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D. L. Barber

J. N. A. Lott

D. A. Harris

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PRACTICAL METHODS FOR IDENTIFICATION OF RICE ENDOSPERM PROTEIN BODIES AND FECAL PROTEIN PARTICLES IN LIGHT MICROSCOPY

D.L. Barber*, J.N.A. Lott* and D.A. Harris[1]

*Department of Biology, McMaster University, Hamilton, Ontario, Canada, L8S 4K1
[1]Mead Johnson Research Center, Bristol-Myers Squibb Company, Evansville, IN 47721, USA

Abstract

Indigestible 1-3 μm protein 'cores', particles that originate from protein bodies of rice endosperm, were examined in bright-field and fluorescence microscopy, using a variety of histological and histochemical procedures. Our application of histological, histochemical and chemical approaches to a study of the feces of animals fed rice-containing diets has resulted in the development of methods for routine identification of insoluble rice fecal protein particles. For the specific identification of rice endosperm storage protein, whether protein bodies, cores or fecal protein particles, aqueous eosin Y stain in conjunction with fluorescence microscopy, and a combination of orange G + aniline blue in bright-field microscopy appear to be reliable methods when used in conjunction with each other.

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Direct inquiries to J.N.A. Lott
Telephone number: (416) 525 9140 Ext. 4589
Telefax Number: (416) 521 2955

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Introduction

The storage proteins of most grains and other seeds are found in well-defined subcellular structures called protein bodies, usually ranging from 1.5-8.0 μm in diameter. While some may appear homogeneous in the light microscope, others often contain protein crystalloids and/or globoid crystals, which are proteinaceous or phosphate storage compounds, respectively (Ashton, 1976; Lott, 1981). Protein bodies in mature rice grains are found in three regions: the aleurone, subaleurone portions of the endosperm, and the embryo (Bechtel and Pomeranz, 1978a). The protein bodies of the aleurone and some in the embryo contain globoid crystals; those in the subaleurone of mature rice grains do not. Most of the work reported here has been carried out on subaleurone protein bodies and their remnants as found in commercial, polished, milled rice and rice products where the aleurone and embryo have been removed (Juliano, 1985).

Chemical analysis of rice protein shows that glutelins, prolamins, globulins and albumins are all present. In chemical terms, plant proteins have been defined in terms of their solubilities (Osborne, 1924). Glutelins are soluble in low concentrations of NaOH, prolamins in dilute alcohols, globulins in dilute solutions of NaCl, and albumins in water. Approximately 60-70% of the total storage protein of rice endosperm consists of various polypeptides of glutelin, while prolamins represent 20-30% (Ogawa et al., 1987, 1989); albumins and globulins in rice are a very small proportion of the total protein content. Up to 15% of rice endosperm protein is indigestible; however, the net protein utilization of rice is as good as that of other cereals, because of its relatively higher biological value (Juliano, 1985). Other cereal grains generally have a preponderance of prolamins, while seed storage proteins of dicot plants frequently consist largely of globulins (Ashton, 1976; Juliano, 1985). At least some of the indigestible protein of rice is identifiable morphologically in the light microscope. Ubiquitous 1-2 μm particles first reported in Japanese sewage by microbiologists (Tanaka et al., 1975a, b)

were identified as remnants of rice protein. These 'fecal protein particles' were further equated with the 'cores' of one or more endosperm protein body types (Bechtel and Pomeranz, 1978b), on the basis of enzyme studies accompanying electron microscopy. Rice protein particles obtained by removing the readily soluble and/or digestible portions of the protein body will here be referred to as 'cores'. From the work of Bechtel and Pomeranz (1978b), cores measuring 1.3 μm were estimated to represent 15-20% of the total volume of the protein bodies in which they were seen.

