Methionine and Sulfate Increase a Bowman−Birk-Type Protease Inhibitor and Its Messenger RNA in Soybeans

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A soybean cotyledon cDNA library was screened for clones representing RNAs that were more abundant in cotyledons grown on methionine-supplemented medium than in those grown on unsupplemented (basal) medium. Three isolated clones encoded the Bowman−Birk protease inhibitor (BBPI). RNA gel blot hybridization confirmed that the BBPI mRNA level is increased by methionine (Met). Met and/or sulfate supplementation increased the amount of protease inhibitor activity in cultured cotyledons. In seeds of greenhouse-grown plants, protease inhibitor activity was increased by the addition of exogenous sulfate to the nutrient solution. Since BBPI and related low molecular weight proteinase inhibitors have an unusually high cyst(e)ine content, these compounds have been hypothesized to function in the storage of reduced sulfur. The data presented here support this hypothesis by providing evidence that additional sulfur as Met or sulfate increases the quantity of these proteins in seeds by increasing their mRNA levels.

Keywords: *Bowman−Birk; Glycine max; protease inhibitor; soybean; sulfate*

INTRODUCTION

Our interest in protease inhibitors arose as a byproduct of our attempts to improve the nutritional quality of soybeans by increasing protein sulfur amino acid content (Thompson and Madison, 1992). It is well-known that legume proteins are deficient in the sulfur-containing amino acids (Larkins, 1981). We found that protein methionine (Met) is increased 22% in cultured immature soybean seeds by the addition of Met to the medium (Thompson et al., 1981) due to the absence of the β-subunit (which lacks Met) of conglycinin related to a decrease in β-subunit mRNA (Holowach et al., 1986). During these investigations, we observed that exogenous Met increased the level of low molecular weight mRNAs in cultured cotyledons as compared to those cultured in basal medium (de Banzie et al., 1986). These low molecular weight mRNAs were predominantly the messenger RNAs for the Bowman−Birk protease inhibitor (BBPI). Soybeans contain at least four other similar small proteins soluble in 60% ethanol that we define as soybean protease inhibitor (SPI). The finding of BBPI mRNA was of considerable interest to us because soybean SPIs are proteins with high cyst(e)ine content (20 mol %) and low Met content (<2 mol %) (Odani and Ikenaka, 1972). Although cyst(e)ine is not an essential amino acid, it can reduce the Met requirement by reducing the need for conversion of Met to cyst(e)ine. Hence, changing the cyst(e)ine content could affect the nutritional value of a protein.

There has been an interest in the protease inhibitors of seeds over the years. This interest has been generated by their negative and positive effects on animals and insects that ingest them. For example, it has been known for a long time that raw soybeans do not support the growth of rats as well as heated soybeans (Osborne and Mendel, 1917). There is evidence that protease inhibitors are a significant cause of this problem with raw beans [for a more detailed discussion see Liener (1994)] since heating inactivates the inhibitors (Liener and Tomlinson, 1981; DiPietro and Liener, 1991). On the other hand, protease inhibitors can prevent cancer (Troll and Kennedy, 1993).

The function of SPIs in legume seeds is unknown. Suggested functions include the following: (1) prevention of consumption by insects, etc. (Ryan, 1981; Sakal et al., 1989); (2) prevention of degradation by endogenous enzymes (Tan-Wilson et al., 1985a); (3) acceptor of reducing power (Kobrehel et al., 1991); (4) storage protein to be utilized during germination (Tan-Wilson et al., 1985b).

The classical BBPI is a double-headed inhibitor, inhibiting both trypsin and chymotrypsin (Birk, 1961). This protein contains 71 amino acids, of which 14 are half-cystine residues, involved in 7 disulfide bonds (Odani and Ikenaka, 1973). The disulfide bonds provide the molecule with rigidity and make the molecule relatively resistant to inactivation (DiPietro and Liener, 1989).

The results of our investigation indicate how the level of SPIs in soybean might be changed in a beneficial manner.

