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Departamento de Genética e Evolução

Isolamento, Purificação, Caracterização e Estudo dos Mecanismos de Regulação das Enzimas Lisina Oxoglutarato Reductase (LOR) e Sacaropina Desidrogenase (SDH) em Arroz (*Oriza sativa* L.)

Salete Aparecida Gaziola

Orientador: Prof. Dr. Ricardo Antunes Azevedo

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do grau de Doutor em Genética e Biologia Molecular, área de concentração Genética Vegetal e Melhoramento.

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ABREVIATURAS

AEC	S-(2-aminoetil)-L- cisteína.
AK	Aspartato quinase.
Ask1	Gene que codifica a AK insensível à retroinibição por lisina.
ATP	Adenosina trifosfato.
Ca^{2+}	Cálcio.
cDNA	DNA complementar ao RNA.
dapA	Gene de *Corynebacterium* que codifica a enzima AK.
DHDPS	Dihidrodipicolinato sintase.
0EGTA	Etileno Glicol bis (β-aminoetil) eter, ácido tetraacético.
GUS	β-glucuronidase
HSDH	Homoserina desidrogenase.
KCl	Cloreto de potássio.
kDa	Unidade de massa molecular aparente.
α-KG	Ácido α-oxoglutárico.
K_m	Constante de Michaelis.
LOR	Lisina 2-Oxoglutarato Reductase.
Lt1	Gene que confere resistência à inibição por lisina e treonina.
LYS1	Sacaropina desidrogenase de levedura formadora de lisina.
LYS9	Sacaropina desidrogenase de levedura formadora d eglutamato.
LYSC	Gene de *Corynebacterium* que codifica a enzima DHDPS.
mRNA	RNA mensageiro.
NAD	Nicotinamida Adenina Dinucleotídeo.
NADP	Nicotinamida Adenina Dinucleotídeo Fosfato.
NADPH	Nicotinamida Adenina Dinucleotídeo Fosfato na forma reduzida.
ORF	Quadro de leitura aberto..
PAGE	Eletroforese em Gel de Poliacrilamida não desnaturante.
PAGE-SDS	Eletroforese em gel de poliacrilamida desnaturante.
SAM	*S*-adenosilmetionina.
SDH	Sacaropina desidrogenase
TRIS	Tris (hidroximetil)-Aminometano
ZLKRSDH	Gene que codifica a LKR/SDH de milho.

RESUMO

Desde que os cereais são deficientes em pelo menos um dos aminoácidos essenciais tais como lisina, treonina, metionina e isoleucina produzindo, portanto, proteínas de baixa qualidade nutricional, torna-se cada vez mais importante o entendimento da via metabólica do ácido aspártico, responsável pela síntese desses aminoácidos. A via de biossíntese tem sido estudada detalhadamente nos últimos 30 anos, utilizando-se como estratégia o uso de mutantes vegetais ou plantas transgênicas resistentes à retroinibição por lisina e treonina. Entretanto, tanto a síntese de lisina, como a degradação deste aminoácido estão relacionados aos níveis de acúmulo de lisina nas sementes. No entanto, a via de degradação de lisina em plantas, tem sido evidenciada apenas na última década. A Lisina Oxoglutarato Reductase (LOR) e Sacaropina Desidrogenase (SDH) são enzimas que catalisam os dois primeiros passos da degradação de lisina pela via da sacaropina. Neste trabalho, as enzimas LOR e SDH foram extraídas de sementes de arroz em desenvolvimento e parcialmente purificadas, envolvendo precipitação com sulfato de amônio, cromatografias de troca iônica e filtração em gel que resultaram em uma recuperação final de 30% para LOR e 24% para SDH. A massa molecular foi estimada através de cromatografia de filtração em gel em 203 kDa. As atividades co-migraram em gel de poliacrilamida não desnaturante e apresentaram uma massa molecular de 202 kDa determinada por Ferguson plots. Uma segunda banda de atividade de LOR e SDH em gel não desnaturante foi observada e a massa molecular foi estimada em 396 kDa, sugerindo uma estrutura multimérica. Os estudos cinéticos mostraram um mecanismo de reação ordenado para LOR, onde o α-oxoglutarato seria o primeiro substrato e a sacaropina seria o último produto. Os resultados observados sugerem a existência de um único polipeptídeo bifuncional contendo as atividades de LOR e SDH. O efeito de Ca^{2+} e força iônica (Tris e KCl) como moduladores foram analisados para atividade das enzimas LOR e SDH. O domínio LOR, mas não SDH do polipeptídeo bifuncional, parece ser modulado por Ca^{2+} e pela força iônica. Além disso, S-(2-aminoetil)-L- cisteína (AEC), um análogo de lisina, foi investigada na sua habilidade em substituir lisina como substrato e como inibidor da atividade de LOR, sendo capaz de substituir apenas parcialmente a lisina como substrato de LOR e podendo também inibir a atividade da LOR na presença de lisina na mistura de reação possivelmente competindo com lisina

no sítio ativo. O efeito da S-adenosilmetionina também foi testado mas não apresentou nenhum efeito regulatório na atividade das enzimas LOR e SDH.

ABSTRACT

Cereal seeds are deficient in some essential amino acids such as lysine, threonine, methionine, isoleucine and others, leading to storage proteins of low nutricional quality. Thus, the understanding of the regulation of the metabolism of these amino acids becomes a important task. The study of lysine biosynthesis in plants is being carried out in detail in the last 3 decades, using as main strategies the isolation of natural occurring mutants, biochemical mutants and more recently, transgenic plants. However, very little is known about the degradation of lysine. Two enzymes, lysine 2-oxoglutarate reductase (LOR) and saccharopine desidrogenase (SDH), which regulate the catabolism of lysine, have been isolated from maize. In this study, both enzymes were extracted from rice developing seeds, partially purified and characterized. For the purification of LOR and SDH, the best results were obtained using ammonium sulphate precipitation, ion exchange and gel filtration chromatography, resulting in a final yield of 30% and 24% for LOR and SDH, respectively. The molecular mass was estimated as 203 kDa by gel filtration chromatography and 202 kDa by Ferguson plots on non denaturing gels. Furthermore, the identification of several bands of activity suggested a multimeric nature for the polypeptide. Both enzymes co-migrated independently of the purification method used, suggesting the existence of a bifunctional polypeptide containing LOR and SDH activities. Both enzyme activities were also shown to be specific to the endosperm. Kinetic studies were consistent with an ordered sequence mechanism for LOR, where 2-oxoglutarate is the first substrate and saccharopine is the last product. LOR activity was shown to be modulated by calcium and ionic strength, but not SDH activity. 2-(aminoethyl)-L-cysteine, a lysine analogue, could partially substitute lysine as a substrate for LOR , but could also inhibit LOR activity in the presence of lysine in the reaction mixture, possibly competing with lysine at the active site. S-adenosylmethionine was also tested for LOR and SDH activities, but did not produce any effect on the enzymes activities.

INTRODUÇÃO

O rápido aumento da população mundial tem levado a urgentes esforços para aumentar a produção e a qualidade nutricional dos alimentos. Entre as fontes de proteínas vegetais, as da semente tem um importante papel devido ao aspecto nutricional.

Dentre os vinte aminoácidos normalmente incorporados em proteínas, somente onze podem ser sintetizados por mamíferos adultos. Os nove restantes, denominados essenciais, devem ser providos pela dieta. As plantas e a maioria das bactérias e fungos têm a capacidade de sintetizar todos os vinte aminoácidos.

Os cereais, de uma forma geral, constituem a maior e muitas vezes, a única fonte de proteínas em países subdesenvolvidos. Dentre os cereais, o trigo, o arroz e o milho são, nesta ordem, os mais importantes. O arroz é fonte de alimento importante em países em desenvolvimento, sendo predominantemente cultivado na Ásia, numa área de aproximadamente 133 mil hectares cuja produção chega a ser cerca de 505 mil toneladas (KHUSH, 1997). No Brasil, a área colhida e a produção correspondem à 3.550 mil hectares e 9.400 toneladas, respectivamente (AGRIANUAL, 1998). O arroz é consumido por mais da metade da população mundial, sendo que o consumo nacional atinge cerca de 11,5 milhões de toneladas ao ano (AGRIANUAL, 1998). Trigo e arroz fornecem cerca de 35% de proteína vegetal para nutrição humana. Apesar de sua grande importância na alimentação humana, os cereais possuem baixo conteúdo protéico, cerca de 10% da massa seca (EARLE *et al.*, 1946) e apresentam baixa qualidade nutritiva devido às deficiencias em aminoácidos essenciais como lisina, treonina e triptofano das proteínas de reserva (MERTZ *et al.*, 1958). Embora o nível protéico em arroz (9%) seja inferior ao do trigo (12%), as proteínas de arroz apresentam um maior valor nutricional entre os cereais, pois possuem os níveis mais elevados de aminoácidos essenciais, incluindo-se a lisina e a treonina.

Embora o arroz tenha altos níveis de aminoácidos essenciais, uma das prioridades para o melhoramento de variedades nesta espécie consiste no aumento do conteúdo desses aminoácidos (KUMAMARU *et al.*, 1997).

Grande progresso tem sido recentemente obtido nos estudos genéticos e bioquímicos do metabolismo do nitrogênio e biossíntese de aminoácidos em plantas. Este é um campo de fundamental importância porque, além de síntese de proteínas, as plantas

utilizam aminoácidos, seus precursores e os produtos do seu catabolismo para muitas atividades metabólicas. Torna-se portanto, bastante interessante e desejável a manipulação da regulação da biossíntese e degradação de aminoácidos.

A degradação da lisina é possivelmente um ponto fundamental para o acúmulo deste aminoácido em sementes (BROCHETTO-BRAGA *et al.*, 1992; KARCHI *et al.*, 1994; GONÇALVES-BUTRUILLE *et al.*, 1996). Estudos da regulação, empregando a expressão de genes quiméricos para síntese de lisina em sementes em desenvolvimento de *N. tabacum* (KARCHI *et al.*, 1994), sugeriram que a síntese e o catabolismo da lisina são regulados coordenadamente durante o desenvolvimento da semente. Em arroz, a via de biossíntese de lisina e treonina foi também estudada (SHAW e KU, 1984; TEIXEIRA *et al.*, 1998), mostrando a presença de duas isoformas de AK, uma sensível à inibição por lisina e uma sensível à inibição por treonina (TEIXEIRA *et al.*, 1998).

O presente trabalho teve por finalidade a obtenção de informações sobre o catabolismo de lisina em arroz com o objetivo de contribuir para um melhor entendimento sobre o acúmulo de lisina durante o desenvolvimento das sementes.

1. Metabolismo de lisina

Várias estratégias tem sido desenvolvidas com o objetivo de melhorar a qualidade nutricional de plantas para um melhor equilíbrio dos aminoácidos essenciais e em particular, do conteúdo de lisina.

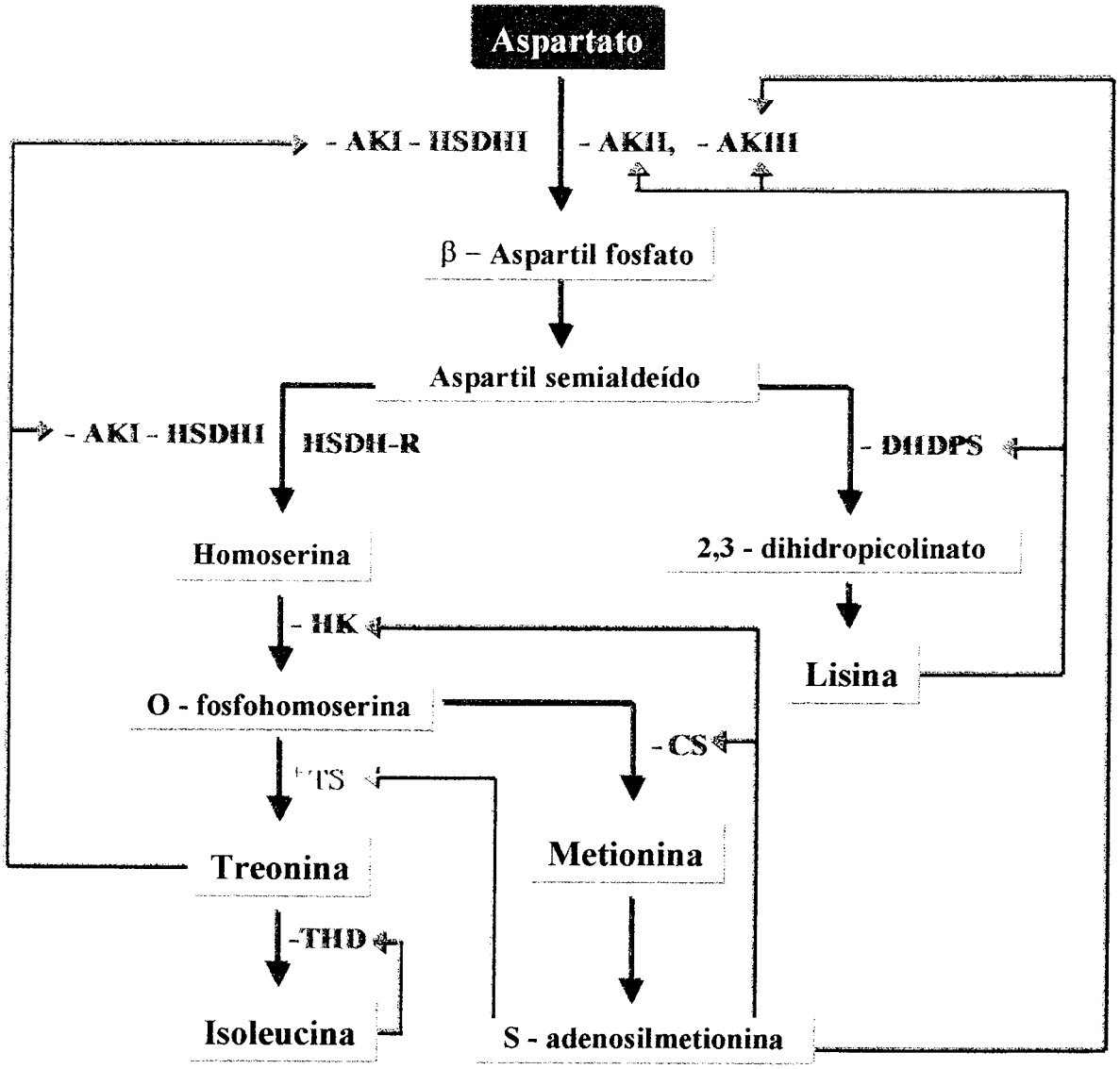
Determinadas vias metabólicas têm recebido atenção especial devido ao seu valor potencial para a melhoria da qualidade nutritiva, em resposta à diferentes formas de estresses e herbicidas.

Os aminoácidos essenciais, lisina, treonina, metionina e isoleucina assim denominados por não serem sintetizados por animais monogástricos, são sintetizados pela via metabólica do ácido aspártico. A compreensão de como se comportam as enzimas envolvidas na síntese e degradação desses aminoácidos é o primeiro passo para uma possível manipulação da via e de seus produtos finais.

Estudos genéticos e bioquímicos têm levado à identificação dos mecanismos de regulação da via metabólica do ácido aspártico, mostrando que várias enzimas são reguladas positiva ou negativamente pelos produtos finais ou seus análogos (Fig. 1) (MADISON e THOMPSON, 1976; SODEK, 1976; ROGNES *et al.*, 1980; SAINIS *et al.*, 1981; BRYAN, 1990a; AZEVEDO *et al.*, 1997) e por interações gênicas (BRENNECKE *et al.*, 1996), controlando desta forma, a síntese de aminoácidos.

1.1. Biossíntese de lisina

Existem duas vias distintas para a síntese de lisina. Em fungos e euglenóides, a lisina é sintetizada envolvendo o ácido α-aminoadípico (AAA) como intermediário. Em bactérias e plantas superiores, a via é caracterizada pela formação do ácido diaminopimélico (DAP) (BONNER e LEA, 1990). O uso de precursores de lisina e intermediários radioativos, confirmaram a síntese de lisina via ácido diaminopimélico (MOLLER, 1974; MIFLIN e LEA, 1977; MILLS *et al.*, 1980). Estudos de incorporação *in vivo* dos precursores marcados foram realizados em endospermas de cevada (MOLLER, 1974), de milho (SODEK, 1976) e cloroplastos isolados de ervilhas (MILLS *et al.*,1980).



Fluxo da via metabólica

Inibições

Ativações

AK: Aspartato Quinase
CS: Cistationina Sintase
DHDPS: Dihidrodipicolinato Sintase
HSDH: Homoserina Desidrogenase
THD: Treonina Desidratase
TS: Treonina sintase

Figura 1: Regulação da via metabólica do ácido aspártico, adaptado de BRYAN, 1990a.

O diaminopimelato é o último intermediário na ramificação específica da via de formação de lisina, tendo o aspartato como precursor inicial. A síntese dos outros três aminoácidos é derivada de outras ramificações da via (Fig. 2; BRYAN, 1990a; GALILI, 1995; AZEVEDO *et al.*, 1997).

- **Aspartato Quinase**

A aspartato quinase (AK) (EC 2.7.2.4.) é a primeira enzima chave da via metábolica do ácido aspártico. Essa via apresenta outros pontos chave, as enzimas Dihidrodipicolinato Sintase (DHDPS) (EC 4.2.1.52) e Homoserina Desidrogenase (HSDH) (EC 1.1.3.), envolvidas especificamente na biossíntese de lisina e treonina, respectivamente.

Algumas enzimas da via do aspartato foram localizadas no cloroplasto (BRYAN, 1990a; GALILI, 1995), sugerindo sua ação nessa organela. A via metabólica inicia-se com a fosforilação do ácido aspártico em β-aspartil fosfato pela enzima AK em uma reação catalisada por ATP/Mg^{2+}. O β-aspartil fosfato é convertido em β-aspartil semialdeído, pela ação da enzima aspartato semialdeído desidrogenase (EC 1.2.1.11). Até este ponto a via é comum para os quatro aminoácidos e a partir daí ramifica-se em dois caminhos que serão discutidos posteriormente. Um deles origina a lisina, que é formada a partir da condensação do β-aspartil semialdeído e piruvato em 2,3-dihidrodipicolinato pela ação da enzima DHDPS (EC 1.3.1.26). O outro caminho da via origina os aminoácidos treonina, isoleucina e metionina. A metionina pode ser convertida em S-adenosilmetionina.

A AK foi extraída, purificada e caracterizada parcialmente em algumas plantas superiores, entre elas, milho, cevada, cenoura, ervilha, soja (AZEVEDO *et al.*, 1997) e arroz (TEIXEIRA *et al.*, 1998). Pelo menos duas isoformas da AK foram identificadas como sensíveis à retroinibição por lisina e outra por treonina (ou pela ação sinergística de lisina e S-adenosilmetionina (SAM)) (ROGNES *et al.*, 1980; AZEVEDO *et al.*, 1992a). Três diferentes isoenzimas da AK foram identificadas em cevada (LEA *et al.*, 1992) e em milho (AZEVEDO *et al.*, 1992a). Entre as diferentes isoenzimas, a forma sensível à inibição por lisina é predominante em tecidos de crescimento rápido (LEA *et al.*, 1979). A isoenzima sensível a treonina foi observada em cevada (BRIGHT *et al.*, 1982), em milho (AZEVEDO *et al.*, 1992a) e em *Coix* (LUGLI e AZEVEDO, 1994). Em arroz, foram detectadas a presença de duas isoformas da AK, uma predominante e sensível à inibição por lisina e outra sensível à inibição por treonina (TEIXEIRA *et al.*, 1998). O primeiro cDNA que codifica a enzima AK sensível à inibição por treonina foi isolado em cenoura (WEISEMANN e MATTEWS, 1993).

