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Formation of dialyzable iron during in-vitro digestion and extraction of mycoprotein

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Abstract

Mycoprotein is the biomass of the fungus *Fusarium venenatum*. The ability of mycoprotein to enhance production of dialyzable iron following in-vitro digestion or extraction was studied, with the aim of identifying the components responsible. Digested mycoprotein, extracted mycoprotein and digested chicken breast muscle all produced about ten times as much dialyzable iron as the control. All of the dialyzable iron from mycoprotein was ferric. Essentially all the dialyzable iron passed through a 1 kDa molecular weight cut-off membrane. Iron binding components were extracted from mycoprotein using weak acid but not water. Extraction of mycoprotein produced both dialyzable and non-dialyzable components, which bound ferric iron. Analysis of the digests and the dialyzable fraction of acid extraction showed that they contained siderophores, which contributed to the formation of dialyzable iron.

Keywords: Mycoprotein; Dialyzable iron; Siderophores

1. Introduction

Iron is an essential micronutrient especially important during growth and development. Most of the iron in the diet is in the non-heme form and is poorly absorbed (Carpenter & Mahoney, 1992). Bioavilability of iron is affected by a number of dietary factors which can interact with the non-heme iron, including proteins and their digestion products (Fairbanks, 1999).

Mycoprotein is the nucleic acid – reduced biomass of the mushroom – like fungus *Fusarium venenatum* (Rodger, 2001; Miller & Dwyer, 2001). Commercial food products containing mycoprotein are marketed as an alternative to meat products, since the biomass has a fibrous texture and has the advantage of being devoid of animal fats and cholesterol.

Despite some nutritional drawbacks, muscle tissue/meat has the ability to enhance the bioavailability of non-heme iron (Bjorn-Rasmussen & Hallbert, 1979), a property not found in other animal proteins (Cook & Monsen, 1976; Hurrell, Lynch, Trinidad, Dassenko, & Cook, 1989) or in plant proteins (Cook, Morck, & Lynch, 1981) and not yet shown in microbial protein. However, preliminary studies with commercial products containing mycoprotein, marketed under the trade-name Quom™, indicated that following digestion they produced dialyzable iron (Shinde & Mahoney, 2004), which is of interest because it is in-vitro indicator of iron bioavailability (Sandberg, 2005).

These products, however, contained small amounts of filler proteins and additives, such as citric acid, which could have influenced the results. The effect of mycoprotein itself on the production of dialyzable iron was studied with the objective of identifying the source/nature of the chelators. The results were also compared with those from chicken muscle, for which mycoprotein is an alternative.

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2. Materials and methods

2.1. Materials

Freeze-dried mycoprotein biomass was obtained from Marlow Foods, Billingham, Cleveland, UK. The mycoprotein was produced by fermentation of *F. venenatum* A3/5 (deposited with the American Type Culture Collection as PTA-2684) as described by Rodger (2001).The freeze-dried biomass contained ~95% solids and ~5% water. It contained 51.3% protein calculated as nitrogen × 6.25; nitrogen was measured by the micro-Dumas method (Ma & Rittner, 1979).The remaining solids were: fibre (25–28%) consisting of chitin, β1–3 and β1–6 glucans; fat (12–14%); water soluble sugars (2–5%) and ash (<5%); according to data supplied by the manufacturer.

Skinless, boneless chicken breast, chilled but not frozen, was obtained from a local supermarket. Dialysis membranes were from Spectrum Labs, Rancho Dominguez, CA, USA. For digestions to measure total dialyzable iron, Spectra/Por 1 membranes with a diameter of 20.4 mm and molecular weight cut-off (MWCO) of 6–8 kDa were used. For size analysis Spectra/Por 7 membranes with varying MWCO were used. All membranes were soaked in deionized water and rinsed several times before use.

2.2. Chemicals

Pepsin, pancreatin, bile extract, PIPES buffer, ferrozine, bovine serum albumin and reagents for the siderophore assay [chrome azurol S(CAS), hexadecyltrimethylammonium bromide(HDTMA), desferrioxamine B and 5-sulfosalicylic acid] from Sigma Chemical (St. Louis, MO USA). All other chemicals were reagent grade. Distilled, deionized water was used throughout.

