PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 626–630 (1978)

Biological Evaluation of Crystalline Fraction I Protein from Tobacco[1] (40110)

B. H. ERSHOFF,* S. G. WILDMAN†, AND P. KWANYUEN†

Institute for Nutritional Studies, Culver City, California 90230 and the †Department of Biology, University of California, Los Angeles, California 90024

Leaf proteins are one of the most abundant protein sources in the world but at present very little leaf protein is used directly for human consumption. Proteins constitute but a small portion of the leaf; and for them to serve as a significant source of protein for man, they must be concentrated. The cost of such concentration has priced these proteins out of serious consideration as a source of human protein in the past. Technological improvements in the concentration of leaf proteins, however, have raised the possibility that leaf proteins may serve as an important source of protein for man in the future.

The distribution of total leaf protein is roughly 50% soluble and 50% insoluble in water. Of the former, approximately one-half consists of a single homogeneous protein whose molecular weight is approximately 500,000 daltons. This protein was called Fraction I protein at the time of its discovery in 1947 but has since been shown to be identical to the enzyme ribulose diphosphate carboxylase (1). This enzyme catalyzes the union of carbon dioxide with other carbon containing compounds during photosynthesis. It is present in all green leaves and is the most abundant, single protein in the world (1). Fraction I protein from tobacco is unique from that derived from other green plants in that only from plants of the genus Nicotiana (of which tobacco is a member) can it be obtained in pure, crystalline form (2). In the salt free, crystalline state, lyophilization produces a tasteless white powder. With the exception of a slightly lower level of methionine its content of essential amino acids equals or exceeds that of the FAO Provisional Pattern (Table I). In the present communication data are presented on the comparative nutritive value of casein and crystalline Fraction I protein from tobacco when fed as the sole source of dietary protein on the weight increment, PER (protein efficiency ratio), hematological findings, serum levels of protein, lipids and other serum constituents, and organ weights of immature male rats.

Materials and methods. Tobacco plants (Nicotiana tabacum) were grown in a green house and were harvested in 2–6 kg fresh-weight batches when they had attained a height of 18–21 inches. Immediately after harvesting, crystals of Fraction I protein were obtained by a slight modification of the method developed by Lowe (3).[2] The tobacco leaves were extracted with 0.1 *M* NaCl and beta mercaptoethanol, the filtered juice heated to 50° for 10 min, centrifuged to remove the insoluble green fraction, and the remaining brown solution passed through a G-25 Sephadex chromatographic column equilibrated with 0.025 *M* Tris–HCl and 0.0005 *M* EDTA, pH 7.6. The soluble leaf proteins eluted in the void volume which was collected. Fraction I protein crystals appeared in the eluate almost immediately and continued to appear during 3 days storage at 5°. The mother liquor was decanted from the layer of crystals which were resuspended and washed in a small volume of glass-distilled water. The suspensions containing 6–18 g (dry weight) of Fraction I protein crystals were examined by phase and dark field microscopy to insure the absence of bacteria. Enough 1 *M* NaCl was added dropwise to completely dissolve the crystals, the solution centrifuged to remove a slight turbidity and the clear, colorless solution placed in cello-

[1] This investigation was supported in part by Research Grant No. AER 76-11051 from the RANN Division of the National Science Foundation. However, any opinions, findings, conclusions or recommendations expressed herein are those of the authors and do not necessarily reflect the views of the National Science Foundation.

[2] The authors are indebted to Dr. Richard Lowe of the University of Kentucky for having made his simplified method for isolating Fraction I protein crystals available for their use prior to publication.

0037-9727/78/1574-0626$01.00/0

TABLE I. ESSENTIAL AMINO ACIDS IN CRYSTALLINE FRACTION I PROTEIN FROM TOBACCO LEAVES COMPARED TO FAO PROVISIONAL PATTERN (GRAMS PER 100 GRAMS OF PROTEIN).

Amino acids	Provisional pattern[a]	Fraction I protein from tobacco[b]
Isoleucine	4.2	4.2
Leucine	4.8	8.8
Lysine	4.2	5.8
Phenylalanine	2.8	4.4
Tyrosine	2.8	4.9
Methionine	2.2	1.6
Threonine	2.8	5.2
Tryptophan	1.4	1.5
Valine	4.2	7.2

[a] Reference (11).

