; Turner, N. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Anal. Biochem.* **1966**, *17*, 278-293.

(40) Yemm, E. M.; Cocking, E. C. Estimation of amino acids by ninhydrin. *Analyst* **1955**, *80*, 209-213.

(41) Lima, S. T. C.; Azevedo, R. A.; Santoro, L. G.; Gaziola, S. A.; Lea, P. J. Isolation of the bifunctional enzyme lysine 2-oxoglutarate reductase-saccharopine dehydrogenase *Amino Acids* **2003**, *24*, 179-186.

(42) Lima, S. T. C.; Azevedo, R. A.; Santoro, L. G. Improved procedures for extraction of lysine 2-oxoglutarate reductase/saccharopine dehydrogenase (LOR/SDH)

enzyme from *Phaseolus vulgaris* cultivars. *New Zeal. J. Crop Hort. Sci.* **2003**, *31*, 261-268.

(43) Brochetto-Braga, M. R.; Leite, A.; Arruda, P. Partial purification and characterization of lysine-ketoglutarate reductase in normal and opaque-2 maize endosperm. *Plant Physiol.* **1992**, *98*, 1139-1147.

(44) Stepansky, A.; Galili, G. Synthesis of the Arabidopsis bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase enzyme of lysine catabolism is concertedly regulated by metabolic and stress-associated signals. *Plant Physiol.* **2003**, *133*, 1407-1415.

(45) Schaeffer, G.W.; Sharpe, F.T. Electrophoretic profiles and amino acid composition of rice endosperm proteins of a mutant with enhanced lysine and total protein after backcrosses for germplasm improvements. *Theor. Appl. Genet.* **1997**, *95*, 230-235.

(46) Munck, L. Barley seed proteins. Symp. Seed proteins. The AVI Publishing Company, Inc. Westport, Connecticut **1972**, p. 144-164.

(47) Sastry, L. V. S.; Paulis, J. W.; Cobb, L. A.; Wall, J. S.; Axtel, J. D. Genetic variability of alcohol-soluble storage proteins in high-lysine sorghums. *J. Agric. Food Chem.* **1986**, *34*, 1061-1067.

(48) Reddy, N. P. E.; Vauterin, M.; Frankard, V.; Jacobs, M. Biochemical characterisation and cloning of alpha-kafirin gene from sorghum (*Sorghum bicolor* L Moench). *J. Plant Biochem. Biotech*. **2001**, *10*, 101-106.

REFERÊNCIAS BIBLIOGRÁFICAS GERAIS

Azevedo, RA, Arana, JL, Arruda, P (1990) Biochemical genetics of the interaction of the lysine plus threonine resistant mutant *Ltr*19* with *opaque-2* maize mutant. Plant Science 70, 81-90.

Azevedo RA, Blackwell RD, Smith RJ, Lea PJ (1992a) Three aspartate kinase isoenzymes from maize. Phytochemistry 31, 3725-3730.

Azevedo RA, Smith RJ, Lea PJ (1992b) Aspartate kinase regulation in maize: evidence for co-purification of threonine-sensitive aspartate kinase and homoserine dehydrogenase. Phytochemistry 31,3731-3734.

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46,395-419.

Azevedo RA, Lea PJ (2001) Lysine metabolism in higher plants. Amino Acids 20, 261-279.

Azevedo RA (2002) Analysis of the aspartic acid metabolic pathway using mutant genes. Amino Acids 22, 217-230.

Azevedo RA, Damerval C, Landry J, Lea PJ, Bellato CM, Meinhardt LW, Le Guilloux M, Delhaye S, Toro AA, Gaziola SA, Berdejo DBA (2003) Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. *European Journal Biochemistry* 270, 4898-4908.

Azevedo RA, Damerval C, Lea PJ, Landry J, Bellato CM, Meinhardt LW, LeGuilloux M, Delhaye S, Toro AA, Gaziola SA, Varisi VA, Gratão PL (2004a) Endosperm protein synthesis and lysine metabolism in distinctopaque maize seed mutants. *Functional Plant Biology*, in press.

Azevedo RA, Lea PJ, Damerval C, Landry J, Bellato CM, Meinhardt LW, LeGuilloux M, Delhaye S, Varisi VA, Gaziola SA, Gratão PL, Toro AA (2004b) Regulation of Lysine Metabolism and Endosperm Protein Synthesisby the Opaque-5 and Opaque-7 Maize Mutations. *Journal of Agricultural and Food Chemistry*, in press.