As part of continuing investigations into the relationship of rice protein structure and digestibility in processed food products, we have developed a combination of light microscopic and chemical methods which may be used to identify indigestible rice protein moieties, whether they arise from intact (raw or processed) or digested rice endosperm. While a considerable body of electron microscopic information on the ultrastructure of rice protein bodies and fecal protein particles exists (see e.g., Tanaka et al., 1975b; Bechtel and Pomeranz, 1978a, b; Krishnan et al., 1986; Ogawa et al., 1987), there is very little information on light microscopic recognition of these structures. For routine investigations of diets or fecal samples in rice-fed animals, the light microscope is a much easier tool to use than the electron microscope, and the following methods may be helpful.

Materials and Methods

Grains and Seeds

Milled rice endosperm, rice flour, and commercially destarched rice flour (hereafter referred to as 'rice protein concentrate') of *Oryza sativa* L. cv Lemont were used in our research. All rice products were supplied by the Mead Johnson Research Center, Evansville, Indiana. For comparison with the Lemont cultivar, hand-shelled grains of Nipponbare and Kameno-o rice cultivars were also examined, as was wild rice (*Zizania palustris*). In addition, mature dry seeds or portions thereof from other plant species were examined. The species included seeds of the legumes: soybean (*Glycine max* L. cv Lee), green pea (*Pisum sativa* L. cv Little Marvel) and peanut (*Arachus hypogaea* L. cv Virginia), which were intact, including the testa. Non-legumes included squash (*Cucurbita maxima* L. cv Warted Hubbard) seeds and coconut (*Cocos nucifera*), which were stripped of their outer seed coats before grinding. Cereal grains of triticale (*Triticale hexaploide* Lart. cv Rosner), corn (*Zea mays* L. cv Golden Beauty), barley (*Hordeum vulgare* L. cv. Himalaya), oat (*Avena sativa*), rye (*Secale cereale* cv Kustro), and wheat (*Triticum vulgare* cv Durum) were intact before grinding. Seed materials were ground to flour in a clean electric coffee mill.

Light Microscopic Procedures

The general procedure for handling all particulate material was as follows: a slurry (1:5 v:v) of seed flour and water was mixed in a small test tube, heated to 90°C for 3 minutes to gelatinize any starch, and a drop smeared between two microscope slides, which were then slid apart to distribute the material evenly on both adjacent surfaces. The smears were allowed to dry in air, fixed in freshly-prepared Carnoy's fixative for 30 seconds, and then stained for 30 seconds to 10 minutes, depending upon individual protocols (Humason, 1972; Pearse, 1968, 1984). Stained preparations were mounted permanently by dehydrating in ethanol, clearing in xylene and mounting in a synthetic resin (DPX; BDH, Inc.).

For sectioning, samples of milled rice endosperm and rice products were fixed either in neutral buffered formalin or a mixture of 1% glutaraldehyde and 3% paraformaldehyde in phosphate buffer. The fixed material was dehydrated slowly, with vacuum, through 95% ethanol and embedded in 2-butoxyethanol methacrylate plastic (PolySciences, Inc., Warrington, PA). Sections were routinely cut at 3 μm and mounted on glass slides, baked at 80°C overnight to remove excess water, and then stained and mounted permanently.

Procedures used to identify rice protein bodies and their components fell into three categories:

a) **Protein-specific stains**: 0.5% aqueous eosin Y (C.I. 45380); 0.5% eosin Y in 95% ethanol; 0.1% aqueous light green SF yellowish (C.I. 42095); 1% aqueous orange G (C.I. 16230) in 5% tannic acid; 0.5% aqueous orange G + 1% aqueous aniline blue WS (C.I. 42780) in a combination normally used as the second component of the Mallory Trichrome stain; orange G + eosin Y; mercury bromphenol blue.

b) **Fluorochromes**: fluorescamine (0.01% in acetone) for protein; o-phthalaldialdehyde (OPT) for aromatic amines; 0.01% aqueous acridine orange (C.I. 46005) for proteins and nucleic acids (Pearse, 1968 and 1984).

c) **Histochemical procedures**: periodic acid-Schiff (PAS) to identify neutral polysaccharides; performic acid-Schiff (PFAS) to demonstrate S-S and S-H groups; nile blue sulphate for neutral lipids and phospholipids, and OPT for catecholamines.

