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METHIONINE ANALOGS INHIBIT PRODUCTION OF β-SUBUNIT OF SOYBEAN 7S PROTEIN

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Key Word Index—*Glycine max*; Leguminosae; soybean; storage protein; methionine; cycloleucine; norleucine; norvaline; *S*-ethylcysteine; *S*-adenosylmethionine.

Abstract—We have previously reported that exogenous methionine inhibits production of the β-subunit of the 7*S* storage protein in cultured soybean cotyledons, and that this inhibition involves lack of functional *m*RNA for the β-subunit. Analogs of methionine were used to study this inhibition. Cycloleucine, norleucine, norvaline and *S*-ethylcysteine treatments prevented accumulation of the β-subunit. The effects of cycloleucine and norleucine on β-subunit synthesis might have been indirect, since these compounds inhibited growth and caused a 2- to 3-fold increase in free methionine concentration. Norvaline did not affect free methionine concentration, but it did inhibit growth. Treatment with a combination of *S*-ethylcysteine and aminoethoxyvinylglycine prevented appearance of the β-subunit without inhibiting growth or raising the *S*-adenosylmethionine concentration. Thus, accumulation of *S*-adenosylmethionine does not appear to mediate the effect of exogenous methionine on β-subunit production. Treatment with *S*-ethylcysteine raised free methionine concentration only 34%, so *S*-ethylcysteine was probably acting directly to inhibit β-subunit production. Measurements of free methionine concentrations in seeds of different sizes, taken from intact plants, suggested that the relatively late appearance of the β-subunit in normal soybean seed development may be due to the presence of high levels of free methionine in very young seeds.

INTRODUCTION

Soybean seeds are widely utilized as a source of protein for the diets of monogastric animals, but the nutritional value of soybean seeds (and other legume seeds) would be enhanced by an increase in their methionine content. We have devised a procedure for culturing immature cotyledons on defined media [1]. The cultured cotyledons grow more rapidly than cotyledons developing on intact plants, and synthesize apparently normal storage proteins [1, 2]. Supplementation of the standard culture medium with methionine increases the dry wt of the cotyledons, the methionine content of the total protein fraction, and aminoacylation of methionyl *t*RNA [3]. The increase in methionine content of the protein fraction is mostly due to an increase in the ratio of 11*S* protein to 7*S* protein [2]. In addition, however, methionine supplementation prevents appearance of the 7*S* β-subunit, which contains no methionine residues [2]. When cotyledons weighing between 20 and 50 mg are placed into standard culture medium, the 7*S* α′- and α-subunits are always prominent within 24 hr, whereas the β-subunit appears only after 2–3 days of growth *in vitro* [2]. Absence of the β-subunit in methionine-fed cotyledons is due to a lack of functional *m*RNA for that polypeptide [4].

This paper reports work aimed at identifying the effector molecule that prevents accumulation of β-subunit *m*RNA in cotyledons cultured in the presence of exogenous methionine. One candidate for the role of effector is AdoMet (*S*-adenosylmethionine). A second possibility is that a metabolite of methionine other than AdoMet is the effector. A third possibility is that methionine itself mediates the effect on gene expression. We have used methionine, analogs of methionine, and an inhibitor of methionine biosynthesis in experiments to select among the above possibilities.

RESULTS

When added to the standard growth medium, cycloleucine (32 mM), norleucine (4 mM), norvaline (4 mM) and *S*-ethylcysteine (4 mM), each prevented accumulation of the 7*S* β-subunit in cultured soybean cotyledons (Fig. 1). The β-subunit was clearly present in the self-paired controls for each of these treatments (data not shown). As with methionine treatment, the effect of the analogs on protein accumulation was specific for the β-subunit (Fig. 1). Of the analogs tested, only norleucine was detectable in hydrolysates of the protein from the treated cotyledons (data not shown).