MATERIALS AND METHODS

Growth of Soybean Plants. Soybean plants (*Glycine max* L. Merr. cv. Provar) grown with different levels of sulfate were grown in a greenhouse in Perlite and watered with a modified

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Hoagland' nutrient solution (Thompson et al., 1977) containing 1.5 mM $MgCl_2$ and either 0.03, 0.3, 2, or 10 mM K_2SO_4. All other soybean plants were grown on a complete nutrient solution (which contained 1.5 mM $MgSO_4$) (Thompson et al., 1977).

In Vitro Cotyledon Culture. In vitro culture of small (20−30 mg) immature cotyledons was as previously described (Thompson et al., 1977). In experiments in which the effects of Met and sulfate were tested, L-Met and sulfate were added to a sulfur-deficient medium containing 1.5 mM $MgCl_2$, to final concentrations of 8.4 and 1.5 mM, respectively, unless stated otherwise. The immature cotyledons were cultured for 7 days at 25 °C.

Construction and Screening of cDNA Library. Total RNA was prepared from cotyledons cultured in vitro in basal medium or Met-supplemented medium as described by Hall et al. (1978). Poly(A)-containing RNA was obtained by oligo-(dT)-cellulose affinity chromatography (Maniatis et al., 1982). Double-stranded cDNA was synthesized from poly(A)-containing RNA prepared from cotyledons grown on Met-supplemented medium and inserted into lambda gt11 using *Eco*RI linkers (Gubler and Hoffman, 1983; Huynh et al., 1985; Okayama and Berg, 1982). The library was plated out, and duplicate sets of filters were prepared. One set was probed with ^{32}P-labeled cDNA from cotyledons grown on basal medium and the other with ^{32}P-labeled cDNA prepared from cotyledons grown on Met-supplemented medium (Benton and Davis, 1977; Maniatis et al., 1982). Phage colonies that showed stronger hybridization with the latter probe were selected for analysis.

cDNA Sequence Determination. cDNA inserts were isolated from lambda gt11 and subcloned into pGEM 3 (Promega, Madison, WI) as *Eco*RI/*Kpn*I or *Sac*I/*Kpn*I restriction fragments. Nucleotide sequences of plasmid DNAs were determined by the dideoxy chain termination method (Sanger et al., 1977), using sequencing kits from Promega and United States Biochemical (Cleveland, OH). Both DNA strands were sequenced. Sequencing primers were T7 primer and lambda gt11 forward and reverse sequencing primers (New England Biolabs, Beverly, MA). Search of the EMBL/GenBank database employed the sequence similarity and alignment programs of IntelliGenetics (Mountain View, CA).

RNA Electrophoresis and Hybridization. RNA was denatured with formaldehyde and electrophoresed in a 1.5% agarose gel. The lane containing molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD) was excised from the gel and stained with ethidium bromide; the remaining RNA was transferred to nitrocellulose (Maniatis et al., 1982). The hybridization probe was prepared by random hexamer primer labeling of linearized plasmid DNA with [α-^{32}P]dATP (Amersham, Arlington Heights, IL, Multiprime kit protocol). Hybridization was in 5× SSC, 0.1% SDS/10 mM sodium phosphate, pH 6.5, 0.02% insoluble poly(vinylpyrrolidone) (Sigma, St. Louis, MO), 0.02% Ficoll 400, and 0.02% bovine serum albumin in 50% deionized formamide. After hybridization at 52 °C for 8 h, filters were washed twice at 20 °C and then twice at 65 °C in 0.3× SSC/0.1% SDS before autoradiography.

Protease Inhibitor Assay. SPI activity was assayed according to a modification of the method of Kakade et al. (1974). The cotyledons were homogenized in 10 volumes of 60% ethanol at room temperature, and insoluble material was removed by centrifugation for 20 min at 8000*g*. The protein precipitated by 2 volumes of ice-cold acetone was collected and dialyzed (molecular weight cutoff ≈ 3500) overnight in water at 4 °C. The inhibitory activity toward bovine trypsin in this fraction (Bowman's fraction) (Bowman, 1946; Odani and Ikenaka, 1977) was measured using benzoyl-DL-arginine *p*-nitroanilide as the substrate and with purified lima bean inhibitor (Sigma) as a reference.

