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EFFECT OF METHIONINE ON GROWTH AND PROTEIN COMPOSITION OF CULTURED SOYBEAN COTYLEDONS

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Abstract—Immature soybean cotyledons were cultured *in vitro* on a 'complete' medium with and without supplementation with methionine. The supplement increased dry wt by 23%. The growth increase indicated that under these conditions the cotyledons could not synthesize methionine rapidly enough to supply the methionine required for maximum protein synthesis. This indication was supported by finding that aminoacylation of methionyl-transfer RNA was increased 18% by methionine supplementation. Supplemental methionine also increased the methionine content of the protein fraction by more than 20%, decreased the arginine content by 11% and significantly affected several other amino acids. These latter results indicate that the amino acid composition of seed protein can be influenced by the supply of amino acids.

INTRODUCTION

We are involved in a program to improve the amino acid composition of soybeans by increasing the methionine content. The possibility that exogenous methionine might affect seed growth and protein composition was tested with soybean cotyledons cultured *in vitro* [1] because methionine can inhibit growth of higher plants [2–4] and liverworts [5]. Hence, we cultured soybean cotyledons on a complete medium, unsupplemented or supplemented with methionine. We now report the effects of supplemental methionine on growth, aminoacylation of transfer RNAs and protein composition of cultured soybean cotyledons.

RESULTS

Growth effects

The effect of added methionine on the growth of soybean cotyledons in culture was measured. Methionine supplementation increased wet wt 34.8% and dry wt 23.6% (Table 1). The greater increase in wet wt demonstrates that methionine supplementation enhanced water uptake and retention more than dry matter accumulation.

The average increase in protein due to methionine in 15 pairs of cotyledons analysed for amino acid composition (see Table 3) was 19%. The increase in dry wt in this subset was 27%, so that the dry wt increase is only partly accounted for by the increase in protein.

When the routine methionine level (8.4 mM) was doubled, growth changed little, indicating that 8.4 mM methionine gives maximal growth and that methionine is not toxic over a range of concentrations. Levels of methionine below 8.4 mM promoted smaller increases in growth. Tests on the addition of aspartic acid, cysteine, threonine, valine, isoleucine, leucine, proline, tyrosine, phenylalanine, histidine, lysine and arginine at several concentrations showed no effect on growth of cotyledons. Hence, the methionine effect appears to be unique.

Table 1. Effect of methionine on the increase in dry wt and wet wt of cultured soybean cotyledons

	Dry	Wet
Number of cotyledon pairs	134	205
Average increase in weight on basal medium	65.7 mg	190 mg
Increase in weight due to added methionine	23.6 ± 1.8%*	34.8 ± 2.4%*
Significance	$P < 0.001$	$P < 0.001$

Pairs of 25 to 50 mg cotyledons were cultured for 6 days at 27° [1]. Dry wt was measured after lyophilization. One cotyledon from an embryo pair was grown in a medium supplemented with 67 μmol of L-methionine. The pairs used for dry wt measurements were a subset of those used for wet wt measurements. The '*t*'-test for paired samples was used to assess statistical significance.

*Mean ± s.e.

Aminoacylation of transfer RNAs (tRNA). One obvious explanation for the effect of methionine on growth was that methionine biosynthesis was slow enough to limit protein synthesis and growth. To test the possibility that the amount of methionine limited protein synthesis, we measured the aminoacylation of methionyl-*t*RNA. Table 2 shows that in 14 comparisons the aminoacylation of methionyl-*t*RNA increased significantly (18%) but aminoacylation of other *t*RNAs did not. Because methionyl-*t*RNA is intermediate in stability among the 20 aminoacyl-*t*RNAs [6], we wanted to know whether the

Table 2. Effect of methionine on the aminoacylation of *t*RNAs from soybean cotyledons

*t*RNA tested	Cotyledons cultured in: Basal medium % Aminoacylated	Cotyledons cultured in: Basal plus methionine % Aminoacylated	% Change due to added methionine	No. of tests	Level of significance
Methionine	56 ± 3.0*	66	+18	14	$P < 0.001$†
Lysine	42	41	−2	2	N.S.‡
Histidine	47	46	−2	4	N.S.
Isoleucine	69	72	+4	2	N.S.
Leucine	71	75	+6	1	N.S.
Phenylalanine	71	67	−6	3	N.S.
Valine	80	77	−4	1	N.S.

In each test, six pairs of cotyledons were grown in basal medium with or without supplementation with 67 μmol of L-methionine.