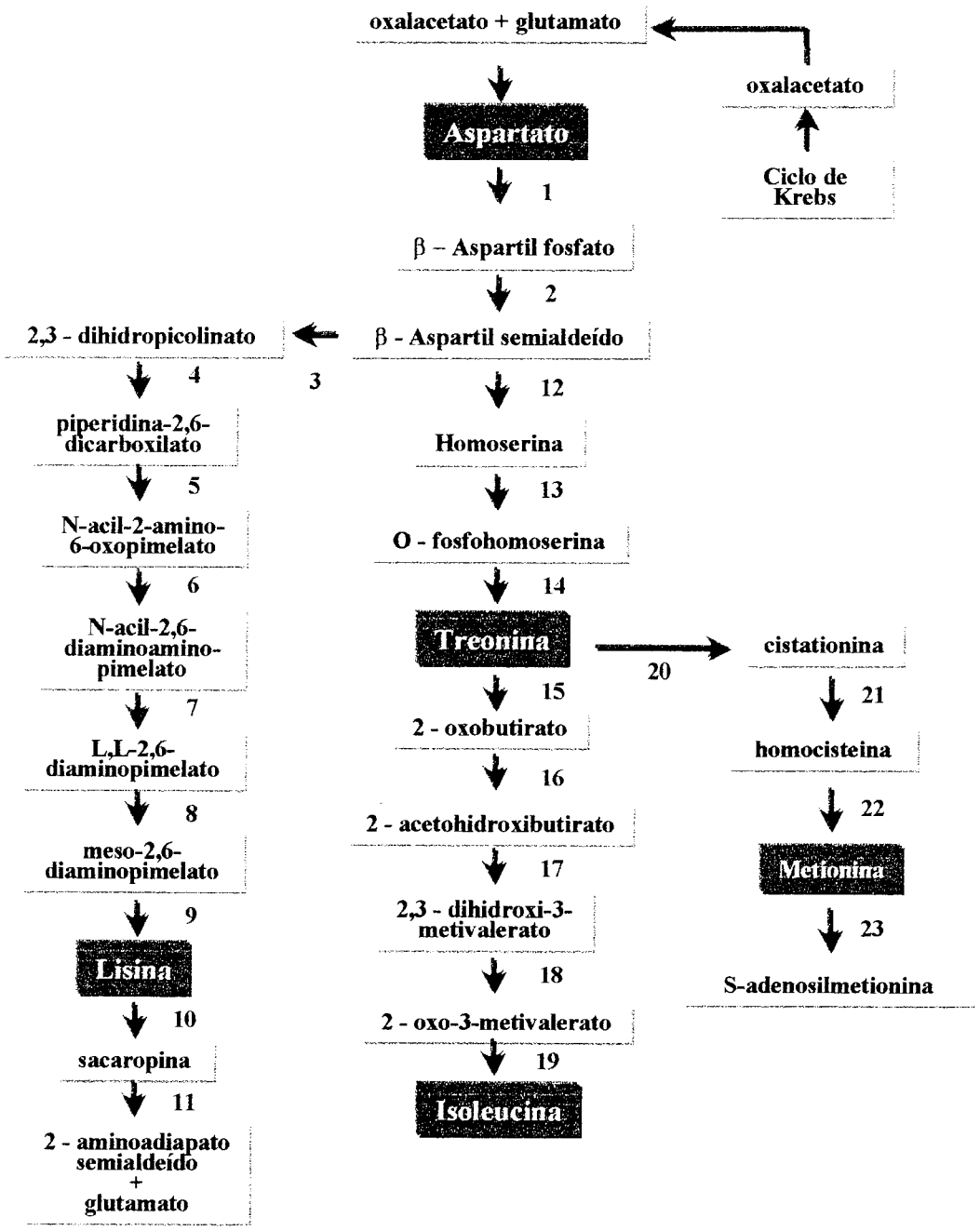


Figura 2: Via metábolica do ácido aspártico, adaptado de BRYAN, J. K. , 1990.
1 Aspartato Quinase; 2 Aspartato Semialdeído Desidrogenase; 3 Dihidrodipicolinato Sintase; 4 Dihidrodipicolinato Redutase; 5 Piperidina Dicarboxilase Acilase; 6 Acildiaminopimelato Aminotransferase; 7 Acildiaminopimelato Deacilase; 8 Diaminopimelato Epimerase; 9 Diaminopimelato Descaboxilase; 10 Lisina Oxoglutarato Redutase; 11 Sacaropina Desidrogenase; 12 Homoserina Desidrogenase; 13 Homoserina Quinase; 14 Treonina Sintase; 15 Treonina Desidratase; 16 Acetolato Sintase; 17 Acetohidroxato Redutoisomerase; 18 Dihidroxato Desidratase; 19 Aminotransferase de Cadeia Ramificada; 20 Cistationa-γ-Sintase; 21 Cistationa-β-Liase; 22 Metionina Sintase; 23 Metionina Adenosil Transferase.

Além da regulação da AK por lisina, treonina e SAM, o envolvimento de Ca^{2+} e calmodulina na regulação da AK têm sido proposto em espinafre (SANE *et al.*, 1984; KOCHHAR *et al.*, 1986; KOCHHAR *et al.*, 1987; KOCHHAR *et al.*, 1998) e muito recentemente em cevada (RAO *et al.*, 1999). Entretanto, existem sérias controvérsias quanto ao potencial de regulação da AK pelos mesmos. KOCHHAR *et al.* (1988) relataram que calmodulina seria uma das subunidades da AK sensível à lisina purificada de folhas de espinafre, apresentando uma massa molecular de 17 kDa. Além disso, os autores mostraram que a atividade da AK é inibida por EGTA (quelante específico de Ca^{2+}) e estimulada pela adição de Ca^{2+}. Em contrapartida, em cenoura (BONNER *et al.*, 1986) e milho (AZEVEDO *et al.*, 1992c) estudos com a AK sensível à lisina altamente purificada, não mostraram nenhuma evidência para um papel regulatório de Ca^{2+} e calmodulina na atividade da AK. Recentemente, RAO *et al.* (1999) relataram que em cevada, a atividade da isoenzima sensível à lisina não é estimulada por Ca^{2+} e calmodulina enquanto que as duas outras isoenzimas, detectadas como sensíveis à treonina, foram ativadas pelos mesmos. Esses resultados contradizem aqueles descritos anteriormente em cevada (LEA *et al.*, 1992).

Resultados para massa molecular das isoenzimas da AK sensível à inibição por lisina mostraram algumas variações. Em cenoura massas moleculares variando entre 100 kDa e 258 kDa foram determinadas (RELTON *et al.*, 1988). Em células em suspensão de milho (DOTSON *et al.*, 1989), a massa molecular da enzima foi estimada, de acordo com cromatografia de filtração em gel e eletroforese em géis desnaturantes e não desnaturantes, em 254 kDa, composta de duas subunidades de 49 kDa e duas de 60 kDa. AZEVEDO *et al.* (1992a) relataram para milho uma massa molecular de 180 kDa para a AK sensível à treonina e aproximadamente 150 kDa para a isoenzima sensível à lisina, ambas determinadas por cromatografia de filtração em gel e eletroforese em gel não desnaturante. Para arroz, a massa das isoenzimas foram estimadas em 163 kDa para a isoforma sensível à lisina e 186 kDa para a isoforma da AK sensível à treonina (TEIXEIRA *et al.*, 1998).

Clones genômicos da AK-HSDH sensíveis à treonina foram isolados em *A. thaliana* e milho (GHISLAIN *et al.*, 1994; MUEHLBAUER *et al.*, 1995). Em *A. thaliana*, o gene que codifica a AK-HSDH sensível à treonina está presente em cópia única e a região codificante é interrompida por 15 íntrons pequenos (78-134 pb). A comparação entre as

sequências do cDNA da AK-HSDH de cenoura e dos genes *thrA* e *metL* de *E. coli* demostraram 80%, 37% e 31,4% de similaridade, respectivamente (GHISLAIN *et al.*, 1994). Entretanto, um cDNA de AK-HSDH isolado de *A. thaliana* por complementação funcional de um mutante de levedura, mostrou uma sequência de nucleotídeos e aminoácidos que difere consideravelmente da sequência previamente descrita por GHISLAIN *et al.* (1994). As análises genéticas para as enzimas de milho mostraram que pelo menos cinco genes codificam a AK; três genes codificam o polipeptídeo bifuncional AK-HSDH e os outros dois genes codificam a aspartato quinase monofuncional (MUEHLBAUER *et al.*, 1994). Posteriormente, dois cDNAs codificando a isoenzima monofuncional de AK sensível a retroinibição por lisina (sem o domínio da HSDH) foram clonados de *A. thaliana* (FRANKARD *et al.*, 1997; TANG *et al.*, 1997b).

Alguns estudos mostraram em cenoura (WILSON *et al.*, 1991), milho (AZEVEDO *et al.*, 1992b) e arroz (TEIXEIRA *et al.*, 1998) a presença de um polipeptídeo bifuncional contendo as atividades de AK-HSDH, sendo ambas atividades inibidas por treonina, como já havia sido descrito anteriormente em *E. coli* (PATTE *et al.*, 1967) e inicialmente proposto em plantas por AARNES e ROGNES (1974).

A enzima HSDH catalisa a primeira reação do ramo da via de biossíntese de treonina, metionina e isoleucina, convertendo o β-aspartato semialdeído em homoserina. Plantas superiores geralmente possuem duas isoenzimas da HSDH, uma forma sensível à inibição por treonina e outra resistente (BRYAN, 1980; LEA *et al.*, 1985). As isoenzimas diferem em tamanho, sendo que a forma sensível a treonina exibe uma massa de 190 kDa e a forma resistente ao redor de 70 kDa (WALTER *et al.*, 1979).

Plantas transgênicas expressando construções quiméricas contendo o promotor da AK/HSDH de *A. thaliana* fundido com GUS como gene reporter mostraram que o gene AK/HSDH está sujeito a regulação temporal e espacial, sendo expresso em tecidos vegetativos, orgãos florais e sementes em desenvolvimento (ZHU-SHIMONI *et al.*, 1997). Além disso, sugerem que a regulação metábolica desse gene opera a nível transcricional (ZHU-SHIMONI *et al.*, 1998).

- **Dihidrodipicolinato Sintase**

Uma ramificação da via metabólica do ácido aspártico está relacionada com a via específica de biossíntese de lisina, que envolve a enzima DHDPS. Dentre as enzimas da via, a DHDPS é a mais fortemente retroinibida pela lisina e tem papel fundamental na regulação da biossíntese deste aminoácido (AZEVEDO *et al.*, 1997). A DHDPS catalisa a condensação do piruvato e β-aspartato semialdeído formando o 2,3-dihidrodipicolinato, que é um intermediário da biossíntese do diaminopimelato e lisina. Esta enzima foi isolada em bactérias e purificada até homogeneidade em *E. coli* (SHEDLARSKY e GILVARD, 1970) e *Bacillus licheniformis* (HALLING e STAHLY, 1976). A DHDPS também foi estudada, purificada e caracterizada em várias plantas tais como milho (CHESHIRE e MIFLIN, 1975; FRISCH *et al.*, 1991a; 1991b), trigo (KUMPAISAL *et al.*, 1987), espinafre (WALLSGROVE e MAZELIS, 1981), ervilha (DEREPPE *et al.*, 1992) e *N. tabacum* (GHISLAIN *et al.*, 1990). O gene que codifica a DHDPS também foi caracterizado em milho (FRISCH *et al.*, 1991b), *Populus* (VAUTERIN e JACOBS, 1994), soja (SILK *et al.*, 1994) e *N. tabacum* (GHISLAIN *et al.*, 1995).

KANEKO *et al.* (1990), a partir de células em suspensão de trigo, purificaram a enzima DHDPS até a homogeneidade e clonaram dois diferentes cDNAs que codificam essa enzima. Comparações entre os clones de milho (FRISCH *et al.*, 1991a) e de trigo (KANEKO *et al.*, 1990) mostraram codificar proteínas de mesmo tamanho contendo 326 aminoácidos. Entretanto, quando sequências de direcionamento de DHDPS de milho e trigo foram comparadas, nenhuma homologia foi encontrada, a não ser na região imediatamente adjacente ao início da proteína madura (AZEVEDO *et al.*, 1997). Análises de hibridação de DNA genômico sugerem que em milho podem existir vários genes que codificam a DHDPS (FRISCH *et al.*, 1991a). Todos os clones caracterizados codificam proteínas de 326 aminoácidos, com exceção de *Populus* que apresenta 327 (VAUTERIN e JACOBS, 1994). Em *Coix lacryma-jobi* (DANTE, 1997), a caracterização de um clone genômico do gene *DapA* que codifica a DHDPS, revelou uma sequência que codifica um polipeptídeo de 377 aminoácidos. Essa sequência mostrou uma ORF interrompida por dois íntrons de aproximadamente 1 e 0,8 Kb e a proteína final contendo 326 aminoácidos que apresentam 95% de similaridade com a de milho (FRISCH *et al.*, 1991a).

Duas diferentes linhas de evidências têm indicado que a enzima DHDPS é o ponto central de controle do ramo da via que leva à biossíntese de lisina em plantas superiores. As análises de mutantes contendo AK insensível ou com sensibilidade reduzida à lisina e/ou treonina, mostraram sempre o acúmulo de treonina, mas não de lisina (BRIGHT *et al.*, 1982; AZEVEDO *et al.*, 1990). Plantas de *N. tabacum* contendo uma DHDPS insensível à inibição por lisina mostraram um acúmulo de lisina apenas em folhas (KARCHI *et al.*, 1994). Entretanto, o acúmulo de lisina em sementes, não tem sido observado.

- **Mutantes Bioquímicos e Plantas transgênicas**

A seleção de mutantes vegetais resistentes à lisina e treonina com alterações no conteúdo desses aminoácidos e que apresentam formas de enzimas menos sensíveis à retroinibição, tem mostrado ser uma importante ferramenta para se obter plantas com maior valor nutritivo, bem como ajudar no entendimento da regulação da via de biossíntese de lisina e treonina.

Estudos com mutantes vegetais, mostraram que as enzimas apresentaram um padrão de inibição alterado e também podem conter níveis aumentados do produto final. Esse sistema, inicialmente demonstrado em microorganismos (DEMAIN, 1970), tem sido demonstrado para a via do ácido aspártico (AZEVEDO *et al.*, 1997). Mutantes bioquímicos podem ser produzidos por mutagenesis de sementes ou células selecionadas em meio seletivo contendo aminoácidos ou seus análogos (GREEN e PHILLIPS, 1974; LEA *et al.*, 1992).

A seleção de mutantes obtidos por cultura de tecidos contendo lisina ou S-(2-aminoetil) L-cisteína (AEC), um análogo de lisina, tem sido um método utilizado para a obtenção de plantas com níveis de lisina alterados. Várias espécies de plantas foram analisadas, utilizando-se essa estratégia, tais como milho (AZEVEDO e ARRUDA, 1995), arroz (SCHAEFFER e SHARPE, 1981; 1987), *N. tabacum* (NEGRUTIU *et al.*, 1984), *Pennisetum americanum* (BOYES e VASIL, 1987), *A. thaliana* (VERNAILLEN *et al.*, 1985) e batata (JACOBSEN, 1986), porém os mutantes obtidos mostraram pouca ou nenhuma alteração nos níveis de lisina em sementes e a enzima DHDPS sensível à inibição por lisina. Em alguns casos, a resistência à AEC está relacionada a sua reduzida ou

inexistente absorção pelas raízes das plantas (BRIGHT *et al.*, 1979; CATTOIR *et al.*, 1983; AZEVEDO e ARRUDA, 1995).

Baseado na mesma idéia geral de desregulação da AK e DHDPS associada ao desenvolvimento de técnicas de transformação de plantas, obteve-se a produção de plantas transgênicas expressando genes de bactérias que codificam as enzimas da via do ácido aspártico caracteristicamente menos sensíveis à retroinibição por lisina e treonina, oferecendo uma nova estratégia para melhorar a produção de lisina e treonina.

Plantas transgênicas de *N. tabacum* expressando uma DHDPS codificada por um gene bacteriano levou a superprodução e acúmulo de lisina nas folhas (SHAUL e GALILI, 1992a). Similarmente, plantas transgências *N. tabacum* expressando no citoplasma e cloroplastos uma AK de bactéria insensível à inibição por lisina, resultaram em uma superprodução de treonina. A superprodução e acúmulo de treonina solúvel foi maior nas plantas transgênicas que expressavam a AK nos cloroplastos (SHAUL e GALILI 1992b). A síntese de lisina e treonina mostraram estar sob a regulação da AK, DHDPS e HSDH. Plantas transgências de *N. tabacum* expressando as enzimas DHDPS e AK de *E. coli* mostraram uma concentração muito superior de lisina solúvel acompanhada pela redução na treonina solúvel quando comparadas as plantas transgênicas expressando separadamente a DHDPS e AK, respectivamente (SHAUL e GALILI, 1993). Estratégia similar foi empregada para cevada (BRINCH-PERDENSEN *et al.*, 1996), soja e canola (FALCO *et al.*, 1995). Em cevada, concentrações superiores de lisina e metionina nas plantas transgências expressando a enzima DHDPS e AK de bactéria foram observados (BRINCH-PERDENSEN *et al.*, 1996). A enzima DHDPS e AK codificadas pelos genes *dap*A e *lys*C de *Corynebacterium*, respectivamente, foram expressas a partir de um promotor semente específico em soja e canola (FALCO *et al.*, 1995). Em ambas as plantas foram observados um grande aumento de lisina solúvel nas sementes com acúmulo de ácido amioadípico em canola e sacaropina em soja (FALCO *et al.*, 1995).

Os resultados obtidos de plantas mutantes e de plantas transgênicas contendo formas de AK e/ou DHDPS menos sensíveis à retroinibição por lisina, mostraram que a DHDPS tem papel fundamental na biossíntese de lisina, enquanto a AK exerce um papel secundário. A AK parece estar principalmente relacionada a regulação geral da via do ácido

aspártico, controlando especificamente os níveis do β-aspartato semialdeído, substrato comum de todos os aminoácidos produzidos pela via. O balanço entre a síntese de lisina e treonina parece ser determinada pela competição entre DHDPS e HSDH pelo β-aspartato semialdeído (SHAUL E GALILI, 1993).

1.2. DEGRADAÇÃO DE LISINA

Além do entendimento de como funcionam as enzimas envolvidas na biossíntese de lisina e treonina, as quais foram estudadas em detalhes e muitas informações estão disponíveis quanto aos aspectos regulatórios, grande ênfase tem sido dada ao estudo do catabolismo de lisina em plantas até então muito pouco conhecido. Recentes estudos têm indicado que o catabolismo de lisina tem papel essencial no acúmulo de lisina em sementes de plantas.

Em microrganismos (JONES *et al.*, 1965; 1966; SAUNDERS *et al.*, 1966; RAMOS *et al.*, 1988) (Fig. 3A) e mamíferos (HUTZLER e DANCIS, 1968; 1970; FJELLSTEDT e ROBINSON, 1975a; NODA e ICHIARA, 1978; MARKOVITZ *et al.*, 1984) a degradação de lisina foi extensivamente estudada.

Está bem estabelecido que os dois primeiros passos na degradação oxidativa da lisina em mamíferos são catalizados pela lisina 2-oxoglutarato redutase (LOR; EC 1.5.1.8, também conhecida como lisina cetoglutarato reductase – LKR) (HUTZLER e DANCIS, 1968; FJELLSTEDT e ROBINSON, 1975a; NODA e ICHIARA, 1978), e sacaropina desidrogenase (SDH; EC 1.5.1.9) (HUTZLER e DANCIS, 1970; FJELLSTEDT e ROBINSON, 1975b). A LOR condensa lisina e 2-oxoglutarato (α-KG) em sacaropina na presença de NADPH, enquanto a SDH hidrolisa a sacaropina em α-aminoadípico-semialdeído e glutamato na presença de NAD ou NADP. Glutamato pode ser reciclado em outros aminoácidos ou ser convertido em 2-oxoglutarato pela glutamato desidrogenase (MELO-OLIVEIRA *et al.*, 1996). O α-aminoadípico semialdeído é convertido em ácido α-aminoadípico e compostos de baixa massa molecular. Mutações no gene da LOR em humanos resultam em uma doença genética severa chamada lisinemia familiar (MARKOVITZ *et al.*, 1984).

A purificação das enzimas de fígado de rato mostrou que a LOR e SDH são polipeptídeos monofuncionais distintos (NODA e ICHIHARA, 1978). Entretanto, evidências de que as atividades da LOR e SDH fazem parte de um único polipeptídeo bifuncional em humanos e animais, foram fornecidas por estudos de placenta humana (FJELLSTEDT e ROBINSON, 1975b) e fígado de boi e babuíno (MARKOVITZ *et al.*, 1984).