2.3. Methods

All glassware were rinsed in 2 N HCl and then rinsed several times with distilled, deionized water before use.

2.4. Digestions

The digestion procedure was based on the method originally described by Miller, Schricker, Rasmussen, and Van Campen (1981) and included modifications designed to reduce the amount of extraneous iron and control the final pH. The details of the procedure have already been described (Vattem & Mahoney, 2005).

Mycoprotein (3.85 g) containing 2.0 g protein was dispersed in 90 ml water. Chicken muscle (8.6 g) containing 2.0 g protein was homogenized in 90 ml water for 3 min, in one minute bursts. After addition of ferric iron (37.5 μmoles/2 g protein) and adjustment to pH 2 the samples were digested with pepsin (pH 2) and then pancreatin/bile (pH 6.5) as previously described (Vattem & Mahoney, 2005). Adjustment of pH after the pepsin digestion was made with a dialysis bag (6–8 kDa MWCO) containing sufficient $NaHCO_3$ to neutralize the titratable acidity (Vattem & Mahoney, 2005); the final pH after pancreatin digestion was 6.5 ± 0.1.

2.4.1. Controls

An iron only control was run using the procedures described above but using water in place of the protein sample.

In order to compensate for endogenous iron in the samples, proteins were digested as above without adding ferric iron. The resulting iron values were subtracted from the values with added iron to correct for the contribution of endogenous iron.

2.4.2. Non-digested samples

Non-digested samples were prepared with mycoprotein and iron using the procedure described above under digestions but omitting the enzymes.

2.5. Extractions

2.5.1. Water and acid extracts

Water extracts were obtained by suspending 1 g mycoprotein in 25 ml water and shaking for 1 h at 37 °C. For acid extracts, the suspension was adjusted to pH 2 using 6 N HCl before shaking. After shaking, the suspension was centrifuged for 10 min at 1750*g* and the supernatant was used to determine iron binding.

For extracting siderophores, a sample was extracted with acid by shaking for 2 h at 37 °C; a dialysis bag (1 kDa MWCO) containing 20 ml 0.01 N HCl was placed in the suspension prior to shaking.

2.5.2. Extraction using the digestion conditions

Samples were extracted using the procedures and conditions of digestion (above) but without enzymes and iron. This was done to extract any iron chelators which were present in the sample before digestion and which would be extracted during digestion.

2.6. Analysis

After the digestion procedure the dialyzate and retentate were centrifuged at 1750*g* for 10 min to remove insoluble iron. Aliquots of the supernatants containing soluble iron were mixed with equal volumes of reducing protein precipitant and of non-reducing protein precipitant (Vattem & Mahoney, 2005) and left overnight. The next day the samples were centrifuged again at 1750*g* for 10 min. to remove insoluble protein. The final supernatants were analyzed for protein and iron.

Protein was measured by the Biuret method (Cooper, 1977) using bovine serum albumin as a standard.

Total and ferrous iron were measured with ferrozine as previously described (Vattem & Mahoney, 2005) using a standard curve generated using $FeC1_3$ (0–5 mg/ml) in the

presence of hydroxylamine hydrochloride. To measure iron binding in extracts, 2 ml of iron solution containing 50 μg Fe^{3+} per ml in 0.1 N HCl were added to 8 ml extract and the pH was adjusted to 2 with 6 N HCl. After 10 min 0.2 M Na_2CO_3 was added dropwise to bring the pH to 6.5 and the final volume was adjusted to 12 ml. The mixture was then centrifuged for 10 min at 1750*g* to remove insoluble iron and the supernatant was analyzed for soluble iron as described above.

Siderophores in the enzymatic digest and the dialyzable acid extract were determined using the universal siderophore assay (Schwyn & Nielands, 1987) using desferrioxamine B as a standard.

Each experiment was repeated three times unless otherwise stated. Each pepsin digestion was followed by two pancreatin digestions of the same sample. Data were analyzed by one-way analysis of variance. Means were compared for significance by Tukey's method with a 95% confidence limit.