*Mean ± s.e.

†Significance calculated by paired '*t*'-test.

‡Not significant at $P = 0.5$.

aminoacylation measurement was meaningful. Two findings indicate that the data are valid. First, per cent aminoacylation was relatively constant among tests. Second, even though histidyl-*t*RNA is more labile than methionyl-*t*RNA [6], its per cent aminoacylation is almost as high as that for methionyl-*t*RNA.

The increase in aminoacylation of *t*RNA by added methionine indicates that methionine production in cotyledons is inadequate for maximum protein synthesis. The constancy in the enhanced aminoacylation of methionyl-*t*RNA and the lack of effect of methionine on aminoacylation of other *t*RNAs indicates that the effect

Table 3. Amino acid content of mature soybeans and cultured cotyledons

Amino acid	Mature beans* (Mol %†)	Cotyledons cultured in: Basal medium (Mol %†)	Cotyledons cultured in: Basal plus methionine (67 μmol) (Mol %†)	% Change due to added methionine
Aspartic acid + asparagine	11.75	11.83 ± 0.11‡	12.19 ± 0.14‡	3.0[a]
Threonine	4.94	3.46 ± 0.03	3.61 ± 0.03	4.2[a]
Serine	7.02	4.77 ± 0.05	4.94 ± 0.04	3.5
Glutamic acid + glutamine	17.51	21.25 ± 0.23	20.82 ± 0.24	−2.1[b]
Proline	5.87	6.20 ± 0.08	6.18 ± 0.06	−0.3
Glycine	7.34	8.10 ± 0.06	8.47 ± 0.08	4.6[a]
Alanine	6.74	7.06 ± 0.04	7.27 ± 0.05	2.9[b]
Valine	4.81	4.79 ± 0.08	4.82 ± 0.10	0.6
Methionine	1.00	0.98 ± 0.03	1.20 ± 0.01	21.9[a]
Isoleucine	4.49	4.37 ± 0.06	4.37 ± 0.03	0.0
Leucine	7.35	8.20 ± 0.08	8.10 ± 0.06	−1.2
Tyrosine	2.90	2.79 ± 0.02	2.79 ± 0.02	0.0
Phenylalanine	4.01	3.92 ± 0.03	3.84 ± 0.02	−2.1[a]
Histidine	2.26	2.65 ± 0.15	2.51 ± 0.12	−5.6[b]
Lysine	5.47	5.03 ± 0.06	5.13 ± 0.06	2.0
Arginine	6.54	4.65 ± 0.25	4.08 ± 0.17	−11.4[a]

*Calculated from data of Yazdi-Samadi *et al.* [35] for Harosoy G3.

†Mol % calculation is based on all amino acids measured and hence excludes cysteine and tryptophan.

‡Mean ± s.e. ($n = 15$).

a and *b* refer to statistical significance between + and − methionine treatment at the 1% and 5% level, respectively. Comparisons were made by paired '*t*'-test on fifteen pairs of cotyledons.

on methionyl-*t*RNA is real. The similarity of the increases in aminoacylation of methionyl-*t*RNA (18%), protein (19%) and dry wt (23%) supports the concept that aminoacylation of methionyl-*t*RNA limited protein synthesis, which in turn limited growth.

The major problem encountered in measuring the extent of aminoacylation of cotyledon *t*RNA was separating the *t*RNA from polysaccharides. Undoubtedly, the values observed are somewhat lower than the actual values *in vivo* due to hydrolysis of the aminoacyl-*t*RNA during isolation, separation from polysaccharides and treatment with periodate. We tested the extent of aminoacylation of two of the *t*RNAs specific for basic amino acids, which are reported to be the most labile, and four *t*RNAs specific for neutral amino acids, reported to be about as stable as methionyl-*t*RNA [6].

Protein composition. Table 3 presents the amino acid composition of the total protein fraction of 15 pairs of soybean cotyledons cultured with and without supplemental methionine. Of the 16 amino acids measured, nine showed a statistically significant difference due to the added methionine. The amino acid composition of the protein was so consistent that even a small difference in composition was statistically significant (e.g. phenylalanine). However, only the changes in methionine and arginine were large enough to have practical importance; methionine increased 21.9% and arginine decreased 11.4%. The increase of methionine has nutritional significance because methionine is the most limiting of the essential amino acids in soybean protein [7]. Possible explanations for the compositional changes are presented later.