Arruda P, Sodek L, Silva WJ. (1982) Lysine-ketoglutarate reductase activity in developing maize endosperm. Plant Physiology 69, 988-989.

Arruda P, Kemper EL, Papes F, Leite A (2000) Regulation of lysine catabolism in higher plants. Trends in Plant Science 5, 324-330.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Bielinski LR, Turner N (1966) Separation and estimation of aminoacids in crude plant extracts by thyn-layer electroforesis and chromatography. Analytical Biochemistry 17, 278-293.

Brandt AB (1975) *In vivo* incorporation of lysine-C^{14} into the endosperm proteins of wild type and high lysine barley. FEBS Letters 52, 288-291.

Bright SWJ, Kueh JSH, Franklin J, Rognes SE, Miflin BJ (1982) 2 genes for threonine accumulation in barley-seeds. Nature 299 (5880), 278-279.

Brinchpedersen H, Galili G, Knudsen S, Holm PB (1996) Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Molecular Biology 32, 611-620.

Brito G Q (1995) Características agronômicas, composição química, qualidade de consumo de selagens de duas variedades e três híbridos de sorgo forrageiro. Dissertação (M.S.), Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 69p.

Brochetto-Braga MR, Leite A, Arruda P (1992) Partial purification and characterization of lysine-ketoglutarate reductase in normal and opaque-2 maize endosperm. Plant Physiology 98,1139-1147.

Chaves AV (1997) Avaliação de cultivares de sorgo (*Sorghum bicolor* L. Moench) para produção de silagem. Dissertação (M.S.) - Universidade Federal de Viçosa, Viçosa, 35p.

Chiba Y, Ishikawa M, Kijima F, Tyson RH, Kim J, Yamamoto A, Mambara E, Leustek T, Wallsgrove RM, Naito S (1999) Evidence for autoregulation of cystathionine γ-synthase mRNA stability in Arabidopsis. Science 286, 1371-1374.

Cunha-Lima ST, Azevedo RA, Santoro LG, Gaziola SA, Lea PJ (2003) Isolation of the bifunctional enzyme lysine 2-oxoglutarate reductase-saccharopine dehydrogenase from *Phaseolus vulgaris*. Amino Acids 24, 179-186.

Deleu C, Coustaut M, Niogert MF, Larpher F (1999) Three new osmotic stress-regulated cDNAs identified by differential display polymerase chain reaction in rapeseed leaf discs. Plant Cell and Environment 22, 979-988.

Elkin RG, Freed MB, Hmaker BR, Zhang Y, Parsons CM (1996) Condensed tannins are only partially responsible for variations in nutrient digestibility of sorghum grains cultivars. Journal of Agricultural and Food Chemistry 44, 848-853.

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Webber P (1995) Transgenic Canola and soybean seeds with increased lysine. Biotechnology 13, 577-582.

Falco SC (2001) Increasing lysine in corn. Amino Acids 21, 57-58.

Feller A, Ramos F, Pierard A, Dubois E (1999) In *Saccharomyces cerevisae*, feedback inhibition of homocitrate synthase isoenzymes by lysine modulates the activation of *LYS* gene expression by Lys14p. European Journal of Biochemistry 261, 163-170.

Fjellstedt TA, Robinson JC (1975) Purification and properties of L-lysine-α ketoglutarate reductase from human placenta. Archives of Biochemistry et Biophysics 168, 536-548.

Ferguson, K A (1964) Starch gel electrophoresis, application to the classification of pituitary proteins and polypeptides. Metabolism- Clinical and Experimental 13, 985-1002.

Fornazier RF, Azevedo RA, Ferreira RR, Varisi VA (2003) Lysine catabolism: flow, metabolic role and regulation. Brazilian Journal of Plant Physiology 15, 9-18.

Galili G, Tang G, Zhu X, Gakiere B (2001) Lysine catabolism: a stress and development super-regulated metabolic pathway. Current Opinion in Plant Biology 4, 261-266.

Galili G, Hofgen R (2002) Metabolic engineering of amino acids and storage proteins in plants. Metabolic Engineering 4, 3-11.

Gaziola SA, Teixeira CMG, Lugli J, Sodek L, Azevedo RA (1997) The enzymology of lysine catabolism in rice seeds. Isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate/saccharopine dehydrogenase bifunctional polypeptide. European Journal of Biochemistry 247, 364-371.

Gaziola SA, Alessi ES, Guimarães PEO, Damerval C, Azevedo RA (1999) Quality protein maize: a biochemical study of enzymes involved in lysine metabolism. Journal of Agricultural and Food Chemistry 47, 1268-1275.