Electrophoresis of polypeptides from cotyledons grown with a range of methionine concentrations indicated that the minimum methionine concentration in the medium required for the prevention of β-subunit

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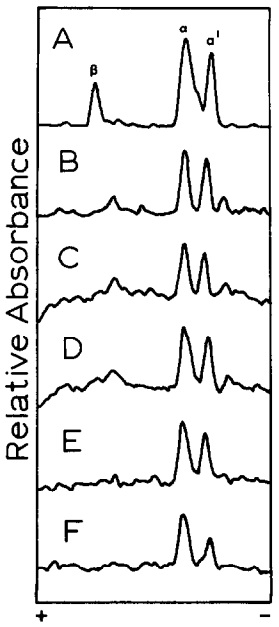

Fig. 1. Densitometer traces of dissociating polyacrylamide electrophoresis gels of protein extracts from immature soybean cotyledons cultured with and without inclusion of methionine or methionine analogs. Control cotyledons (A) were cultured on standard growth medium. Compounds tested for inhibition of β-subunit accumulation were added to the standard growth medium. Treatments were as follows: (B) 4 mM methionine; (C) 4 mM *S*-ethylcysteine; (D) 4 mM norleucine; (E) 4 mM norvaline; (F) 32 mM cycloleucine. The β-subunit was clearly present in the self-paired controls for each treatment (data not shown).

production was between 0.1 and 0.5 mM (Table 1). The minimum concentration of cycloleucine required to prevent appearance of the β-subunit was between 16 and 32 mM, whereas the minimum concentration of norleucine, norvaline and *S*-ethylcysteine required for the same effect was between 2 and 4 mM. All of the analogs except *S*-ethylcysteine inhibited growth at concentrations that prevented β-subunit accumulation (Table 1). Cycloleucine, norleucine and *S*-ethylcysteine each caused increases in free methionine concentration in the cotyledon tissues, and all of the analogs which prevented β-subunit accumulation also raised AdoMet concentrations by factors ranging from 1.2 to 2.0 (Table 1). When aminoethoxyvinylglycine (AVG), an inhibitor of methionine biosynthesis [5], was fed simultaneously with *S*-ethylcysteine (4 mM), the concentration of free methionine was still 1.3 times the control level, but the AdoMet concentration was at the control level, while growth was normal and appearance of the β-subunit was prevented.

Methioninol (2-amino-4-methylthiobutanol) (4 mM), 2-methylmethionine (4 mM) and 2-aminobutyrate (4 mM) did not affect accumulation of the β-subunit (data not shown). No effects on growth, β-subunit accumulation, or intracellular concentrations of free methionine or AdoMet were obtained by including 0.5 mM AdoMet in the culture medium (data not shown). The effects of ethionine and trifluoromethionine on β-subunit production could not be determined because of the extreme toxicity of these compounds.

Inclusion of the branched-chain aliphatic amino acids, valine, isoleucine and leucine (4 mM each) in the medium with norleucine, norvaline, or cycloleucine, fully relieved growth inhibition caused by those compounds, but also allowed normal accumulation of the β-subunit. Inclusion of the branched-chain amino acids (4 mM each) also allowed production of the β-subunit in the presence of *S*-ethylcysteine (4 mM). No β-subunit appeared in cotyledons fed 4 mM methionine, despite simultaneous feeding of the branched-chain amino acids.

In seeds on intact plants, the concentration of free methionine drops dramatically at approximately the same time that the β-subunit appears in normal seed development (Fig. 2).

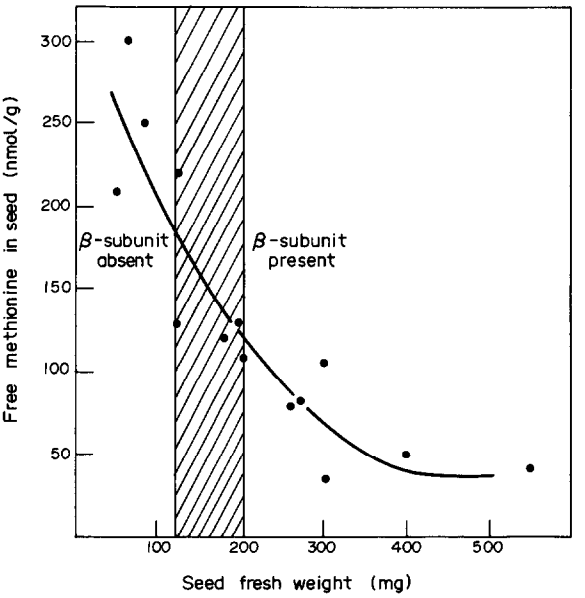

Fig. 2. Correlation between appearance of 7*S* β-subunit and decline in free methionine concentration in soybean seeds from intact, greenhouse-grown plants. Hatched area represents seed fr. wt range where β-subunit is first detectable.

