DISTRIBUTION OF SOLUBLE AMINO ACIDS IN MAIZE ENDOSPERM MUTANTS

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ABSTRACT: For human nutrition the main source of vegetable proteins are cereal and legume seeds. The content of total soluble amino acids in mature endosperm of wild-type, opaque and floury maize (*Zea mays* L.) mutants were determined by HPLC. The total absolute concentration of soluble amino acids among the mutants varied depending on the mutant. The o11 and o13 mutants exhibited the highest average content, whereas o10, fl3 and fl1 exhibited the lowest average content. In general, the mutants exhibited similar concentrations of total soluble amino acids when compared to the wild-type lines, with the clear exception of mutants o11 and fl1, with the o11 mutant exhibiting a higher concentration of total soluble amino acids when compared to its wild-type counterpart W22 and the fl1 mutant a lower concentration when compared to its wild-type counterpart Oh43. For methionine, the mutants o2 and o11 and wild-type Oh43 exhibited the highest concentrations of this amino acid. Significant differences were not observed between mutants for other amino acids such as lysine and threonine. The high lysine concentrations obtained originally for these mutants may be due to the amino acids incorporated into storage proteins, but not those present in the soluble form.
Key words: *Zea mays*, lysine, threonine, methionine

DISTRIBUIÇÃO DE AMINOÁCIDOS SOLÚVEIS EM ENDOSPERMAS MUTANTES DE MILHO

RESUMO: A principal fonte de proteínas vegetais para alimentação humana e animal é fornecida pelos grãos de cereais e leguminosas. O conteúdo de aminoácidos solúveis totais foi determinado por HPLC em endospermas de milho (*Zea mays* L.) normal e mutantes opaco e floury. A concentração de aminoácidos solúveis totais variou entre os mutantes. Os mutantes o11 e o13 se destacaram com médias superiores, enquanto que mutantes o10, fl3 e fl1 apresentaram as menores médias. De maneira geral, para a concentração total de aminoácidos solúveis, os grãos dos mutantes foram similares aos seus tipos selvagens com exceção dos mutantes o11 e fl1 sendo que o primeiro apresentou valor maior que seu tipo selvagem W22, enquanto que o fl1 teve valor menor que o Oh43. Para metionina, os mutantes o2 e o11 e o tipo selvagem Oh43 apresentaram as mais altas concentrações deste aminoácido. Valores similares foram observados entre os mutantes e os tipos selvagens para concentração de outros aminoácidos analisados, tais como lisina e treonina. As altas concentrações sugeridas originalmente para estes mutantes devem ser devidas aos níveis destes aminoácidos incorporados nas proteínas de reserva, mas não na forma solúvel.
Palavras-chave: *Zea mays*, lisina, metionina, treonina

INTRODUCTION

The main source of proteins in plants is grains of legumes and cereals. Among cereals, maize and rice represent the staple food for most part of the population of Latin American countries, Africa and Asia. Brazil is one of the most important maize producers of the world, with a production of 33.038.068 Mt (FAO, 2000). However, maize is a relatively poor cereal when it comes to the quality of its protein, because it has limiting amounts of two essential amino acids, lysine and tryptophan (Azevedo et al., 1997).

Among the amino acids normally found in proteins, only 11 can be synthesized by adult animals. The other amino acids, referred to as essential amino acids, must be acquired through the diet. Plants, most bacteria and fungi have the ability to synthesize all twenty amino acids. Among the essential amino acids, lysine is synthesized through the aspartic acid metabolic pathway, which also leads to the formation of threonine, methionine and isoleucine (Azevedo et al., 1997; Azevedo & Lea, 2001). Regulation of the aspartic acid pathway occurs by negative or positive feedback from key enzymes (Molina et al., 2001). Some of them, such as aspartate kinase (AK), homoserine dehydrogenase (HSDH) and dihydrodipicolinate synthase (DHDPS), involved in lysine and threonine synthesis, were identified as major points of carbon flow control, through the pathway. The lysine

synthesis branch that begins with DHDPS is followed by the degradation pathway that comprises the action of the bifunctional enzyme lysine 2-oxoglutarate reductase/ saccharopine dehydrogenase (LOR/SDH) (Azevedo et al., 1997; Gaziola et al., 1997; Gaziola et al., 2000; Azevedo & Lea, 2001).

