found to be an efficient method of removing the antinutritional factors in *Citrullus* seeds.

Experiment 3. Results of experiment 3 indicate that feeding whole *Citrullus* seeds is not as growth depressing as feeding the meal. Feeding up to 15% seeds in the diet had no significant effect on growth. Birds consuming 10–15% *Citrullus* seeds consumed significantly more feed than the controls, and as a result feed efficiency was significantly reduced. In a previous report (Sawaya et al., 1983) it was shown that the oil extracted from *Citrullus* seeds was free from any antinutritional factor and in fact was equal in its nutrient value to corn oil when fed to growing chicks.

Experiment 4. Results of experiment 4 confirm the findings of experiment 3 and show no significant differences in growth, feed consumption, or feed efficiency of chicks consuming up to 20% *Citrullus* seeds in their diet. However, it is not recommended that *Citrullus* seeds be fed at such high levels in practical poultry formulations since this brings up the fiber level of the ration to over 10% and thus reduces feed utilization drastically. They can easily be incorporated in broiler rations at levels of 5–10% of the ration if all nutrients are kept constant. On the basis of data presented in this study, the undecorticated seed as well as the meal contain significant amounts of protein, oil, and ash but a lower amount of nitrogen-free extract. Although both the seed and meal contain substantial amounts of protein, the crude fiber content of both the seed and meal is high. Consequently, the seed would have to be decorticated prior to extraction or the meal processed further to make it more suitable as a stock feed. Purified protein can be isolated from such products for possible use in human food formulations. However, the meal contains certain growth-depressing factors for chicks, and more studies are needed to determine the detoxification procedure in order to exploit the full potential of *C. colocynthis* seed protein. These seeds are reported to be heavily consumed by humans in some African countries (Afolabi et al., 1985), and processing prior to consumption is regarded as the possible way for detoxification.

Registry No. Na, 7440-23-5; K, 7440-09-7; Ca, 7440-70-2; Mg, 7439-95-4; P, 7723-14-0; Fe, 7439-89-6; Zn, 7440-66-6; Cu, 7440-50-8; Mn, 7439-96-5.

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Isolation and Characterization of the Major Fraction (2S) of *Madhuca* (*Madhuca butyraceae*) Seed Proteins

T. Shanmugasundaram and L. V. Venkataraman*

A method is described for isolating the major protein of *Madhuca* seeds in a homogeneous form. Its $S_{20,w}$ value was found to be 2S. It contains 4.1% carbohydrate and no phosphorus. The 2S protein consists of at least two subunits. Circular dichroism measurements indicate that this protein consists of 35% α helix, 38% β structure, and 27% random coil.

Madhuca (*Madhuca butyraceae*) (family Sapotaceae) is a minor oil seed of tree origin in India. The seed contains about 5–8% protein and 50–60% fat, which is being used in soap and cosmetic industries. the defatted flour, though a good source of protein, is not edible due to the presence of saponins (Wealth of India, 1952). In this laboratory, we have undertaken a systematic study on *Madhuca* seed proteins to obtain information on their chemical, physicochemical, nutritional, and functional properties.

Madhuca seed protein consists of three fractions with $S_{20,w}$ values of 2S, 10S and 15S (Shanmugasundaram and

Central Food Technological Research Institute, Mysore 570 013, India.

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Venkataraman, 1982). No detailed study of the protein fractions appears to have been made. In this paper, we describe a method for isolating the 2S protein, which constitutes about 76% of the total proteins, and also some of its physicochemical characteristics.

MATERIALS AND METHODS

***Madhuca* Seeds.** *M. butyraceae* seed kernels were obtained from the National Botanical Research Institute, Lucknow. The meal was prepared as described earlier (Shanmugasundaram and Venkataraman, 1982).

Isolation of the Major Fraction. The protein from the meal was extracted in water by a meal to solvent ratio of 1:10 (w/v). The slurry was stirred for 1 h at room temperature and centrifuged at 3700*g* for 10 min. To the supernatant was added $(NH_4)_2SO_4$ to 60% saturation, and the solution was stirred kept at room temperature (~28 °C) for 20 min. The precipitate was removed by centrifugation, and the clear solution was dialyzed against water for 12 h; the retentate was lyophilized. About 75 mg of the lyophilized protein dissolved in 1 M NaCl was loaded on a Sepharose 6B column (1.5 × 100 cm) that was previously equilibrated with 1 M NaCl. The protein was eluted with 1 M NaCl, and the fractions from 165 to 240 mL were collected, pooled together, dialyzed against water, and lyophilized. To test the homogeneity of the preparation, 3 mL of the protein solution containing 40 mg of the protein was loaded on the Sepharose column (1.5 × 100 cm). The protein was eluted as above. Three-milliliter fractions were collected, and the absorbance was measured at 280 nm.