Para a forma nativa da LOR/SDH em mamíferos foi determinada uma massa molecular aparente de 468 kDa, sugerindo ser um tetrâmero com subunidades idênticas de 115 kDa observado em gel desnaturante (MARKOVITZ *et al.*, 1984). Proteólise limitada do polipeptídeo purificado de 115 kDa determinou a separação em dois fragmentos com massas moleculares de 62,7 kDa e 49,2 kDa, conservando a atividade da LOR e SDH, respectivamente. Pela manutenção da atividade de cada fragmento, concluiu-se que os domínios são separados e funcionam de maneira independente (MARKOVITZ *et al.*, 1987). Em placenta humana, a massa molecular foi estimada em 480 kDa para o polipeptídeo contendo as atividades LOR e SDH (FJELLSTEDT e ROBINSON, 1975a).

A enzima LOR purificada de fígado de rato teve a massa molecular estimada em 230 kDa e 52 kDa, para as formas nativa e desnaturada, respectivamente. Isso sugere que a enzima ativa é um tetrâmero de subunidades de tamanho similares (NODA e ICHIHARA, 1978). As enzimas LOR e SDH de fígado de rato parecem estar localizadas na matriz mitocondrial (BLEMINGS *et al.*, 1994), enquanto que em fígado de boi e babuíno as enzimas foram extraídas da fração mitocondrial (MARKOVITZ *et al.*, 1984).

Em fungos e leveduras, as enzimas LOR e SDH se apresentam como monômeros de 49 kDa e 73 kDa que são codificados por dois genes *lys*1 e *lys*9, respectivamente (FELLER *et al.*, 1999; RAMOS *et al.*, 1988; BORELL *et al.*, 1984).

LOR e SDH em plantas

Em contraste à via de biossíntese de lisina, que parece operar nos plastídeos, as enzimas envolvidas nos dois primeiros passos do catabolismo de lisina em plantas parecem estar localizadas no citoplasma, pois a proteína não contém sequências de direcionamento para mitocôndria ou cloroplasto em *A. thaliana* ou milho (EPELBAUM *et al.*, 1997; CORD-NETO, 1998).

A maioria das informações sobre o catabolismo de lisina em plantas superiores foi obtida a partir de estudos de incorporação e metabolismo de aminoácidos marcados com [^{14}C]. Experimentos com lisina marcada com [^{14}C] resultaram na incorporação do carbono em ácido α-aminoadípico, ácido glutâmico, sacaropina e ácido diaminopimélico (AZEVEDO *et al.*, 1997). Em sementes em desenvolvimento de milho e cevada, o [^{14}C] de lisina marcada foi incorporado em ácido glutâmico, prolina e sacaropina (SODEK e

WILSON, 1970; BRANDT, 1975; ARRUDA *et al.*, 1982). A identificação de alguns produtos de reação evidenciaram o catabolismo de lisina via sacaropina (Fig. 3B). A detecção e caracterização da atividade de LOR em sementes em desenvolvimento de milho foi a primeira evidência enzimática para a operação da via da sacaropina em plantas (ARRUDA *et al.*, 1982).

Em plantas, a primeira enzima da via de degradação da lisina, LOR, foi caracterizada em milho (ARRUDA *et al.*, 1982; ARRUDA e DA SILVA, 1983; BROCHETTO-BRAGA *et al.*, 1992) e apresenta altas taxas de atividade em endosperma em desenvolvimento, sendo específica deste tecido. A atividade da LOR muito reduzida no mutante opaco-2, está correlacionada com a diminuição dos níveis de zeína neste mutante (ARRUDA *et al.*, 1982; BROCHETTO-BRAGA *et al.*, 1992). O gene *opaco-2*, é um transativador de genes de zeína de 22 Kd e pode estar envolvido na regulação do gene que codifica a enzima LOR em endosperma de milho (ARRUDA *et al.*, 1982; BROCHETTO-BRAGA *et al.*, 1992; CATHERINE DAMERVAL, comunicação pessoal). Por outro lado, a diminuição na atividade de redutase, causada pelo gene *opaco-2*, pode, em parte, explicar os elevados níveis de lisina encontrados em endosperma opaco-2. Recentes estudos com variedades de milho normais, opaco-2 e QPM (Quality Protein Maize), produzidas pela introdução de genes modificadores mantendo as características químicas do mutante opaco-2, sugerem que a introdução desses genes intensificou o efeito do gene *opaco-2* nas atividades de LOR e SDH. A redução da atividade das enzimas também levou a um aumento na disponibilidade de lisina solúvel (GAZIOLA *et al.*, 1999). Além disso, o padrão de expressão temporal do gene que codifica a LOR/SDH de milho, denominado *ZLKRSDH*, em endospermas normais e opaco-2, mostraram que a mutação opaco-2 reduz drasticamente a transcrição desse gene, reduzindo os níveis de polipeptídeos LOR/SDH e a atividade enzimática (KEMPER *et al.*, 1999).

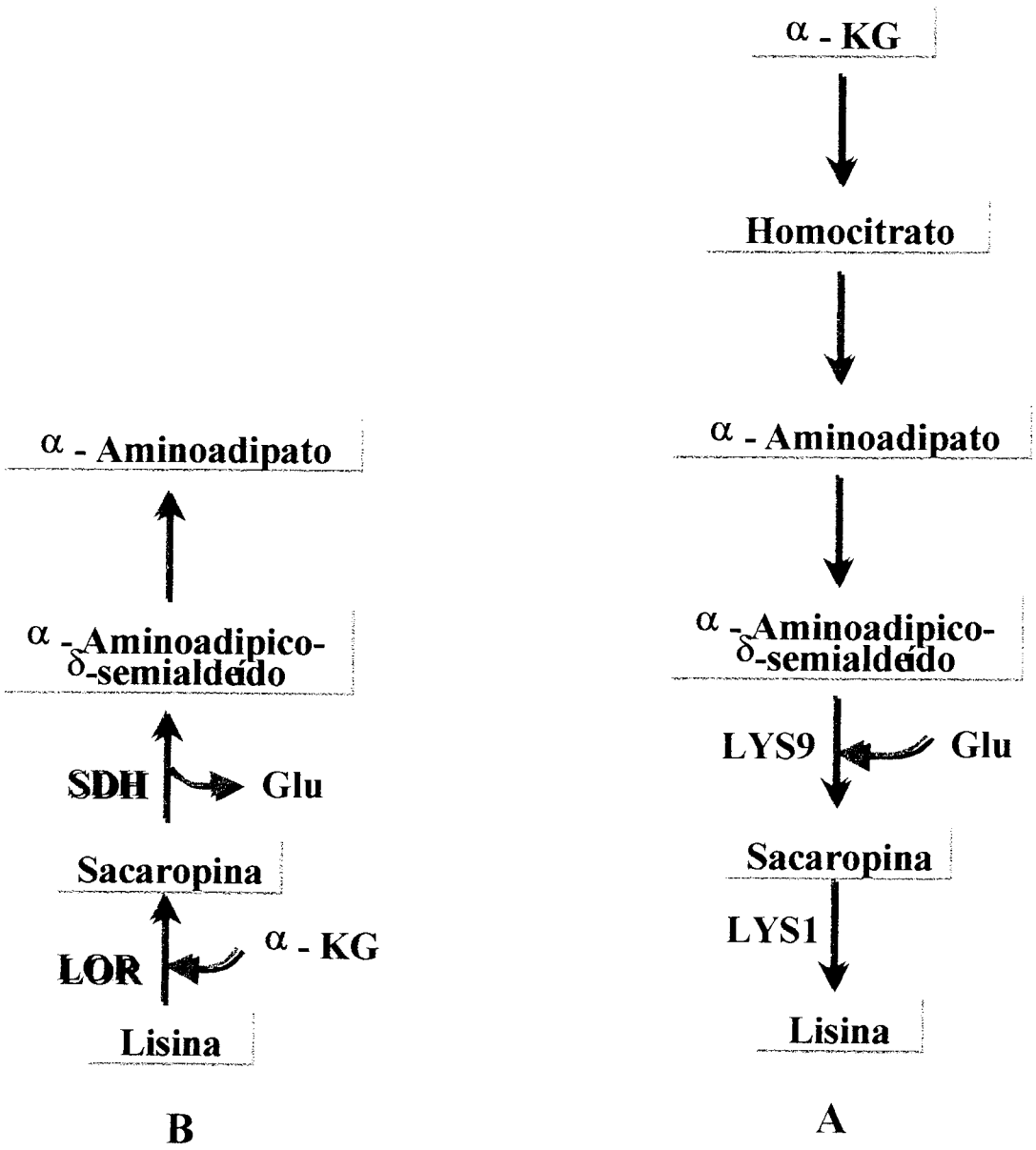


Figura 3: Via da sacaropina para degradação (B) de lisina em plantas e para síntese (A) em leveduras (BROCHETTO-BRAGA *et al.*, 1992; RAMOS *et al.*, 1988). GLU, glutamato; α-KG, α-oxoglutarato; LYS9, sacaropina desidrogenase; LYS1, sacaropina desidrogenase formadora de lisina; LOR, lisina oxoglutarato redutase; SDH, sacaropina desidrogenase.

Estudos bioquímicos e genéticos mostraram que em plantas, similarmente aos mamíferos, as enzimas LOR e SDH estão contidas em um único polipeptídeo bifuncional (GONÇALVES-BUTRUILLE *et al.*, 1996; EPELBAUM *et al.*, 1997; MIRON *et al.*, 1997; TANG *et al.*, 1997a). As evidências que suportam essa hipótese foram obtidas da co-purificação de ambas enzimas, à taxas constantes, em colunas cromatográficas e também pela detecção de suas atividades em géis de poliacrilamida.

KARCHI *et al.* (1994) expressaram as enzimas DHDPS e AK de bactéria, insensíveis à retroinibição por lisina em sementes em desenvolvimento de *N. tabacum*. O acúmulo de lisina solúvel foi duas vezes maior em plantas transgênicas quando comparado às plantas controle. Entretanto, sementes maduras das plantas transgênicas não apresentaram acúmulo de lisina. Este resultado foi explicado pelo aumento na atividade da LOR nas plantas transgênicas em relação aos controles, sugerindo que as enzimas envolvidas na síntese e catabolismo de lisina são coordenadamente reguladas durante o desenvolvimento da semente.

A análise de plantas transgênicas de soja e canola expressando os genes *dap*A e *lys*C de *Corynebacterium* codificando respectivamente, as enzimas AK e DHDPS em suas sementes, demonstraram um grande aumento no acúmulo de lisina livre nas sementes (FALCO *et al.*, 1995), diferentemente dos resultados obtidos em *N. tabacum* (KARCHI *et al.*, 1994). Contudo, embora as atividades de LOR ou SDH não tenham sido medidas, foram observados acúmulos dos intermediários do catabolismo de lisina, ácido α-amino adípico e sacaropina, em canola e soja, respectivamente (FALCO *et al.*, 1995).

GONÇALVES-BUTRUILLE *et al.* (1996), a partir da caracterização da SDH de sementes em desenvolvimento de milho, apresentaram evidências de que as atividades da LOR e SDH estão localizadas em dois domínios funcionalmente independentes e que existe na forma nativa como um dímero de 260 kDa. Em SDS-PAGE, as atividades de LOR e SDH co-migraram em uma banda única de 125 kDa.

Digestão com elastase separou o polipeptídeo bifuncional de 125 kDa em cinco bandas de 35 a 65 kDa, sendo que somente a banda de 65 e a de 57 kDa contém os domínios de LOR e SDH, respectivamente. Notavelmente, após proteólise limitada das enzimas LOR/SDH de milho, polipeptídeos derivados da SDH inibiram a atividade enzimática da LOR (KEMPER *et al.*, 1998).

O primeiro trabalho de clonagem do gene que codifica as enzimas LOR e SDH revelou que o gene ocupa 6,2 Kb do genoma de *A. thaliana,* possui uma ORF de 3,16 Kb e é interrompido por 24 íntrons pequenos entre 68 e 352 pb, possuindo um domínio amino correspondente à LOR e um domínio carboxi homólogo a SDH (EPELBAUM *et al.*, 1997). As análises mostraram que em *A. thaliana* não existe nenhum elemento regulatório *opaco 2,* o qual parece estar envolvido na regulação da LOR em endospermas de milho (BROCHETTO-BRAGA *et al.*, 1992). Em endospermas imaturos de milho, a análise de sequências do cDNA, designado *ZLKRSDH*, que codifica a enzima bifuncional LOR/SDH revelou uma proteína de 117 kDa e uma ORF de 1056 aminoácidos (KEMPER *et* al., 1999). A proteína apresenta 44, 46 e 72% de similaridade quando comparadas à proteína de rato, *C. elegans* e *A. thaliana,* respectivamente (CORD-NETO, 1998).

Em *Arabidopsis,* TANG *et al.* (1997) obtiveram evidências da presença de duas isoenzimas de SDH demonstradas através de dois picos de atividade, eluído de uma coluna de troca iônica, um contendo as atividades de LOR/SDH e o outro somente a atividade de SDH. Além disso, análises de hibridação de DNA genômico e mRNA sugeriram que as duas isoenzimas são traduzidas a partir de dois mRNAs distintos que são produzidos por um único gene em um mecanismo ainda desconhecido.

Entretanto, em milho, a transcrição do gene produz cinco espécies de transcritos, dos quais, um em maior abundância, codifica uma proteína de 117 kDa (CORD-NETO, 1998), homológa à proteína LOR/SDH de *A. thaliana* (TANG *et al.*, 1997). Os quatro transcritos alternativos possivelmente não são traduzidos (CORD-NETO, 1998), diferentemente de *A. thaliana* (TANG *et al.*, 1997a).

- **Regulação das enzimas LOR e SDH de plantas**

A regulação do catabolismo da lisina ainda não está claramente entendida. Em sementes pode ser regulado não somente por suas propriedades bioquímicas, mas possivelmente por complexos controles transcricionais, traducionais e pós-traducionais dos genes que codificam as enzimas LOR e SDH (TANG *et al.*, 1997a). Parece que LOR e SDH controlam processos bastante complexos e seus aspectos estruturais e funções celulares diferem entre várias espécies eucarióticas.

Os resultados apresentados indicam que um aumento de lisina solúvel nas sementes de *N. tabacum* pode estimular a atividade da enzima LOR envolvida na via de degradação de lisina (KARCHI *et al.*, 1994). Por outro lado, a mutação opaco-2 está relacionada com a diminuição na atividade da LOR e com o aumento dos níveis de lisina encontrados em sementes de milho em desenvolvimento (BROCHETTO-BRAGA *et al.*, 1992; CORD-NETO, 1998; GAZIOLA *et al.*, 1999). Essa atividade reduzida da LOR/SDH pode em parte, ser responsável pelo alto conteúdo de lisina nos endospermas de milho opaco-2.

Outro mecanismo de regulação da atividade da LOR dependente de lisina parece estar relacionado com uma cascata intracelular de sinais mediada por Ca^{2+} e fosforilação de proteínas (MIRON *et al.*, 1997; KARCHI *et al.*, 1995), além de altas concentrações de sais (KEMPER *et al.*, 1998). Trabalhando com sementes de tabaco, Karchi *et al.* (1995), observaram que a atividade de LOR pode ser estimulada pela adição de lisina exógena e que tais efeitos foram reduzidos significativamente quando tratados com EGTA e que tal efeito inibitório pode ser revertido pela adição de Ca^{2+}. Em milho, a atividade de LOR, mas não de SDH, parece ser modulada por Ca^{2+}, força iônica e calmodulina (KEMPER *et al.*, 1998).

Além disso, tratamentos *in vitro* com fosfatase alcalina levando à defosforilação da LOR/SDH de sementes em desenvolvimento de soja causou uma inibição na atividade da LOR de uma maneira dependente de lisina (a ligação da lisina à LOR é essencial para sua desfosforilação com fosfatase alcalina). Nenhum efeito foi observado sobre a atividade de SDH (MIRON *et al.*, 1997). Esses resultados sugerem que em sementes, a LOR ativa é uma fosfoproteína e que é modulada pela ação alternada de proteínas quinases e fosfatases.

A expressão do gene que codifica as enzimas LOR e SDH em sementes é altamente regulada, provavelmente, aos níveis transcricional e pós-transcricional em resposta aos teores de lisina solúvel. Em geral, a superprodução de lisina pode ser obtida pela alteração da sensibilidade à inibição por lisina, mas o acúmulo desse aminoácido em sementes de cereais requer, possivelmente a manipulação das enzimas LOR e/ou SDH. Estudos detalhados dessa regulação e a identificação de mecanismos de transdução de sinais que estimulam a atividade da LOR representam um grande desafio.

APRESENTAÇÃO DOS TRABALHOS E OBJETIVOS

Esta tese é constituída de um trabalho preliminar, um trabalho central de caracterização, ambos publicados, e um outro, submetido à publicação. O objetivo geral foi purificar parcialmente, caracterizar a nível bioquímico e estudar os mecanismos de regulação bioquímica das enzimas lisina oxoglutarato redutase (LOR) e sacaropina desidrogenase (SDH) em arroz de sequeiro. O trabalho central trata da purificação e caracterização parcial das enzimas, enquanto o segundo está relacionado ao estudo dos mecanismos de regulação das enzimas LOR e SDH de arroz.

Trabalho 1: **Enzyme isolation and regulation with lysine biosynthesis and degradation in developing seeds of rice.**

Salete A Gaziola, Cristiana M. G. Teixeira, Akihiko Ando, Ladaslav Sodek e Ricardo A Azevedo. Int. Rice Research Notes, IRRN 21: 27-28, 1996.

Objetivos:

As enzimas LOR e SDH, envolvidas na degradação de lisina, não tinham sido até então estudadas em arroz. Considerando que o arroz é de grande importância na alimentação humana e que os cereais, de uma maneira geral, apresentam baixo conteúdo de lisina, tomamos como objetivo purificar e caracterizar parcialmente essas enzimas, buscando informações que pudessem contribuir para o entendimento dessa via em plantas. Para tanto, as seguintes etapas foram realizadas:

- Determinar o tecido de maior atividade das enzimas LOR e SDH.
- Determinar o estágio de desenvolvimento das sementes de maior atividade das enzimas LOR e SDH.

Trabalho 2: **The enzymology of lysine catabolism in rice seeds. Isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate reductase/saccharopine dehydrogenase bifunctional polypeptide.**

Salete A Gaziola, Cristiana M. G. Teixeira, Juverlandi Lugli, Ladaslav Sodek e Ricardo A. Azevedo. Eur. J. Biochem. *247*, 364-371, 1997.

Objetivos:

- Purificação das enzimas LOR e SDH de sementes em desenvolvimento de arroz, ou seja, do tecido de maior atividade enzimática.
- Determinação da massa molecular.
- Estudos cinéticos.
- Identificação das atividades enzimáticas em gel não desnaturante.

Trabalho 3: **Degradation of lysine in rice seeds: Effect of calcium, ionic strength, *S*-adenosylmethionine and S-(2-aminoethyl)-L-cisteine on lysine 2-oxoglutarato reductase-saccharopine dehydrogenase bifunctional enzyme.**

S. A. Gaziola, L. Sodek, P. Arruda, P. J. Lea e R. A. Azevedo. Submetido (1999).

Objetivos:

Além de isolar e caracterizar as enzimas de arroz, procedemos aos estudos de alguns mecanismos envolvidos na regulação das enzimas. Os objetivos deste trabalho foram:

- Determinação de pH ótimo para a LOR e SDH.
- Estudar os efeitos de cálcio, EGTA; força iônica (Tris e KCl); e S-adenosilmetionina (SAM) na regulação das enzimas LOR e SDH de arroz.
- Estudar S-2-aminoetilcisteína (AEC), análogo de lisina, como substrato e inibidor da atividade de LOR.

Enzyme isolation and regulation with lysine biosynthesis and degradation in developing seeds of rice

S. A. Gaziola and C. M. G. Teixeira, Genetics Department, State University of Campinas, Brazil; A. Ando, Genetics Department, University of Sao Paulo, Brazil; L. Sodek, Plant Physiology Department, State University of Campinas, Brazil; and R. A. Azevedo, Genetics Department, University of Sao Paulo

The seeds of cereals are nutritionally deficient in certain essential amino acids. Aspartic acid serves as a common precursor of several amino acids, including lysine (Lys), threonine (Thr), methionine (Met), and isoleucine (Ile). As yet, the aspartic acid pathway has not been studied in rice.

We are currently investigating the following enzymes in rice seeds: aspartate kinase (AK), the first enzyme of the pathway, and homoserine dehydrogenase (HSDH), which plays a major role in the biosynthesis of threonine, together with lysine ketoglutarate (LKR) and saccharopine dehydrogenase (SDH), both of which are involved in the catabolism of lysine.