The amino acid composition of the protein agrees reasonably well with published data on mature beans (Table 3). The most striking differences are in the contents of threonine, serine, glutamic acid plus glutamine, and arginine. Possibly these differences reflect differences in maturity or variety of beans.

DISCUSSION

We thought that the growth stimulation by added methionine might be due to a deficiency of sulfur (as sulfate) in the basal medium. This possibility was ruled out when a decrease in sulfate in the medium by a factor of five did not affect growth or protein content of soybeans (data not included). Sulfur fertilization increased yield and sulfur amino acid content of seed protein ([8, 9] and references cited therein), when sulfur supplies were limiting in the control treatments.

The profound effects of methionine on growth and amino acid composition of protein indicated that methionine was readily absorbed by the cotyledons. Nevertheless, we measured the non-protein methionine content of cotyledons after 6 days in culture. In one test, the methionine-supplemented cotyledons had *ca* 14 times as much non-protein methionine as unsupplemented cotyledons (14.9 vs 1.1 nmol per g wet wt). Undoubtedly the difference would have been greater at earlier stages when cotyledons were small and before methionine was utilized. Clearly methionine was readily absorbed.

The increases in growth of cotyledons and aminoacylation of methionyl-*t*RNA engendered by methionine indicated that the rate of methionine biosynthesis limited the rate of protein synthesis and growth. This indication raised the questions of whether the addition of methionine would affect the growth of seeds on a plant or whether other amino acids might have limiting biosynthetic rates. Preliminary results showed that providing methionine to roots of a soybean plant did not enhance growth of either vegetative parts or the seeds. This is not surprising because cotyledon culture is an artificial situation in which cotyledons grow faster than they do on a plant [1]. The ready availability of nutrients in culture should have made growth maximal. Furthermore, we do not know whether transport of methionine to the seed from other parts of the plant supplements methionine synthesis in the seed. The fact that other amino acids do not enhance growth when added *in vitro* indicates that their rates of formation in the seed are adequate even when the rate of protein synthesis is abnormally high. Possibly controls on the rate of methionine biosynthesis and/or the relatively complicated biosynthetic pathway [2] restrict methionine synthesis more than that of other amino acids. There is evidence that methionine regulates its own synthesis. For example, Dougall [10] presented isotope competition data to this effect. Carlson [11] and Zenk [12] isolated tobacco cells that were resistant to methionine antagonists (methionine sulfoximine and ethionine, respectively) and contained elevated levels of uncombined methionine. Sloger and Owens [13] found that ethionine-resistant *Chlorella* had elevated levels of methionine, cysteine and cystathionine. Possibly mutants are resistant because they lack the feed-back regulation that methionine (or a metabolite) causes in the wild-type cells.

It has been known for some time that added or excess lysine and threonine can limit the growth of plants and that this limitation can be overcome by methionine [2]. These findings clearly indicate that lysine plus threonine can reduce methionine synthesis, as might be expected, because all three amino acids are derived from aspartic acid. We have no good explanation for the lack of growth inhibition by methionine in soybean cotyledons while *Lemna* [2], *Mimulus* [3] and maize [4] are inhibited.

We considered two other questions. First, was methionine directly responsible for growth effects, or was it converted to some compound that was directly responsible? Second, would some possible precursor of methionine be equally effective? The effect of methionine was not due to its conversion to ethylene [14] because we previously [1] showed that, over a wide range of concentrations, chloroethanephosphonic acid (an ethylene precursor) did not affect growth and protein synthesis. Similarly, neither *S*-adenosylmethionine nor other potential sources of methyl groups (choline, betaine) affected growth (data not included). Nor did the possible products of methionine metabolism, spermidine and spermine, affect growth. We also tested potential precursors (serine and glycine) as well as actual methionine precursors (aspartic acid, homoserine, homocysteine, cysteine and sulfide) and found no effect on growth. One tentative conclusion from the latter observation is that limitation on methionine synthesis lies at the step where homocysteine is methylated. Methionine sulfoxide, but not methionine sulfone, increased growth indicating that methionine sulfoxide was reduced to methionine by the cotyledons.