Polyacrylamide Gel Electrophoresis. The method of Reisfeld et al. (1962) was used with 7.5% gels and 0.01 M β-alanineacetic acid buffer of pH 4.5. Electrophoresis was done at a constant current of 2 mA/tube for 90 min. The gels were stained with Coomassie Brilliant Blue for 3 h and destained with a solution containing acetic acid, methanol, and water in the ratio 75:50:875.

Ultracentrifugation. The sedimentation velocity experiment was done by using a 1% solution in 0.1 M phosphate buffer of pH 7.6 containing 1 M NaCl at room temperature (~28 °C) at 59800 rpm in a Spinco Model E analytical ultracentrifuge. Photographs were taken at 15-min intervals of centrifugation and the $S_{20,w}$ was calculated by the standard procedure (Schachman, 1959).

Carbohydrate Content. Carbohydrate was estimated by the method of Montgomery (1961).

Phosphorus Content. Phosphorus content was estimated by method of Taussky and Shorr (1953).

Amino Acid Analysis. The sample containing 20 mg of protein (N × 6.25) was hydrolyzed in vacuo with 10 mL of 6 N HCl containing 0.1% phenol at 110 °C for 24 h. The hydrolysate was evaporated in a rotary vacuum evaporator to remove excess acid and dissolved in 0.2 M sodium citrate buffer (pH 2.2). Amino acid analysis was carried out by a LKB α amino acid analyzer, and the amino acid composition is expressed as grans/100 g of protein.

Absorption Spectrum. The absorption spectrum of the protein was recorded in a Perkin-Elmer double-beam spectrophotometer in the 240–330-nm range.

Fluorescence Spectrum. The emission spectrum was measured in the range 300–400 nm after excitation at 280 nm using protein solution having an absorbance of 0.01 at 280 nm.

SDS-Polyacrylamide Gel Electrophoresis. The method of Laemmeli (1970) was used with RNase (14300), lysozyme (18400), trypsinogen (24000), pepsin (34700), egg albumin (45000), and BSA (66000) as molecular weight markers.

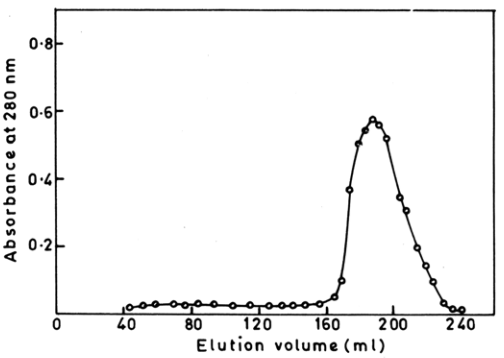

Figure 1. Gel filtration pattern of 2S protein in 1 M NaCl solution.

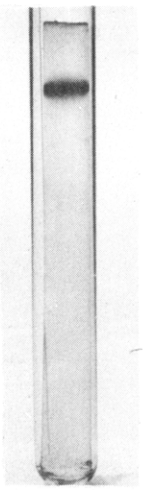

Figure 2. Gel electrophoresis pattern of 2S protein (0.01 β-alanineacetic acid buffer of pH 4.5).

Circular Dichroism (CD) Spectrum. CD measurements were made in a Jasco J20 automatic spectropolarimeter equipped with a xenon are lamp using a 1-mm quartz cell. The measurements were made with solutions having an absorbance of less than 2 at 280 nm. Molar residue ellipticity was calculated by the standard procedure (Adler et al. 1973). A value of 115 for the mean residue weight was assumed. The α helical content of the protein was estimated by the method of Greenfield and Fasman (1969), and the proportion of the β structure was estimated by the method of Sarkar and Doty (1966).

RESULTS AND DISCUSSION

The homogeneity of the protein prepared by the method described earlier (Materials and Methods) was determined by gel filtration, gel electrophoresis, and ultracentrifugation. In gel filtration (Figure 1), the *Madhuca* protein gave a broad peak eluting at 180 mL. In gel electrophoresis, a single sharp band was observed (Figure 2). A single broad peak, which is characteristic of low molecular weight proteins, was observed in the ultracentrifuge studies (Figure 3). The $S_{20,w}$ value was found to be 2S. Thus, a homogeneous protein could be obtained by our method.

Carbohydrate and Phosphorus Contents. The carbohydrate content of the 2S protein was 4.1%, and it did not contain any phosphorus.