Storage proteins present in maize endosperm can be classified, based on their solubility (Osborne & Mendel, 1914; Landry & Moureaux, 1970), as albumins, globulins, prolamins (called zeins) and glutelins. Zeins are the most abundant proteins as they represent 60% or more of the total protein content in the endosperm (Gaziola et al., 1999).

Many endosperm mutations are characterized by a change in the synthesis of storage proteins in maize seeds (Molina et al., 2001). The most studied and utilized mutants in breeding programs aiming at maize protein quality have been the opaque-2 (o2) (Gaziola et al., 1999) and the floury-2 (fl2) (Nelson et al., 1965). The *o2* gene has been mapped on chromosome 7 and segregates as a simple recessive gene; the *fl2* gene, however, is co-dominant and is located in chromosome 4 (Kodrzycki et al., 1989).

The o2 mutant differs from wild-type maize in the amount and distribution of endosperm proteins, with a decrease in the proportion of zeins and a large increase in glutelins, globulins and albumins (Gaziola et al., 1999). The total amount of amino acids in the endosperm of the mutant lines is also higher than in the wild-type genotypes that were their ancestors (Sodek & Wilson, 1971), with a specific increase especially in lysine and a decrease in glutamic acid, proline and leucine (Sodek & Wilson, 1971). The fl2 mutant has higher concentration of glutelins, with little effect on zeins, resulting in a slight increase in lysine concentration as compared to wild-type genotypes (Sodek & Wilson, 1971). Other studies aimed at characterizing the opaque-7 (o7) mutant indicated high amino acid levels in the mutant (Ma & Nelson, 1975). In a similar way, high lysine levels have been reported in the floury-3 (fl3) mutant as compared to wild-type maize (Ma & Nelson, 1975).

Even though the *o2* gene is able to change favorably the amount of amino acids in the grains of maize, its use in agriculture has been limited due to the reduced yield and undesirable grain characteristics, such as low consistence and a farinaceous endosperm that retains water, affecting harvest and contributing to an increase of the susceptibility to diseases (Gupta et al., 1970; Loesch et al., 1976). As a consequence of this, research has been directed toward selecting aspartic acid metabolic pathway mutants with enzymes less sensitive to feedback inhibition, by means of mutagenic agents or cell cultures supplemented with toxic levels of amino acids or their analogs (Azevedo et al., 1997; Azevedo & Lea, 2001; Azevedo, 2002). Such mutants have shown variation relative to some amino acids, but not to lysine. Transgenic plants have been produced based on the same principle of biochemical mutants, but plants with a high lysine content in the grains were again not obtained (Azevedo, 2002).

This information suggests that the amount of lysine in cereal grains could be dependent upon the manipulation of the enzymes LOR and SDH, which are involved in lysine degradation (Molina et al., 2001), as observed for the o2 mutant, which has reduced activity for these enzymes when compared to wild-type genotypes (Brochetto-Braga et al., 1992).

In this work, we have analyzed the levels of total soluble amino acids and the aspartic acid metabolic pathway products: lysine, threonine and methionine, in mature grains of different mutant genotypes of maize o1, o2, o5, o7, o10, o11, o13, fl1, fl2 and fl3, which have never been characterized in detail in regard to any aspect related to amino acid metabolism, and particularly to lysine.

MATERIAL AND METHODS

Anthers and grains of different lines of maize were utilized. Grains of the mutant genotypes opaque (o1, o2, o5, o7, o10, o11 and o13) and floury (fl1, fl2 and fl3) and their respective wild-types were provided by the Maize Genetics Cooperation Stock Center (Urbana, Illinois, USA). The wild-type genotypes utilized as control for the mutant lines were Oh43 for o1, o2, o13, fl1 and fl2; B37 for o7; W22 for o10, o11 and o13; B79 for o5; and WT3 for fl3. The wild-type maize inbred line N6, from the Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, maize seed bank, was also utilized as an internal control. Plants were grown in a glasshouse and auto-pollinated, during the summer of 2001, and ears with dry grains were harvested for amino acid quantification. Anthers were collected before anthesis and stored at -80°C for further amino acid analysis.