DISCUSSION

The free methionine and AdoMet concentrations in cotyledon tissue cultured with 0.5 mM exogenous methionine were only 3.2-fold and 1.3-fold higher than in controls, though the β-subunit did not appear. These results indicate that the mechanism by which methionine feeding prevents production of the β-subunit, does not involve large increases in the sizes of the endogenous pools of free methionine or AdoMet.

Upon finding that several methionine analogs prevented appearance of the β-subunit, we wondered whether those analogs were acting in place of methionine, or affecting β-subunit production by raising the free methionine concentration in the treated cotyledons. The data here do not allow us to discount the possibility that norleucine and cycloleucine prevented the appearance of the β-subunit by raising the intracellular concentration of free methionine. Their inhibitory effect on growth also complicates interpretation of results. The increases in free methionine concentration (fr. wt basis) in cotyledons treated with cycloleucine and norleucine, might have been

Table 1. Summary of results of methionine-feeding and methionine analog-feeding experiments

Treatment	Conc in medium (mM)	β-Subunit produced	Approx fr. wt gain % of control	Free Met conc % of control	AdoMet conc % of control
Control	—	Yes	100	100* ± 9, $n = 9$	100† ± 3, $n = 6$
Methionine	0.1	Yes	120	88 ± 26, $n = 3$	93 ± 2, $n = 2$
Methionine	0.5	No	120	323 ± 22, $n = 2$	126 ± 6, $n = 4$
Methionine	1.0	No	120	499 ± 4, $n = 2$	148 ± 5, $n = 2$
S-Ethylcys	4	No	100	134 ± 12, $n = 4$	201 ± 3, $n = 2$
S-Ethylcys	4	No	100	133 ± 14, $n = 3$	99 ± 6, $n = 3$
plus AVG	0.1				
Cycloleu	32	No	50	333 ± 15, $n = 2$	120 ± 11, $n = 2$
plus val	4				
Norleu	4	No	50	227 ± 8, $n = 2$	185 ± 27, $n = 2$
Norval	4	No	60	83 ± 6, $n = 2$	156 ± 8, $n = 2$
Cycloleu,	32	Yes	80	276 ± 30, $n = 3$	154 ± 30, $n = 3$
val, leu, ile	4				
Norleu,	4	Yes	100	174 ± 3, $n = 2$	91 ± 12, $n = 2$
val, leu, ile	4				
Norval,	4	Yes	100	100 ± 12, $n = 2$	86 ± 2, $n = 2$
val, leu, ile	4				

*118 nmol/g (fr. wt).

†9.4 nmol/g (fr. wt).

All compounds tested were added to the standard medium, which was used to culture cotyledons for data designated 'Control' on the table. The culture period was six days in each experiment. AVG was filter-sterilized; other compounds tested were autoclaved. Protein subunit analysis was by dissociating polyacrylamide gel electrophoresis [2]. Free methionine concentrations were determined as described [3]. AdoMet concentrations were determined as described in ref. [9].

a reflection of unabated rates of methionine biosynthesis in the presence of lower-than-normal fr. wt gain of those cotyledons. However, the results of experiments in which simultaneous addition of the branched-chain protein amino acids and cycloleucine and norleucine restored normal growth without preventing an increase in free methionine concentrations, suggest otherwise. A more likely explanation for the increases in free methionine concentration is that cycloleucine and norleucine raised the concentration of free amino acids in general, by inhibiting protein synthesis. This idea is supported by the fact that free phenylalanine and free leucine concentrations were elevated several fold in cotyledons treated with cycloleucine and norleucine (data not shown). The small increases in AdoMet concentrations caused by these two analogs may have resulted from the elevated free methionine concentrations. Exogenous valine (4 mM) was routinely included with cycloleucine to partially counteract cycloleucine toxicity [6]. Although norvaline treatment prevented appearance of the β-subunit without elevating free methionine concentration in the cotyledons, the norvaline data must be regarded with reservation, because of the growth inhibition in those experiments.

It seems unlikely that the 30% increase in free methionine concentration caused by *S*-ethylcysteine treatment was preventing appearance of the β-subunit, although the possibility is not ruled out. It is clear that *S*-ethylcysteine prevented appearance of the β-subunit in some way other than elevation of AdoMet concentration in the cotyledons, since treatment with *S*-ethylcysteine plus AVG did not inhibit growth and did not change the AdoMet concentration from that in control cotyledons.

