Effect of Sulfur Deficiency on the Synthesis of α-Setarin, a Methionine-Rich Protein of Italian Millet

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ABSTRACT

Seeds of Italian millet (*Setaria italica* (L.) Beauv.) variety K 221-1 were grown in sand culture supplied with low (0.0125 m*M*), medium (0.125 m*M*), and high (1.25 m*M*) concentrations of sulfur (S) as magnesium sulfate, and seeds were analyzed for the amounts of α-setarin synthesized. Plants raised under sulfur-deficient conditions showed clear visual symptoms of sulfur deficiency, and the α-setarin synthesis was reduced considerably as shown by crossed immunoelectrophoresis, rocket immunoeletrophoresis, and avidin-biotin micro titer enzyme-linked immunosorbent assay (AB-microELISA). Immunochemical techniques were also instrumental in the present study in identifying storage proteins among other millets that cross react with the antibodies raised against α-setarin.

Setarin, the alcohol-soluble protein of Italian millet (*Setaria italica* (L.) Beauv.) is relatively rich in methionine, an essential amino acid. We recently isolated and characterized a methionine-rich protein, α-setarin, from the prolamin fraction of Italian millet endosperm flour (Naren and Virupaksha 1990).

Several workers have shown that sulfur nutrition has an influence on the synthesis of sulfur-rich storage proteins of lupin (Gillespie et al 1978), wheat (Castle and Randall 1987), and peas (Millerd et al 1979). In the present study, we describe the effect of sulfur deficiency on the synthesis of α-setarin. This paper also illustrates the application of quantitative immunochemical methods to the investigation of α-setarin synthesis in millet.

MATERIALS AND METHODS

Plant Culture Methods

Seeds of Italian millet, variety K 221-1, were used for the isolation of α-setarin and as a source of sulfur-deficient grains. Plants were raised in sand culture in two rows, 10 cm apart, in Bakelite pots. After periodic thinning of plant populations, 10 plants in each pot were retained to maturity. Plants received modified complete Hoagland's nutrient medium (Epstein 1972) for six weeks. Magnesium sulfate served as the sulfur source, and from the sixth week onwards until two weeks before harvest the following sulfur regime was maintained: low sulfur (0.0125 m*M* $MgSO_4$, 0.4 ppm), medium sulfur (0.125 m*M* $MgSO_4$, 4 ppm), and high sulfur (1.25 m*M* $MgSO_4$, 40 ppm).

Chemical Analyses

N was estimated by the microKjedahl method (Bailey 1972) and S was estimated after wet ashing of samples (Blanchar et al 1965).

Immunochemical Methods

α-Setarin was prepared by cryoprecipitation of prolamin extracts (Naren and Virupaksha 1990). Antiserum against α-setarin was raised in rabbits. A particulate suspension of 1 mg of α-setarin in 1% (w/v) Triton X-100 mixed with an equal volume of Freund's complete adjuvant was injected intramuscularly followed by biweekly booster doses of 0.5 mg of α-setarin suspension in Triton X-100.

The prolamin fraction (setarin II) was extracted from the defatted whole seed flour with 70% (v/v) ethanol, 0.6% (w/v) sodium acetate, 0.5% (v/v) 2-mercaptoethanol, 0.5% (w/v) sodium dodecyl sulfate (SDS) solvent system (EtOH/NaOAC/MSH/SDS). The extract was used as the antigen preparation in immunoassays. Purified α-setarin was dissolved in EtOH/NaOAC/MSH/SDS solvent and used as standard antigen for quantification in immunoassays.

Double immunodiffusion was performed in petriplates according to the procedure of Ouchterlony (1958) and crossed immunoelectrophoresis was conducted according to Talwar (1983). Rocket immunoelectrophoresis was carried out using the procedure of Weeke (1973). A linear relationship between the area of the immunoprecipitin peak (rocket area) and α-setarin concentration over a 1-7 μg range was demonstrated. Avidin-biotin micro titer enzyme-linked immunosorbent assay (AB-microELISA) was performed as described by Rao et al (1983). Affinity purified anti-rabbit IgG-biotin conjugate suitably diluted with serum diluent buffer was used in conjunction with horseradish peroxidase-avidin conjugate. MicroELISA plates were read at 492 nm in a microELISA reader (Dantech Lab Inc., Alexandria, VA). One ELISA unit was arbitrarily defined as equivalent to an absorbance of 1 at 492 nm.