Soluble amino acids were extracted as described by Azevedo et al. (1990), using 0.05 g of anthers. Samples were homogenized with a glass rod in 50 μL of Milli-Q water. Then, the extracts were centrifuged twice at 15,000 rpm for 15 min at 4°C and the supernatants were utilized for the amino acid analyses.

Silica gel-polyester TLC plates measuring 20 cm x 20 cm (Sigma-Aldrich, St. Louis, MO, USA) were utilized for the soluble amino acid qualitative analysis. Ten μL of anther free amino acid extracts were applied onto the chromatography plates. The plates were activated at 80°C for 60 min prior to the application of the samples. The chromatograms were developed in a glass chamber containing a solution of butyl alcohol, acetone, ammonium hydroxide, and deionized water (50:50:25:10 mL). After separation of the amino acids, the plates were dried in a ventilated fume cupboard for 48 h at room temperature. The amino acids spots were revealed with

a 0.2% ninhydrin solution in acetone (w/v). Amino acid standards were included for amino acid identification, with a special interest in lysine.

The procedure for amino acid extraction by HPLC in reverse phase was based on the technique published by Bielesky & Turner (1986), with some modifications. A refined flour was obtained by macerating 0.1 g of grain; the flour was homogenized in 2.0 mL of a MCW extraction solution (12.0 mL methanol, 5.0 mL chloroform and 3.0 mL bidistilled water) and incubated for 12 h at 4°C. The extracts were centrifuged at 2,500 rpm for 20 min at 4°C and the supernatant was mixed with 0.5 mL chloroform and 0.75 mL bidistilled water. The solution was centrifuged once again and the supernatant was maintained at 38°C for 2 h. After this period, the supernatant was frozen and submitted to freeze-drying for 48 h. For each freeze-dried sample, 250 μL of milli-Q water were added. The amino acid solution was then filtered through a 0.22 μm membrane (Millipore Ind. e Com. Ltda, São Paulo, SP, Brazil) to be utilized in a final concentration of 125 nmol mL^{-1}. The HPLC analyses used 20 μL of the amino acid solution and 40 μL of o-phthaldialdehyde solution (OPA).

The soluble amino acids were separated and analyzed by reverse-phase HPLC. A Spherisorb ODS-2 C18 column was utilized with 0.8 mL min^{-1} elution for a linear gradient formed by solutions of 65% methanol and pH 7.5 phosphate buffer (50 mM sodium acetate, 50 mM disodium phosphate, 1.5 mL acetic acid, 20 mL tetrahydrofuran and 20 mL methanol in 1 L water). The gradient increased the proportion of methanol from 20 to 28% between 0 and 5 min, from 28 to 58% between 5 and 35 min, from 58 to 75% between 35 and 40 min, 75 to 95% between 40 and 56 min, 95 to 96 % between 56 and 60 min, and 96 to 100 % between 60 and 61 min. The amino acids eluting from the column were monitored by a Shimatdzu (model RF350) fluorescence detector operating on a 250 nm excitation wavelength and a 480 nm emission wavelength. Twenty μL of the amino acid solution and 40 μL of the OPA solution were mixed. After 2 min, 20 μL of the amino acids derivatized with OPA were injected into the HPLC detector. Data are expressed as nmol mL^{-1} of supernatant and as %mol of the total amino acids recovered (excluding proline that does not form a derivative with OPA). A standard solution containing all amino acids was derivatized with OPA as described above and utilized for column profile and elution sequence identification.