Amino Acid Composition. The amino acid composition of the 2S protein is shown in Table I. The protein consisted of a higher amount of glutamic acid (30.1%), cystine (3.2%), and arginine (9%) like the low molecular weight proteins of sunflower, rape seed, and linseed (Schwenke et al., 1973; Youle and Huang, 1981; Madhusudhan, 1984).

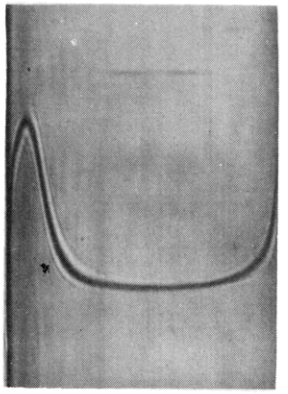

Figure 3. Sedimentation velocity pattern of 2S protein in 0.1 M phosphate buffer containing 1 M NaCl solution, pH 7.6. Photograph taken after 15 min at 59 780 rpm, bar angle 70°; sedimentation proceeds from left to right.

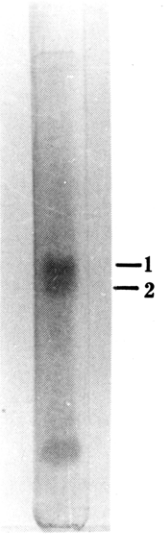

Figure 4. SDS-polyacrylamide gel electrophoresis pattern of the 2S protein.

Table I. Amino Acid Composition of the 2S Protein of *Madhuca* Seed[a]

amino acid	compn, g/16 g of N	amino acid	compn, g/16 g of N
aspartic acid[b]	7.2	valine	4.9
threonine	3.0	isoleucine	2.2
serine	4.5	leucine	6.5
glutamic acid[c]	30.1	tyrosine	1.5
proline	4.2	phenylalanine	4.5
glycine	5.5	histidine	2.0
alanine	4.6	lysine	4.5
cystine	3.2	arginine	9.0
methionine	0.8		

[a] Values are averages of two independent determinations. [b] Includes aspargine. [c] Includes glutamine.

SDS-PAGE. This experiment was performed to determine the subunit structure of the protein. SDS-PAGE pattern of the 2S protein showed two bands marked as 1 and 2 (Figure 4). The molecular weights corresponding to the bands 1 and 2 were 30 200 and 26 300, respectively.

Absorption Spectrum. The UV absorption spectrum of the 2S protein was typcial of a protein with its maximum absorption at 278 nm and minimum at 255 nm (Figure 5). The ratio of absorbance of 280 nm to that of 260 nm was 1.1. This corresponded to a value of 2.5% nucleic acid (Layne, 1957). However, the protein had no phosphorus, showing the absence of nucleic acid contamination in the preparation.

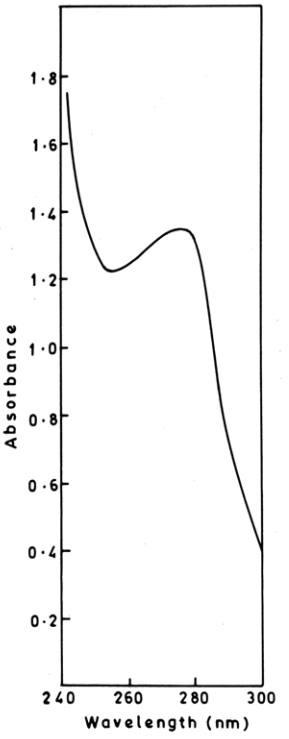

Figure 5. Ultraviolet absorption spectrum of 2S protein in 1 M NaCl solution.

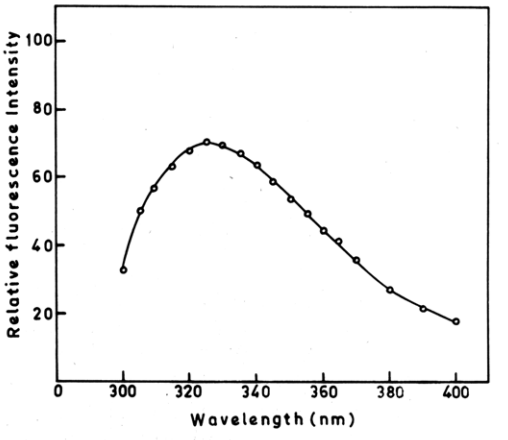

Figure 6. Fluorescence emission spectrum of 2S protein in 1 M NaCl solution.