Gaziola SA, Sodek L, Arruda P, Lea PJ, Azevedo RA (2000) Degradation of lysine in rice seeds, Effect of calcium, ionic strength, S-adenosylmethionine and S-2-aminoethyl-L-cysteine on the lysine 2-oxoglutarate reductase-saccharopine dehydrogenase bifunctional enzyme. Physiologia Plantarum 110, 164-171.

Gonçalves-Butruille M, Szajner P, Torigoi E, Leite A, Arruda P (1996) Purification and characterization of the bifunctional enzyme lysine-ketoglutarate reductase saccharopine dehydrogenase from maize. Plant Physiology 110, 765-771.

Hamaker BR, Kirleis AW, Mertz ET, Axtell JD (1986) Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. Journal of Agricultural and Food Chemistry, 34:647-649.

Heremans B, Jacobs M (1994) Selection of *Arabidopsis thaliana* (L.) Heynt. Mutants resistant to aspartate-derived amino acids and analogues. Plant Science 101, 151-162.

Heremans B, Jacobs M (1995) Threonine accumulation in a mutant of Arabidopsis thaliana (L.) Heynt. With an altered aspartate kinase. Journal of Plant Physiology 146, 249-257.

Heremans B, Jacobs M (1997) A mutant of (*Arabidopsis thaliana* L.) Heynh. With modified control of aspartate kinase by threonine. Biochemical Genetics 35, 139-153.

Hesse H, Kreft O, Maimann S, Zeh M, Willmitzer L, Hofgen R (2001) Approaches towards understanding methionine biosynthesis in higher plants. Amino Acids 20, 281-289.

Hinnebusch AG (1988) Mechanism of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cervisiae*. Microbiological Reviews 52, 248-273.

Karchi H, Miron D, Benyaacov S, Galili G (1995) The lysine-dependent stimulation of lysine catabolism in tobacco seed requires calcium and protein-phosphorylation. Plant Cell 7, 1963-1970.

Karchi H, Shaul O, Galili G (1994) Lysine synthesis and catabolism are coordinately regulated during tobacco seed development. Proceedings of the National Academy of Science of the United States of America 91, 2577-2581.

Kemper EL, Cord-Neto G, Capella AN, Gonçalves-Butruille M, Azevedo RA, Arruda P (1998) Structure and regulation of the bifunctional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase in maize. European Journal of Biochemistry 253, 720-729.

Kemper EL, Cord-Neto G, Papes F, Martinez-Moraes KC, Leite A, Arruda P (1999) The role of opaque-2 on the control of lysine degrading activities in developing maize endosperm. Plant Cell 11, 1981-1994.

Kim SA, Kwak JM, Jae SK, Wang MH, Nam HG (2001) Overexpression of the *ATGluR2* gene encoding an *Arabidopsis* homologue of mammalian glutamate receptors impairs calcium utilization and sensitivity to ionic stress in transgenic plants. Plant Cell Physiology 15, 74-84.

Laber B, Maurer W, Hanke C, Grafe S, Ehler S, Messerschimidt A, Clausen T (1999) Characterization of recombinant *Arabidopsis thaliana* threonine synthase. European Journal of Biochemistry 263, 212-221.

Lam HM, Chui J, Hsieh MH, Meisel L, Oliveira IC, Shin M, Coruzzi G (1998) Glutamate-receptor genes in plants. Nature 396, 125-126.

Lea PJ, Blackwell RD, Azevedo RA (1992) Analysis of barley metabolism using mutant genes. In, Shewry PR (ed) Barley, genetics, biochemistry, molecular biology and biotechnology, CAB International, Wallingford, pp 181–208.

Lee SI, Kim HU, Lee YH, Suh SC, Lim YP, Lee HY, Kim HI (2001) Constitutive and seed-specific expression of a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene leads to increased free lysine in rice seeds. Molecular Breeding 8, 75-84.

Lefèvre A, Consoli L, Gaziola SA, PellegrinoA N, Azevedo RA, Damerval C (2002) Dissecting the opaque-2 regulatory network using transcriptome and proteome approaches along with enzyme activity measurements. Scientia Agricola 59, 407-414.

Lugli J, Campbell A, Gaziola SA, Smith RJ, Lea PJ, Azevedo RA (2002) Enzymes of lysine metabolism from *Coix lacryma-jobi* seeds. Plant Physiology and Biochemistry 40, 25-32.

McDonald P, Henderson AR, Heron S (1991) The biochemistry of silage. 2 ed. Marlow, Chalcombe Publications, 340p.