The analysis of variance was performed with the aid of the SAS software (SAS Institute, 1993), and the means of variables presenting significance in the analysis were compared by the Duncan test. The %mol of methionine X data were transformed in $\sqrt{X+10}$, and the nmol mL^{-1} total amino acid and methionine data Y were transformed in *Ln (Y + 10)*. These transformations were chosen based on the distribution pattern of the data. The experimental design was completely randomized with three replicates for the mutant lines. The wild-type controls were quantified with one replicate and for this reason they were excluded from the statistical analyses; however, they were included in the tables of means to allow comparisons between magnitudes of values. The criterion arbitrated for comparison between magnitudes of values involving wild-type lines was based on the least significant difference obtained with mutant lines for the Duncan test, without ascribing a statistical confidence to these magnitude comparisons.

RESULTS AND DISCUSSION

The o2 mutant has been characterized for agronomic and biochemical aspects, including amino acids and storage proteins. However, very little or no information is available for several other mutants related to the opaque and floury classes of mutations. This is the first report presenting the characterization of maize endosperm mutants for absolute and relative amounts of soluble amino acids in grains.

Differences were observed for total amino acids (prob. > F= 0.0155) and methionine levels (prob. > F= 0.0115) between mutant grains, but were not observed for lysine (prob. > F= 0.7602) and threonine (prob. > F= 0.8518) levels. For lysine and threonine, the magnitudes of the values between mutants and wild-types were similar. With regard to proportions of amino acids in the grain, differences between mutants were detected for methionine (prob. > F= 0.0142), but not for threonine (prob. > F= 0.7633) and lysine (prob. > F= 0.7600).

In terms of total amino acid amounts (Table 1), grain from lines o11 (1898.84 nmol mL^{-1}) and o13 (1758.80 nmol mL^{-1}) were prominent with higher means, while grain from lines o10 (592.57 nmol mL^{-1}), fl3 (493.67 nmol mL^{-1}) and fl1 (479.30 nmol mL^{-1}) contained the lowest means. In general, the mutant grains had values similar to their wild-types with the exception of o11 and fl1. The first had a value higher than the wild-type W22, whilst fl1 was lower than Oh43. No differences were observed between mutant genotypes at 1%.

As to absolute levels of soluble methionine (Table 1), at 5%, grain from lines o2 (64.53 nmol mL^{-1}) and o11 (36.79 nmol mL^{-1}) were superior with the highest means, while the lowest means were observed for the mutants o7 (11.27 nmol mL^{-1}) and o1 (10.63 nmol mL^{-1}). Most mutants had values close to their wild-type counterparts, except o11 and o13, which exhibited higher values than those of line W22. The observed performance of o2 grains was superior to the o10, fl2, fl1, fl3, o7 and o1 mutants at 1%.

With regard to relative values of methionine at 5% (Table 1), mutants o2 (3.65% mol) and fl1 (3.37% mol), o10 (2.99% mol) and fl3 (2.78% mol) can be considered superior, while the smallest means occurred for lines o5

Table 1 - Total soluble amino acids and soluble methionine means, expressed as absolute values (nmol mL^{-1}), and in the case of methionine also as relative values (%mol), in grain of wild-type and mutant maize genotypes.