[b] Calculated from amino acid analyses contained in reference (12).

phane tubing and dialyzed at 5° against 10 vol of glass-distilled water which was changed 3 times at 24-hr intervals to remove NaCl. The recrystallized protein was transferred to flasks and the protein lyophilized to produce a white, nonhygroscopic powder. Approximately 800 g of dry Fraction I protein crystals from tobacco were prepared for the present study. The latter was obtained from the processing of 300 kg of fresh tobacco leaves.

Twenty male rats of the Sprague–Dawley strain ranging from 48–52 g in body weight were divided into two comparable groups of 10 animals each. These were fed diets similar to the AOAC Protein Evaluation Diets (4) but differed as to the source of protein. Rats in group I were fed ANRC Reference Casein[3] and those in group II Fraction I protein from tobacco as the sole source of dietary protein which was incorporated at a 10% level (based on N content × 6.25) in the test diets. Sucrose was employed as the source of dietary carbohydrate. Animals were placed in individual metal cages with raised screen bottoms and were provided the above diets and water *ad libitum*. Animals were fed daily, and all food not consumed 24 hr after feeding was discarded. Food consumption was determined daily for each rat for 28 days. Animals were weighed once weekly. After the 28th day of feeding, rats were fasted overnight. In the morning they were anesthetized with sodium pentobarbital, and 5 ml of blood was withdrawn from each rat by heart puncture. Two ml were taken for hematological determinations; serum was separated from the remaining 3 ml and analyzed with a Technicon SMAC high speed computer controlled Biochemical Analyzer[4] for serum levels of protein, lipids and other serum constituents. The internal organs were examined grossly, and organ weights were determined. The data were treated statistically by Student's t test (5).

Results. Weight increment and PER. The average weight increment and PER of rats in the two dietary groups after 7, 14, 21 and 28 days of feeding are indicated in Table II. Both the weight increment and PER of rats fed the diet containing crystalline Fraction I protein from tobacco (group II) were significantly greater at each of the above time periods than that of rats fed the casein-containing diet (group I).

Hematological findings. The hematological findings of rats in the two dietary groups after 28 days of feeding are indicated in Table III. No significant differences in Hb, Hematocrit, RBC, WBC, MCHC, MCH, MCV and differential cell count were observed between the two groups.

Serum levels of proteins, lipids and other constituents. Serum levels of protein, lipids and other constituents for rats in the two dietary groups after 28 days of feeding are indicated in Table IV. Serum levels of protein, cholesterol, triglyceride, glucose, urea, creatinine, total bilirubin, inorganic phosphorus, sodium and chloride did not differ significantly between the two groups. Serum level of albumin, the A/G ratio, calcium and carbon dioxide were slightly but significantly greater in rats fed the diet containing crystalline Fraction I protein from tobacco than in rats fed the casein-containing diet. Serum globulin and potassium levels, however, were slightly but significantly greater in rats fed the casein-containing diet than in those fed the diet containing crystalline Fraction I protein from tobacco.

Organ weights. Organ weights of rats in the two dietary groups on the 29th day of the experiment are indicated in Table V. The weight of the liver, kidneys, spleen, thymus and seminal vesicles was significantly greater

[3] ANRC Reference Casein, Sheffield Chemical Co., Norwich, N.Y.

[4] Technicon Corporation, Tarrytown, N.Y.

TABLE II. COMPARATIVE EFFECTS OF CASEIN AND CRYSTALLINE FRACTION I PROTEIN FROM TOBACCO ON THE WEIGHT INCREMENT AND PER OF RATS (10 ANIMALS PER GROUP).

	Casein-containing diet (group I)	Fraction I protein-containing diet (group II)
Initial body weight[a]	48.8 ± 0.34	48.8 ± 0.56
Avg. weight increment (g) after the following days of feeding:[a]		
7 days	18.1 ± 0.80	25.7 ± 1.29[b]
14 days	51.5 ± 1.69	65.2 ± 1.87[b]
21 days	80.8 ± 2.40	100.6 ± 2.25[b]
28 days	114.4 ± 3.25	139.1 ± 2.78[b]
Avg. food consumption (g) after the following days of feeding:		
7 days	66.4	75.6
14 days	167.7	189.3
21 days	281.0	324.9
28 days	404.5	461.4
PER after the following days of feeding:[a]		
7 days	2.73 ± 0.07	3.40 ± 0.08[b]
14 days	3.07 ± 0.06	3.44 ± 0.03[b]
21 days	2.88 ± 0.06	3.10 ± 0.03[c]
28 days	2.83 ± 0.04	3.01 ± 0.02[b]

[a] Mean ± SE of the mean.
[b] Significantly different ($P < 0.001$) from group I value.
[c] Significantly different ($P < 0.01$) from group I value.