The preparations were examined in both bright-field microscopy and in a Leitz epifluorescence unit using a fluorescein filter and barrier filters of 520 nm. Color photographs were prepared from Kodak Ektachrome slide film.

Table 1: STAINING REACTIONS OF RICE PROTEIN BODIES

PROCEDURE	REACTIONS	
	pbs/cores	bacteria
For Bright-Field Microscopy		
Eosin Y 0.5% aqueous	salmon-pink	salmon-pink
Eosin Y 0.5% alcoholic	salmon-pink	salmon-pink
Light green SF yellowish	u/s	green
Orange G + tannic acid	yellow	yellow
Orange G + tannic acid + eosin Y	yellow	salmon-pink
Orange G + aniline blue	yellow	blue
Mercury bromphenol blue	blue	blue
Performic acid - Schiff	mauve/pink	u/s
For Fluorescence Microscopy		
Eosin Y 0.5% aqueous	yellow/white	faintly yellow
Eosin Y 0.5% alcoholic	yellow/white	faintly yellow
Fluorescamine	white	white
O-phthalaldialdehyde	u/s	u/s
Acridine orange 0.01%	yellow	variable

Legend pbs - protein bodies; u/s - unstained

Periodic acid-Schiff stained starch magenta-pink; other structures were unstained.

Feeding Experiments

In these experiments, some of the seeds and grains mentioned above, both whole and ground, were used as diet items in addition to rice endosperms. In a preliminary experiment a hamster was fed a diet of whole rice and rice flour/protein concentrate prepared as a cake, or squash or peanut flour for three consecutive days. Fecal pellets were collected on Days 2 and 3 of the trial. For the succeeding four days of the week, the animal was returned to commercial hamster food. Fecal pellets were collected both before the beginning of the feeding trials and on Day 7, to represent the 'control' condition. The fecal pellets were air-dried, then ground in a mortar and suspended in water before being smeared on slides, fixed and stained. Later, rats were used in a single-blind feeding experiment using rice-, lactalbumin- or ovalbumin-containing diets. The fecal pellets from all rats on a particular regime were pooled and freeze-dried before being examined as described above.

Isolation Procedures

To establish the specificity of staining procedures for rice protein cores and fecal protein particles, as opposed to the intact protein bodies, experiments were undertaken to isolate cores from the protein bodies, and later to dissolve them.

Isolation of rice protein cores was routinely carried out using the commercially destarched rice protein concentrate, because of its high protein concentration. To produce cores of insoluble rice protein which were morphologically equivalent to fecal protein particles, the soluble proteins were removed by sequential extraction from the rice protein concentrate. One gram of water-washed protein concentrate was suspended in 5 ml 0.1 M NaOH for 15-30 minutes, twice, to dissolve soluble glutelins, neutralized with 1 M HCl, washed in distilled water and then treated with a five-fold excess of 70% ethanol or 60% 2-propanol to remove soluble prolamins. The aggregates of 1-3 μm particles obtained were distinguishable from fecal protein particles only by their relative purity and tendency to aggregate. The prepared cores were precipitated centrifugally, resuspended in distilled water as a slurry, and smeared on glass slides, fixed and stained as described above.

Formic acid or performic acid (formic acid + hydrogen peroxide + sulfuric acid) was used to dissolve protein cores and fecal protein particles (Resurreccion and Juliano, 1981; Pearse, 1984).