Genotype	Original mean	Transformed Mean*	5%	1%
Soluble amino acids				
---------- nmol mL^{-1} ----------				
Oh43	2501.35	7.829		
Opaco-11	1898.84	7.554	a	A
Opaco-13	1758.80	7.478	a	A
Opaco-2	1674.76	7.429	ab	A
Opaco-5	1174.30	7.077	abc	A
Floury-2	748.06	6.631	abc	A
Opaco-1	725.03	6.600	abc	A
Opaco-7	646.55	6.487	c	A
Opaco-10	592.57	6.401	c	A
B79	501.07	6.237		
Floury-3	493.67	6.222	c	A
Floury-1	479.30	6.191	c	A
B37	442.40	6.115		
W22	292.28	5.711		
WT3	207.21	5.381		
Soluble methionine				
---------- nmol mL^{-1} ----------				
Opaco-2	64.53	4.311	a	A
Oh43	64.48	4.311		
Opaco-11	36.79	3.846	ab	AB
Opaco-13	29.40	3.674	bc	AB
Opaco-5	27.97	3.637	bc	AB
Opaco-10	17.66	3.320	bc	B
Floury-2	16.06	3.260	bc	B
Floury-1	15.89	3.254	bc	B
Floury-3	14.91	3.215	bc	B
B79	12.68	3.121		
WT3	11.36	3.061		
Opaco-7	11.27	3.057	c	B
Opaco-1	10.63	3.027	c	B
W22	10.17	3.004		
B37	5.29	2.727		
Soluble methionine				
---------- %mol ----------				
WT3	5.48	3.934		
Opaco-2	3.65	3.685	a	A
W22	3.48	3.672		
Floury-1	3.37	3.657	ab	AB
Opaco-10	2.99	3.605	abc	AB
Floury-3	2.78	3.575	abcd	AB
Oh43	2.58	3.547		
B79	2.53	3.540		
Floury-2	2.07	3.474	bcd	AB
Opaco-11	2.00	3.463	bcd	AB
Opaco-5	1.82	3.438	cd	AB
Opaco-13	1.82	3.438	cd	AB
Opaco-7	1.74	3.426	cd	B
Opaco-1	1.56	3.400	d	B
B37	1.19	3.345		

Means followed by different letters indicate differences at the used probability levels.

*Used transformations: soluble amino acids and methionine (nmol mL^{-1}): $y=(\ln x + 10)$; soluble methionine (%mol): $y = (x + 10)^{1/2}$

(1.82% mol), o13 (1.82% mol), o7 (1.74% mol) and o1 (1.56% mol). Most mutants produced levels similar to those shown by their wild-types, except for o11, o1 and o13, which exhibited lower values than those of their wild-types W22 and Oh43. At 1%, o2 exhibited higher relative amounts of methionine only when compared to mutants o7 and o1.

Maize anther extracts were also utilized to carry out qualitative analyses of free lysine by TLC (Figure 1), in an attempt to preliminarily identify highly significant variations in the concentration of lysine in the different mutant genotypes in relation to the wild-types. The results obtained did not allow a clear discrimination between different genotypes, at least in regard to levels of free lysine, since this technique is basically used to observe very contrasting patterns as, for example, those observed for the maize mutant ask1, which accumulated 144 times more threonine in the anther as compared to wild-type maize, allowing a previous identification in the field of plants with genotype ask1, in order to utilize exclusively ask1 plants in further crossings (Azevedo et al., 1990).

The analyses performed by TLC and HPLC were carried out to characterize differences among mutant genotypes as well as among the original wild-type genotypes. Differences were observed in total amino acid levels and in methionine between mutants. However, for the amino acids lysine and threonine, no differences were observed.

An analysis of the proportion of methionine relative to the total amino acids in the grain, suggests a different distribution of soluble amino acids among the lines under study, considering that the soluble amino acids represent between 6-8 % of the total amino acids in the wild-type grain. It would be interesting to analyze the soluble amino acids fraction, as well as the amino acids incorporated into storage proteins in the grain, both

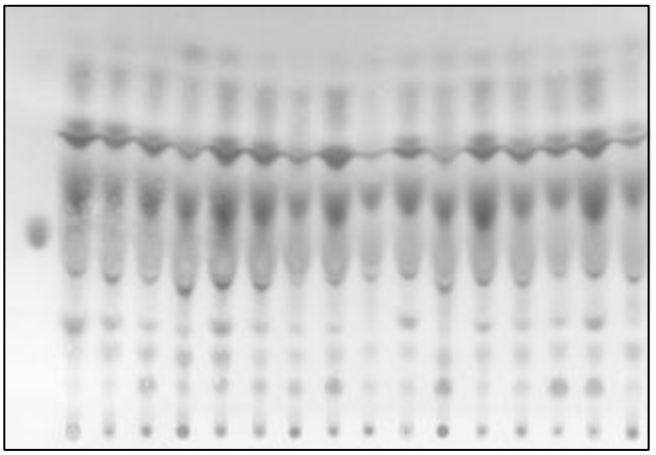

Figure 1 - Soluble lysine analysis of anthers of wild-type and mutant maize genotypes by thin layer chromatography (TLC). Lines represent (1) N6-ESALQ; (2) W22; (3) Oh43; (4) WT3; (5) B79; (6) B37; (7) o1; (8) o2; (9) o5; (10) o7; (11) o10; (12) o11; (13) o13; (14) fl1; (15) fl2; (16) fl3.