All of the enzymes were extracted from the developing seeds, roots, and leaves of rice cultivar IAC165, and LKR and SDH only from the cotyledons and developing seeds of soybean in Tris and phosphate buffers containing DTT, EDTA, PMSF, PVP, KCl, and glycerol. The enzymes were partially purified with an ammonium sulfate (0-60% saturation) precipitation step and desalted on Sephadex G25 or G50 columns. Enzyme activities were measured using the methods of Azevedo et al (1992a, 1992b) and Brochetto-Braga et al (1992).

All three stages presented AK and HSDH activities, with stage 2 showing higher levels of activity. Lys (5 mM) was the major inhibitor of AK activity, with inhibition varying from 30 to 90% among the stages tested (Table 1). Thr (5 mM) only slightly (4-12%) inhibited AK activity, and Lys or Thr did not inhibit HSDH (Table 1). Lys predominantly inhibited AK in rice as has been observed in all of the plant species

Table 1. *Aspartate kinase* (AK) (nkat mg prot^{-1}) and Homoserine dehydrogenase (HSDH) (nmol min^{-1} mg prot^{-1}) activities extracted from developing seeds of rice.

Enzyme/treatment	Developing seeds		
	Stage 1	Stage 2	Stage 3
AK			
Control	0.046	0.070	0.055
+Thr	0.044	0.061	0.052
+Lys	0.004	0.036	0.042
+Lys + Thr	0.003	0.025	0.040
HSDH			
Control	1.389	0.704	0
+Thr	0.849	0.610	0

Table 2. Lysine ketoglutarate (LKR) and saccharopine dehydrogenase (SDH) activities (nmol min^{-1} mg prot^{-1}) extracted from rice and soybean tissues.

Plant tissue	LKR activity	SDH activity
Rice		
Developing seeds (stage 1)	4.6	5.0
Developing seeds (stage 2)	9.1	4.2
Developing seeds (stage 3)	0	2.0
Root	0	0
Leaf	0	0
Soybean		
Cotyledons	0.24	1.06
Developing seeds	5.10	3.16

tudied so far (Lea et al 1992), with the xception of *Coix lacryma-jobi*.

It is currently difficult to assure that an soenzyme sensitive to Thr is present in rice eds. For HSDH, we did not observe a hr-sensitive form of the enzyme, as olated in other plant species studied. hese results suggest that a bifunctional zyme containing Thr-sensitive AK-SDH activities, such as the enzyme olated from some higher plants (Azevedo al 1992b), is not present in rice.

LKR and SDH were extracted from rice d soybean tissues using phosphate and is buffers. The inclusion of PVP is sential to maintain both activities ependent of the buffer system used. The esence of KCl at 50 mM also helped to aintain the activity. LKR and SDH tivities were observed in developing eds at three different stages. However, KR and SDH activities were not present in ots and leaves (Table 2). In soybean, both KR and SDH activities were observed in veloping seeds and cotyledons (Table 2).

These preliminary tests showed that both enzymes are very sensitive to proteases and phenolic compounds during extraction. High concentration of salts in the buffer also appears to increase enzyme activity. The evidence suggests that both enzymes are seed-specific in rice but not in soybean.

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The enzymology of lysine catabolism in rice seeds

Isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate reductase/saccharopine dehydrogenase bifunctional polypeptide

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In plant, the catabolism of lysine has only been studied in some detail in maize. The enzymes lysine 2-oxoglutarate reductase (also known as lysine α-ketoglutarate reductase; LOR) and saccharopine dehydrogenase (SDH), which convert lysine into saccharopine, and saccharopine into glutamic acid and 2-aminoadipate 6-semialdehyde, respectively, were isolated from immature rice seeds and partially purified through a three-step purification procedure involving ammonium sulphate precipitation, and anion-exchange and gel-filtration chromatographies, leading to a final yield of 30% for LOR and 24% for SDH. The molecular masses estimated by gel-filtration chromatography on a Sephacryl S200 column and by native non-denaturing PAGE using Ferguson plots were 203 kDa for both enzymes by gel-filtration and 202 kDa for both enzymes by native non-denaturing PAGE. A second band of LOR and SDH activities on native gels was observed for both enzymes with an estimated molecular mass of 396 kDa, which indicated a multimeric structure. Kinetic studies were consistent with an ordered sequence mechanism for LOR, where 2-oxoglutarate is the first substrate and saccharopine is the last product.

The results observed for the LOR/SDH activity ratios during purification, the copurification in all three steps, the molecular masses, the relative mobilities on native non-denaturing gels and the p*I* estimated for LOR and SDH suggest the existence of a bifunctional polypeptide containing LOR and SDH activities.

Keywords: lysine 2-oxoglutarate reductase; saccharopine dehydrogenase; lysine catabolism; rice; *Oriza sativa* L.

Lysine is one of the essential amino acids synthesized by the aspartic acid metabolic pathway in higher plants (Azevedo et al., 1997; Lea et al., 1992). This pathway has been well characterized in some plant species (Azevedo et al., 1990; Azevedo et al., 1992a–c; Brennecke et al., 1996; Bright et al., 1982; Bryan, 1990a; Dotson et al., 1989; Galili, 1995; Heremans and Jacobs, 1995; Muehlbauer et al., 1995; Wilson et al., 1991), especially with regard to the regulation of lysine biosynthesis (Azevedo et al., 1997). A better understanding of this process is important for future genetic-manipulation studies that lead to an overproduction and accumulation of lysine in plant seeds.

Previous studies have provided evidence for three important enzymatic steps for the control of lysine biosynthesis. The first step of the pathway is catalysed by the enzyme aspartate kinase, involving the conversion of aspartic acid to β-aspartyl phosphate. Aspartate kinase has been well characterized in several plant species and shown to exist in up to three isomeric forms. Two of the isoforms may be inhibited by lysine alone or synergistically with *S*-adenosylmethionine, and the third may be inhibited by threonine (Azevedo et al., 1992a; Bright et al., 1982; Muehlbauer et al., 1995; Rognes et al., 1980). The second potential point of control is the step catalysed by the enzyme homoserine dehydrogenase, which is responsible for the conversion of β-aspartyl semialdehyde to homoserine in the presence of the coenzymes NADH or NADPH (Bryan, 1990a). Two homoserine dehydrogenase isoenzymes, one threonine resistant and the other threonine sensitive, have been characterized in plants (Azevedo et al., 1992b; Bryan, 1990a,b). Data indicate that the threonine-sensitive aspartate kinase and homoserine dehydrogenase are part of a single bifunctional polypeptide (Azevedo et al., 1992b; Muehlbauer et al., 1995; Wilson et al., 1991). The third point of control involves dihydrodipicolinate synthase, an enzyme that catalyses the conversion of β-aspartyl phosphate to 2,3-dihydrodipicolinate, the first step unique to the branch of lysine biosynthesis (Wallsgrove and Mazelis, 1981). Dihydrodipicolinate synthase has been characterized in some plant species and shown to be inhibited by low concentrations of lysine (Dereppe et al., 1992; Frisch et al., 1991; Ghislain et al., 1990; Kumpaisal et al., 1987; Matthews and Widholm, 1978; Mazelis et al., 1977; Wallsgrove and Mazelis, 1981). The enzyme is thought to be the major control point in the synthesis of lysine (Azevedo et al., 1997).

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Abbreviations. LOR, lysine 2-oxoglutarate reductase; SDH, saccharophine dehydrogenase.

Enzymes. Saccharopine dehydrogenase (NAD$^+$, L-lysine-forming) (EC 1.5.1.8); saccharopine dehydrogenase (NADP$^+$, L-glutamate-forming) (EC 1.5.1.9); aspartate kinase (EC 2.7.2.4); homoserine dehydrogenase (EC 1.1.1.3); dihydrodipicolinate synthase (EC 4.2.1.52).

Biochemical mutants containing enzymes altered in their inhibition by lysine, threonine and *S*-2-aminoethyl-L-cysteine (a lysine analogue) have been obtained for several plant species (Azevedo et al., 1997; Galili, 1995; Lea et al., 1992). Although increased concentrations of threonine and lysine have been encountered in some of these mutants (Hibberd and Green, 1982; Negrutiu et al., 1984), the grain concentration of lysine remained unaltered (Azevedo and Arruda, 1995; Azevedo et al., 1990; Hibberd and Green, 1982). When the *Escherichia coli lysC* gene was fused to a chloroplast transit peptide and the seed-specific promoter of the bean phaseolin gene, and transformed into tobacco plants, the plants exhibited a 14–17-fold increase in the soluble threonine concentration of the seed. However when the *E. coli dapA* gene was expressed in tobacco seeds, there was no evidence of increased soluble lysine in the mature seed (Azevedo et al., 1997; Karchi et al., 1994). These results suggest that for lysine accumulation to occur in seeds, the manipulation of catabolism could be important, as well as biosynthesis.

However, very little is known about lysine catabolism in plants. Most of the information present in the literature have been obtained from experiments with mammals (Fellows, 1973; Fellows and Lewis, 1973; Fjellstedt and Robinson, 1975a,b; Hutzler and Dancis, 1968). In mammalian tissues, lysine is condensed with 2-oxoglutarate into saccharopine in a reaction catalysed by the enzyme lysine 2-oxoglutarate reductase (LOR) with NADPH as a cofactor. In a second step, saccharopine is converted to 2-aminoadipate 6-semialdehyde and glutamic acid in a reaction catalysed by the enzyme saccharopine dehydrogenase (SDH), which can use NAD^+ or $NADP^+$ as a cofactor (Hutzler and Dancis, 1970; Fjellstedt and Robinson, 1975b). Both enzymes from mammalian tissues have been well characterized. Evidence that LOR and SDH activities are part of a single polypeptide has been provided by studies with human placenta (Fjellstedt and Robinson, 1975b), and baboon and cattle livers (Markovitz et al., 1984). On the other hand, Noda and Ichihara (1978) working with rat liver, demonstrated that LOR and SDH are two separate enzymes.

The presence of LOR and SDH activities in plant tissues suggests that the catabolic pathway may be similar to that in animals. LOR and SDH have been partially purified and characterized from maize endosperm (Brochetto-Braga et al., 1992; Gonçalves-Butruille et al., 1996). Both enzymes carried out similar reactions and exhibited similar properties to the mammalian enzymes. Gonçalves-Butruille et al. (1996) presented evidence suggesting that in maize LOR and SDH activities are specific to endosperm tissue and are located in two functionally independent domains, which exists in the native form as a dimer of 260 kDa. Apart from the studies with maize, LOR activity has been determined in developing tobacco seeds and shown to be induced by lysine (Karchi et al., 1994).

The metabolic pathways for the biosynthesis and degradation of lysine have not been characterized for rice. In this study, we present data on the partial purification and characterization of LOR and SDH from immature rice seeds. Evidence is presented for the possible existence of a bifunctional protein in the developing rice grain containing both activities, as reported previously for maize.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma, except the Bio-Rad protein reagent, which was supplied by Bio-Rad Laboratories. The Sephacryl S-200 gel-filtration resin was purchased from Pharmacia.

Plant material. The IAC-165 upland rice variety, kindly donated by Dr Akihiko Ando (Departamento de Genética, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo), was used for the extraction, isolation and purification of LOR and SDH enzymes.

Plants were grown in the field and immature rice seeds (milky stage) were harvested directly into liquid nitrogen and stored at −70 °C for further analysis.

Enzyme assays. LOR activity was assayed routinely spectrophotometrically in a 1.0-ml cuvette at 30 °C by following the oxidation of NADPH by the change in absorbance at 340 nm over 15 min, with appropriate adjustments for a lysine-free control. The assay mixture contained 100 mM potassium phosphate, pH 7.0, 20 mM L-lysine, 10 mM 2-oxoglutarate neutralized with KOH, 0.14 mM NADPH, and enzyme sample in a 0.9-ml assay. SDH activity was measured routinely spectrophometrically in a 1.0-ml cuvette by following the rate of substrate-dependent reduction of NAD^+ to NADH monitored at 340 nm at 30 °C over 15 min, with appropriate adjustments for a saccharopine-free control. The reaction mixture contained 100 mM Tris/HCl, pH 8.4, 2 mM saccharopine, 2 mM NAD^+, and enzyme in a 0.9-ml assay. Activities were expressed as nmol NADPH/NAD^+ oxidized/reduced · min^{-1} · ml^{-1}.

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

Enzyme purification. Unless stated otherwise, all procedures were carried out at 4 °C. All buffers were prepared in Milli-Q water (Millipore).

Immature rice seeds (250 g) were ground with a pestle and mortar in liquid nitrogen. The powder was homogenized in 500 ml 100 mM potassium phosphate, pH 7.0, 50 mM KCl, 1 mM EDTA, 1 mM DL-dithiothreitol, 10% (by vol.) glycerol (buffer A) containing 0.1 mM phenylmethylsulfonyl fluoride, and 5% (mass/vol.) insoluble polyvinylpyrrolidone. The homogenate was filtered through six layers of gauze, and centrifuged at 15000 *g* for 30 min to remove cell debris. The supernatant was adjusted to 20% ammonium suphate saturation by gently stirring for at least 1 h. The suspension was centrifuged at 15000 *g* for 30 min and the supernatant subjected to a second ammonium sulphate precipitation at 45% saturation for 1 h with continuous stirring. After centrifugation at 15000 *g* for 30 min, the sedimented proteins were dissolved in 10 ml buffer A. The sample was loaded onto a Sephadex G-25 column (2.6 cm × 20 cm) equilibrated with 100 mM Tris/HCl, pH 7.4, 1 mM DL-dithiothreitol, 1 mM EDTA and 10% (by vol.) glycerol (buffer B) and run under gravity. The desalted sample was loaded onto a DEAE-Sephacel column (2.6 cm × 12 cm; flow rate 0.5 ml/min) equilibrated in buffer B and connected to a chromatography Bio-Rad Econo System. The column was developed by washing out the unbound fraction with 100 ml buffer B followed by a linear gradient from 0 to 500 mM KCl (180 ml) at 1 ml/min. Fractions of 3 ml were collected and used for LOR and SDH activity determinations. Fractions containing both enzyme activities were pooled and concentrated with 70% saturated amonium sulphate. After stirring for 1 h, the sample was centrifuged at 15000 *g* for 30 min, and the precipitated proteins were dissolved in buffer B to 4 ml. 3.5 ml of the sample were applied to a Sephacryl S-200 gel-filtration column (2.6 cm × 86 cm) equilibrated in buffer B. The column was developed with buffer B at 0.35 ml/min, and fractions of 3.5 ml were collected and assayed for LOR and SDH activities. Fractions containing enzyme activity were combined and used for the kinetics studies.

PAGE. Electrophoresis was carried out under native non-denaturing condition in gels (Bio-Rad Mini Gel System) containing 7% polyacrylamide with a 4% stacking gel. A constant current of 10 mA/gel was applied for 90 min and the temper-

Table 1. Protocol for purification of LOR and SDH from 250 g frozen rice immature seeds. Sequential steps of LOR and SDH purification from one of several preparations.

Purification step	Protein	LOR			SDH			Activity ratio (LOR/SDH)
		Total activity	Specific activity	Yield	Total activity	Specific activity	Yield	
	mg	nmol · min^{-1} · ml^{-1}	nmol · min^{-1} · ml^{-1} · mg protein^{-1}	%	nmol · min^{-1} · ml^{-1}	nmol · min^{-1} · ml^{-1} · mg protein^{-1}	%	
Crude extract	1028	1984	1.93	100	1748	1.70	100	1.13
20–45% $(NH_4)_2SO_4$	227.9	1764	7.74	89	1404	6.16	80	1.26
DEAE-Sephacel	27.1	319	11.77	16	263	9.70	15	1.21
Sephacryl S-200	8.2	593	72.32	30	425	51.83	24	1.40

ature set to 4 °C. Electrophoresis buffers and gels were prepared as described by Laemmli (1970) except that SDS was excluded.

LOR and SDH activity stain. LOR activity staining was performed by incubating a trimmed slice of a native PAGE gel in 100 mM Tris/HCl, pH 7.4, 20 mM L-lysine, 10 mM 2-oxoglutarate and 0.3 mM NADPH, at 30 °C for 30 min in the dark. Another slice was incubated in the same reaction mixture but without L-lysine. LOR activity was detected by inspection of the gel slices under ultraviolet light (260 nm).

SDH activity staining was performed by incubating gel slices in 100 mM Tris/HCl, pH 8.4, 4 mM saccharopine, 2 mM NAD^+, 0.05% (mass/vol.) NitroBlue Tetrazolium and 0.1 mM phenazine methosulphate at 30 °C for 30 min, in the dark. The control assay contained the reaction mixture without saccharopine. Reaction mixtures for both procedures were prepared immediately before use.

Protein staining. Gels were also stained for protein with silver nitrate as described by Oakley et al. (1980) or with Coomassie Blue R250.

LOR and SDH electroelution from native non-denaturing gels. Fractions containing LOR and SDH activities eluted from the gel-filtration chromatography column were combined and applied to a 7% polyacrylamide native non-denaturing gel. Part of the gel was stained for SDH activity and the corresponding position of the band stained for SDH activity was cut from the gel and the slice (1 mm) used for electroelution in a Bio-Rad Electro-Eluter. Electroelution was carried out at 400 V and 60 mA for 5 h at 4 °C. After the elution was completed, the eluted sample was collected in the membrane cap in 600 µl buffer B. Aliquots of the electroeluted sample were used for protein determination, and assays of LOR and SDH activities, and were applied to a 7% polyacrylamide native non-denaturing gel and stained with Coomassie Blue R250.

LOR and SDH molecular-mass determination. The molecular mass of LOR and SDH was determined by gel electrophoresis under native non-denaturing conditions and by gel-filtration on a Sephacryl S-200 column (2.6 cm×86 cm) as described above. The determination by electrophoresis was carried out as described in the Sigma bulletin MKR-137. An aliquot of the sample prepared for gel-filtration chromatography containing high LOR and SDH activities and non-denatured proteins (Sigma Kit MW-ND-500), was applied to a series of individual native gels with 5–10% polyacrylamide. The lanes containing LOR and SDH were trimmed and stained for enzyme activities and the relative mobilities for each concentration of polyacrylamide recorded. Molecular-mass markers were stained with Coomassie Blue R250 and their relative mobility values recorded.

For gel-filtration determinations, the following molecular-mass markers were used: blue dextran to determine the void volume of the column (2000 kDa), catalase (232 kDa); sweet potato β-amylase (200 kDa); yeast alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa); bovine erythrocyte carbonic anhydrase (29 kDa); and horse heart cytochrome *c* (12.4 kDa). The determinations were carried out simultaneously with the partial purification of LOR and SDH as described above.

Preparative IEF. An aliquot of an extract after desalting on a Sephadex G-25 column was diluted to a 5 mM salt with Milli-Q water and made to 10% (by vol.) glycerol. To this diluted sample, 1.25% (by vol.) Bio-Lyte ampholyte pH 3–10 was added and the final volume adjusted to 55 ml. Loading the sample into the Rotofor Cell (Bio-Rad Laboratories) was accomplished by injecting the sample into the focussing chamber prior to the run. The electrolyte for the anode was 100 mM H_3PO_4 and for the cathode was 100 mM NaOH. Focussing was carried out for 2 h at 2 °C at constant power (1500 V and 100 mA). Focussed proteins were collected in 20 fractions simultaneously by means of a vacuum pump, and the fractions were analysed for LOR and SDH activities.

Kinetic studies. The partially purified LOR and SDH enzymes were used for kinetic studies. Activities were monitored continuously to obtain initial-velocity data. The K_m values were determined graphically from the double-reciprocal plots of activity against the variable substrate concentration, maintaining the remaining substrates at the routine assay concentrations (close to saturation). The K_m determined here is a close approximation to that defined by Dalziel (1969), where K_m is the concentration that gives half the true maximum rate in the presence of infinitely large concentrations of the other substrates. Inhibitor studies were performed as for K_m, except for the inclusion of two fixed inhibitor concentrations in the assay.

RESULTS

Purification of LOR and SDH from immature rice seeds. LOR and SDH were partially purified from immature rice seeds. The three-step purification procedure is outlined in Table 1. Very high losses of LOR and SDH activites were observed during extraction. The presence of 5% (mass/vol.) insoluble polyvinylpyrrolidone and 10% (by vol.) glycerol in the extraction buffer, and glycerol in the running buffers was crucial to maintain LOR and SDH activities.