Cyst(e)ine Analysis. Uncombined cyste(i)ne was measured as pyridylethylcysteine after treatment of an extract with 4-vinylpyridine (Cavins and Friedman, 1970). Uncombined amino acids were extracted with 80% (v/v) ethanol as described (Holowach et al., 1984b). The dried extract was dissolved in water, and an aliquot suitable for analysis was diluted to 250 μL with water; 40 μL of 1 M Tris-Cl, pH 8.4, was added followed by 1 μL of β-mercaptoethanol. After 10 min at room temperature, 3 μL of 4-vinylpyridine (Aldrich Chemical Co., Milwaukee, WI) was added to convert cysteine to pyridylethylcysteine. After 30 min, 10 μL of mercaptoethanol was added to remove unreacted vinylpyridine. The sample was acidified with 290 μL of 0.5 M HCl, and the entire sample was analyzed on a Beckman model 119CL amino acid analyzer using a 0.35 M Na^+, pH 5.12, buffer (Moore et al., 1958).

RESULTS

As part of our program to raise the sulfur amino acid content of soybeans, we have investigated the effects of Met on mRNA levels in cultured soybean seeds. cDNA libraries prepared from cotyledons cultured in basal or Met-supplemented media were screened with labeled cDNA prepared from cotyledons cultured on basal medium and with labeled cDNA prepared from cotyledons cultured on Met-supplemented medium.

Three clones (1-1, 6-4, and 6-7), which hybridized preferentially with the labeled cDNA from cotyledons cultured on Met-supplemented medium, were isolated. The inserts of these clones were sequenced and found to differ only in the length and location of the poly(A) tail. The 3′-untranslated regions of clones 1-1 and 6-4 were 14 and 12 residues, respectively, shorter than that of clone 6-7. The nucleotide sequence of clone 6-7 has been deposited in the GenBank as Accession U11260.

The predicted amino acid sequence of clone 6-7 was found to be identical to that of the soybean BBPI (Odani and Ikenaka, 1977), except for the presence of an additional C-terminal tyrosine residue. A missing deoxyadenylate in our sequence resulted in the terminating TAA codon becoming a TAC codon for tyrosine, which is immediately followed by a TAA termination sequence. The nucleotide sequence of the coding region was 94% identical to that of a BBPI cDNA clone sequenced by Hammond et al. (1984, 1985) and 97% identical to the sequence reported by Baek and Kim (1993). All of the differences in the nucleotide sequence in the coding region occurred in the third ("wobble") position of the codon and did not affect the amino acid sequence. The 3′-nontranslated region of clone 6-7 was identical to that of Baek and Kim for 120 nucleotides. These two sequences, however, showed little similarity (16%) to the 3′-untranslated region reported by Hammond et al. (1984). The 3′-nontranslated region of clone 6-7 contained two polyadenylation signal sequences (AATAAA), in contrast to the single partial sequence (AATAA) reported by Hammond et al. (1984).

Northern blot analysis using clone 6-7 demonstrated that the insert was derived from an RNA that is increased by Met (Figure 1). The clone hybridized to a single RNA band considerably smaller than the 18S ribosomal RNA band. The hybridization to total RNA from cotyledons cultured in basal medium was only ∼15% as strong as that detected using RNA from cotyledons cultured in Met-supplemented medium. The effect of Met on the level of SPI mRNA was very similar to the effect of Met on the level of protease inhibitor activity in cotyledons cultured in vitro (∼7-fold increase in both cases). Trypsin inhibitor activity was assayed in a partially purified fraction (Bowman's fraction) derived from a 60% ethanol soluble material that precipitated with 67% acetone. This purification separates double-headed protease inhibitors from the Kunitz