Results

Morphology of Rice Protein Bodies

Protein bodies of sectioned endosperm (Fig. 1), untreated flour or rice protein concentrate were approximately 3-6 μm in diameter, and generally circular or oval in section. Fecal protein particles and cores were spherical or broadly oval as well, measuring 1-3 μm (Fig. 2). In preparations stained with aqueous eosin Y for 10 minutes and examined under fluorescence microscopy, the rice protein bodies and fecal protein particles had a biphasic structure consisting of a brightly fluorescent rim encircling a less-fluorescent central region. With staining times increased to 45 minutes or longer, the biphasic structure was lost, and the protein body/core was uniformly brightly fluorescent. In wet smears (temporary preparations), the fecal protein particles were oval or spherical and morphologically resembled cocci or broad bacilli, but they were refractive, heterogeneous in size, and generally lacked the Brownian movement characteristic of bacteria. Rice protein concentrate that had been chemically treated to remove prolamins and glutelins consisted of clumps of cores which could not be disrupted with ultrasonic treatment alone. Fecal protein particles, on the other hand, were never clumped. Apart from these differences, cores and fecal protein particles were morphologically similar. There was no evidence that the destarching of rice flour had any effect on the size or staining qualities of the protein bodies in the rice protein concentrate, or on the cores derived from it.

Other seed flours examined showed a variety of sizes of protein bodies, ranging from amorphous or very finely particulate to spheres 6-8 μm in diameter, but only *Oryza sativa* protein bodies were always spherical, and gave the characteristic yellow-white fluorescence with eosin.

Staining Reactions

Bright-field Microscopy A summary of staining and histochemical reactions produced by rice protein bodies is seen in Table 1. In bright-field microscopy, the most consistent reaction differentiating seed protein bodies, rice cores, and rice fecal protein particles from similarly-shaped bacteria or other bits of debris was the orange G + aniline blue combination. Rice protein cores and fecal protein particles were pale to bright yellow, and bacteria were generally blue, with other material staining various shades of blue (Fig. 2). Protein bodies and cores from both raw and cooked rice also stained yellow with orange G + aniline blue, but these preparations generally were free from bacterial contamination.

Eosin Y + orange G stained all rice cores yellow and bacteria pink, but it was sometimes hard to differentiate color in the very small particles. Other procedures, while they stained the cores and fecal protein particles, colored bacteria the same way, so it was very difficult to differentiate rice from bacteria. Mercury bromphenol blue stained sectioned protein bodies in different shades of blue, but individual protein bodies stained uniformly, i.e., without differences in density. The central part of the intact protein body did not color more deeply than the periphery.

Histochemistry The periodic acid-Schiff (PAS) procedure for 1:2-glycol groups gave a brilliant magenta reaction in the starch grains, but failed to react with rice protein bodies. On the other hand, the protein bodies gave uniform mauve to pink reactions to performic acid-Schiff (PFAS), which demonstrates S-S or S-H bonds.

Figure Legends

Photographs 1, 2, 3, and 5 taken under oil immersion.

Fig. 1. Subaleurone rice protein bodies (pb) in sectioned whole grain; a variety of sizes is evident (arrows). Starch grains (S) are dark-staining and polygonal. Stain: PAS and hematoxylin.

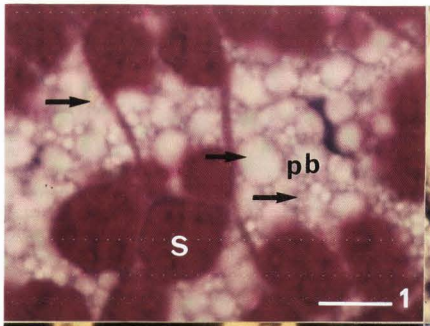
Fig. 2. Preparation from slurry of fecal material showing both fecal protein particles (large arrows) and bacteria (small arrows) stained by orange G + aniline blue. In some cases the fecal protein particles show adherent bacteria. Stain: Orange G + aniline blue.

Fig. 3. Fecal protein particles and bacteria seen in fluorescence microscopy. The brightly fluorescent fecal protein particles show clearly against the dark background (fpp); bacteria are less fluorescent (arrows). Stain: Aqueous eosin Y.

Fig. 4. Cell walls (arrows) remaining after the slurry of chemically prepared cores of rice protein bodies was treated with 'performic' acid for 15 minutes. Acid-mounted, temporary, unstained preparation.