TABLE III. HEMATOLOGICAL FINDINGS OF RATS FED HIGHLY PURIFIED DIETS CONTAINING EITHER CASEIN OR FRACTION I PROTEIN FROM TOBACCO AS THE SOLE SOURCE OF PROTEIN (10 ANIMALS PER GROUP).

Hematological constituents	Casein-containing diet (group I)[a]	Fraction I protein-containing diet (group II)[a]
Hemoglobin, g/dl	14.1 ± 0.12	14.2 ± 0.09
Hematocrit, %	41.3 ± 0.67	42.3 ± 0.20
Red blood cells, millions per ml[3]	6.62 ± 0.12	6.78 ± 0.07
White blood cells, thousands per ml[3]	6.5 ± 0.66	5.7 ± 0.21
MCHC, %	34.5 ± 0.58	34.0 ± 0.12
MCH, mcmcg	20.8 ± 0.24	20.9 ± 0.17
MCV, cu. mic.	61.3 ± 0.78	61.3 ± 0.52
Polys, %	8.0 ± 1.19	7.2 ± 0.94
Lymph, %	92.0 ± 1.32	92.8 ± 1.03

[a] Mean ± SE of the mean.

TABLE IV. SERUM LEVELS OF PROTEIN, LIPIDS AND OTHER CONSTITUENTS OF RATS FED PURIFIED DIETS CONTAINING CASEIN OR CRYSTALLINE FRACTION I PROTEIN FROM TOBACCO AS THE SOLE SOURCE OF PROTEIN (10 ANIMALS PER GROUP).

Serum constituents	Casein-containing diet (group I)[a]	Fraction I protein-containing diet (group II)[a]
Total protein, g/dl	5.7 ± 0.07	5.8 ± 0.07
Albumin, g/dl	4.5 ± 0.04	4.9 ± 0.06[b]
Globulin, g/dl	1.2 ± 0.05	0.9 ± 0.03[b]
A/G ratio	3.99 ± 0.17	5.53 ± 0.17[b]
Cholesterol, mg/dl	126 ± 3.72	121 ± 2.43
Triglyceride, mg/dl	55 ± 7.98	41 ± 6.22
Glucose, mg/dl	107 ± 20.88	85 ± 3.88
Urea nitrogen, mg/dl	9.3 ± 0.90	8.0 ± 0.53
Creatinine, mg/dl	0.5 ± 0.02	0.5 ± 0.02
Total bilirubin, mg/dl	0.2 ± 0.00	0.2 ± 0.01
Calcium, mg/dl	10.6 ± 0.10	11.0 ± 0.06[b]
Inorganic phosphorus, mg/dl	8.9 ± 0.36	8.7 ± 0.16
Sodium, mEq/L	139 ± 1.81	142 ± 0.93
Potassium, mEq/L	6.6 ± 0.21	5.8 ± 0.11[c]
Chloride, mEq/L	99 ± 0.60	99 ± 0.31
Carbon dioxide, mEq/L	23 ± 0.45	25 ± 0.20[b]

[a] Mean ± SE of the mean.
[b] Significantly different ($P < 0.001$) from group I value.
[c] Significantly different ($P < 0.01$) from group I value.

in rats fed the diet containing crystalline Fraction I protein from tobacco than in rats fed the casein-containing diet. When the weight of these organs was expressed as weight per 100 g body weight, however, a difference between the two dietary groups was no longer manifest. No significant differences were observed between the two groups in heart, adrenal and testes weight. No differences were observed grossly in the appearance of the various organs between rats in the two dietary groups.

Discussion. Present findings indicate that tobacco which in the past has been grown primarily for smoking purposes can be processed to produce a water soluble crystalline

TABLE V. ORGAN WEIGHTS OF RATS FED HIGHLY PURIFIED DIETS CONTAINING EITHER CASEIN OR FRACTION I PROTEIN FROM TOBACCO AS THE SOLE SOURCE OF PROTEIN (10 ANIMALS PER GROUP).