The effect of methionine on protein composition shows that the level of either a free amino acid or its aminoacylated *t*RNA can affect protein synthesis or

degradation. A possible mechanism for a shift in composition can be suggested. Since DNA specifies indirectly the amino acid composition of the proteins formed, we must assume that the added methionine affects transcription or translation. Since our data show that added methionine increased the aminoacylation of methionyl-*t*RNA, it is conceivable that in the absence of added methionine the rate of synthesis of some polypeptide chains was limited by the availability of charged methionyl-*t*RNA. Clearly, limitation of the rate of synthesis by lack of methionyl-*t*RNA would be less severe for polypeptide chains with low levels of methionine than for chains with higher levels. Hence, the proportion of methionine-rich polypeptide chains might be enhanced by the addition of methionine. Methionine is a unique amino acid in that it is involved in the initiation of polypeptide synthesis [15]. However, it is assumed that all polypeptide chains require methionine for initiation and would be equally affected by a lack of charged methionyl-*t*RNA. The polypeptide chains of soybean storage proteins do not contain methionine at the amino terminal end [16, 17]; apparently the methionine is eliminated after initiation and completed polypeptide chains could be devoid of methionine.

The soybean seed differs from vegetative tissues in that 70% of its protein is storage protein [18] whose only known function is to supply amino acids during germination of the seed. Thus, differences in amino acid composition of the seed probably are due to differences in the storage protein fraction. The amino acid composition of the storage protein, within wide limits, is not critical to the plant because the germinating seed can carry out amino acid interconversions. The soybean seed has two major groups of storage proteins—conglycinin (7S proteins) and glycinin (11S proteins) [18]. Glycinin contains more methionine and less arginine [19] than conglycinin [17, 20]; hence, a change in the ratio of conglycinin to glycinin could alter the methionine content of the cotyledon protein. Indeed, we have preliminary evidence that addition of methionine to the media increases the ratio of glycinin to conglycinin (data not shown). Changes in this ratio would also be expected to affect the levels of the other amino acids, as we observed (Table 3).

We considered the possibility that amino acids other than methionine might affect protein composition. We did not investigate this possibility because of the amount of work involved. However, we consider it unlikely that supplementation with other amino acids would affect protein composition because they have no effect on growth. The latter fact indicates that the rate of synthesis of amino acids other than methionine is not limiting and hence storage protein synthesis and composition are not likely to be altered.

Effects of nutrients on composition of protein have been reported. Leavitt and Ryan [21] reported that a mutant of the yeast *Pichia* had at least 70% more lysine in its protein than the wild type, but they did not report data to explain their results. In *Chlorella vulgaris*, Soldatini *et al.* [22] observed that the protein fraction was enriched up to 50% in methionine when sulfite was added to the standard medium. Sulfur (as sulfate) should have been adequate but possibly accelerated utilization of sulfite enhanced methionine production. However, specific effects of sulfite were indicated on photosynthetic CO_2 fixation rate and products of photosynthesis.

In experiments similar to ours, Blagrove *et al.* [23] observed in *Lupinus angustifolius* that the sulfur supply to plants during growth affected the sulfur content of seed protein. Increasing the sulfur supply to sulfur-deficient plants more than doubled seed-protein sulfur content. They further showed that the ratio of the major seed storage proteins differed between high- and low-sulfur plants. Specifically, the ratio of β-conglutin to α- and γ-conglutins was less in high- than in low-sulfur seeds. This was consistent with the fact that β-conglutin had no methionine and a low cystine content [24]. These results were extended by amino acid analysis of whole seed and globulins of high- and low-sulfur seeds. Cystine and methionine were larger fractions of total amino acid residues in high- than in low-sulfur seeds. In these investigations [23], sulfur supply did not affect protein content of seeds. Apparently sulfur supply controlled the relative amounts of storage proteins, but not the overall protein level, which was regulated by available nitrogen [25]. On the other hand, Blagrove *et al.* [23] imposed a sulfur-limiting condition, whereas we did not. Hence, the mechanisms for the shift in composition might differ.

Vegetative tissues, in contrast to seeds, did not show an effect of sulfur supply on protein composition [26], presumably because the proteins in vegetative tissues have essential metabolic functions. The inconstant composition of storage protein has significant nutritional implications because legume seeds are important sources of protein for humans [27]. Our results and those of Blagrove *et al.* [23] have additional implications because legume seeds are generally deficient in methionine [28] and because these studies show that sulfur nutrition can effect the level of methionine in protein.

EXPERIMENTAL

Plant material and culture. Soybean plants (*Glycine max* L. Merrill—cv Provar) were grown in the greenhouse in a mixture of sand and soil on a modified Hoagland's nutrient soln [1]. Pods containing immature beans were harvested and the embryos (50–100 mg fr. wt) were removed aseptically. Axes and seed coat were removed. One cotyledon from an embryo was placed in 8 ml of basal medium [1] while the other cotyledon was placed in the same medium supplemented with methionine (normally 67 μmol). The cotyledons were incubated 6 days under described conditions [1]. Cotyledons then were rinsed, blotted, weighed and frozen. In many cases, the cotyledons were lyophilized and reweighed.

