α- and β-Setarins: Methionine-Rich Proteins of Italian Millet (*Setaria italica* (L.) Beauv.)

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ABSTRACT

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Differential cryoprecipitation was employed for the purification of two methionine-rich proteins, α-setarin and β-setarin from the alcohol-soluble prolamin fraction (setarin II) of Italian millet flour. Both α- and β-setarins were low molecular weight polypeptides rich in sulfur amino acids. Methionine accounted for eight residues each out of a total of 63 and 71 amino acid residues in α- and β-setarin, respectively. The peptide map pattern of cyanogen bromide cleaved α-setarin suggests that the methionine residues were randomly distributed throughout the polypeptide chain.

The alcohol-soluble prolamin of Italian millet (*Setaria italica* (L.) Beauv.), referred to here as setarin, is the major storage protein in the millet endosperm. Setarin has a nutritionally unfavorable amino acid composition, being severely deficient in lysine and tryptophan (Monteiro et al 1982). However, it is relatively rich in methionine compared with the other solubility classes of Italian millet endosperm protein, albumins-globulins and glutelins. Setarin consists of several electrophoretically distinct species of polypeptides (Monteiro et al 1982), and it is not known whether any or all of these proteins are rich in methionine. Melcher and Fraij (1980) were able to isolate a protein fraction rich in methionine from zein (the prolamin of corn) by cryoprecipitation of corn extracts. Therefore, it was of interest to isolate methionine-rich proteins from setarin and study their chemical properties.

Italian millet constitutes a staple food crop for a sizable section of the population in India. Millet-based diets are usually supplemented with pulses, which are also deficient in sulfur amino acids, cysteine, and methionine. Supplementation of foods with L-methionine to achieve balance is not entirely satisfactory for consumer acceptability because it imparts a strong cabbage note with an unsavory aftertaste (Damico 1975). Isolation and characterization of proteins rich in sulfur amino acids and identification of the genes responsible for their synthesis may provide a basis for improving protein quality through gene manipulation, which has so far been beyond the traditional methods of plant breeding. Unlike the work with corn and other cereals, where considerable progress has been made in the identification of genes responsible for prolamin synthesis (Wilson and Larkins 1984), very little is known about the genetic aspects of setarin biosynthesis. In this communication we describe the purification and characterization of α- and β-setarin methionine-rich proteins of Italian millet.

MATERIALS AND METHODS

Preparation of Seed Material

Italian millet variety K 221-1 was grown at the University of Agricultural Sciences Farm, Bangalore, and harvested in August 1986. Farm yard manure (6,000 kg/ha) was added to the field two to three weeks before sowing, and 30 kg of N/ha and 15 kg of P_2O_5/ha were applied at the time of sowing. Millet seeds were ground in a coffee mill and sieved to pass through a 0.375-mm sieve. Flour was defatted by stirring at ambient temperature (27° C) with acetone; the solvent was removed by suction filtration and air-dried.

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Isolation of Setarins

Three setarin fractions were isolated from the prolamin fraction of Italian millet by a modified cryoprecipitation procedure of Melcher and Fraij (1980) as shown in Figure 1. All extractions were done at ambient temperature for 30 min, and supernants were recovered by centrifugation at 2,000 × *g* for 10 min. Samples were frozen overnight at −20° C and cryoprecipitates were recovered by centrifugation at −10° C at 2,000 × *g* for 10 min.

Analytical Methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% acrylamide gels was performed according to Laemmli (1970). Bovine serum albumin (66K), ovalbumin (43K), soybean trypsin inhibitor (20.1K), lysozyme (14.3K), and insulin β-chain (3.5K) were used as standard marker proteins.