LOR was purified approximately 38-fold with a final yield of 30%, while SDH was purified approximately 31-fold with a final yield of 24%. An increased yield was obtained after the

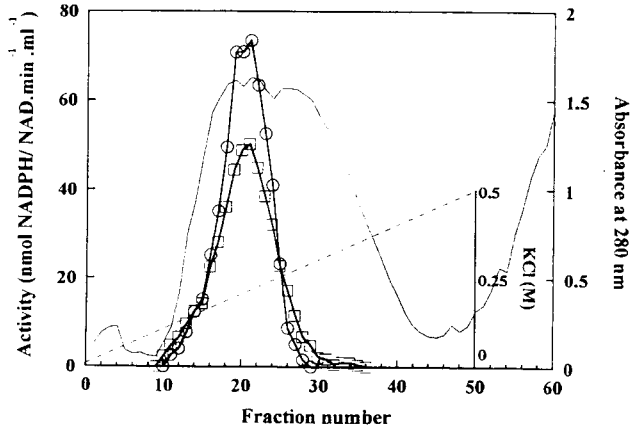

Fig. 1. Purification of LOR and SDH by anion-exchange chromatography on a DEAE-Sephacel column. Elution profile of LOR (○) and SDH (□) activities. The protein elution profile as measured at 280 nm (——) is shown. A desalted sample from the ammonium-sulphate-precipitation step was applied to the column (2.6 cm×12 cm), equilibrated with buffer B. Elution was performed in the same buffer with a linear KCl gradient (– – –) as indicated. The flow rate was 1 ml/min. Fractions of 3 ml were collected and assayed for LOR and SDH activities.

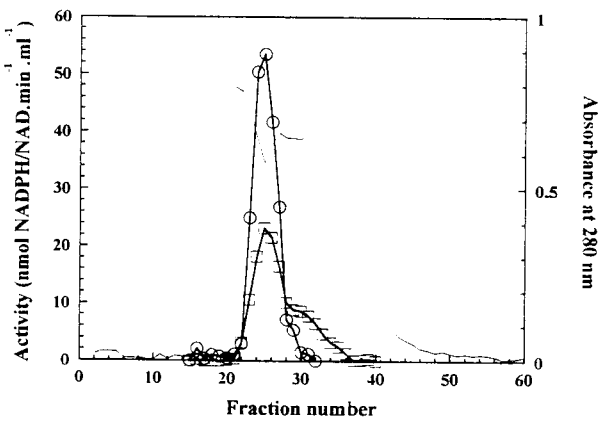

Fig. 2. Purification of LOR and SDH by gel-filtration chromatography on a Sephacryl S-200 column. Elution profile of LOR (○) and SDH (□) activities. The protein elution profile as measured at 280 nm (——) is shown. Partially purified LOR and SDH from the anion-exchange-chromatography step was concentrated by ammonium sulphate precipitation (70% saturation). The sedimented proteins were dissolved in buffer B (4 ml) and 3.5 ml were loaded onto the Sephacryl S-200 column (2.6 cm×86 cm) equilibrated in buffer B. Elution was performed in the same buffer at a flow rate of 0.35 ml/min. Fractions of 3.5 ml were collected and assayed for LOR and SDH activities.

ammonium sulphate step after the crude extract had been desalted and assayed. The crude extract was characterized by a high rate of non-specific oxidation of NADPH during the LOR assay, which was reduced sharply after the ammonium-sulphate-purification step. Both enzyme activities were recovered during the ammonium-sulphate-precipitation step in the 20–45% fraction. After desalting the suspended pellet on a Sephadex G-25 column, the sample was loaded onto a DEAE-Sephacel column and eluted with a linear gradient from 0 to 500 mM KCl. The LOR and SDH elution profiles from the anion-exchange-chromatography step are shown in Fig. 1. The peaks of LOR and SDH activities were eluted by the same concentration of KCl (160 mM). Rechromatography of the enzymes on DEAE-Sephacel with a linear gradient from 100 mM to 350 mM KCl, did not improve the purification and resulted in losses of both enzyme activities. LOR and SDH activities eluted in the same fractions from the gel-filtration column (Fig. 2). One peak of LOR activity and one peak of SDH activity were observed.

LOR and SDH activity stain. One band was observed in native gels stained for LOR activity (Fig. 3). A slower-migrating band of LOR activity was also observed; however, it only appeared in some experiments. Gels incubated with the control assay mixture did not indicate the presence of any non-specific bands (Fig. 3).

Other lanes of the same gel when stained for SDH activity also produced one band that exhibited identical mobility and level of activity as the band stained for LOR activity (Fig. 3). As seen in the gels stained for LOR activity, a slower-migrating band of SDH with much lower SDH activity appeared in some experiments. Both bands were saccharopine dependent, since the control assay mixture did not stain for activity (Fig. 3).

Molecular-mass determination. The molecular masses of LOR and SDH were determined by gel-filtration chromatography (Sephacryl S-200) and by native non-denaturing PAGE. For gel-filtration chromatography, one peak of LOR activity was observed. The molecular mass was estimated to be 203 kDa (Figs 2 and 4). For SDH, one peak of activity, although broader than the LOR peak, was observed, which eluted with the 203-kDa LOR peak. This result was consistent for ten experiments of gel-filtration chromatography.

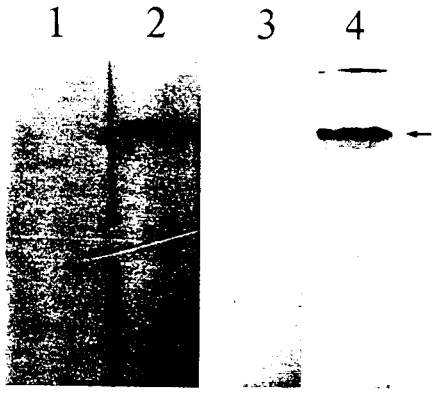

Fig. 3. Activity staining of LOR and SDH. Partially purified LOR and SDH from the anion-exchange chromatography step was concentrated by ammonium sulphate precipitation (70% saturation). 1 ml of the sedimented proteins dissolved in buffer B (4 ml) was desalted on a Sephadex G-25 column (1.8 cm×10 cm) equilibrated in the same buffer. Aliquots of 40 µl were used for LOR and SDH activity staining. Lane 1, specific activity staining for LOR activity, but the substrate, lysine, was excluded from the reaction mixture; lane 2, stained for LOR activity; lane 3, specific activity staining for SDH, but the substrate, saccharopine, was excluded from the reaction mixture; lane 4, stained for SDH activity. The arrow indicates the LOR/SDH activity band.

The molecular mass in native non-denaturing gels was determined by the use of Ferguson plots (Ferguson, 1964). Two bands of LOR and SDH stained for activity in all gel concentrations with the molecular mass markers, which were stained with Coomassie blue R250. In all native non-denaturing gels, the two bands of LOR exhibited identical mobilities to the two bands staining for SDH activity. The band showing higher LOR and SDH activities had an estimated molecular mass of 202 kDa, whereas the other band, presenting much lower LOR and SDH activities, corresponded to a molecular mass of 396 kDa (Fig. 5).

LOR and SDH electroelution. The electroelution of the SDH activity band from the gels was very efficient, and LOR and SDH activities were recovered in the electroeluted sample. Re-

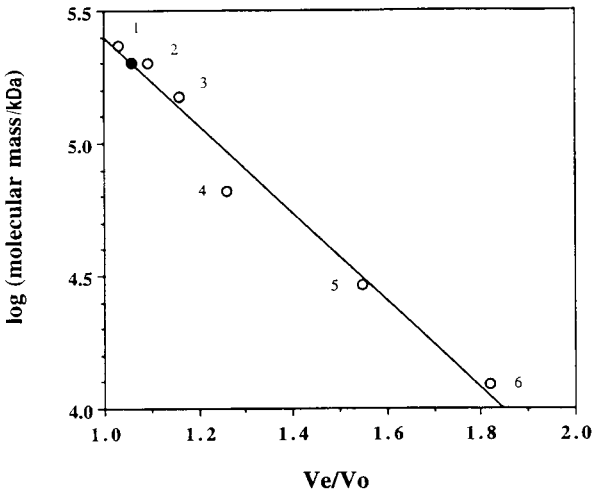

Fig. 4. LOR and SDH molecular-mass determination by gel-filtration chromatography on a Sephacryl S-200 column. Molecular-mass markers (○): 1, catalase (232 kDa); 2, sweet potato β-amylase (200 kDa); 3, yeast alcohol dehydrogenase (150 kDa); 4, bovine serum albumin (66 kDa); 5, bovine erythrocyte carbonic anhydrase (29 kDa); 6, horse heart cytochrome *c* (12.4 kDa). 203 kDa LOR/SDH (●).

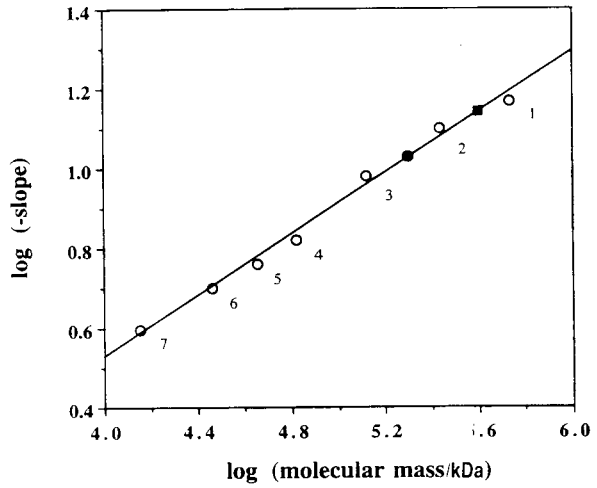

Fig. 5. Ferguson plots for LOR and SDH molecular-mass determination by native non-denaturing PAGE. Molecular-mass markers (○) for native non-denaturing PAGE were applied individually to a series of native non-denaturing gels containing 5–10% polyacrylamide. For each gel concentration, a sample containing LOR and SDH activities was applied. Molecular-mass markers are indicated (○): 1, urease (545 kDa, hexamer); 2, urease (272 kDa, trimer); 3, bovine serum albumin (132 kDa, dimer); 4, bovine serum albumin (66 kDa, monomer); 5, chicken egg albumin (45 kDa); 6, carbonic anhydrase (29 kDa); 7, bovine milk α-lactalbumin (14.2 kDa). 202-kDa LOR/SDH band (●); 396-kDa LOR/SDH band (■).

application of this electroeluted sample to a native non-denaturing gel produced two bands: a major band corresponding to the 202-kDa LOR and SDH activity bands (the electroeluted band); and a minor band corresponding to the 396-kDa LOR and SDH activity bands observed in some experiments (Fig. 6).

p*I*. Protein samples containing LOR and SDH activities were submitted to IEF in a Rotofor Cell. LOR and SDH activities were recovered, after focussing in a pH 3–10 gradient, in the same fractions with pH 5.0–7.0. The p*I* was estimated to be 6.0 for both enzymes.

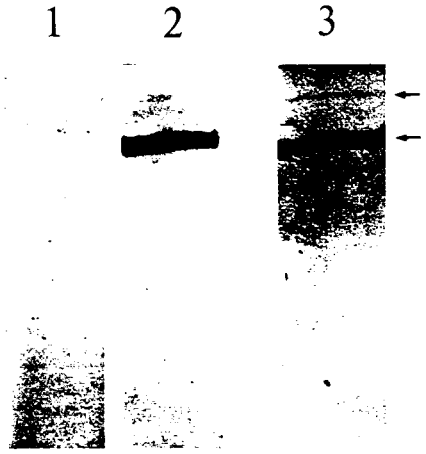

Fig. 6. SDH activity staining and electroelution from a native non-denaturing gel. An aliquot of a sample of combined fractions containing LOR and SDH activities from the gel-filtration step was applied to a 7% polyacrylamide native non-denaturing gel. A trimmed slice of the gel was stained for SDH activity, and the corresponding area of the SDH activity band was electroeluted. Lane 1, stained for SDH activity but the substrate, saccharopine, was excluded from the reaction mixture; lane 2, stained for SDH activity; lane 3, the two protein bands stained with Coomassie Blue R250 obtained by electroelution of the SDH band stained in lane 2. The arrows indicate the 202-kDa and 396-kDa LOR/SDH bands.

Table 2. Kinetic studies of rice LOR and SDH. The K_m values were determined graphically from the double-reciprocal plots of activity against the variable substrate concentration, maintaining the remaining substrates fixed at the routine assay concentrations (close to saturation). The K_m value determined here is therefore a close approximation of that defined by Dalziel (1969), where K_m is the concentration that gives half the true maximum rate in the presence of infinitely large concentrations of the other substrates. Inhibitor studies were performed in the same way, except for the inclusion of two different but fixed inhibitor concentrations in the assay. Values given are means ± SE (n = 3).

Parameter	Claculated value
	mM
K_m (lysine)	4.480 ± 1.140
K_m (2-oxoglutarate)	0.790 ± 0.160
K_m (NADPH)	0.032 ± 0.011
K_m (saccharopine)	0.130 ± 0.023
K_m (NAD)	0.490 ± 0.078
K_i of saccharophine (lysine)	2.000 ± 0.230
K_m of saccharophine (lysine)	0.250 ± 0.060

Kinetic studies. Data for the K_m values of the substrates lysine, 2-oxoglutarate and NADPH (LOR) and saccharopine and NAD (SDH) are shown in Table 2. Inhibitor constants for some of the reaction products are also shown. In the LOR reaction, saccharopine was a competitive inhibitor towards 2-oxoglutarate and non-competitive with regards to lysine. SDH was strongly inhibited by 2-aminoadipate, but much less so by the other product, glutamic acid, when both were present at 5 mM. Neither of the products of the SDH reaction produced clear inhibitory mechanisms. 2-oxoglutarate, although not a product, was found to inhibit the SDH reaction.

DISCUSSION

In maize endosperm, LOR activity appears to be related to the rate of lysine catabolism, in that the high-lysine mutant

opaque-2 exhibited a reduced level of lysine catabolism (Sodek and Wilson, 1971) and a lower activity of LOR (Brochetto-Braga et al., 1992). The lower lysine requirement for the synthesis of reserve protein in normal maize endosperm may underlie the higher rate of lysine breakdown. Lysine has been shown to induce the activity of LOR in tobacco (Karchi et al., 1994). In rice, LOR and SDH activities were lower, on a fresh-mass basis, than those obtained for maize. This difference might be related to a relatively lower rate of lysine degradation in rice seeds, considering the higher overall lysine content of rice grain compared with that of maize. This suggestion is strongly supported by the findings reported by Falco et al. (1995), who constructed transgenic canola and soybean plants with increased activities of bacterial aspartate kinase and dihydrodipicolinate synthase, leading to the overproduction and accumulation of soluble lysine in the seeds. Falco et al. (1995) suggested that the accumulation of lysine in canola and soybean seeds is controlled not only by the rate of synthesis, but also by the rate of breakdown. Lysine-overproducing lines of canola were shown to accumulate 2-aminoadipic acid in the seeds, whilst the lysine-overproducing lines of soybean accumulated saccharopine. The high concentrations of soluble lysine and the accumulation of saccharopine and 2-aminoadipic acid would suggest that in the seeds of soybean and canola (as opposed to tobacco) the enzymes of lysine catabolism are present in low abundance.

Insoluble polyvinylpyrrolidone and glycerol were shown to be essential in the extraction buffer for the maintenance of LOR and SDH activities. The purification procedure presented in this paper resulted in a lower degree of purification compared with the those of enzymes purified from maize endosperms. However, the recovery was considerably higher for both enzymes. For rice LOR, we obtained a 30% yield compared with 0.6% for maize endosperm (Gonçalves-Butruille et al., 1996), whereas for rice SDH we obtained 24% yield compared with 2% for maize endosperm (Gonçalves-Butruille et al., 1996).

In maize (Gonçalves-Butruille et al., 1996), as shown previously in mammals (Markovitz et al., 1984; Markovitz and Chuang, 1987), LOR and SDH activities appear to be part of a single bifunctional polypeptide. Our data are consistent with this characteristic. A single peak of LOR activity and a single coincident peak of SDH activity were eluted in the same fractions at 160 mM KCl (Fig. 1). Copurification of LOR and SDH was also observed during gel-filtration chromatography on Sephacryl S-200 (Fig. 2).

Identical relative mobility values were obtained after native non-denaturing PAGE for the two bands staining for LOR activity with those staining for SDH activity. Further evidence for an LOR/SDH bifunctional protein in rice was obtained by IEF studies, where the two enzyme activities were recovered in the same fractions, corresponding to a p*I* of 6.0. Furthermore, the LOR/SDH activity ratio of approximately 1.2 obtained during the purification of the enzymes was very similar in all steps and was constant in all experiments that have been carried out with this purification procedure (Table 1). Such a constant ratio would be expected for a bifunctional enzyme.

Although the existence of an LOR/SDH bifunctional protein, such as in maize, seems probable in rice, based on the results presented, some differences were observed for the enzymes from maize and rice. In rice, although LOR and SDH activities were recovered in the same range of ammonium sulphate saturation (20–45%), this range was different from that determined for the maize enzyme (35–60% saturation; Brochetto-Braga et al., 1992), but very similar to the solubility properties of human LOR, which is recovered between 26.5% and 35% ammonium sulphate. The LOR/SDH activity ratio varied from 1.0 to 3.6 during the purification of the enzyme from maize (Gonçalves-Butruille et al., 1996) compared with 1.2–1.4 observed for rice. In maize, one peak of LOR/SDH activity was observed during the gel-filtration step on Superdex 200 HR with an estimated molecular mass of 260 kDa (Gonçalves-Butruille et al., 1996), suggesting that the enzyme exists as a dimer, since only one major band with an estimated molecular mass of 125 kDa was detected on SDS/PAGE (Gonçalves-Butruille et al., 1996). In rice, on a Sephacryl S-200 gel-filtration column, LOR activity eluted with SDH activity (Fig. 2). The molecular mass of the rice enzyme was estimated to be 203 kDa, which is different from the values of 140 kDa (Brochetto-Braga et al., 1992) and 125 kDa (Gonçalves-Butruille et al., 1996) estimated for maize LOR by native non-denaturing PAGE and SDS/PAGE, respectively, of 260 kDa estimated for maize LOR/SDH by gel-filtration on a Superdex 200 HR column (Gonçalves-Butruille et al., 1996) and of 480 kDa for the mammalian enzyme (Fjellstedt and Robinson, 1975a).

Two bands for LOR and SDH were observed in some preparations when the samples were applied to native non-denaturing gels. The molecular masses determined by Ferguson plots as 202 kDa and 396 kDa for the LOR/SDH bands indicate that the 202-kDa band may represent the holoenzyme and that the 396-kDa band may represent a dimeric or tetrameric molecule, since the subunit composition of the rice bifunctional polipeptide has not been determined. In maize, the gel-filtration step and the native gel stained for LOR or SDH showed only one peak or band with molecular masses of 260 kDa (Gonçalves-Butruille et al., 1996) and 140 kDa (Brochetto-Braga et al., 1992), respectively.

The electroelution of the band that stained for enzyme activity may be useful as a final purification step. Analysis of the electroeluted sample by PAGE produced two bands, one corresponding to the same 202-kDa LOR/SDH electroeluted band as expected, and the other corresponding to the 396-kDa LOR/SDH. The latter may, therefore, be a multimeric form resulting from the association of the 202-kDa LOR/SDH band.

Although product-inhibition studies did not produce clear inhibition mechanisms for SDH, studies with LOR did. That the product saccharopine was competitive with respect to the substrate 2-oxoglutarate and non-competitive with respect to lysine is consistent with an ordered sequence mechanism where 2-oxoglutarate is the first substrate to join and saccharopine is the last product to leave the enzyme · substrate complex (Cleland, 1963). This mechanism is similar to that proposed for the human placenta LOR (Fjellstedt and Robinson, 1975a) but different from the maize enzyme where saccharopine was competitive towards lysine and non-competitive towards 2-oxoglutarate (Brochetto-Braga et al., 1992). The K_m for 2-oxoglutarate (0.8 mM) was similar for maize and rice LOR but somewhat higher than that found for the human enzyme. The K_m for lysine, however, was similar (4.5 mM) for rice and human LOR, and somewhat lower than that for the maize enzyme.

Conclusion. We have partially purified and characterized two enzymes, LOR and SDH, that regulate lysine catabolism in rice seeds. These two enzymes had previously only been purified and partially characterized from maize endosperms in plant species. We have demonstrated the copurification of both enzymes independent of the purification procedure used, which allows us to suggest the existence of a bifunctional protein containing LOR and SDH activities. Apart from the information obtained concerning purification and characterization, the activities observed for both enzymes in a plant that contains high levels of lysine compared with other cereals, provides further evidence that the regulation of lysine catabolism may be essential for lysine accumulation. Genetic manipulation leading to transgenic plants with

altered LOR and SDH activities or antisense LOR/SDH, in association with alterations in the biosynthesis of lysine, may be the key to lysine overproduction and accumulation.