Preparation of samples for amino acid analysis. Frozen or lyophilized cotyledons were ground in a Potter–Elvehjem all-glass homogenizer with 80% EtOH at room temp. The homogenate was transferred to a 15 ml conical centrifuge tube and centrifuged at 2000 *g* for 15 min. The supernatant was removed and the residue was repeatedly extracted. The air-dried residue was extracted with 2 ml and 1 ml of 1 M NaOH at room temp.; phases were separated by centrifugation. Then the residue was heated for 10 min in 1 ml of 1 M NaOH. After centrifugation, the residue was washed with 1 M NaOH and the vol. adjusted to 5 ml. The protein content of this extract was measured by reaction of hydrolysed material with ninhydrin [29]. An aliquot containing *ca* 5 mg of protein was placed in a test-tube (18 × 175 mm) along with 1 μmol of *p*-fluorophenylalanine (as int. standard) and excess HCl. Water was removed in an air stream. The protein was hydrolysed by heating in an evacuated, sealed tube with 1 ml of 3 M mercaptoethanesulfonic acid [30] (Pierce Chemical Co.) for 48 hr at 120°. The hydrolysate was adjusted to

pH 1–1.5 with satd Na_3 citrate soln, diluted to 2 ml, and filtered through a 0.4 μm filter. Although the unusual hydrolysis conditions gave complete recovery of methionine (101 ± 2%) and minimal humin formation (as judged by lack of dark-colored material [31]) cysteine and tryptophan were destroyed, but the recovery of other amino acids was 90% or greater.

Amino acid analysis. The amino acids were measured on a Beckman model 119CL amino acid analyser equipped with an integrator. We separated amino acids by using Na citrate buffer, pH 3.25 (Na^+ = 0.2 M, 1% *i*-PrOH) for 40 min, Na citrate buffer, pH 4.12 (Na^+ = 0.2 M) for 26 min and Na citrate buffer, pH 6.40 (Na^+ = 1.0 M) for 29 min using Beckman W-3 resin. The column was started at 50° and raised to 65° after 20 min. This modified program improved separation of methionine from the buffer-breakthrough peak and isoleucine peak. Standard solns were analysed every third run. All samples were analysed at least 4 times with good reproducibility. We measured 16 amino acids separately. Amino nitrogen content of the hydrolysate was measured by ninhydrin with alanine as the standard [29]. The amino nitrogen content was 105–115% of the total amount of individual amino acids as measured on the amino acid analyser. This agreement indicates that the amino acid data are valid.

Extraction of tRNA. tRNA was extracted from soybean cotyledons by a modification of procedure II of ref. [32]. Medium B [32] was used to homogenize the beans in a cold mortar and pestle. Diethylpyrocarbonate was omitted as it did not affect either yield or purity of the tRNA. The RNA pellet was resuspended in 0.1 M sodium acetate, pH 4.5, and centrifuged at 20 000 g for 20 min. RNA was reprecipitated with 2 vol. of EtOH and centrifuged after standing for 30 min at −15°. Total time was *ca* 5 hr and yield of tRNA was 1–1.5 mg per 3 g of cotyledons.

Periodation of tRNA. Unaminoacylated tRNA was inactivated by treatment with 2.5 mM $NaIO_4$ in 0.1 M acetate, pH 4.5, for 15 min at room temp. The decrease in *A* at 232 nm [33] upon the addition of ethylene glycol was used to verify that periodate was still present at the end of the incubation.

Measurement of amino acid acceptor activity. The periodate-treated and control (untreated) tRNA solns in 0.1 M acetate, pH 4.5, were treated with 3 vol. of 2 M Tris, pH 8.9, for 30 min at 37° to remove attached amino acids. Acceptor activity was then determined by use of a crude yeast enzyme prepn [34] and various [^{14}C] amino acids. Activity was maximized for the amount of enzyme used, Mg^{2+} concn and time of incubation at 37°. An aliquot of the incubation soln was placed on a filter paper disc, fixed in cold 10% TCA and washed with 5% TCA (2×), EtOH, EtOH–Et_2O (1:1) and Et_2O and counted with a liquid scintillation spectrometer.

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