3. Results

3.1. Digestion

The effect of mycoprotein and chicken muscle on the production of dialyzable iron is shown in Fig. 1. Digested chicken and mycoprotein produced very similar amounts of dialyzable iron, about 10 times that of the control. However, non-digested mycoprotein produced about as much dialyzable iron as the digested sample. For both mycoprotein samples, essentially all the dialyzable iron was ferric, whereas for chicken about one-third of the dialyzable iron was ferrous.

The effect of these samples on production of total ferrous iron is shown in Fig. 2. In the control, the ferrous iron represented about 10% of the total soluble iron. The mycoprotein samples produced less ferrous iron than the control, whereas chicken produced about twice as much as the control.

Following digestion of mycoprotein, the amount of iron dialyzed at each molecular weight cut-off (MWCO) is shown in Fig. 3. There was no significant difference in the amount of dialyzable iron at each MWCO indicating that all the dialyzable iron was small enough to pass the 1 kDa MWCO. The amount of dialyzable ferrous iron was less than 5% of the total dialyzable iron for each MWCO (8 kDa: 2.33 ± 0.45 μg; 3.5 kDa: 1.50 ± 0.62 μg; 2 kDa: 1.83 ± 0.55 μg; 1 kDa: 0.43 ± 0.11 μg) and was not considered significant.

Analysis of the digested mycoprotein in the absence of added iron showed that it contained siderophores at a level of 288 ± 5 μmoles desferrioxime equivalents/g mycoprotein.

3.1.1. Extractions

The ability of water and acid extracts of mycoprotein to solubilize iron is indicated in Fig. 4. The water soluble extract produced less soluble iron than the control but the acid extract solubilized about three times as much as the control, indicating that it contained iron binding components.

Mycoprotein was extracted using the digestion conditions (acid and then neutral) but without enzymes or iron and the extracts tested for the ability to solubilize iron. The results are shown in Fig. 5. Both dialyzable and non-dialyzable fractions solubilized more than two times as

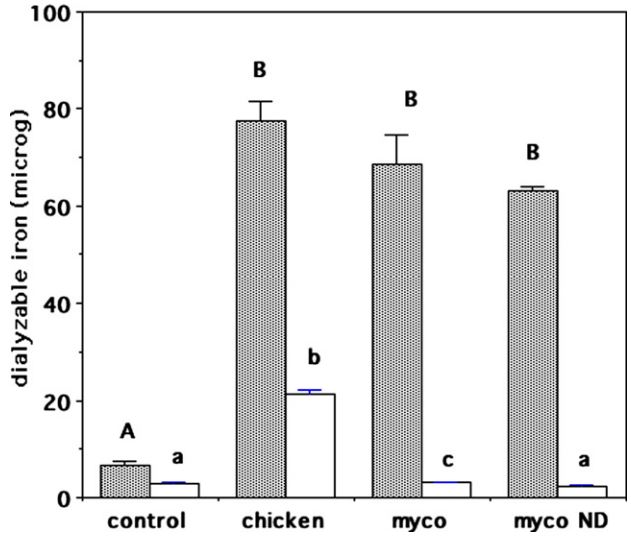

Fig. 1. Production of dialyzable iron by mycoprotein and chicken:■ total dialyzable iron; □ ferrous dialyzable iron. myco, mycoprotein; myco ND, non-digested mycoprotein. Values are means ± SD ($n = 6$). Means in the same group (□ or ■) without a common letter differ at $p < 0.05$. Where no error bar is shown, the variance was too small to register.