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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

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Abstract

Lysine biosynthesis has been extensively studied and the regulatory enzymes characterized in some of the most important crop plants, however, much less is known about the lysine degradation pathway. Lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) have recently been partially purified and characterized from plants, and have been shown to exist as a single bifunctional polypeptide. We have further characterized these enzymes from rice endosperm in relation to Ca^{2+} and ionic strength modulation. Optimum pH values of 7.0 and 8.0 were obtained for LOR and SDH, respectively. The LOR domain of the polypeptide was modulated by Ca^{2+} and ionic strength, whereas the SDH domain was not. It would appear that the modulation by Ca^{2+} and ionic strength of LOR is a common feature among plant LOR enzymes. *S*-adenosylmethionine (SAM) did not produce any significant effect on either enzyme activity, indicating that it only plays a role in the regulation of lysine biosynthesis. The effect of *S*-2-aminoethyl-L-cysteine (AEC) as both a substrate and an inhibitor of LOR activity was also tested. AEC was shown to partially substitute for lysine as a substrate for LOR, but was also able to inhibit LOR activity, possibly competing with lysine at the active site. The higher K_m for AEC compared to lysine may reflect a lower binding affinity for AEC.

1. Introduction

Lysine is one of the essential amino acids synthesized via the aspartic acid metabolic pathway in plants [1]. The low concentration of this amino acid in cereal seeds is considered a major nutritional limitation and a great deal of attention has been devoted to overcoming this problem, which so far has proved to be a difficult task.

In order to understand the factors governing lysine formation and accumulation in plants, key enzymes responsible for the regulation of the aspartic acid metabolic pathway have been isolated and characterized in several plants species [1,2]. However, most of the research carried out up to the present time, has concentrated almost entirely on the enzymes controlling the biosynthesis of lysine, with special attention being paid to: aspartate kinase (AK, EC 2.7.2.4), that catalyzes the phosphorylation of aspartic acid to β-aspartyl phosphate and dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52), which is the first enzyme after the branch point of the aspartic acid pathway.

The study of these enzymes and those of threonine and methionine synthesis, has provided a clear view of their involvement in regulatory aspects of the aspartic acid metabolic pathway, allowing strategies to be elaborated in order to obtain plants with increased concentration of lysine and threonine. Biochemical mutants containing enzymes with altered regulatory properties have been selected *in vitro*, but although the threonine concentration has been shown to be considerably elevated in the majority of plant tissues analysed, all of them have failed to exhibit over-accumulation of lysine in the seed [3-9].

These results indicated that another hurdle was still to be overcome. Parallel work on the maize opaque-2 mutant and the activity of enzymes involved in lysine degradation, has strongly suggested that in order to achieve lysine accumulation in

seeds, the catabolism of lysine must be reduced [10]. In the opaque-2 mutant, the activities of two enzymes, lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8; also designated lysine α-ketoglutarate reductase [LKR]) and saccharopine dehydrogenase (SDH, EC 1.5.1.9), were shown to be strongly reduced, thus increasing the availability of lysine to be incorporated into storage proteins and or allowing more lysine to accumulate in the soluble form in the maize endosperm. In maize seeds, the developmental pattern of LOR activity is co-ordinated with nitrogen input and zein synthesis indicating a common regulation of lysine degradation and prolamine accumulation [11]. Falco et al. [12] working with transgenic plants accumulating lysine in the seeds, demonstrated that the intermediates of lysine catabolism, α-aminoadipic acid and saccharopine, accumulated in canola and soybean seeds, respectively, indicating a strong role of LOR and SDH in the degradation of lysine even in these plants.

LOR and SDH have been isolated and purified from maize [13] and rice [14] endosperms. In both plant species, LOR and SDH were shown to be specific to the endosperm and part of a single bifunctional polypeptide. The regulation of LOR, SDH, HSDH and AK activities by the *opaque-2* maize gene has also been investigated [4,15,16]. In fact, zein and LOR genes are under transcriptional regulation by the opaque-2 bZIP factor [17]. Recent studies have shown that LOR activity can be activated by Ca^{2+}, high salt concentrations, organic solvents and Mg^{2+} in maize [18] and by Ca^{2+} and protein phosphorylation in tobacco seeds [19]. Ca^{2+} was also shown to modulate enzymes involved in lysine metabolism such as AK from spinach leaves [20], but this was not confirmed in carrot [21], maize [22] and rice [23].

Some other compounds have also been shown to play an important role in the regulation of the aspartate pathway. *S*-adenosylmethionine (SAM) is an end point amino

acid, which has a regulatory influence on the pathway and is important in methionine cycling [1,24]. SAM can markedly stimulate the activity of threonine synthase (TS, EC 4.2.99.2) [25,26] and with lysine can synergistically inhibit AK activity in plants [27,28]. *S*-2-aminoethyl-L-cysteine (AEC), a lysine analogue, has been used for the selection of mutants with enzymes altered in their feedback inhibition patterns to lysine [8,29-32] and can substitute for lysine in proteins [1].

In this paper, we present the characterization of rice LOR-SDH with respect to optimum pH, Ca^{2+} and ionic strength modulation, and effects of inhibitors/or activators of aspartate pathway enzymes.

2. Materials and methods

2.1. Plant Material

The IAC 165 rice upland variety was used in this study for the extraction and partial purification of the LKR-SDH bifunctional enzyme. Plants were grown in the field and immature seeds (milky stage) were harvested and stored frozen at -80°C.

2.2. LOR and SDH extraction and partial purification

The procedure adopted in this work was essentially as described by Gaziola et al. [14] with minor modifications and the elimination of one purification step (gel filtration). The following steps were performed at 4°C. Frozen immature rice seeds were extracted in three volumes of 100 mM potassium phosphate buffer, pH 7.0, containing 50 mM KCl, 1 mM EDTA, 1 mM DL-dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 10% (w/v) glycerol and 5% (w/v) insoluble polyvinylpyrrolidone (buffer A). The homogenate was first filtered through gauze and centrifuged at 15000*g* for 30 min to remove cell debris. The supernatant was adjusted to 20% ammonium sulphate saturation by gently stirring for 30 min and subjected to centrifugation at 15000*g* for 30 min. The supernatant was collected and adjusted to 50% ammonium sulphate saturation. After 30 min of continuous stirring, the mixture was centrifuged at 15000*g* for 30 min, and the precipitated proteins dissolved in minimum volume of buffer A, minus phenylmethylsulphonyl fluoride and insoluble polyvinylpyrrolidone. The sample was then applied to a Sephadex G-25 column (2.6 x 25 cm) previously equilibrated with 100 mM Tris/HCl buffer, pH 7.4, 1 mM DTT, 1 mM EDTA and 10% (v/v) glycerol (buffer

B), and run under gravity. The desalted sample was loaded onto a DEAE-Sephacel column (2.5 x 10 cm; 1 ml/min) equilibrated with excess of buffer B. LOR-SDH was eluted from the column with a 0-500 mM KCl linear gradient in buffer B. Fractions containing LOR-SDH activities were combined and used for LOR and SDH analyses.

2.3. LOR and SDH assays

LOR and SDH activities were measured exactly as described by Gaziola et al. [14]. Activities were expressed as nmol $NADPH/NAD^+$ oxidized/reduced/min/ml Assays were performed with and without the compound to be tested in the blank, to ensure that any possible interference was being detected and taken into account. Each enzyme was assayed at the optimum pH required for maximum activity. SDH activity staining on non-denaturing PAGE gels was performed exactly as described previously by Gaziola et al. [14].

2.4. Effect of pH, Ca^{2+}, ionic strength, SAM and AEC on LOR and SDH activities

LOR and SDH assays were performed in a pH range of 6.3 - 8.4 for LOR and 7.0 - 8.4 for SDH. The effect of Ca^{2+} on LOR and SDH activity was determined by adding 1 mM EGTA to the control assay mixture and increasing concentrations of $CaCl_2$ (0 - 150 μM) to the control mixture plus EGTA. The effect of ionic strength on LOR and SDH activities was determined in two steps; initially the concentration of Tris was tested (0 - 300 mM) and then a varying concentrations of KCl (0 - 500 mM), with a fixed Tris concentration. Varying concentrations of SAM (0 - 2 mM) were added to the assays and the activity determined using blanks containing the corresponding SAM

concentrations. AEC at 0 - 30 mM was tested as a substrate of LOR in the absence of lysine and as an inhibitor in the presence of 20 mM lysine. The K_m value for AEC was determined as described by Gaziola et al. [14].

2.5. Protein Determination

Protein concentrations of the samples were determined by the method of Bradford [33] using bovine serum albumin as a standard.

3. Results

3.1. Isolation and partial preparation of LOR-SDH bifunctional enzyme

The bifunctional enzyme LOR-SDH was extracted and partially purified from developing rice seeds by anion exchange chromatography as described previously [14]. A combined fraction containing LOR and SDH activities was used in all experiments. No significant alterations in the elution and activity patterns of LOR-SDH were observed in our preparations when compared to previous experiments [14] (data not shown).

3.2. Optimum pH for LKR and SDH

In previous reports on animal and plant LOR and SDH activities, different assay pH values have been used, we therefore compared the activities of rice LOR and SDH at different pH values. Fig. 1A shows that the pH value of 7.4, which was originally used for rice LOR [14], was not the optimum. By reducing the pH of the assay to 7.0, an increase in 87% of LOR activity was observed. Similarly, the optimum pH for the rice SDH was also tested (Fig. 1B). A pH value of 8.0 produced a slight increase of 10% in SDH activity when compared to pH 8.4, that had been used previously for rice and maize SDH [14].

3.3. Effect of Ca^{2+} on LOR and SDH activities

Some enzymes, including maize LOR are modulated by Ca^{2+} [18,34,35]. To assess whether the rice LOR and SDH activities could be modulated by Ca^{2+}, LOR and SDH activities were determined in assay mixtures containing EGTA or $CaCl_2$. The addition of 1 mM EGTA decreased LOR activity by 21%, whereas the addition of 1.6 mM $CaCl_2$ plus 1 mM EGTA produced an increase in LOR activity by 43%, as compared to the control (Fig. 2A). The addition of a low concentration of $CaCl_2$ (10 μM) to the control assay, keeping EGTA at 1 mM led to a stronger inhibition of LOR activity (37% of the control) (Fig. 2B). Further increases in $CaCl_2$ concentration up to 150 μM in the assay mixture restored LOR activity to the control levels (Fig. 2B).

As has been shown previously for maize SDH [18], rice SDH activity was not influenced by $CaCl_2$ (Fig. 3). In contrast to the results observed for the LOR domain of the LOR-SDH bifunctional enzyme (Fig. 2), we did not observe any alteration in the activity pattern of SDH in the presence of EGTA alone, $CaCl_2$ alone or in combination with the same varying $CaCl_2$ concentrations as described for LOR (Fig. 2).

3.4. Effect of ionic strength on LOR and SDH activities

Enzyme modulation by ionic strength has been shown for maize LOR activity [18]. We have also tested this possibility for the rice LOR-SDH bifunctional enzyme. LOR activity was initially assayed with varying concentrations of Tris, and the optimum concentration was determined as 100 mM (Fig. 4A). This finding prompted us to assay LOR activity in varying concentrations of KCl, but keeping Tris at 10 mM. After a 100% increase in LOR activity up to a KCl concentration of 100 mM, activity was

strongly reduced at higher KCl concentrations, being almost completely inhibited at 500 mM KCl (Fig. 4B). On the other hand, LOR activity was also increased by concentrations up to 50 mM KCl, when the Tris concentration was 100 mM (Fig. 4C).

As performed for LOR, SDH activity was also tested for modulation by ionic strength (Fig. 5). In contrast to LOR, the variation observed for SDH was much less, although higher levels of SDH activity were also observed from 100 to 300 mM Tris (Fig. 5A). When Tris was kept at 100 mM in the buffer system, KCl concentrations from 0 to 200 mM did not result in any significant alteration in SDH activity, while a 500 mM KCl concentration caused a 20% reduction in SDH activity (Fig. 5B).

3.5. Effect of AEC on LOR activity

First we analysed the efficiency of AEC as a substrate for LOR. The data clearly show that AEC can substitute for lysine as substrate, although less effectively (Fig. 6A). At 20 mM, which is the assay concentration of lysine, LOR activity with AEC was only 52% of that with lysine. The K_m value for AEC of 17 mM was determined graphically from the double-reciprocal plots of activity against variable substrate concentration, maintaining the remaining substrates fixed at the routine assay concentrations (close to saturation). The kinetic data also showed that V_{max} with AEC was almost identical to that obtained with lysine (2.23 and 2.22, respectively). To further verify the effect of AEC on LOR activity, AEC was then tested in combination with lysine in the assay mixture. Additions of up to 10 mM AEC to the assay mixture containing 20 mM lysine led to a reduction of total LOR activity (lysine plus AEC dependent NADPH oxidation) which then stabilized with higher concentrations of AEC (Fig. 6B).

3.6. Effect of SAM on LOR and SDH activities

SAM, a methionine derivative, is an important regulator of some of the enzymes involved in the aspartic acid metabolic pathway [27]. We tested LOR and SDH activities in the presence of SAM to verify whether SAM might also play a regulatory role in lysine degradation. However, the addition of SAM (0 to 2 mM) to the assay mixture for LOR and SDH assays did not show any alteration in enzyme activities (Fig. 7).

3.7. Multimeric forms of SDH

Non-denaturing PAGE gels were used to identify possible variants of SDH in rice endosperm. The DEAE-Sephacel partially purified protein samples were applied to non-denaturing PAGE gels, which were then stained for SDH activity. Several forms of SDH were observed in rice, while a control sample of maize exhibited a single predominant band of SDH activity (Fig. 8).

4. Discussion

LOR and SDH enzymes have been characterized in rat liver [36] and human placenta [37,38]. In plants species, LOR and SDH has also been recently studied in maize, rice, tobacco and *Arabidopsis* [12,13,18,19,39,40]. The interest in plant LOR and SDH is due to the importance of these enzymes in the control of lysine degradation in seeds and to the relative limited success in producing cereals seeds with high levels of lysine.

In a previous report, the rice LOR and SDH enzymes were purified and shown to exist as a bifunctional polypeptide [14] in a similar manner to maize [13]. We have observed in this study, that the pH previously used for rice LOR [14], is not the optimum. By reducing slightly the pH from 7.4 to 7.0, an increase of 87% in LOR activity was obtained. This result confirms the optimum pH of 7.0 previously determined for maize LOR [10]. An alteration of pH from 8.4 to 8.0 also produced an increase in SDH activity, although to a lesser extent, which is lower than the optimum pH of 8.5 for maize SDH [13]. Dehydrogenases have been shown to exhibit high pH optima often close to 10 when measured in the NAD→NADH direction [41]. These results suggest that the maize and rice enzymes, although clearly similar have different kinetic properties and molecular masses [14].

Ca^{2+} is an important messenger in plant signal transduction [42] and is known to modulate several enzymes [34]. In maize, LOR, but not SDH, activity has been shown to be modulated by Ca^{2+} and ionic strength [18]. The Ca^{2+} modulation of LOR activity in maize, may be a result of a possible modulation of an inhibitory domain [18]. A low affinity Ca^{2+} binding site is present in both maize and *Arabidopsis* protein sequences [40,43]. In rice, it was observed that only LOR activity is regulated by Ca^{2+}, but not

SDH, as described for maize [18]. Although the general pattern of Ca^{2+} modulation of rice and maize LOR-SDH enzymes are very similar, when the same Ca^{2+} and EGTA concentrations were used, they basically differ in terms of the extent of the Ca^{2+} effect, so that the addition of EGTA to the rice LOR assay reduced the activity by 21% (Fig. 2), whereas in maize this reduction may be of 70% depending on the salt concentration of the buffer system [18]. The result suggest that modulation by Ca^{2+} may be a common feature of plant LOR.

A regulatory role of Ca^{2+} for other enzymes involved in lysine metabolism has been proposed. In spinach, HSDH and AK were shown to be stimulated by Ca^{2+}, with one of AK subunits being calmodulin [44]. In barley, such a regulatory role for Ca^{2+} and calmodulin has also been proposed recently [45]. However, several other reports for carrot [21], maize [22] and rice [23] did not confirm these findings.

Calmodulin has also been shown to regulate glutamate decarboxylase by binding to inhibitory domains which once deleted produce a fully active enzyme insensitive to Ca^{2+}-calmodulin *in vitro* [46,47]. Although not tested in this study, it is possible that rice LOR activity could also be affected by calmodulin, since calmodulin antagonists were shown to inhibit almost completely the Ca^{2+}-dependent LOR activity of maize [18].

Apart from modulation of LOR activity by Ca^{2+} in maize [18] and tobacco seeds [19], the lysine dependent stimulation of lysine degradation also requires protein phosphorylation in tobacco seeds [19]. The maize LOR activity was also shown to be modulated by ionic strength, but not the SDH activity [18]. Rice LOR activity was here shown to be modulated by ionic strength, whereas SDH was not affected significantly. Curiously, the maximum LOR activity that was obtained with 100 mM Tris, could be increased further by a higher concentration of KCl (Fig. 4). Such a general effect of salt

concentration may be explained by stabilization of the holoenzyme as suggested for the maize enzyme, since both plant species exhibited identical patterns [18]. However, the combined results of Tris and KCl suggest that both can stimulate LOR activity in a different manner, that is not restricted to the general ionic strength of the assay. In plants, HSDH is present in two forms, threonine-resistant and threonine-sensitive [48]. Bryan [49] has shown in maize that the threonine-sensitive isoenzyme can be regulated differentially under physiological conditions such as K^+ concentration. Thus, it may be possible that KCl is somehow specifically affecting rice LOR activity. The absence of any significant effect of ionic strength on the rice SDH domain of the bifunctional enzyme remains unclear, but is in accordance with the maize SDH [18].

AEC is a lysine analogue, which can substitute for lysine in proteins and cause similar inhibitory effects [8,29]. AEC was tested as possible inhibitor and for its ability to substitute lysine as substrate for LOR activity in the spectrophotometric assay. The results for rice LOR activity clearly showed that AEC can substitute for lysine as a substrate, but less effectively (Fig. 6A), despite of the fact that the same V_{max} was obtained for both substrates. The higher K_m for AEC would therefore reflect a lower substrate binding capacity for AEC. When AEC is present in the assay with lysine, LOR activity was inhibited (Fig. 6B). However, the inhibition of LOR activity by AEC is complex since the assay procedure (NADPH oxidation) measures the activities for both substrates. The observed "inhibition" could reflect a lower transformation rate with AEC as a substrate, compared to that with lysine as a substrate. An assay in which only the lysine-specific reaction were measured would be expected to reveal AEC as a competitive inhibitor of lysine. Such an assay is, however, technically difficult to perform with the precision necessary for the kinetics studies. It is noteworthy that AEC could not substitute for lysine as a substrate, nor inhibit LOR activity of maize [10]. On

the other hand, our data for rice LOR are very similar to those for the human placenta enzyme [37]. This confirms our previous report [14] that kinetic properties of rice LOR were similar to the human enzyme but different from that of maize. A more detailed study of the kinetics involving AEC and lysine in relation to LOR has not been reported. However, the only other lysine analogue that has had kinetics studies performed, is lysine-*p*-nitroanilide [37], which indicated that this inhibitor is competitive with respect to 2-oxoglutarate and non-competitive with respect to lysine, also suggesting that this inhibitor binds either at the 2-oxoglutarate site or saccharopine site [37]. The use of amino acid analogues as experimental tools for biochemical-genetic studies in higher plants and bacteria has been used widely [8,50]. AEC has been used instead of lysine to select AK and DHDPS enzymes insensitive to lysine inhibition [51,52]. AK isoenzymes have been isolated from rice and tested for AEC inhibition [23,53]. The lysine-sensitive AK isoenzyme could also be inhibited by AEC, but not to the same extent as lysine [23].