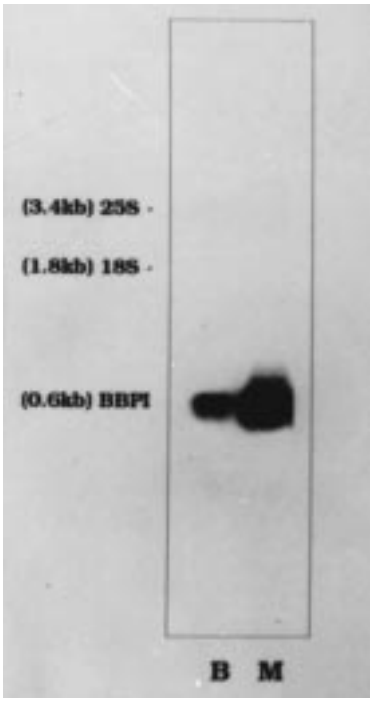

Figure 1. Hybridization of ^{32}P-labeled cDNA clone 6-7 to blot transfer of RNA extracted from soybean cotyledons cultured in basal (B) or Met-supplemented (M) medium. Twenty micrograms of total RNA was electrophoresed in each lane. On the left-hand side the positions of the 18S and 25S ribosomal RNAs are shown.

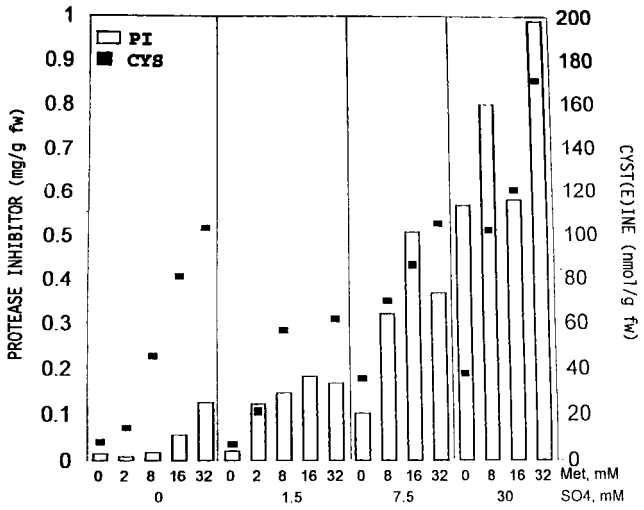

Figure 2. Effect of Met and sulfate concentrations on the level of protease inhibitors and uncombined cyst(e)ine in cultured soybean cotyledons. Culture media contained the indicated concentrations of Met and sulfate. Protease inhibitor concentrations and cyst(e)ine contents were assayed as described under Materials and Methods. The results are from a single experiment that are typical of a large number of analyses.

protease inhibitor (Bowman, 1946; Odani and Ikenaka, 1977).

Raising the Met and/or sulfate level in the medium increased the amount of trypsin inhibitor activity as much as 70-fold (Figure 2). The reduced response to Met in the absence of added sulfate is not due to limiting sulfur, since cultured cotyledons can grow at a maximum rate using Met as their sole sulfur source (Holowach et al., 1984a).

Figure 2 also shows the uncombined cyst(e)ine content of the cultured cotyledons. Since SPI contains such a

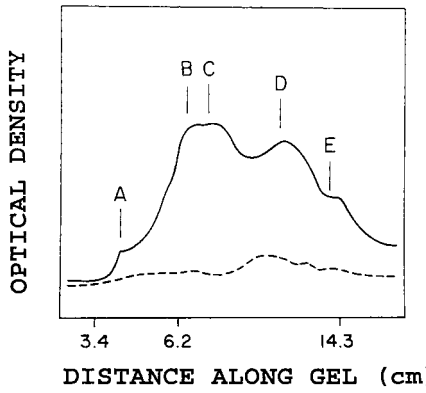

Figure 3. Effect of Met on the level of proteins from cultured cotyledons soluble in 60% ethanol (Bowman's fraction) (Bowman, 1946). Ethanol soluble protein from equivalent amounts of cotyledons that had been cultured with and without supplemental Met were separated by SDS electrophoresis through a 15% polyacrylamide gel, silver-stained (Blum et al., 1987), and scanned with an LKB scanning densitometer. Lines with numbers show positions and molecular weights of marker proteins electrophoresed on the same gel. Lines show positions of protein peaks, as determined by the LKB GELSCAN program. Three experiments gave similar results. The solid line shows the proteins for the cotyledons grown with supplemental Met, and the dashed line shows the proteins for cotyledons grown on basal medium.