Fig. 5. Area of the same preparation photographed in Figure 3, also under fluorescence microscopy, after treatment for 15 minutes with performic acid, restaining, and remounting. Bacteria (arrows) remain unchanged, but rice fecal protein particles are either dissolved or indistinguishable. Stain: Aqueous eosin Y.

Scale Bars = 10 μm.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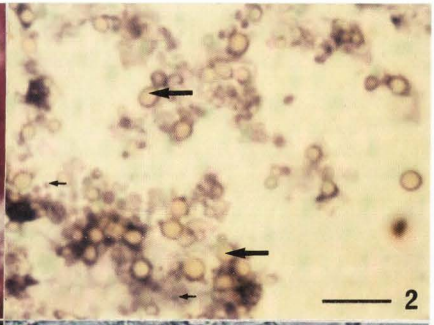
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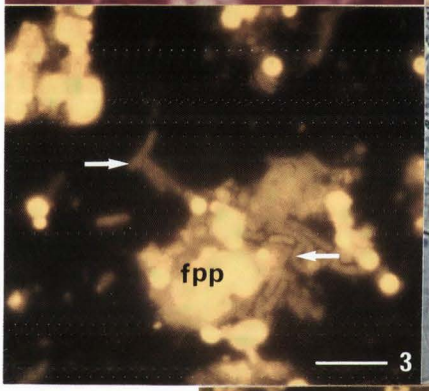
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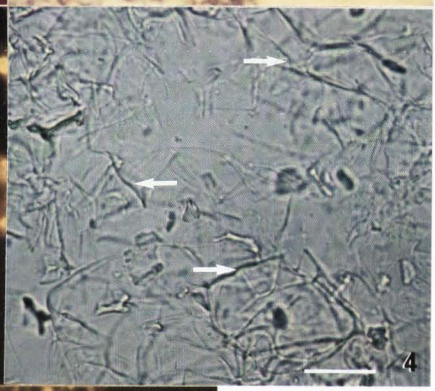
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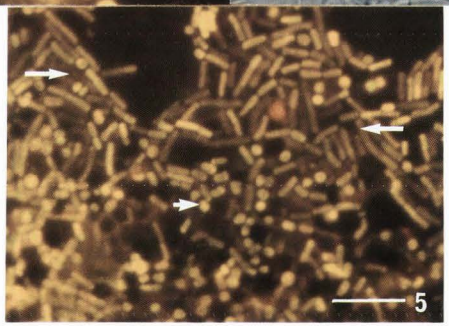
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The number of protein bodies in the unembedded slurry preparations treated with PFAS was drastically reduced after the procedure, whereas there was no apparent loss of material in sectioned preparations. In the latter, the protein bodies were uniformly pale pink. Nile blue sulphate for neutral lipids and phospholipids stained sectioned cell walls blue, a phospholipid reaction, but gave no other reactions in the endosperm of polished rice or rice products.

Fluorescence Microscopy Preparations stained for bright-field microscopy were also examined in fluorescence microscopy. In fluorescence microscopy, protein bodies/cores and fecal protein particles of rice stained with 0.5% aqueous eosin Y fluoresced brilliantly yellow-white against a generally dark background (Fig. 3). The starch of all the seed flours examined when uncooked also gave a brilliant greenish-white fluorescence; the reaction was largely abolished after the starch was gelatinized by moist heat. In any case, the shades of color between fluorescent starch and protein were quite different, even for rice. In some preparations of intact rice protein concentrate, the spherical protein bodies were surrounded by an amorphous material fluorescing orange-red. Fecal protein particles in the eosin-stained preparations were especially clear because they were not aggregated. Occasionally cocci fluoresced a fainter yellow-white; when fecal particles and bacteria were adjacent in a preparation, there was little difficulty in distinguishing one from the other. Coccoid bacteria were also all the same size (about 1 μm), while the fecal protein particles ranged from 1-3 μm in diameter. Bacilli were also commonly seen in these preparations (Fig. 3).