McLean WC, de Romaña GL, Placko RP, Graham GG (1981) Protein quality and digestibility of sorghum in preschool children: Balance studies and plasma free amino acids. Journal of Nutrition 111, 19-28.

Markovitz PJ, Chuang, DT (1987) The bifunctional aminoadipic semialdehyde synthase in lysine degradation. Journal of Biological Chemistry 262, 9353-9358.

Mazur B, Krebbers E, Tingey S (1999) Gene discovery and product development for grain quality traits. Science 285, 372-375.

Medici, L. O.; Azevedo, R. A.; Smith, R. J.; Lea, P. J. The influence of nitrogen supply on antioxidant enzymes in plant roots. *Funct. Plant Biol.* **2004**, *31*, 1-9.

Miron D, Ben-Yaacov S, Karchi H, Galili G (1997) *In vitro* dephosphorylation inhibits the activity of soybean lysine-oxoglutarate reductase in a lysine-regulated manner. Plant Journal 12, 1453-1458.

Miron D, Ben-Yaacov S, Reches D, Schupper A, Galili G (2000) Purification and characterization of bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase from developing soybean seeds. Plant Physiology 123, 665-663.

Molina SMG, Gaziola SA, Lea PJ, Antunes RA (2001) Manipulação de cereais para acúmulo de lisina em sementes. Scientia Agricola 58, 205-211.

Moulin M, Deleu C, Larher F (2000) L-Lysine catabolism is osmo-regulated at the level of lysine-ketoglutarate reductase and saccharopine dehydrogenase in rapeseed leaf discs. Plant Physiology and Biochemistry 38, 577-585.

Muehlbauer GJ, Somers DA, Matthews BJ, Gengenbach BG (1994) Molecular genetics of the maize (*Zea mays* L.) aspartate kinase-homoserine dehydrogenase gene family. Plant Physiology 106, 1303-1312.

Muller M, Knudsen S (1993) The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant Journal 4, 343-355.

Munk L, (1972) Barley seed proteins. Symp. Seed proteins. The AVI Publishing Company, Inc. Westport, Connecticut. P. 144-164.

Nogueira FAS (1995) Qualidade das silagens de híbridos de sorgo de porte baixo com e sem teores de taninos e de colmo seco e suculento, e seus padrões de fermentação, em condições de laboratório. Dissertação (MS), Escola de Veterinária da UFMG, Belo Horizonte, 78p.

Oria MP, Hamaker BR, Shull JM (1995a) In vitro protein digestibility of developing and mature sorghum grain in relation to a-,b- and g-kafirin dissulfide crosslinking. Journal of Cereal Science 22, 85-93.

Oria MP, Hamaker BR, Shull JM (1995b) Resistance of sorghum a-,b- and g-kafirin to pepsin digestion. Journal of Agricultural and Food Chemistry 43, 2148-2152.

Ravanel S, Gakiere B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. Proceedings of the National Academy of Science of the United States of America 95, 7805-7812.

Reddy NPE, Vauterin M, Frankard V, Jacobs M (2001) Biochemical characterisation and cloning of alpha-kafirin gene from sorghum (*Sorghum bicolor* L Moench). Journal of Plant Biochemistry and Biotechnology 10, 101-106.

Rocha VR, Gonçalves LC, Rodrigues JAS, Brito AF, Rodriguez NM, Borges I (2000) Avaliação de sete genótipos de sorgo [*Sorghum bicolor* (L.) Moench] para produção de silagem. I- Características agronômicas. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 52, 506-511.

Sastry LVS, Paulis JW, Cobb LA, Wall JS, Axtell JD (1986) Genetic variability of alcohol-soluble storage proteins in high-lysine sorghums. Journal of Agricultural and Food Chemistry 34, 1061-1067.

Shelton MJR, Couch F, Hole JH, Jones RE, Leighton CM, Lyman and Briggs JK (1951) Grain sorghum byproduct feeds for farm animals. Texas Agronomical Experimental Station Bulletin, 743.

Shaul O, Galili G (1992b) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia col*i. Plant Physiology 100, 1157-1163.

Shaul O, Galili G (1993) Concerted regulation of lysine and threonine synthesis in tobacco plants expressing bacterial feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Molecular Biology 23, 759-768.

Shull JM, Watterson JJ, Kirleis AW (1991) Proposed nomenclature for the alcohol soluble proteins (kafirins) of *Sorghum bicolor* (L.) Moench based on molecular weigth, solubility and structure. Journal of Agricultural and Food Chemistry 39, 83-87.