RESULTS AND DISCUSSION

Plants were raised on 0.4, 4.0, and 40 ppm S in the nutrient medium representing deficient, adequate, and more than required levels of S, respectively. Plants receiving deficient levels of S showed symptoms of deficiency, with the onset of maturity that intensified towards the end of maturity. Some of the visual symptoms exhibited by deficient plants were stunted growth and yellowing of leaves, whereas the plants given optimal or adequate levels of S were healthy in appearance. Sulfur deficiency also led to a decrease in the dry weight of plants, 1,000-kernel weight, and other physiological parameters (Table I). Sulfur concentration in the seeds was low in millet samples grown in sulfur-deficient medium, whereas S levels in samples from plants given adequate or high sulfur levels were not affected. However, N levels in the seeds of the plants were not affected by levels of S treatment, with the result that N/S ratios were quite high in the seeds of plants given low S. This ratio is a critical value for plant nutrition. Furthermore, Randall et al (1981) considered grain S content important for breadmaking quality in wheat in addition to its role in determining the nutritional status of the grain. Sulfur nutrition does alter the grain quality in Italian millet by increasing the N/S ratio when inadequate S is supplied.

Quantitative estimates of the major changes in sulfur-rich proteins (particularly α-setarin) as a result of deficiencies of S were made by the application of immunochemical techniques. Prolamin (setarin II) extracts prepared from plants given different sulfur treatments were used in crossed immunoelectrophoresis (CIE) to obtain semiquantitative estimates. Figure 1 shows the tracing of the immunoprecipitation lines obtained with the pro-

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TABLE I
Physiological Parameters of Italian Millet (Variety K 221-1) Grown under Different levels of Sulfur Nutrition[a]

Sulfur	Dry Weight (g)	Grain Yield (g)	Harvest Index (%)	1,000-Kernel Weight (g)	Grain S (%)	Grain N (%)	N/S
Low	22.8	3.0	13.17	3.007	0.08	2.25	28.13
Medium	26.0	3.9	16.96	3.325	0.12	2.20	18.33
High	26.2	4.1	15.54	3.387	0.12	2.30	19.25
F test[b]	NS	**	**	NS	**	**	**
CD[c] at 1%	...	0.086	0.456	...	0.00812	0.0115	0.106

[a]The three treatments were subjected to five replications and the average values are shown.
[b]NS = Nonsignificant; ** = highly significant.
[c]Critical difference.

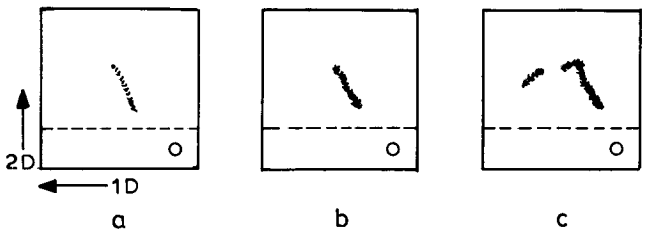

Fig. 1. Tracings of crossed immunoelectrophoresis patterns of Italian millet (variety K 221-1): low-sulfur seeds (**a**), medium-sulfur seeds (**b**), high-sulfur seeds (**c**). Rabbit antiserum against α-setarin was used.

lamin preparations from plants treated with different levels of sulfur. In sulfur-deficient seeds, a weak immune arc is seen, and the intensity of this immune arc increased with adequate and more than adequate levels of sulfur treatments (Fig. 1A–C). The latter, in addition, showed another faint immune precipitin line that could not be discerned in plants treated with 0.4 and 4 ppm S. The immune lines obtained from control (field-grown) seeds were similar to those seen in seeds raised on 4 ppm S in pot culture (tracings not included). Since the antiserum used in CIE analysis was raised against α-setarin, it is evident from these results that the synthesis of α-setarin is depressed under conditions of sulfur deficiency.

Quantitative estimates of α-setarin levels of seeds from different sulfur treatments were made by rocket immunoelectrophoresis (RIE). A standard curve was constructed from a plot of α-setarin concentrations (0–7 μg) versus the areas under the rockets (results not shown), and the amounts of α-setarin in the crude extracts of Italian millet seeds were quantified by reference to the standard curve (Table II). The results indicated that seeds treated with low levels of sulfur had 0.56% α-setarin, representing 7.72% of the total extractable prolamin fraction. Plants raised on adequate levels of S as well as the control plants had 0.7% α-setarin, 9.33% of the total extractable prolamin fraction. Plants treated with high sulfur levels (40 ppm) in growth medium had 0.72% α-setarin, 9.58% of the total extractable prolamin fraction. Thus, it is evident that under conditions of S deficiency, the level of α-setarin, the major sulfur-rich protein of the prolamin fraction of Italian millet, is reduced considerably. Levels of sulfur less than 0.4 ppm in the nutrient medium might reduce the α-setarin levels in the prolamin fraction even more drastically if the plants could be raised to the stage of inflorescence, seed setting, and maturity. However, since the minimum level of S in the growth medium required to raise Italian millet plants to maturity in pot cultures is not known, levels of S lower than 0.4 ppm were not investigated. CIE and RIE have been used extensively in studies of seed storage proteins of *Vicia faba* by Millerd et al (1978) and pea seed proteins by Guldager (1978). The results of the present work are in agreement with those of Randall et al (1979), who provided convincing evidence on the specific effects of sulfur deficiency on the storage protein synthesis in *Pisum sativum* using CIE profiles. However, these investigators used multivalent antiserum raised against protein body extracts of *Pisum sativum,* whereas in the present work monospecific antiserum raised against α-setarin was used. Randall et al (1979) further demonstrated the suitability