Fluorescamine-treated rice examined in the fluorescence microscope also resulted in a bright white reaction from protein bodies but adjacent non-protein material also fluoresced. Protein bodies treated with mercury bromphenol blue fluoresced a dark red color, but showed no distinction among sizes or types of protein body; the fluorescence, moreover, was very difficult to see. No other procedures tried in this study resulted in specific fluorescence of rice protein cores or fecal protein particles.

Treatment of chemically-prepared protein cores with formic/performic acids resulted in the removal of most cores, leaving behind a latticework of cell wall structures (Fig. 4) which provided structural support for the aggregates. When these acids were applied to fecal preparations containing indigestible rice protein cores, the cores disappeared, leaving fecal bacteria (Fig. 5).

Feeding Experiments with Other Plant Proteins

When other seed/grain flours were examined after orange G + aniline blue staining, only squash flour contained spherical or oval protein bodies stained deep yellow; they were slightly larger than those of rice. The squash protein bodies were also strongly fluorescent after eosin Y staining. Other seed/grain proteins showed a diffuse yellow staining with orange G, indicating the presence of protein, and a faint yellow-to-orange fluorescence after eosin Y. None of the other flour products, except squash, had any structures which could be confused morphologically with rice protein bodies.

Examination of the hamster fecal pellets showed that only a rice diet resulted in feces with particles that fluoresced after eosin Y staining, or yellow particles after orange G + aniline blue. Feces examined from feeding experiments with seeds/grains other than rice, including squash, appeared no different from the control.

In single-blind feeding experiments, where the diets themselves were examined microscopically, the diets containing rice showed the reactions to orange G + aniline blue and to eosin Y characteristic of rice, and except for a few fluorescent particles identified as 'rice cores' in lactalbumin-containing diets, identifications matched the presence of rice. However, the fluorescent particles seen in the lactalbumin-containing diet substances were absent from the comparable fecal samples. Only rats fed on rice diets showed fecal protein particles in microscopic preparations.

Discussion

The intent of this work was to discover procedures which stain rice protein bodies and cores differently from other components of rice seeds, and from the protein bodies of other seeds. During the course of these experiments, we discovered several compounds which color rice endosperm protein bodies, cores and fecal protein particles. In fact, any stain for acidophilic proteins successfully demonstrated rice protein. The mercury bromphenol blue technique identifies 'protein', but it fails to differentiate among types of protein. In rice, some procedures preferentially stained cytoplasmic protein outside the storage bodies, e.g., light green SF yellowish. For bright-field microscopy, the orange G + aniline blue combination comprising the second stage of the Mallory Trichrome stain differentiated between the protein bodies and other components in cooked preparations or fecal samples, and was especially useful for studies of fecal smears. This particular color combination also provided the greatest possible contrast between rice protein particles and other components of the smear. For example, although eosin and orange G selectively stained rice cores and fecal protein particles yellow and bacteria pink in bright-field microscopy, those two shades are sufficiently similar to be difficult to distinguish in small structures, and provide little contrast for photography.

In the fluorescence microscope, the best method for identifying rice protein particles as distinct from protein bodies of other seeds was found to be 0.5% aqueous eosin Y, chemically identified as tetrabromofluorescein. The observation is empirical; it is not clear why rice protein, of all the cereal proteins studied, should give such a strong reaction to the stain while others fluoresce only weakly under identical conditions. The brightness of the fluorescence is not related to the depth of eosin staining as seen in bright field microscopy for any of the seeds studied. Only a few 1-2 μm cocci in fecal smears showed fluorescence with the same brightness as rice protein bodies, and they could be differentiated from the rice by their uniform size and generally more modest excitation. As well, they failed to stain with orange G in bright-field preparations. Other fluorochromes, including alcoholic solutions of eosin Y tested, lacked specificity, were more expensive, and demanded more technical expertise than the aqueous eosin Y procedure. The presence of a few fluorescent particles in lactalbumin-containing diets was unexpected, and their cause unknown. They may have been a particular type of bacteria not seen in the other animals or experiments, or even contamination of the diet by material containing rice protein bodies; at any rate, no fecal protein particles were seen in the feces of rats fed a lactalbumin-containing diet.