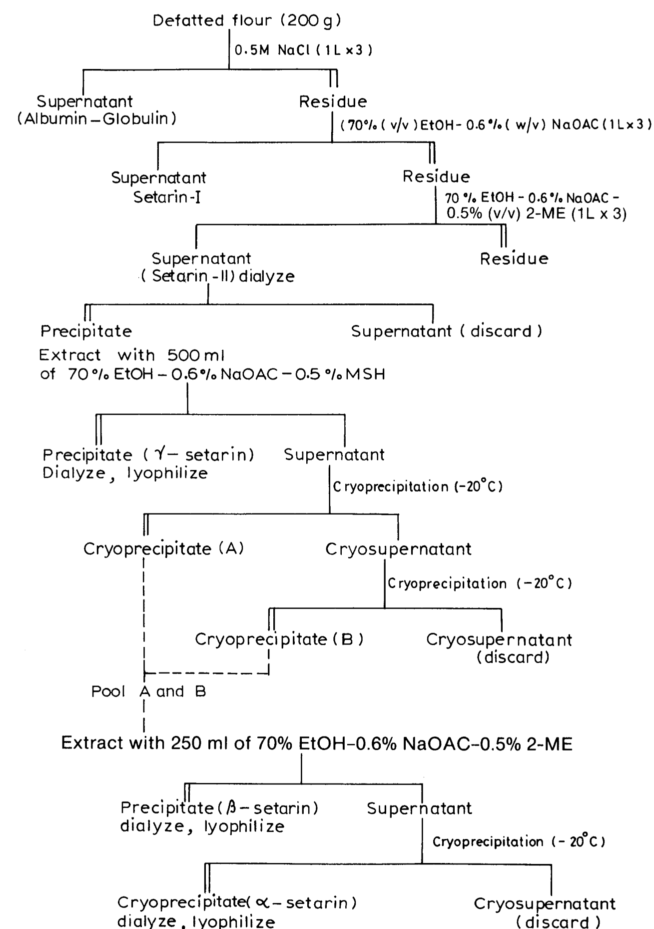

Fig. 1. Scheme of isolation of methionine-rich proteins. EtOH = ethanol, NaOAC = sodium acetate, 2-ME = 2-mercaptoethanol.

TABLE I
Sequential Extraction of Italian Millet (variety K 221-1) Protein Fractions[a]

Solvent System/ Protein Fraction	Yield[b] (%)	Nitrogen[c] (%)	Protein (%N × 6.25)	Sulfur[d] (%)
Whole flour	···	1.98	12.34	0.10
0.5*M* NaCl				
Albumin + globulin	1.43	12.00	75.00	0.10
70% Ethanol/0.6% sodium acetate				
Setarin I	6.60	12.50	78.13	0.19
70% Ethanol/0.6% sodium acetate/2-mercaptoethanol				
Setarin II	2.24	13.50	85.40	0.29
γ-setarin	0.23	12.50	78.13	0.36
β-setarin	0.09	15.00	93.75	0.69
α-setarin	0.14	15.82	98.86	0.79

[a]Values are averages of duplicate determinations. Duplicates did not differ by more than 5%.
[b]Yield of protein fraction (g/100 g of flour).
[c]Nitrogen was estimated according to the micro-Kjeldahl method (Bailey 1972).
[d]Sulfur was estimated after wet digestion with a mixture of nitric acid and percholoric acid (Blachar et al 1965).

Nitrogen in samples was estimated by micro-Kjeldahl analysis (Bailey 1972) and expressed as protein (N × 6.25, %) on a moisture-free basis. Sulfur was estimated after digestion of samples with nitric acidic acid/perchloric acid mixture according to Blachar et al (1965).

Weighed amounts of protein samples were hydrolyzed with 6*N* HCl in vacuo at 110°C for 22 hr for amino acid analysis by the procedure of Spackman et al (1958) on an LKB 4004 amino acid analyzer.

Peptide mapping on cyanogen bromide (CNBr) cleaved (Gross 1967), performic acid oxidized (Hirs 1967) protein samples was performed on Whatman No. 3 filter paper according to Bennett (1967). First dimensional electrophoresis was run in acetate-formate buffer (pH 1.9) for 90 min at 1,500 V, and second dimension ascending chromatography was run in a solvent system of *n*-butanol, pyridine, acetic acid, and water (9:16:10:7.2).