Singh R, Axtell JD (1973) High lysine mutant gene (*hl*) that improves protein quality and biological value of grain sorghum. Crop Science 13, 535-539.

Sodek L, Wilson CM (1970) Incorporation of leucine-C^{14} into protein in the developing of normal and opaque-2 corn. Archives of Biochemistry et Biophysics 140, 29-38.

Stepansky A, Galili G (2003) Synthesis of the Arabidopsis bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase enzyme of lysine catabolism is concertedly regulated by metabolic and stress-associated signals. Plant Physiology 133, 1407-1415.

Tang G, Miron D, Zhu-Shimoni JX, Galili G (1997) Regulation of lysine catabolism through lysine-oxoglutarate reductase and saccharopine dehydrogenase in *Arabidopsis*. Plant Cell 9, 1305-1316.

Tang G, Zhu X, Gskiere B, Levanony H, Kahana A, Galili G (2002) The bifunctional *LKR/SDH* locus of plants also encodes a highly active monofunctional lysine-ketoglutarate reductase using a polyadenylation signal located within an intron. Plant Phisiology 130, 147-154.

Toro AA, Medici LO, Sodek L, Lea PJ, Azevedo RA (2003) Distribution of soluble amino acids in maize endospem mutants. Scientia Agricola 60, 91-96.

Vasal SK (1994) High quality protein corn. In, Hallawer AR (ed) Specialty Corns, CRC Press., p. 79-120

Weaver AC, Hamaker BR, Axtell JD (1998) Discovery of Grain Sorghum germ plasm with high uncooked and cooked in vitri protein digestibilities. Cereal Chemistry 75, 665-670.

Wilson BJ, Gray AC, Matthews BF (1991) Bifunctional protein in carrot contains both aspartate kinase and homoserine dehydrogenase activities. Plant Physiology 97, 1323-1328.

Wilkinson JM, (1978) The ensiling of forage maize: Efects on composition and nutritive value. Forage Maize. London Agricultural Research Council. p 201-237.

Yemm EM, Cocking EC (1955) Estimation of amino acids by ninhidrin. Analyst. 80, 209-213.

Zago CP (1999) Silagem de sorgo. In: Anais do 7º simpósio sobre nutrição de bovinos - Alimentação Suplementar, 7. Piracicaba, FEALQ, p.47-68.

Zhu X, Tang G, Galili G (2000) Characterization of the two saccharopine dehydrogenase isozymes of lysine catabolism encoded by the single composite *AtLKR-SDH* locus of *Arabidopsis*. Plant Physiology 124, 1363-1372.

Zhu XH, Tang GL, Galili G (2002) The activity of the Arabidopsis bifunction lysine-ketoglutarate reductase/saccharopine dehydrogenase enzyme of lysine catabolism is regulated by functional interaction between its two enzyme domains. Journal of Biological Chemistry 277, 49655-49661.

CONCLUSÕES GERAIS E PERSPECTIVAS PARA O FUTURO

O genótipo Massa 03 – Dow Agroscience constitui um material onde a purificação e caracterização das enzimas relacionadas ao catabolismo da lisina é trabalhosa devido à instabilidade e baixa atividade destas enzimas. A possibilidade dessa característica ser extensiva à espécie ainda precisa ser investigado.

Entretanto, o estudo deste genótipo contribuiu para um melhor entendimento do metabolismo da lisina e sua relação com o acúmulo em sementes, uma vez que as baixas atividades puderam ser correlacionadas com a concentração de lisina, no sentido de causa (baixa atividade de LOR) e efeito (maior acúmulo de lisina).

Embora estudos relativos à dinâmica de incorporação de lisina não foram realizados, foi possível com base nos dados obtidos, ter uma idéia de como ocorre a incorporação de vários aminoácidos essenciais, naquilo que diz respeito as suas proporções relativas ao ácido aspártico, precursor dos mesmos desatacando um direcionamento para síntese da lisina propriamente dita.

Também concluímos que este material apresenta um bom potencial para ser explorado em programas de melhoramento e propriamente para ser utilizado na alimentação animal e humana, devido à presença de lisina em concentrações relativamente altas (compatíveis aos mutantes para alta concentração de lisina do ICRISAT).

Como perspectiva, podemos afirmar que ainda há muito a ser estudado em relação ao papel fisiológico do catabolismo da lisina e principalmente, que futuras análises sejam cromatográficas, que possam dar continuidade ao que já foi realizado

até aqui, ou moleculares, visando o estudo da expressão gênica da LOR neste genótipo podem confirmar como podem estar presentes diferentes isoformas e seu papel na regulação do fluxo metabólico.