qualitative and quantitatively, and then compare them to the amino acid profile of a standard protein of animal origin with high biological value, for example, cow milk or chicken egg proteins. This would allow supplementary and relevant information to be obtained in relation to the amino acid profile as well as to the biological value of proteins in the lines under study.

Higher values of soluble lysine in the mutants were not observed when compared to their wild-type counterparts, which is different from other previously published reports, in which consistent differences have been observed, with higher amounts of lysine in the mutants (Sodek & Wilson, 1971; Gaziola et al., 1999; Wang & Larkins, 2001). Most research has been carried out in order to provide information on the total soluble amino acid content of maize endosperm, where the levels of soluble amino acids and amino acids incorporated into proteins are considered collectively (Ma & Nelson, 1975; Balconi et al., 1998). In a recent paper, Wang & Larkins (2001) reported high soluble lysine concentrations in the mature endosperm of line W64Ao2, as compared to wild-type lines.

In the case of the floury mutants, the endosperm of fl2 had a lower proportion of soluble amino acids (Sodek & Wilson, 1971) and a higher amount of total amino acids (Sodek & Wilson, 1971). No information concerning the concentrations of soluble amino acids is available for the endosperm of fl3, but from acid hydrolysis of storage proteins (Ma & Nelson, 1975) and colorimetric techniques for lysine quantification (Balconi et al., 1998), increases in the proportion of lysine relative to the amounts present in wild-type endosperm have been observed. Balconi et al. (1998) also determined higher levels of lysine in A69yo1 and A69yo11 endosperms.

More pronounced differences in the amounts of soluble amino acids between mutants and wild-type inbred lines have been observed during the development of the endosperm, as a consequence of amino acid transportation from the phloem to endosperm cells, as well as synthesis *in situ* (Silva & Arruda, 1979). In the developing o2 endosperm, the high lysine levels (Silva & Arruda, 1979; Gaziola et al., 1999) coincide with the decreased synthesis of zein proteins (Sodek & Wilson, 1971; Yau et al., 1999) and with the lower activity of the bifunctional enzyme LOR/SDH (Brochetto-Braga et al., 1992; Gaziola et al., 1999). However, the activity of this bifunctional protein during the maturation process of the grain would degrade the lysine not incorporated into proteins, decreasing the soluble concentration even further (Galili, 1995).

No differences in the amount of soluble threonine have been recorded in this study, which agrees with results previously obtained in R802o2 (Sodek & Wilson, 1971) and W64Ao2 (Wang & Larkins, 2001) maize endosperms; nonetheless, these data differ from the results obtained for the endosperm of Oh545o2, which exhibited significant increases in soluble threonine (Wang & Larkins, 2001). This suggests two key aspects that reveal the importance of the interaction between the *o2* gene and the genetic background where it will be expressed and where its phenotypic effects will be evaluated.

A broader characterization of soluble and total amino acid concentrations incorporated into proteins must be performed, together with other biochemical and molecular analyses, to allow a better understanding of the patterns of regulation and control of metabolic pathways that govern the metabolism of essential amino acids, especially lysine, tryptophan, threonine and methionine.

CONCLUSIONS

The observations that no variations in soluble lysine content were detected in the maize endosperm mutants analyzed is contrary to what initially had been suggested when these mutants were isolated. These results can probably be explained by a change in the storage distribution pattern of proteins in these mutants, such as the observed for mutant o2, suggesting that the presumed increase in lysine is due to the amounts incorporated into storage proteins, but not that in the soluble form.

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