SDH can be easily detected on non-denaturing PAGE gels stained for SDH activity [13,14]. Since both LOR and SDH activities are present in a bifunctional polypeptide, the staining for SDH provides information on the native form of the enzyme. Using this procedure, one major band of SDH activity has been observed in maize [13] and rice [14], although a second band of SDH activity was detected in rice extracts [14]. In this study, at least four forms of SDH were observed in rice seeds in contrast to one major band for maize (Fig. 8). Although the results presented in the literature based on Ca^{2+}, ionic strength and kinetic properties seems to suggest that SDH regulation is very similar between rice and maize, the unequivocal formation of multimeric forms of SDH in rice extracts, suggests that this possibility should be further investigated, by testing the SDH bands for the presence of the LOR activity. Since the

native form of maize LOR-SDH is a dimer [18], these multimeric forms could be the result of interactions of different isoforms present in the rice variety used. Furthermore, LOR-SDH has been shown to be phosphorylated in both *Arabidopsis* [40] and maize [43], which indicates that the multimeric forms might also be the result of different phosphorylated forms present in the enzyme extract.

The effect of SAM on the LOR and SDH activities has never been tested previously, despite the fact that it plays a central role in the aspartic acid biosynthetic pathway. SAM can strongly stimulate TS activity and inhibit AK alone or synergistically with lysine in plants [1,24], thus taking part in the regulation of the biosynthesis of lysine and methionine. The lack of any inhibition or stimulation observed for rice LOR and SDH activities in the presence of SAM, clearly suggests that SAM is not involved in lysine degradation. This result is supported by the use of a wide range of SAM concentrations in the experiments, since SAM has been shown not to have any effect on AK activity up to 0.2 mM, but can strikingly stimulate AK activity up to and above 1 mM SAM in *Arabidopsis* [9].

The amount of information on LOR and SDH enzymes currently available in the literature is still small, when compared to other key enzymes of the aspartic pathway and has only been obtained from three plant species. It seems likely, that although LOR and SDH are part of a single polypeptide, the LOR domain differs in several of its properties between these plant species, while SDH exhibits similar patterns of structure and regulation.

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Figure Legends

Fig. 1. Determination of optimum pH for rice LOR (A) and SDH (B) activities. A DEAE-Sephacel partially purified LOR-SDH sample was used in the assays for LOR and SDH, which were performed in triplicate in two separate experiments. Enzyme activities are expressed in nmol NADPH/min/ml/mg prot. for LOR and in nmol NAD/min/ml/mg prot. for SDH.

Fig. 2. $CaCl_2$ effect on LOR activity. In (A), LOR control activity (1) was tested in the presence of 1 mM EGTA (2) and in the presence of 1 mM EGTA plus 1.6 mM $CaCl_2$ (3). In (B), LOR control activity (●) was tested in the presence of 1 mM EGTA (■) and then with increasing concentrations of $CaCl_2$ with a fixed 1 mM EGTA concentration (○). The data are the result of five separate experiments and enzyme activity measured in triplicate. Enzyme activity is expressed in nmol NADPH/min/ml/mg prot.

Fig. 3. $CaCl_2$ effect on SDH activity. In (A), SDH control activity (1) was tested in the presence of 1 mM EGTA (2) and in the presence of 1 mM EGTA plus 1.6 mM $CaCl_2$ (3). In (B), SDH control activity (●) was tested in the presence of 1 mM EGTA (■) and then with increasing concentrations of $CaCl_2$ with a fixed 1 mM EGTA concentration (○). The data are the result of five separate experiments and enzyme activity measured in triplicate. Enzyme activity is expressed in nmol NAD/min/ml/mg prot.

Fig. 4. Effect of ionic strength on LOR activity. In (A), LOR activity was tested in varying concentrations of Tris. In (B), LOR activity was tested in varying concentrations of KCl, but keeping the Tris concentration fixed at 10 mM. In (C), LOR

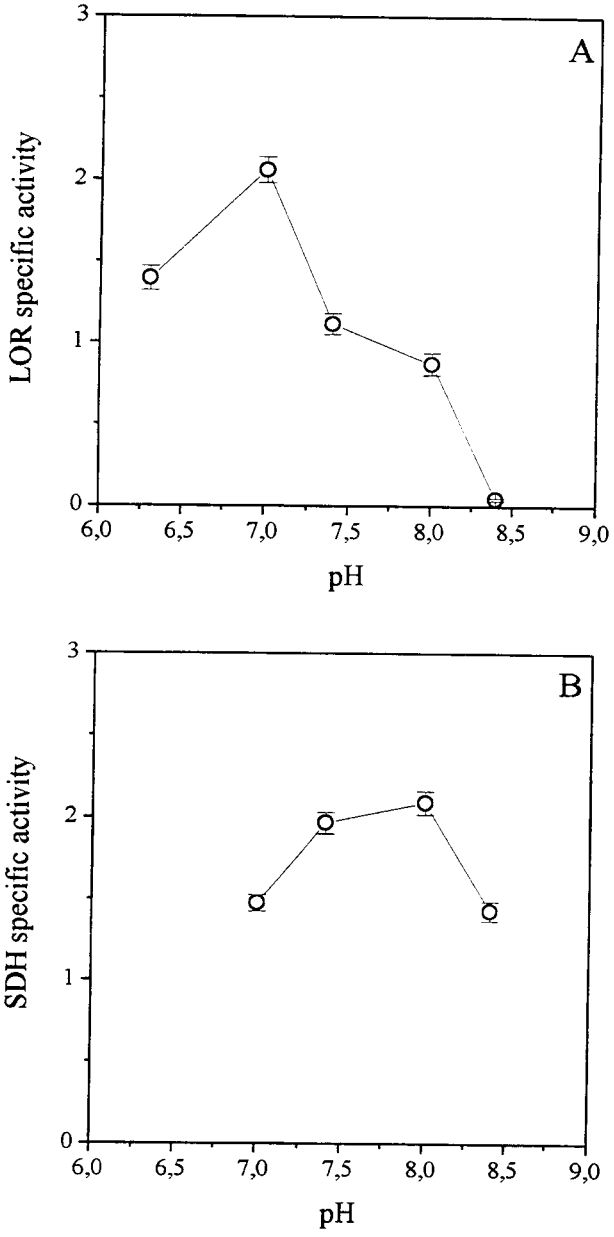
activity was tested in varying concentrations of KCl, but keeping the Tris concentration fixed at 100 mM. The data are the result of five separate experiments and enzyme activity measured in triplicate. Enzyme activity is expressed in nmol NADPH/min/ml/mg prot.

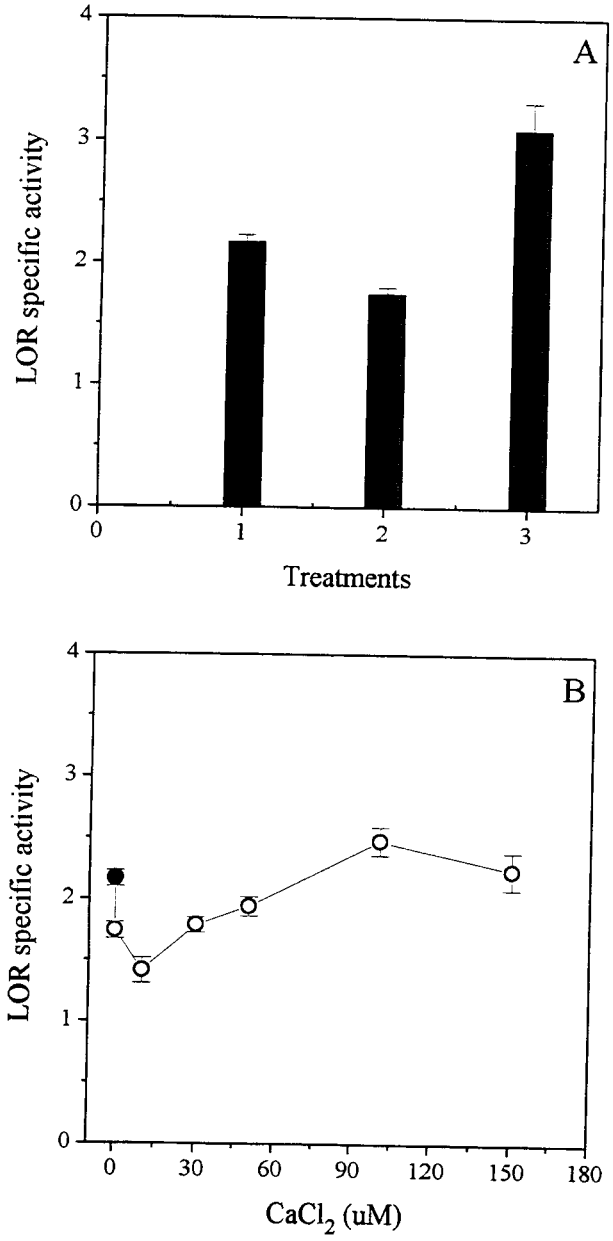
Fig. 5. Effect of ionic strength on SDH activity. In (A), SDH activity was tested in varying concentrations of Tris and in (B) SDH activity was tested in varying concentrations of KCl, but keeping the Tris concentration fixed at 100 mM. The data are the result of five separate experiments and enzyme activity measured in triplicate. Enzyme activity is expressed in nmol NAD/min/ml/mg prot.

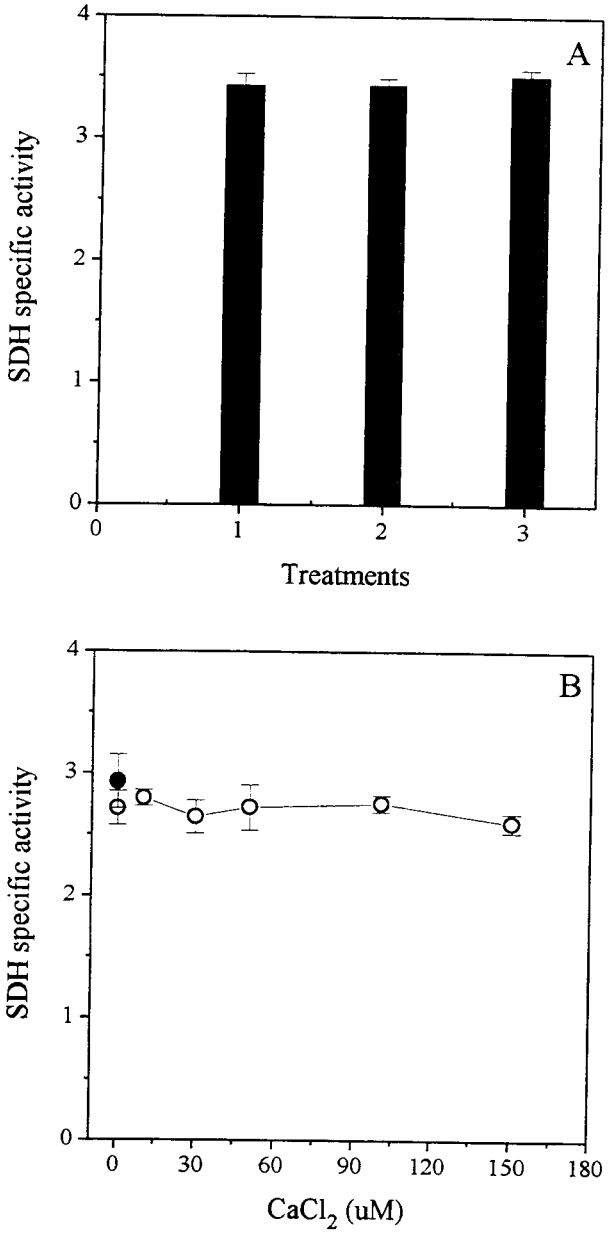
Fig. 6. Determination of the effect of AEC on LOR specific activity. In (A), LOR control assay using lysine (20 mM) as substrate (●) was tested by substituting lysine with varying concentrations of AEC (○). In (B), the standard LOR assay containing a fixed 20 mM lysine concentration was performed with increasing concentrations of AEC in the assay. The data are the result of six separate experiments and enzyme activity measured in triplicate. Enzyme activity is expressed in nmol NADPH/min/ml/mg prot.

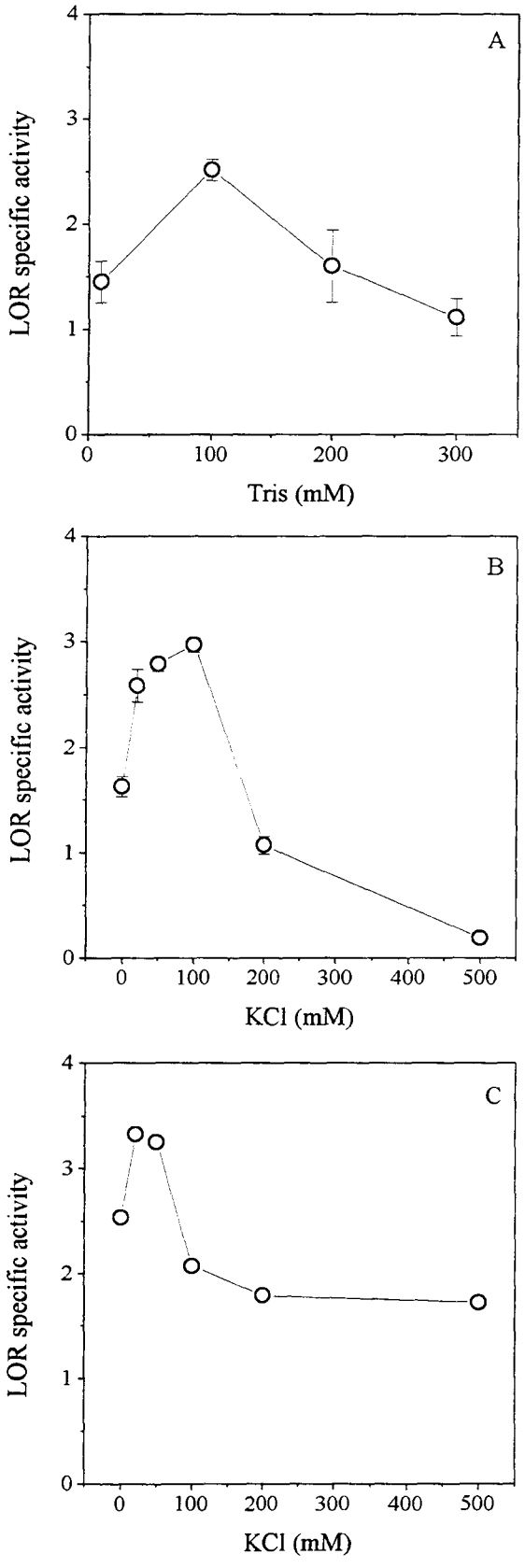
Fig. 7. Effect of SAM on LOR and SDH specific activities. LOR activity (A) and SDH activity (B) were determined in the presence of varying concentrations of SAM. The data are the result of three separate experiments and enzyme activities measured in triplicate. Enzyme specific activities are expressed in nmol NADPH/min/ml/mg prot. for LOR and nmol NAD/min/ml/mg prot. for SDH.

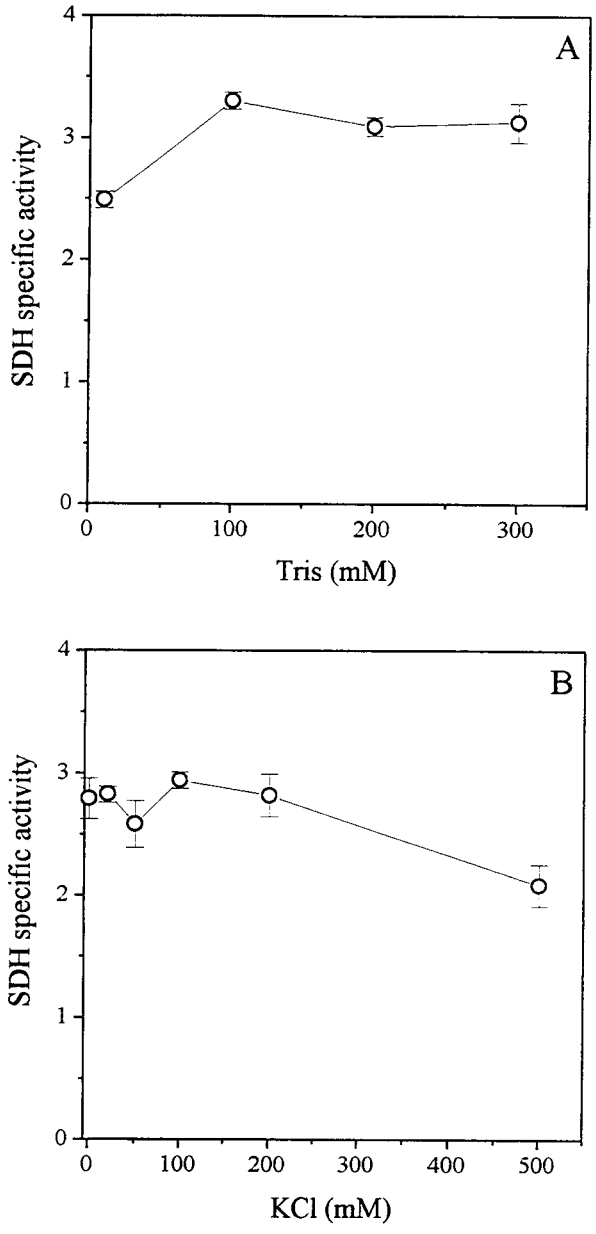
Fig. 8. SDH activity staining in non-denaturing PAGE. LOR-SDH partially purified from maize (lane 1) and rice endosperms (lane 2) was applied to non-denaturing 7% gels and stained for SDH activity.

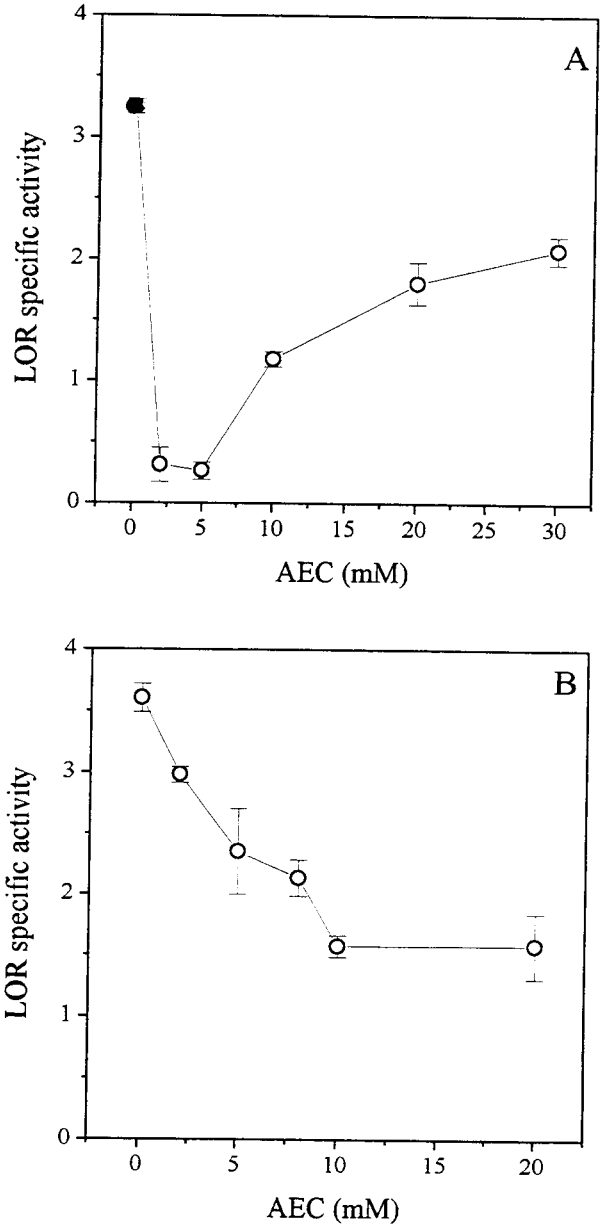

A
LOR specific activity
3
2
1
0
6,0
6,5
7,0
7,5
8,0
8,5
9,0
pH
B
SDH specific activity
3
2
1
0
6,0
6,5
7,0
7,5
8,0
8,5
9,0
pH