Finalmente, ainda podem ser realizados estudos em sementes em desenvolvimento, análogos aos conduzidos com o genótipo Massa 03, para os mutantes do ICRISAT, além de buscar um genótipo “normal” como controle que eventualmente apresente atividades mais altas das enzimas do catabolismo de lisina e relacioná-los com a concentração de aminoácidos, reforçando dessa maneira o papel do catabolismo no controle do acúmulo de lisina em grãos de cereais. Vale ressaltar que a aquisição de um genótipo controle está sendo obtida junto aos bancos de germoplasma, a qual será eventualmente analisada, pelo menos para o conteúdo de lisina e os dados obtidos incluídos no manuscrito a ser submetido à publicação.

APÊNDICE

Detalhamento de Material e Métodos

Obtenção do material vegetal

Foram utilizadas sementes imaturas de sorgo [*Sorghum bicolor* (L.) Moench var. massa 03]. As plantas foram gentilmente cedidas pelo Prof. Dr. Luis Gustavo Nussio do Departamento de Nutrição Animal da ESALQ – USP. O sorgo foi plantado no início de setembro e a coleta das panículas foi realizada em meados de dezembro com as plantas apresentando aproximadamente 100 dias de idade.

Obtenção de um segundo lote de sementes imaturas de sorgo Massa 03

Posteriormente foi obtido um segundo lote de sementes imaturas de sorgo MASSA 03 nas mesmas condições já mencionadas (cerca de 100 dias após a emergência), gentilmente cedidas pela Dow Agroscience / Jardinópolis – SP, coletadas no dia 23/12/2002.

Obtenção de sementes maduras de mutantes de sorgo

Alguns genótipos de sorgo com alta concentração de lisina foram obtidos junto ao banco de germoplasma da Índia ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). Solicitamos amostras de sementes de nove acessos de sorgo caracterizados como alta lisina: IS11167, IS5603, IS16227, IS25792, IS10477, IS22204, IS16210, IS16199 e IS11758.

Essas sementes nos foram gentilmente cedidas e posteriormente procedemos à realização do plantio desses materiais com a finalidade de se obter maiores quantidades de sementes e se avaliar o comportamento desses acessos dentro da realidade climática de nossa região.

Na estufa, foram semeadas em cada vaso três sementes, sendo que após a emergência, somente duas plantas foram cultivadas até a maturidade. Para cada acesso foram plantados cinco vasos e como controle, também foram plantados cinco vasos com a variedade MASSA 03.

Coleta do material vegetal imaturo

Foram colhidas porções basais, intermediárias e apicais das panículas, com intervalo de 3 a 4 dias entre cada coleta, sendo então obtido material em três estágios de maturação diferentes de sementes em desenvolvimento, as quais foram congeladas em nitrogênio líquido imediatamente após a coleta no próprio campo, com auxílio de

um galão de 35 L de nitrogênio e caixas de isopor, onde os três estágios foram separados. O material foi então estocado a –80 ºC.

Extração de LOR e SDH

A extração das enzimas foi realizada a 4ºC. Sementes imaturas de sorgo congeladas a-80 ºC foram utilizadas para extração na proporção de 1:4 (m/v) de tecido vegetal, para tampão 100 mM de fosfato de potássio, pH 7.0, 50 mM KCl, 1 mM EDTA, 1 mM DL-ditiotreitol (DTT), 0.1 mM fenilmetilsulfonilfluoreto (PMSF), 15% de glicerol e 10% (m/v) de polivinilpirrolidona (PVPP). O material foi macerado em mortar com nitrogênio líquido e homogeneizado em tampão. O homogeneizado foi filtrado em dupla camada de gaze e centrifugado a 15000*g* por 30 minutos e o sobrenadante foi imediatamente utilizado para os procedimentos de isolamento e purificação.

Isolamento e purificação da LOR e SDH

Inicialmente o extrato bruto foi submetido ao fracionamento, utilizando-se diferentes faixas de precipitação com sulfato de amônio subsequentemente. As faixas utilizadas foram as de 20 a 40%, 40 a 60%, 60 a 80%. Com outro extrato bruto foram da mesma forma, utilizadas faixas de 0 a 30%, 30 a 50% e 50 a 70%. O sobrenadante resultante da primeira centrifugação para clarificação então foi ajustado para faixa de precipitação de 40 a 70% (determinada após o fracionamento), que foi então adotada como padrão por concentrar a maior atividade das enzimas. Posteriormente o extrato

foi utilizado para os passos seguintes de purificação. Neste aspecto, modificamos o gradiente linear na cromatografia de troca iônica de 0 a 300 para 0 a 250 Mm de KCl e aumentamos o tamanho da fração de 3 para 4 mL, na tentativa de concentrar mais a enzima e tornar o procedimento como um todo, mais rápido, para tentar compensar a instabilidade da enzima e perdas de atividade. Não houve diferença significativa entre os experimentos.