TABLE II
Estimation of the α-Setarin Levels of Italian Millet Prolamin Extracts by Rocket Immunoelectrophoresis (RIE) and AB-microELISA Assays[a]

Treatment	α-Setarin Levels: RIE (% of prolamin)[b]	α-Setarin Levels: AB-microELISA (ELISA units)[c]
Control[d]	9.33 (100.0)	0.55 (100.0)
Low sulfur	7.72 (82.7)	0.47 (82.7)
Medium sulfur	9.33 (100.0)	0.53 (100.0)
High sulfur	9.58 (102.0)	0.56 (101.8)

[a]Prolamin was extracted from whole seed flour with EtoH/NaOAC/MSH/SDS solvent using a flour-to-solvent ratio of 1:10 (w/v).
[b]Average of three replications; replicates did not differ by more than 5%.
[c]Average of four replications; replicates did not differ by more than 5%. ELISA units are as defined in the text.
[d]Control consists of field-grown millet seed samples. Values in parenthesis are percentage of control taken as 100.

of RIE to evaluate the effects of deficiency of certain nutrients such as sulfur on the production of storage proteins, namely legumin, in peas. They concluded that legumin levels in pea cotyledons decreased when low S (0.2 ppm) nutrition was given to plants.

AB-microELISA was another immunotechnique used to quantify the relative amounts of α-setarin in the prolamin fractions of Italian millet samples raised on different levels of S. Table II gives the results in arbitrary ELISA units. Millet seeds treated with low S levels have lower ELISA units than the seeds treated with 4 or 40 ppm S and the control (field grown) samples (Table II). AB-microELISA results thus substantiate the results of CIE and RIE. It is also of interest to note that pearl millet (*Pennisetum americanum* L.) prolamin fraction contains considerable amounts of a protein capable of cross reacting with antisera raised against α-setarin in the AB-microELISA assay. The amount of cross-reacting material in the crude extracts of the prolamin fraction of pearl millet by this assay was 0.189 ELISA units. ELISA is a sensitive technique, and Craig et al (1980) demonstrated that it is possible to estimate as little as 10 ng of storage proteins using ELISA. This technique has been used extensively in quality control of beer and in studies on storage protein synthesis in barley (Vaag 1985). Windemann et al (1982) used an ELISA technique to quantitate reactions to gliadin antisera.

Because AB-microELISA assays revealed the presence of a protein in pearl millet immunologically similar to α-setarin in Italian millet, it was of interest to screen other millet and cereal seeds for the presence of cross-reactive material by the double immunodiffusion methods of Ouchterlony (1958). Double immunodiffusion confirmed the presence of a strongly cross-reactive material against the α-setarin antisera in the prolamin fraction of the pearl millet, whereas finger millet, kodo millet, Japanese barnyard millet, and proso millet prolamin fractions gave much less intense immunoprecipitin lines (results not shown). Cereals such as wheat, maize, rice, sorghum, and barley did not show the presence of any immunereactive protein in the alcohol-soluble protein fraction. It appears from these results that pearl millet is phylogenetically more closely related to Italian millet than other

millets. Serology is probably most effective in the investigation of relatedness or otherwise of members of different taxa. It is assumed that the intensity of precipitin lines in the cross reaction of an antigen with antibody is an indication of the degree of similarity between the homologous and heterologous antigens and hence the taxanomic affinity (Vaughan 1983). Immunodiffusion tests conducted by Festenstein et al (1984) using the antiserum against C-hordein, the prolamin protein of barley, showed that most often prolamins belonging to the subfamily Festucoideae of the Graminae—such as wheat, maize, and barley—react with the C-hordein antiserum, whereas the prolamins of the subfamily Panicoideae, to which millets belong, do not react. Our results show that antiserum against α-setarin does not exhibit cross reactivity with the prolamin fraction of cereals (maize, rice, sorghum, barley, and wheat) but does with the prolamin of millets, thus complementing the results of Festenstein et al (1984). It is worth investigating whether the cross-reactive material in the prolamin fraction of pearl millet has structural homology with α-setarin and whether it has a high content of sulfur amino acids.

CONCLUSIONS

We demonstrated that adequate sulfur nutrition is required for the synthesis of α-setarin, a storage prolamin of *Setaria italica* that is rich in sulfur amino acids. Quantitation of the levels of α-setarin deposited in seeds under sulfur-deficient and sulfur-sufficient conditions was possible using appropriate immunochemical techniques. In the present work, effects of sulfur deficiency on the synthesis of β- and γ-setarins were not investigated. Earlier studies show that in all the species so far investigated the sulfur-rich polypeptides are quantitatively reduced by sulfur deficiency (Randall et al 1979, Randall and Wrigley 1986).

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