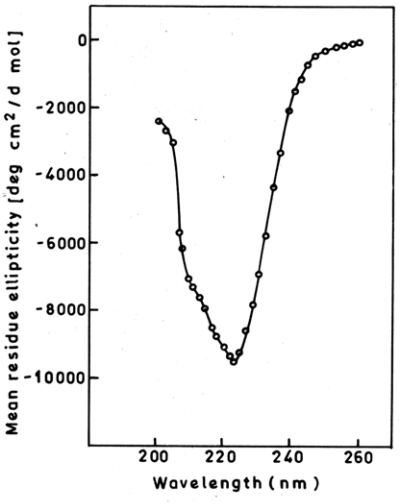

Figure 7. CD spectrum of 2S protein in 0.02 M phosphate buffer, pH 7.6.

Fluorescence Spectrum. The fluorescence emission spectrum of the protein gave a maximum at 325 nm

(Figure 6), suggesting that the contribution of tryptophan is greater than that of tyrosine (Teale, 1960).

CD Spectrum. The CD spectrum of the protein in the region 200–260 nm is given in Figure 7. It consisted of a shoulder at 208 nm and minimum at 224 nm. Calculation of the α helical content of the 2S protein from its ellipticity value at 208 nm using the equation of Greenfield and Fasman (1969) showed the value to be 35%. When the proportion of the β structure was estimated by the method of Sarkar and Doty (1966) taking a mean residue ellipticity value of 23 000° cm^2/dmol at 218 mm for 100% β structure, the proton was found to contain 38% β structure. Thus, the major structures appeared to be α helix and β structure. Many other low molecular weight vegetable proteins are known to consist predominantly of α helix and β structure (Schwenke et al., 1973; Madhusudhan, 1984).

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Hydrolysis of Poly-L-methionyl Proteins by Some Enzymes of the Digestive Tract

Hubert F. Gaertner and Antoine J. Puigserver*

Polymerization of L-, [^{35}S]-L-, and D,L-methionine on casein by the *N*-carboxy anhydride method allowed us to prepare protein derivatives in which 50–70% of the lysyl residues were acylated by methionine polymers of an average chain length of 5–12 residues. In vitro digestibility of resulting polymethionylcaseins was investigated by successive incubation with pepsin, pancreatic endopeptidases, and intestinal aminopeptidase and subsequent amino acid analysis of hydrolysates. Methionine was readily released from covalently attached polymers although hydrolysis was significantly decreased with increasing chain length. Hydrolysis of casein itself was independent of the extent of modification of the lysyl residues whereas D,L-polymethionine chains were not well hydrolyzed. Analysis of peptic and chymotryptic hydrolysates using high-performance liquid chromatography showed that trimethionine and comparable amounts of di-, tri- and tetramethionine were released by each of the corresponding enzymes, respectively. These peptides were in turn good substrates for intestinal aminopeptidase. Polymethionyl proteins must therefore have potential nutritional applications.

INTRODUCTION

The methionine content of various food proteins has recently been shown to be enhanced by covalent attachment of poly-L-methionine through the *N*-carboxy anhydride method (Puigserver et al., 1982; Gaertner and Puigserver, 1984a). Protein α- and ϵ-amino groups are known to act as initiators in the polymerization of *N*-carboxy-α-amino acid anhydrides, leading to the formation of protein derivatives in which a number of poly(amino acid) chains of variable length are covalently attached to some of the lysyl residues (Glazer et al., 1962; Wellner et al., 1963). By contrast, the *N*-hydroxysuccinimide ester method resulted in the exclusive formation of a single isopeptide bond between each lysyl ϵ-amino group and the amino acid (Puigserver et al., 1979a).

The *N*-carboxy anhydride method has the obvious advantage of considerably increasing the amount of covalently linked amino acids even in proteins with low lysine levels. However, since as much methionine as 30% of casein weight could be covalently attached in the form of polymethionine of an average chain length of 8 residues, solubility of the protein derivative was drastically decreased (Gaertner and Puigserver, 1984a). Initial rates of hydrolysis by α-chymotrypsin and trypsin in vitro were decreased as compared to untreated casein, probably because of both steric hindrance preventing an efficient hydrolysis of the polypeptide chain and the presence of acylated lysyl residues inhibiting trypsin action. All these findings might suggest that covalent attachment of polymethionine chains to casein would result in a significant decrease of the overall digestibility of the protein derivatives in vivo. It was therefore important to test this hypothesis to see whether or not such a procedure was of value in improving protein quality.

The aim of this study was to determine in vitro digestibility of casein modified by using the *N*-carboxy anhydride method under different experimental conditions and subsequent availability of the polymerized methionine. To determine the hydrolytic processes for covalently linked polymethionine chains, digestions with a number of individual enzymes were carried out and the resulting data compared to those previously obtained with the synthetic

Centre de Biochimie et de Biologie Moléculaire du CNRS, 13402 Marseille Cedex 9, France.

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