Amino terminal analysis of proteins by dansylation was performed according to Gray (1967) using dansyl chloride.

RESULTS AND DISCUSSION

Sequential extraction of the defatted millet flour according to the scheme shown in Figure 1 resulted in three major protein fractions, namely, albumin-globulin, setarin I (true prolamin), and setarin II (prolamin-like). Yields by weight, protein, and sulfur content of various protein fractions are summarized in Table 1. Further fractionation of the setarin II protein by cryoprecipitation gave three protein preparations designated as γ-, β-, and α-setarin, based on their increasing solubility in the solvent system of 70% ethanol (v/v), 0.6% sodium acetate (w/v), and 0.5% 2-mercaptoethanol (v/v), γ-setarin being the least soluble. α-Setarin had the highest sulfur content of the three (Table I) and was also comparatively pure because it contained only about 1% non-proteinaceous matter. It is clear that cryoprecipitation could be successfully employed to isolate proteins enriched in sulfur amino acids. Melcher and Fraij (1980) reported the enrichment of methionine-rich polypeptides of zein II protein fraction by cryoprecipitation. Modification of their cryoprecipitation procedure gave a higher degree of purification of one of the sulfur-rich polypeptides of the setarin II prolamin in the present work.

Amino acid compositions of setarin II, α-setarin, and β-setarin are shown in Table II. α-Setarin had high amounts of the sulfur amino acids methionine and cysteine. Both α- and β-setarin contained considerably higher amounts of the sulfur amino acids than setarin II; the levels of methionine in α- and β-setarin were 7.3- and 6.5-fold higher, respectively. Melcher and Fraij (1980) reported that the methionine-rich polypeptide fraction of corn they isolated by cryoprecipitation was 6.4-fold higher in methionine than in zein I polypeptide. The amino acid compositions of α- and β-setarin were similar with respect to most amino acids; however, α-setarin had significantly lower amounts of glutamic acid, proline, and lysine and higher amounts of leucine and phenylalanine. Setarin II was distinct from both α- and β-setarin in possessing negligible amounts of serine, tyrosine, and cysteine but had much higher levels of alanine, leucine, and glutamic acid. The amino acid composition of polypeptides present in the cryosupernatant after recovery of α-setarin was not examined.

TABLE II
Amino Acid Composition of Setarin II, α-Setarin, and β-Setarin Fractions (mol/100 mol)

Amino Acid	Setarin II	α-Setarin[a]	β-Setartin[a]
Aspartic acid	6.03	4.76 (3)	4.23 (3)
Threonine	Traces	4.76 (3)	4.23 (3)
Serine	0.05	3.17 (2)	4.23 (3)
Glutamic acid	22.27	14.29 (10)	18.31 (13)
Proline	10.85	11.11 (7)	14.08 (10)
Glycine	6.88	4.76 (3)	4.23 (3)
Alanine	16.54	11.11 (7)	9.80 (7)
1/2 Cystine[b]	Traces	4.76 (3)	4.23 (3)
Valine	5.58	3.17 (2)	2.82 (2)
Methionine[b]	1.74	12.69 (8)	11.27 (8)
Isoleucine	3.79	1.59 (1)	2.82 (2)
Leucine	16.38	9.52 (6)	7.04 (5)
Tyrosine	0.04	3.17 (2)	2.82 (2)
Phenylalanine	5.78	7.94 (5)	5.63 (4)
Histidine	1.52	1.59 (1)	1.40 (1)
Lysine	1.75	Traces	1.40 (1)
Arginine	0.80	1.59 (1)	1.40 (1)
N recovery, %	79.50	86.66	82.66

[a]Values in parenthesis indicate the number of residues based on arginine.
[b]Chromatograms did not show any cysteic acid or methionine sulfoxide in the hydrolyzates. Tryptophan could not be detected by the spectrophotometric method.