Ensaio para LOR e SDH

As atividades de LOR e SDH foram mensuradas como descrito por Gaziola *et al.* (1997) com algumas modificações, em tampão de ensaio Tris-HCl, com pH 7,4 para LOR e 8,4 para SDH. (100 mM Tris, 15% glicerol). A mistura de reação em um volume final de 900 μL foi constituída de 33 mM de L-lisina (100 μl de uma solução estoque 0,3 M), 16,6 mM de α- cetoglutarato (50 μl de uma solução 0,3 M) e 0,23 mM de NADPH para LOR; de 2mM NAD e 2 mM de sacaropina para SDH. O início da reação foi dado adicionando 120 μlL do extrato enzimático. A atividade foi verificada pelo acompanhamento da queda na absorbância em 340 nm, à 30 ºC durante 15 minutos. No controle a atividade residual foi medida pela oxidação inespecífica do NADPH sem adição do substrato lisina (para LOR) e da redução do NAD sem adição do substrato sacaropina (para SDH). Para o cálculo da atividade observou-se a diferença entre a absorbância no tempo zero e em 15 minutos, descontando-se os controles (branco) As atividades foram expressas em nmol NADPH/NAD^+ oxidado/reduzido/min/mL/mg de

proteína. Uma unidade de atividade foi definida como a quantidade necessária de enzima para ocorrer a oxidação de 1 nmol de NADPH ou NAD/ minuto.

Determinação de proteína

A concentração de proteína em todas as amostras foi determinada utilizando-se o Kit da Bio-Rad, segundo o método de Bradford, (1976) e soro albumina bovina (BSA) como padrão. Os valores foram determinados em relação à curva padrão de concentrações conhecidas através de regressão linear. Foram determinadas duas curvas: a) de 0.1 a 1 mg/mL, com intervalos de 0.1 mg/mL e b) de 0.01 a 0.1 mg/mL com intervalos de 0.01 mg/mL. Foram considerados valores dentro da faixa de maior linearidade das curvas.

Cromatografia de troca iônica em DEAE-Sephacel - Gradiente Linear

Para a cromatografia de troca iônica com eluição por gradiente linear foi utilizada uma coluna preparativa de DEAE-Sephacel de 2,5 x 12 cm equilibrada com tampão A com um fluxo de 1mL/min. O precipitado protéico foi ressuspenso em um volume mínimo de tampão A e então dessalinizado em coluna de exclusão molecular com G-25. O extrato dessalinizado foi aplicado na coluna, a qual foi eluída com tampão A, de 0 a 250 mM de cloreto de potássio (KCl). Frações de 3 ou 4 mL foram coletadas e utilizadas para os ensaios enzimáticos para LOR e SDH e para estimar a quantidade de proteína eluída através da medida da absorbância em 280 nm ou pelo método de Bradford (1976). A coluna foi eluída sequencialmente com tampão A 1M de KCl para

retirar as proteínas que eventualmente não eluíram de 0 a 250 mM de KCl e a fração correspondente foi coletada seguida também de ensaio enzimático. Em novos experimentos, a fração eluída de gradiente de 50 a 250 mM foi coletada e imediatamente precipitada com Sulfato de Amônio. Para posteriormente ser eluída em outras colunas como Q-Sepharose, S-200 e Superose.

Extração de aminoácidos

Os aminoácidos foram extraídos segundo o método descrito por Bielinski & Turner (1966) para posterior análise qualitativa em HPLC.

Para 1g de material fresco, foi acrescentado 10 ml de solução MCW (600 ml Metanol, 250 ml Clorofórmio, 150 ml H_2O). O material foi macerado e homogeneizado e após centrifugação, foi acrescentado 1 ml Clorofórmio + 1,5 ml H_2O, para cada 4 ml sobrenadante. Aguardou-se separação de fases e foi utilizada a fase hidrossolúvel para purificação de aminoácidos.