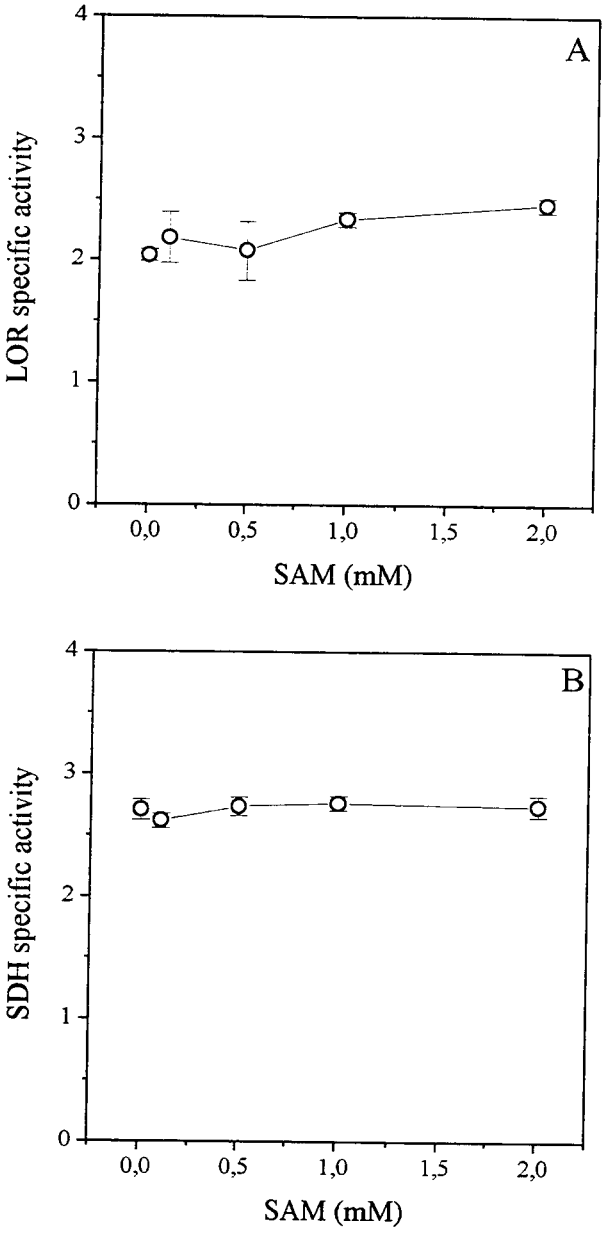

A
LOR specific activity
Treatments
0
1
2
3
4
B
LOR specific activity
$CaCl_2$ (uM)
0
30
60
90
120
150
180

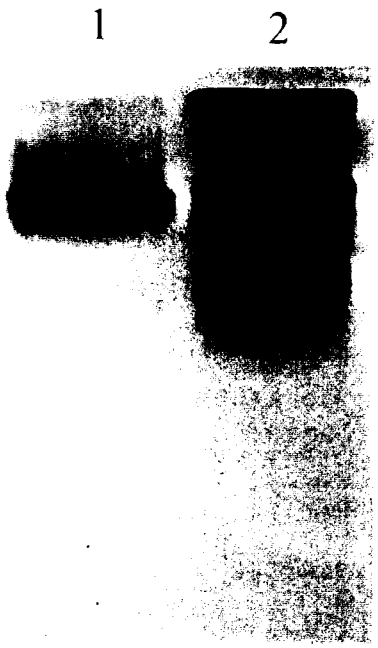

A
SDH specific activity
Treatments
0
1
2
3
4
B
SDH specific activity
$CaCl_2$ (uM)
0
30
60
90
120
150
180


A
LOR specific activity
Tris (mM)
B
LOR specific activity
KCl (mM)
C
LOR specific activity
KCl (mM)


A
SDH specific activity
Tris (mM)
0
100
200
300
0
1
2
3
4
B
SDH specific activity
KCl (mM)
0
100
200
300
400
500
0
1
2
3
4


A
LOR specific activity
AEC (mM)
B
LOR specific activity
AEC (mM)


A
LOR specific activity
SAM (mM)
0
1
2
3
4
0,0
0,5
1,0
1,5
2,0
B
SDH specific activity
SAM (mM)
0
1
2
3
4
0,0
0,5
1,0
1,5
2,0


1
2

DISCUSSÃO

As enzimas envolvidas na degradação de lisina em mamíferos foram caracterizadas como sendo LOR e SDH (HUTZLER e DANCIS, 1968; 1970; FJELLSTEDT e ROBINSON, 1975a; NODA e ICHIHARA, 1978; MARKOVITZ *et al.*, 1984). Em plantas, um grande interesse tem sido voltado para a via de biossíntese de certos aminoácidos essenciais (lisina, treonina, metionina e isoleucina) e de degradação de lisina (via do ácido aspártico). Este interesse justifica-se principalmente porque, na sua maioria, os cereais são deficientes em pelo menos um desses aminoácidos. A primeira caracterização da enzima LOR, em plantas, foi feita em milho (ARRUDA *et al.*, 1982; ARRUDA e DA SILVA, 1983; BROCHETTO-BRAGA *et al.*, 1992). A enzima SDH foi estudada posteriormente em endospermas de milho em desenvolvimento (GONÇALVES-BUTRUILLE *et al.*, 1996). Trabalhos subsequentes de caracterização bioquímica e genética das enzimas LOR e SDH em plantas foram realizados (KARCHI *et al.*, 1994; EPELBAUM *et al.*, 1997; MIRON *et al.*, 1997; TANG *et al.*, 1997a; KEMPER *et al.*, 1998; CORD-NETO, 1998; GAZIOLA *et al.*, 1999).

Estudos de *pH in vitro*, mostraram 7,0 e 8.0 como *pH* ótimos para as atividade de LOR e SDH de arroz, respectivamente. Considerando que as enzimas LOR e SDH parecem estar localizadas no citoplasma (EPELBAUM *et al.*, 1997; CORD-NETO, 1998), seria esperado encontrar um *pH* ótimo próximo de 7,3 - 7,6 determinado para o citoplasma. O *pH* ótimo de uma enzima *in vitro* não é necessariamente idêntico ao *pH* do meio em que normalmente se encontram *in vivo*, podendo estar um pouco acima ou abaixo desse valor. A atividade catalítica das enzimas pode ser regulada ao menos em parte por variações do *pH* do meio em que se encontram *in vivo*. Em milho, o *pH* ótimo para LKR e SDH foi determinado em 7,0 e 9,0, respectivamente (GONÇALVES-BUTRUILLE *et al.*, 1996). Em placenta humana a atividade de LOR foi medida em *pH* 7,35 e a SDH mostrou uma curva característica com atividade ótima no *pH* 8,5 (FJELLSTEDT e ROBINSON, 1975a,b).

Em arroz, os padrões de degradação de lisina mostraram-se diferentes, sendo as atividades das enzimas reduzidas quando comparadas com as atividades de milho (BROCHETTO-BRAGA *et al.*, 1992; GONÇALVES-BUTRUILLE *et al.*, 1996; GAZIOLA *et al.*, 1999), o que poderia explicar níveis mais altos de lisina nesta espécie.

Em endosperma de milho, a atividade da LOR está essencialmente relacionada ao conteúdo de lisina, já que o mutante *opaco-2*, que apresenta altos níveis de lisina, exibiu nível reduzido de degradação deste aminoácido (SODEK e WILSON, 1970) com uma atividade menor da LOR (BROCHETTO-BRAGA *et al.*, 1992). A introdução de genes modificadores em algumas variedades de milho, nas quais as características químicas do mutante *opaco-2* foram mantidos, mostrou que tais genes intensificaram o efeito do gene *opaco-2* nas atividades de LOR e SDH (GAZIOLA *et al.*, 1999). A expressão do gene *ZLKRSDH,* que codifica a enzima LOR/SDH no mutante *opaco-2*, é muito reduzida em comparação aos endospermas normais, diminuindo a quantidade de polipeptídeos LOR/SDH e a atividade enzimática (CORD-NETO, 1998).

A redução da atividade das enzimas também levou a um aumento na disponibilidade de lisina solúvel (GAZIOLA *et al.*, 1999), a qual induziu a atividade da LOR em *N. tabacum* (KARCHI *et al.*, 1994).

A purificação e caracterização parcial destas enzimas mostraram que ambas as atividades residem em um único polipeptídeo bifuncional, de acordo com o que foi descrito para milho (GONÇALVES-BUTRUILLE *et al.*, 1996), *A. thaliana* (EPELBAUM *et al.*, 1997; TANG *et al.*, 1997) e soja (MIRON *et al.*, 1997). Entretanto, um polipeptídeo monofuncional, além do polipeptídeo bifuncional LOR/SDH, contendo a atividade de SDH foi também observado em *A. thaliana* (TANG *et al.*, 1997a).

A existência de polipeptídeos bifuncionais é um fato conhecido em plantas, assim como em bactérias, leveduras e animais. Em *E. coli* já foi comprovada a existência de um polipeptídeo contendo as atividades de AK e HSDH sensíveis à treonina (PATTE *et al.*, 1967). Também na via do ácido aspártico, as enzimas AK e HSDH fazem parte de um único polipeptídeo bifuncional em cenoura (WILSON, 1991), milho (AZEVEDO *et al.*, 1992c) e arroz (TEIXEIRA *et al.*, 1998).

Em humanos, as atividades de LOR e SDH estão contidas no polipeptídeo bifuncional aminoadípico semialdeído sintase e só puderam ser separados por proteólise parcial (MARKOVITZ e CHUANG, 1987).

Proteínas contendo dois diferentes domínios enzimáticos responsáveis por reações em sequência na via metabólica, apresentam algumas vantagens em relação a polipetídeos monofuncionais. Esse arranjo possibilita que os produtos/substratos sejam direcionados à

próxima enzima sem que ocorra desvios para outra parte da via, como no caso da LOR e SDH que catalisam o primeiro e segundo passo da degradação de lisina em plantas. Outra vantagem dos polipeptídeos bifuncionais é a regulação conjunta de sua síntese. As duas atividades são submetidas aos mesmos mecanismos de controle gênico, como por exemplo em processos de amplificação gênica, superproduzindo a proteína bifuncional, modificando os níveis de ambas as atividades na célula (WAHL *et al.*, 1979).

A massa molecular da enzima bifuncional LOR/SDH de arroz foi estimada em 203 e 202 kDa, em coluna de filtração em gel Sephacryl S200 e Ferguson plots, respectivamente. Uma segunda banda com atividade de LOR e SDH foi observada em gel não desnaturante com uma massa molecular estimada, por Ferguson plots, em 396 kDa. Estudos subsequentes mostraram pelo menos quatro formas de SDH em gel não desnaturante em contraste com uma única banda para milho. Em *A. thaliana,* mostrou-se também a presença de um polipeptídeo monofuncional com atividade de SDH (TANG *et al.*, 1997a). A presença de formas multiméricas de SDH deverão posteriormente ser investigadas na presença da atividade de LOR.. Valores distintos de massa molecular de 140, 117 e 125 kDa foram encontrados para milho em PAGE (BROCHETTO-BRAGA *et al.*, 1992; GONÇALVES-BUTRUILLE *et al.*, 1996; KEMPER *et al.*, 1999) e 260 kDa em coluna de filtração em gel Superdex 200HR (GONÇALVES-BUTRUILLE *et al.*, 1996).

A fosforilação de proteínas parece regular a atividade de LOR/SDH em *A. thaliana* (TANG *et al.*, 1997), soja (MIRON *et al.*, 1997) e milho (KEMPER *et al.*, 1999), indicando que formas multiméricas podem resultar de diferentes formas fosforiladas presentes no extrato enzimático. Em vista disso, posssivelmente, poderíamos encontrar tanto a forma bifuncional da enzima LOR/SDH, bem como, formas monofuncionais da enzima SDH.

O Ca^{2+} é um mensageiro secundário importante envolvido na cascata de transdução de sinais em plantas (MUTO, 1992), controlando diversos processos celulares e podendo modular a atividade de muitas enzimas (ROBERTS *et al.*, 1992). A calmodulina, por sua vez, quando ativada pela ligação de Ca^{2+} aos seus sítios ativos, parece modificar a atividade das enzimas ou outras proteínas-alvo na célula. O envolvimento do Ca^{2+} tem sido proposto também para as enzimas da biossíntese de lisina. Em espinafre, a enzima AK sensível à lisina e a HSDH mostraram ser estimuladas por Ca^{2+}, sendo que a calmodulina parece ser uma subunidade da enzima AK sensível à lisina, além de estimular sua atividade

(KOCHHAR *et al.*, 1998). Entretanto, em cenoura (BONNER *et al.*, 1986), milho (AZEVEDO *et al.*, 1992) e arroz (LUGLI *et al.*, 1999), Ca^{2+} e calmodulina parecem não ter nenhum efeito regulatório nas atividades das isoenzimas da AK. Em arroz, a enzima LOR, mas não a SDH, parece ser ativada por Ca^{2+}, sendo inibida por EGTA, de uma maneira que a atividade é restabelecida pela adição de Ca^{2+}. Esses resultados são similares aos encontrados em milho (KEMPER *et al.*, 1998). Embora o padrão de modulação por Ca^{2+} das enzimas de arroz e milho sejam semelhantes sob as mesmas concentrações de Ca^{2+} e EGTA utilizadas, o efeito da adição de EGTA ao ensaio da LOR em arroz reduziu a atividade em 21%, enquanto que em milho a redução foi de 70% (KEMPER *et al.*, 1998). Um possível sítio de ligação para Ca^{2+} não foi detectado em milho (KEMPER *et al*, 1998) embora a sequência da proteína LOR/SDH de *A. thaliana* tenha revelado um possível sítio de ligação (TANG *et al.*, 1997a). KEMPER *et al.* (1998) sugeriram que em milho a ativação da LOR por Ca^{2+} e força iônica (Tris e KCl), resulte em um efeito estabilizador da forma dimérica da enzima, contudo a não modulação da SDH não pôde ser explicada.

A calmodulina parece estar envolvida na regulação da atividade de LOR em milho (KEMPER *et al.*, 1998) observado pela inibição de sua atividade por dois antagonistas da calmodulina estruturalmente diferentes. Embora não tenha sido estudada em arroz, a calmodulina poderia também estar envolvida na regulação da atividade da enzima LOR nesta espécie.

Além deste aspecto, altas concentrações de sais parecem estimular a atividade de LOR mas não tão significativamente a atividade de SDH em arroz. O efeito da indução da atividade das enzimas LOR e SDH por força iônica em arroz, foi claramente demonstrado pela variação crescente nas concentrações de Tris e de KCl dos tampões no meio de reação. A atividade máxima da LOR foi obtida com Tris na concentração de 100 mM, que pôde ser aumentada pela adição de KCl. O efeito da concentração de sais e K^+ pode estar relacionado a regulação da atividade da enzima sob certas condições fisiológicas, tal como relatado para a enzima HSDH sensível à treonina de milho (BRYAN, 1990b), que é regulada diferencialmente pela concentração de K^+. Em milho, somente o domínio LOR da enzima bifuncional LOR/SDH mostrou ser ativado (KEMPER *et al.*, 1998).

Entre os mais variados análogos de lisina, a AEC foi testada para a capacidade de substituir a lisina como substrato para LOR e como possível inibidor da atividade da

enzima em arroz. A enzima LOR de arroz foi inibida por AEC e na ausência de lisina, a AEC pode funcionar parcialmente como substrato. O K_m estimado para AEC (17 mM) foi diferente do K_m obtido para lisina (4,5 mM), mostrando que a LOR tem maior afinidade para lisina do que para AEC. Isto é consistente com os resultados obtidos, onde a atividade da LOR na presença de AEC, nas mesmas condições do ensaio normal, não apresentou os mesmos níveis de atividade da enzima na presença de lisina. No entanto, a mesma V_{max} foi obtida para ambos os substratos separadamente, mostrando uma eficiência similar de reação. Quando a AEC está presente no ensaio com lisina, a atividade da LOR foi inibida. Entretanto, a inibição da atividade da LOR por AEC é um processo difícil de avaliar desde que o ensaio mede a oxidação do NADPH para os dois substratos. A inibição observada com AEC como substrato poderia refletir uma taxa de transformação mais baixa quando comparada com lisina como substrato. Os diferentes valores de K_m determinados para AEC e lisina, sugerem que a LOR apresenta menor afinidade para AEC. Contudo, como já descrito na literatura, a AEC pode substituir a lisina em proteínas, bem como causar efeitos inibitórios similares àqueles de lisina (AZEVEDO e ARRUDA, 1995; JACOBSEN, 1986). Esses resultados são similares aos descritos para LOR de placenta humana (FJELLSTEDT e ROBINSON, 1975a), mas diferentes aos resultados obtidos em milho, que mostraram que a AEC não pode substituir a lisina como substrato bem como inibir a enzima LOR (BROCHETTO-BRAGA *et al.*, 1992). Estudos cinéticos de inibição envolvendo AEC e lisina em relação à LOR nunca foram relatados, sugerindo que, como em arroz, os resultados não sejam compatíveis pois a AEC além de ser um inibidor também funcionaria como substrato. A escolha da AEC dentre os vários análogos de lisina, se deve a AEC ter sido utilizada em todos os estudos enzimáticos de regulação da via do ácido aspártico. Por exemplo, a AEC foi utilizada como ferramenta na seleção das enzimas AK e DHDPS resistentes à inibição por lisina (NEGRUTIU *et al.*, 1984; HEREMANS e JACOBS, 1997). Além disso, isoenzimas da AK foram isoladas de arroz e testadas para AEC, sendo que a isoenzima AK sensível à lisina pode ser inibida por AEC, mas não com a mesma intensidade que é inibida por lisina (LUGLI *et al.*, 1999).

No caso da SAM, nenhum efeito sobre a atividade das enzimas LOR e SDH foi observado em arroz. Por outro lado, a SAM pode inibir a enzima AK pela ação sinergística com a lisina (ROGNES *et al.*, 1980; AZEVEDO *et al.*, 1992a), assim como pode estimular

a atividade da treonina sintase (MADISON *et al.*, 1976; THOEN *et al.*, 1978; GIOVANELLI *et al.*, 1984). É importante lembrar que todas estas enzimas estão em uma mesma via metabólica. Este resultado sugere que a SAM deva estar envolvida na regulação da via do ácido aspártico, principalmente na biossíntese de treonina e lisina, mas não na degradação de lisina.

A via do aspártico tem sido, por mais de 30 anos, estudada detalhadamente no que se refere à biossíntese de lisina e treonina, além de metionina e isoleucina. Contudo, a via de degradação, essencialmente de lisina, vem recentemente despertando a atenção dos pesquisadores e, portanto, permanece ainda pouco esclarecida. Em sementes, a produção e acúmulo de lisina são regulados não somente pela biossíntese, mas também pela sua taxa de catabolismo. A degradação de lisina também é um importante processo regulatório em mamíferos, pois quando ocorrem problemas nesse processo devido, por exemplo, à mutações nas enzimas LOR/SDH, podem aparecer doenças como a hiperlisinemia familiar.

Os trabalhos aqui apresentados procuram contribuir para a elucidação de alguns mecanismos e pontos de regulação da via de degradação de lisina em arroz, um cereal de grande importância econômica em nosso país e na maioria dos países subdesenvolvidos. O gene que codifica a enzima LOR/SDH parece ser regulado à níveis transcricionais e pós-transcricionais em resposta aos níveis de lisina livre. Estudos mais detalhados da expressão do gene que codifica a enzima LKR/SDH e dos mecanismos de transdução de sinais que estimulam a atividade da enzima LOR representam novas metas de pesquisa. Neste sentido, a análise da variabilidade genética em cultivares de arroz seria de grande interesse, uma vez que os trabalhos poderiam ser direcionados no sentido de se avaliar o comportamento das enzimas envolvidas no catabolismo, regulação e acúmulo deste aminoácido, tanto ao nível bioquímico dentro do metabolismo primário, como ao nível genético e molecular.

CONCLUSÕES

1. As enzimas LOR e SDH de arroz mostraram ser específicas de endospermas imaturos de arroz e apresentaram-se com atividade enzimática reduzida, quando em comparação a outras plantas, sugerindo que catabolismo deste aminoácido seja reponsável pelos níveis um pouco mais elevados de lisina neste cereal.

2. Os resultados indicaram que as enzimas LOR e SDH fazem parte de um polipeptídeo bifuncional e possui uma massa molecular de aproximadamente 200 kDa.

3. A enzima LOR apresentou um mecanismo ordenado de reação, onde o α-oxoglutarato seria o primeiro substrato e a sacaropina o primeiro produto a ser liberado.

4. A atividade da enzima LOR mostrou ser modulada por Ca^{2+} e estimulada por sais como Tris e KCl, ao contrário do que ocorreu com a atividade de SDH.

6. AEC pôde substituir parcialmente lisina como substrato, porém necessitando de mais substrato dado o K_m mais alto.

7. As atividades de LOR e SDH não mostraram ser reguladas pela S-adenosilmetionina (SAM).

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