	Casein-containing diet (group I)		Fraction I protein-containing diet (group II)	
Organ	Organ wt.[a]	Organ wt. per 100 g body wt.	Organ wt.[a]	Organ wt. per 100 g body wt.
Liver, g	5.61 ± 0.20	3.4	6.14 ± 0.09[b]	3.3
Kidneys, g	1.35 ± 0.05	0.8	1.58 ± 0.03[c]	0.8
Spleen, g	0.49 ± 0.02	0.3	0.57 ± 0.01[d]	0.3
Thymus, g	0.51 ± 0.03	0.3	0.62 ± 0.03[c]	0.3
Heart, g	0.72 ± 0.03	0.4	0.77 ± 0.02	0.4
Adrenals, mg	36.4 ± 1.4	22.3	41.4 ± 2.3	22.0
Testes, g	2.33 ± 0.05	1.4	2.40 ± 0.03	1.3
Seminal vesicles, g	0.33 ± 0.02	0.2	0.44 ± 0.02[d]	0.2

[a] Mean ± SE of the mean.
[b] Significantly different ($P < 0.05$) from group I value.
[c] Significantly different ($P < 0.01$) from group I value.
[d] Significantly different ($P < 0.001$) from group I value.

protein of high biological value. The latter (crystalline Fraction I protein) is composed entirely of amino acids (6). In contrast to the Fraction I protein isolated from the leaves of other plant species which contain up to 5% carbohydrate (7), crystalline Fraction I protein from tobacco contains less than 0.0005% carbohydrate as measured by gas–liquid chromatography-mass spectra analyses (6). The absorption spectra of crystalline Fraction I protein from tobacco shows no evidence of nucleic acids, visible pigments or materials which absorb at wave lengths greater than 300 namometers (8). It contains no minerals with the exception of the sulphur present in the sulphur-containing amino acids (9). In view of the above and because of its high nutritional value as a source of protein, it has the potential of being developed into important therapeutic products for the treatment of a number of medical conditions. It would appear to be particularly valuable for the feeding of patients with various types of renal disease in which sodium and/or potassium intake must be rigorously controlled (10). Since it is readily soluble in water, it can be incorporated in low residue nutritionally complete liquid diets for the treatment of patients with pylorospasms, gastrointestinal fistula, short bowel syndrome, tumors of the esophagus and gastrointestinal tract, pancreatitis and other diseases involving maldigestion and malabsorption. Further studies are indicated to determine the effects of graded levels of crystalline Fraction I protein in the diet on length of survival and the incidence and severity of pathological changes associated with aging, its effect on reproductive and lactation performance, immunocompetence, resistance to stressor agents and possible toxic manifestations resulting from its long time ingestion in various species of experimental animals.

Summary. Weanling male rats were fed purified diets containing 10% protein in the form of either casein or twice crystallized Fraction I protein from tobacco. Findings indicated that the average weight increment and PER (protein efficiency ratio) of rats fed the diet containing Fraction I protein from tobacco were significantly greater throughout a 28-day experimental period than that of rats fed the diet containing casein. No significant differences in hematological findings were noted between rats in the two dietary groups.

1. Kawashima, N., and Wildman, S. G., Ann. Rev. Plant Physiol. **21**, 325 (1970).
2. Kawashima, N., and Wildman, S. G., Biochim. Biophys. Acta **229**, 240 (1971).
3. Lowe, R. H., FEBC Letters **78**, 98 (1977).
4. AOAC Methods of Analysis, 12th edition, Association of Official Agricultural Chemists, Washington, D.C., Section 43.183, pg. 857 (1975).
5. Steele, R. G. D., and Torrie, J. H., "Principles and Procedures of Statistics." McGraw-Hill, New York (1960).
6. Sakano, K., Partridge, J. E., and Shannon, L. M., Biochim. Biophys. Acta **329**, 339 (1973).
7. Akazawa, T., Saio, K., and Sugiyama, N., Biochem. Biophys. Res. Commun. **20**, 114 (1965).
8. Sakano, K., and Wildman, S. G., Plant Sci. Letters **2**, 273 (1974).

9. Chollet, R., Anderson, L. L., and Hovsepian, L. C., Biochem. Biophys. Res. Commun. **64,** 97 (1975).
10. Comty, C. M., J. Amer. Diet. Assn. **53,** 439 (1968).
11. Food Agr. Organ. U.N. Protein requirements. FAO Nutrition Studies No. 16, Rome (1957).
12. Kawashima, N., Kwok, S. Y., and Wildman, S. G., Biochim. Biophys. Acta **236,** 578 (1971).

Received December 8, 1977. P.S.E.B.M. 1978, Vol. 157.