Para extração dos aminoácidos incorporados em proteínas. O mesmo procedimento descrito para extração de aminoácidos solúveis foi utilizado, porém, após centrifugação e separação de fases, o precipitado foi utilizado para a hidrólise. A hidrólise foi feita com HCl 6N, a 110 ºC em estufa, durante 24h. Para determinar a relação de proteínas e a quantidade de HCl a utilizar, foi feita uma quantificação prévia, ressuspendendo um dos precipitados com NaOH 0,1 M e determinando a concentração de proteínas do ressuspenso pelo método de Bradford (1976), e daí então, para cada 10 mg de proteínas, utilizado 4 ml de HCl.

Análise quantitativa de aminoácidos - Dosagem de aminoácidos solúveis livres totais

As concentrações dos aminoácidos totais foram quantificadas nas sementes maduras nos diferentes estádios de maturação já citados, segundo o método descrito por Yenm & Cocking (1955). Feita a extração dos aminoácidos como já descrito anteriormente, uma alíquota dessa solução foi analisada para aminoácidos solúveis totais (Yenm & Cocking, 1955). Uma curva foi feita utilizando-se leucina como padrão nas concentrações de 40, 80, 120, 160 e 200 nmol/mL. Uma fração solução de aminoácidos foi colocada em um tubo de ensaio completando o mesmo para 1000 mL. Tanto para análise das amostras desconhecidas quanto para a curva padrão acrescentou-se 0,5 mL de tampão citrato de sódio (0,2 M, pH 5,0), 0,2 mL de reativo de ninidrina (5% em metilglicol) e 1 mL de KCN (2% de uma solução 0,01 M em metilglicol). O tubo de ensaio foi coberto com esferas de vidro para evitar evaporação. Então os tubos foram deixados em banho-maria a 100°C durante 20 minutos. Logo em seguida os tubos foram resfriados em água corrente, a temperatura ambiente e o volume foi completado para 4 mL com etanol 60% (1,3 mL) A leitura em espectrofotômetro dos padrões e das amostras contra o branco foi feita em 570 nm. Conhecida a concentração de aminoácidos das amostras, estas foram então submetidas ao HPLC.

Separação e análise da composição de aminoácidos e quantificação de lisina solúvel nas sementes em HPLC

Para a separação e determinação quantitativa de aminoácidos livres dos genótipos de sorgo, foi utilizada a cromatografia líquida de alto desempenho (HPLC) fase reversa após derivatização com *o*-ofitdialdeído (OPA) (Marur *et al.*, 1994), utilizando-se uma coluna Spherisorb ODS-2 (C18). Os derivados OPA foram detectados por fluorescência. Foram utilizados 500 mg de sementes imaturas de sorgo obtidas da homogeneização em 5 mL de MCW (metanol: clorofórmio: água, na proporção 12:5:3). A mistura foi deixada overnight a 4°C e então foi centrifugada a 6000 rpm por 20 minutos. O sobrenadante foi separado, ao qual foi adicionado 1 mL de clorofórmio e 1,5 mL de água para cada 4 mL de MCW (no nosso caso, adicionamos 2,5 mL de clorofórmio e 3,75 mL de água). Nova centrifugação foi realizada e retirado cuidadosamente a fase aquosa formada. A solução foi colocada durante 1 h a 38°C e em seguida centrifugada a 15000 rpm durante 5 minutos. Logo após, foram retirados 20 µl e colocados em um tubo *eppendorf* adicionando-se 60 µl do reagente OPA. O reagente OPA foi preparado dissolvendo-se 50 mg de OPA em 1 mL de metanol e misturando-se a 6,5 mL de tampão borato-NaOH (ácido bórico 2,4% p/v em H_2O; pH ajustado com NaOH 2N). No dia de uso, 5 µL de 2-mercaptoetanol foram adicionados a 625 µL de OPA. Após exatamente 2 minutos (os derivados glicina (GLY) e lisina (LYS) são instáveis), injeta-se 10 µL correspondente a cada genótipo ou padrão no aparelho de HPLC, iniciando a eluição da mistura em um gradiente de 20-100% do tampão “A”

(Na_2HPO4. 7H2O 50 mM, CH_3COONa 50 mM, 1,5 mL de CH_3COOH (pH 7,25), 20 mL de tetrahidrofurano e 20 mL de metanol em um volume final de 1 L) e tampão “B” (metanol 65%) num fluxo de 0,8 mL/min. O gradiente foi programado para aumentar linearmente a proporção de “B” em relação ao “A”. Os derivados aminoácidos-OPA foram detectados por um monitor de fluorescência (Shimazdu) ajustado para λ de excitação de 265 nm e para λ de emissão de 480 nm. As concentrações de aminoácidos nas amostras foram determinadas pela área dos picos integrados, comparados aos picos de um padrão na concentração de 250 nmol/mL.