Setarin II, α-, β-, and γ-setarins were analyzed by SDS-PAGE under reducing conditions (Fig. 2). The electrophoretic patterns of setarin II revealed several polypeptides in the molecular weight range of 43.6–7.9K. α-Setarin showed a single electrophoretic band of low molecular weight (7.9K). β-Setarin also gave a major band corresponding to a molecular weight of 9.1K. On the other hand, γ-setarin consisted of three polypeptides of 7.9K, 21.8K, and 36.3K, of which the low molecular weight polypeptide was the most prominent. α-Setarin appeared to be electrophoretically homogeneous in SDS-PAGE.

Estimates of the minimum molecular weights of α- and β-setarin could be made from the amino acid compositions (number of residues) of these polypeptides. These estimates of molecular weights, 7.4K for α-setarin and 8.3K for β-setarin, are in close agreement with the molecular weight estimates arrived at by SDS-PAGE. Compared to other methionine-rich proteins, α- and β-setarin are low molecular weight polypeptides. Paulis and Wall (1971) obtained methionine-enriched polypeptides of 17.5K from

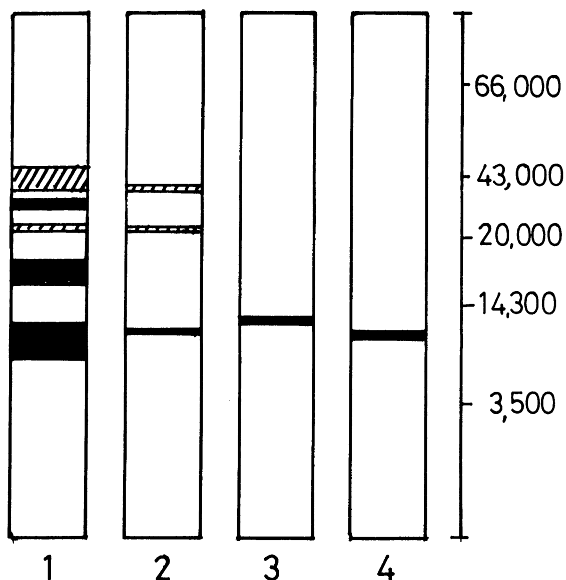

Fig. 2. Diagrammatic representation of the electrophoretic patterns of protein fractions of Italian millet (variety K 221-1) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Setarin II (**1**), γ-setarin (**2**), β-setarin (**3**), and α-setarin (**4**). Each track was loaded with 30 μg of protein

alcohol-soluble glutelin by gel filtration. A-secalin of rye was reported to have a polypeptide of molecular weight 10K that is rich in methionine (Charbonnier et al 1980). In sorghum prolamin, Beckwith and Jones (1972) showed that a 17K polypeptide is present that has 10% of its amino acid residues as methionine.

N-terminal analysis of α-setarin showed that methionine was present at the amino terminal end of the polypeptide. However, a very small amount of leucine was also detected as N-terminal residue of α-setarin, which was perhaps contributed by a contaminating polypeptide that coprecipitated with α-setarin during cryoprecipitation. Thus, α-setarin also appeared to be a fairly homogeneous polypeptide by the criteria of N-terminal analysis.

Amino acid analysis of α-setarin revealed that it had eight methionine residues out of a total of 63 amino acid residues and that β-setarin had eight residues of methionine out of of 71 amino acid residues. Nearly 60% of the methionine in the setarin II fraction can be accounted for by the methionine in α and β-setarin (Table I). It was of interest to gain an insight into the distribution of these methionine residues in the polypeptide chain. Separation of the CNBr-cleaved, performic acid oxidized α-setarin by two-dimensional peptide mapping showed at least nine ninhydrin positive spots of varying intensities on the peptide map. It appears from these results that the large number of methionine residues present in α-setarin are randomly distributed along the peptide chain and not clustered.

CONCLUSIONS

α- and β-Setarin constitute the major sulfur-rich proteins of the Italian millet prolamin fraction. They have rather similar amino acid profiles and molecular sizes. Molecular biology of setarin biosynthesis, and especially the synthesis of methionine-rich proteins of Italian millet, needs to be further investigated now that a simple procedure for isolation of the sulfur-rich proteins of Italian millet has been worked out.

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