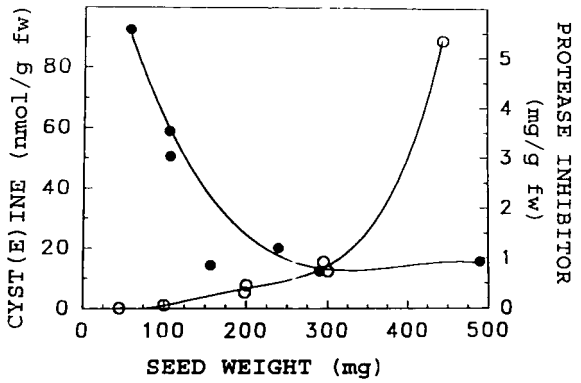

Figure 4. Uncombined cyst(e)ine content (solid circles) and accumulation of protease inhibitor (open circles) in soybean seeds grown on plants grown in the greenhouse on nutrient solution containing 1.5 mM sulfate.

large mole percent of cyst(e)ine (~20%), the uncombined cyst(e)ine was measured (Figure 2) to see if it correlated with the protease inhibitor content. In general, the cyst(e)ine level was higher at elevated Met levels but was not well correlated with protease inhibitor level.

SDS−polyacrylamide gel electrophoresis of the protease inhibitor fractions revealed that at least five low molecular weight proteins were more abundant in cotyledons cultured on Met-supplemented medium than on basal medium (Figure 3).

The relationship between uncombined (nonprotein or free) cyst(e)ine concentration and SPI content in developing soybean seeds (Figure 4) shows that accumulation of SPI was not directly correlated with the cyst(e)ine level. A possible intrepretation of these data is that seeds smaller than ~300 mg are developmentally unable to accumulate significant amounts of SPI.

The effect of sulfate supply on the level of protease inhibitors in the Bowman' fraction in seeds produced on greenhouse-grown plants is shown in Figure 5. No signs of sulfate deficiency were observed, and plant growth and seed production did not differ significantly

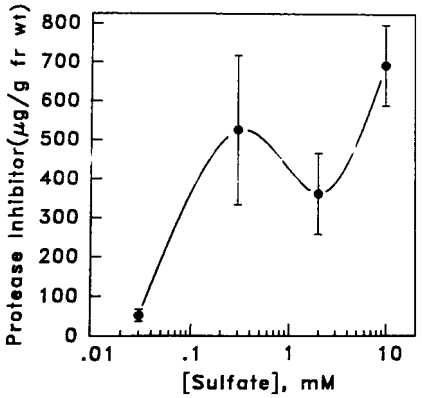

Figure 5. Effect of sulfate supply on protease inhibitor content of soybean seeds. Plants were grown in a greenhouse in Perlite fertilized twice a week with a nutrient solution containing 0.03, 0.3, 2, or 10 mM sulfate. Seeds (14−21; 260−310 mg in weight) were harvested from plants grown at each of the sulfate levels. Protease inhibitor concentration was determined in triplicate as described under Materials and Methods. Bars represent standard error of the mean.

at any sulfate level tested. The level of protease inhibitor in the Bowman fraction from immature seeds was found to be lower in the seeds from the plants receiving 0.03 mM sulfate, indicating that the SPI level was limited by sulfur supply.