Resurreccion and Juliano (1981) identified a higher concentration of sulphur-containing amino acids in the insoluble cores of rice protein bodies than in the rest of the soluble protein, and suggested the S-S bonds were responsible for the indigestibility of the cores. Our observations, on the other hand, showed a uniform distribution of stains over all embedded rice protein bodies, including the PFAS technique which is specific for S-S bonds (Pearse, 1984). In sectioned material, actual differences might be missed because of the thin methacrylate sections used. When the PFAS procedure was applied to unembedded material, there was a general reduction in the number of protein bodies and cores when they were exposed to the performic acid, but formic acid is a known solvent of rice protein cores (Resurreccion and Juliano, 1981). This observation, more than the histochemical reactions, supports the hypothesis that S-S bonds in the protein bodies/cores are involved in the structural integrity of these particles. Our histochemical observations on sectioned material do not indicate regional differences in the location of demonstrable S-S bonds, inasmuch as one protein body type does not color darker with PFAS than another. Cores and fecal protein particles give no histochemical indications they are composed of material other than protein. While there may be oxidation of other components of the core which renders them available to further breakdown by strong formic acid, the mechanism(s) remain enigmatic.

The combination of bright-field and fluorescence procedures should be useful for investigations of rice protein body structure and digestion in monogastric animals. Individually, each method may be insufficient for the untrained observer, but used together, the identification of rice protein particles is reliable even when the rice has been combined with other grains or when fecal preparations are examined.

Acknowledgements

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Discussion with Reviewers

M. Smart: The authors discussion where the insoluble proteins are located in rice endosperm protein bodies. Have they, or has anyone else, evaluated the possibility that the spherical protein bodies obtained by exhaustive extraction or those collected from fecal material are spontaneous artifacts produced during extraction/ digestion?

B.O. Juliano: The authors may have overlooked the fact that in rats, and probably also in man, indigestible rice protein bodies exist only after cooking, although the large spherical protein bodies ... [Type I] ... are less digested by pepsin than crystalline protein bodies ... [Type II] ... even in the raw grain. By contrast, pronase readily corrodes 80% by volume of the ... [Type I] ..., but not ... [Type II] ... (Bechtel and Pomeranz, 1978b). Eggum et al. (1977) demonstrated reduction of true protein digestibility of milled rice from 99.7% to 88.6% on cooking of three rices.

Authors: On the face of it, Dr. Juliano's comment might be considered to be the answer to Dr. Smart's question, with rice protein cores and fecal protein particles being regarded as artifacts brought about by heat treatment of raw rice. However, it should be pointed out that the above assertions, and those statements supported by Eggum et al. (1977) for 99.7% digestibility of raw rice protein, are based entirely upon chemical analysis of rat feces; there is no indication in that work that the feces from rats fed raw (but milled) rice were examined microscopically for fecal protein particles. In our opinion there is no morphological evidence that rice protein cores or fecal protein particles are simply processing artifacts; indeed, circumstantial evidence exists in several places in favor of a 'natural' occurrence of such indigestible material. For one, the work of Bechtel and Pomeranz (1978b) was carried out on raw rice, and this work gives enzymatic evidence that at least a part of one type of protein body contains material consistently inaccessible to proteases. For another, the rice flour we used has been subject only to milling, not cooking, and from both chemical extraction (done at room temperature) and feeding experiments it is clear that, depending upon the material, unextractable or indigestible protein particles are present. In fact, the fundamental question of whether rice protein particles are present in the feces of any animal species fed raw rice has never been addressed. Since our work dealt exclusively with 'processed' rice and rice products (either milled, enzymatically treated, or already cooked), we did not set out to explore the possibility of the absence of indigestible particles in one or more of these products. The present paper represents the first published information on the results of deliberately extracting soluble proteins chemically from rice in order to study the remnants.

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