Changes in the level of aminoacylation of methionyl *t*RNA have been shown to affect gene expression in yeast [7], but since *S*-ethylcysteine was not detectable in protein hydrolysates and did not inhibit growth, it is unlikely that *S*-ethylcysteine was acting on β-subunit production through such a mechanism. An amino acid analog that is recognized at any step in the *t*RNA aminoacylation process would almost certainly be sufficiently toxic to cause growth inhibition.

The data reported here indicate that inhibition of β-subunit production by methionine is not mediated by an increase in AdoMet concentration in the tissue. It appears that *S*-ethylcysteine substitutes for methionine in preventing appearance of the β-subunit. Thus it is likely that methionine and *S*-ethylcysteine are themselves effector molecules in this system.

It is puzzling that in the case of 0.5 mM exogenous methionine, the β-subunit does not appear, while in the case of cycloleucine plus branched-chain amino acids, normal β-subunit accumulation does occur, even though the concentration of free methionine in the cotyledons is similar in both cases. Compartmentation of methionine may be involved. It has been shown that methionine-dependent, cultured cancer cells synthesize normal amounts, or above normal amounts of methionine. Such cells lack the ability to use endogenously synthesized methionine for AdoMet synthesis, but readily use exogenous methionine to synthesize AdoMet [8]. Another possible explanation is that the branched-chain amino acids somehow counteract the methionine-inhibition of β-subunit production. The role of valine, isoleucine and leucine in the system is not clear, but they could have affected the uptake of methionine or the analogs.

Cotyledons developing *in vitro* are in an artificial

environment, and so their behavior is not necessarily an accurate reflection of physiological events in seeds developing on plants. However, Fig. 2 shows that the concentration of free methionine in developing seeds drops dramatically around the time that the β-subunit normally appears in the seed. This suggests that the relatively late appearance of the β-subunit in normal soybean seed development could be due to the presence of high levels of free methionine in very young seeds.

EXPERIMENTAL

Sources of materials. AdoMet, cycloleucine, L-norvaline, L-norleucine, DL-methioninol, 2-methyl-DL-methionine and 2-aminobutyric acid were from Sigma Chemical Co. *S*-Ethyl-L-cysteine was from Mann Research Laboratories. AVG was a gift from Hoffmann-LaRoche.

Cotyledon culture. Cotyledons (weighing 15–40 mg each) of immature soybean seeds taken from greenhouse-grown plants (*Glycine max* L. Merr. cv. Provar) were cultured as previously described [1], except that 125 ml flasks with 40 ml of medium and 10 cotyledons per flask were used rather than small jars with 8 ml of medium and a single cotyledon. In all cases, compounds tested were incorporated into the standard medium. AVG was sterilized by filtration. Cotyledons were harvested after 6 days *in vitro*. Harvested cotyledons were analysed immediately or stored at −80°.

Analytical procedures. Dissociating polyacrylamide gel electrophoresis of proteins was as previously described [2]. The dried gels were photographed and the prints were scanned with an LKB laser densitometer equipped with a recording integrator. Procedures for amino acid analysis [3] and AdoMet measurement [9] have been described.

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REFERENCES

1. Thompson, J. F., Madison, J. T. and Muenster, A. E. (1977) *Ann. Bot.* **41**, 29.
2. Holowach, L. P., Thompson, J. F. and Madison, J. T. (1984) *Plant Physiol.* **74**, 576.
3. Thompson, J. F., Madison, J. T., Waterman, M. A. and Muenster, A. E. (1981) *Phytochemistry* **20**, 941.
4. Creason, G. L., Holowach, L. P., Thompson, J. F. and Madison, J. T. (1983) *Biochem. Biophys. Res. Commun.* **117**, 658.
5. Datko, A. H. and Mudd, S. H. (1982) *Plant Physiol.* **69**, 1070.
6. Abshire, C. J. and Pineau, R. (1967) *Can. J. Biochem.* **45**, 1637.
7. Cherest, H., Surdin-Kerjan, Y. and DeRobichon-Szulmajster, H. (1971) *J. Bacteriol.* **106**, 758.
8. Coalson, D. W., Mecham, J. O., Stern, P. H. and Hoffman, R. M. (1982) *Proc. Nat. Acad. Sci. U.S.A.* **79**, 4248.
9. Creason, G. L., Madison, J. T. and Thompson, J. F. (1985) *Phytochemistry* **24**, 1151.