Many compounds other than sulfate were added to the cotyledon culture medium in an attempt to increase the uncombined cyst(e)ine content of the cotyledons. Among the compounds tried were cysteine, cystine, glutathione, glutathione ethyl ester, sodium sulfide, homocysteine, homocystine, homocysteine thiolactone, *N*-acetylcysteine, serine, *O*-acetylserine, cysteine ethyl ester, cystine−bis(glycine), and (−)-2-oxo-4-thiazolidinecarboxylic acid [which is readily converted to cysteine by endogenous 5-oxoprolinase (Williamson et al., 1982)]. Most of the compounds that significantly increased the free cysteine content also inhibited growth. None of these compounds, except for *N*-acetylcysteine, caused the SPI level to increase significantly. *N*-Acetylcysteine increased the cysteine content, but it also increased the uncombined Met level. It is possible that some of these compounds were not readily absorbed by the immature seeds.

DISCUSSION

The lack of homology between the 3′-noncoding region of the BBPI cDNA described by Hammond et al. (1985) and two other sequences (Odani and Ikenaka, 1972; Baek and Kim, 1993) suggests that BBPI is encoded by at least two genes. It is also possible that there is only one BBPI gene and that the 3′-noncoding region varies with the cultivar.

Although SPI has slightly more Met than total seed protein (1.41 versus 1.16−1.25 mol %) and Met increases SPI, calculations show that the increase in SPI cannot contribute significantly to the 22% increase in protein Met caused by the addition of Met to the culture medium (Thompson et al., 1981). Changes in the amounts of 7S and 11S proteins account for most of the increased protein Met (Holowach et al., 1984b).

The ability to produce relatively large amounts of sulfur-rich seed storage proteins when reduced sulfur is readily available would increase the amount of sulfur-containing amino acids in the seed without severely restricting protein production when sulfur is limiting. Formation of the SPI family of protease inhibitors may provide a means for storing excess sulfur, in addition to the accumulation of sulfate and glutathione in the vacuole [reviewed by Rennenberg (1984)]. The ability of these protease inhibitors to play a dual role as an inducible storage protein and a protease inhibitor may have adaptive value.

The data in Figure 2 show that sulfate is a more effective stimulator of SPI synthesis than is Met. For example, 30 mM sulfate in the medium results in about 4 times more SPI than does 32 mM Met. However, at any given sulfate concentration, the addition of Met resulted in a further increase in the SPI content.

Figure 2 also indicates that free cyst(e)ine is not the effective elicitor of protease inhibitor synthesis. Again, comparing the uncombined cyst(e)ine content of cotyledons cultured with 32 mM Met or 30 mM sulfate shows that there is no correlation between SPI level and uncombined cyst(e)ine content, since growth with Met resulted in almost 3 times more cyst(e)ine and only one-fourth the SPI content. Perhaps sulfate and some form of reduced sulfur are both required for the maximal stimulation of SPI synthesis. It is also possible that total uncombined cyst(e)ine is not a good indicator of effective cyst(e)ine because local concentrations may be more important.

Also, in this regard, Figure 4 shows that the uncombined cyst(e)ine content of soybean seeds grown on the plant is decreasing at the same time that protease inhibitor is increasing rapidly. This finding indicates that cyst(e)ine does not provide the impetus for SPI synthesis. In contrast to our results, Macnicol and Randall (1987) concluded that cysteine is likely to be the signal that is involved in stimulating the synthesis of legumin mRNA in pea cotyledons recovering from sulfur deficiency.

At least five different proteins soluble in 60% ethanol ranging in apparent size from 4000 to 13000 were more abundant in cotyledons grown in Met than in cotyledons grown on basal medium (Figure 3). Whereas we have made no attempt to identify the individual peaks, their size and ethanol solubility make it possible that they are members of the Bowman−Birk family of protease inhibitors since there is evidence for several members of this family in soybeans (Hwang et al., 1977; Tan-Wilson et al., 1985a,b; Odani et al., 1987).

ABBREVIATIONS USED

SPI, soybean protease inhibitor; BBPI, Bowman−Birk protease inhibitor; cyst(e)ine, cysteine plus half-cystine; Met, methionine; SSC, 150 mM NaCl/15 mM sodium citrate, pH 7; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; fw, fresh weight.

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