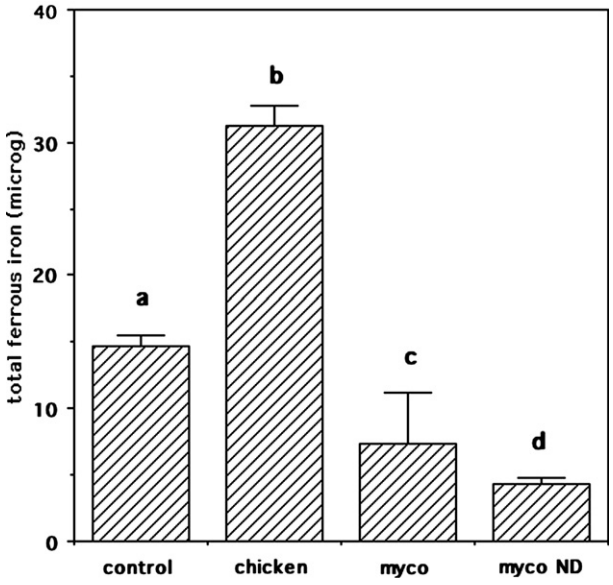

Fig. 2. Production of total ferrous iron by mycoprotein and chicken. myco, mycoprotein; myco ND, non-digested mycoprotein. Values are means ± SD ($n = 6$). Means without a common letter differ at $p < 0.05$.

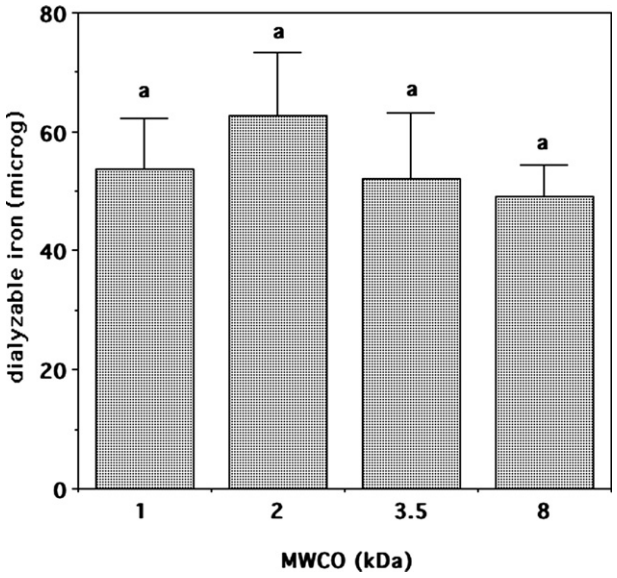

Fig. 3. Size of the dialyzable iron from digestion of mycoprotein. MWCO, molecular weight cut-off of dialysis bags Values are means ± SD ($n = 6$). Means without a common letter differ at $p < 0.05$.

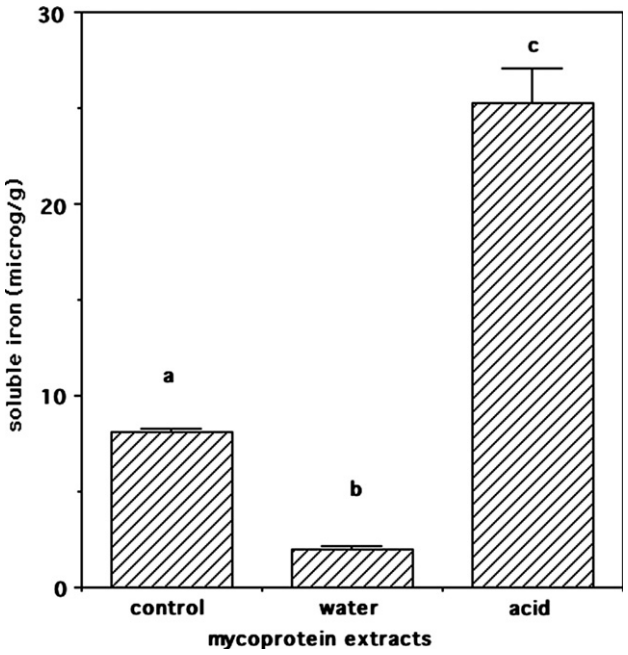

Fig. 4. Production of soluble iron by water and acid extracts of mycoprotein Values are means ± SD ($n = 3$). Means without a common letter differ at $p < 0.05$.

much iron as the control. These results indicated that both low (dialyzable) and high (non-dialyzable) molecular weight compounds were extracted that could bind iron. Both the dialyzable and non-dialyzable extracts contained some peptide material. After extraction using the digestion conditions the mycoprotein residue was subjected to enzymatic digestion with iron and the results compared to non-extracted samples. Extraction prior to digestion led to a 63% drop in dialyzable iron. The results confirmed that

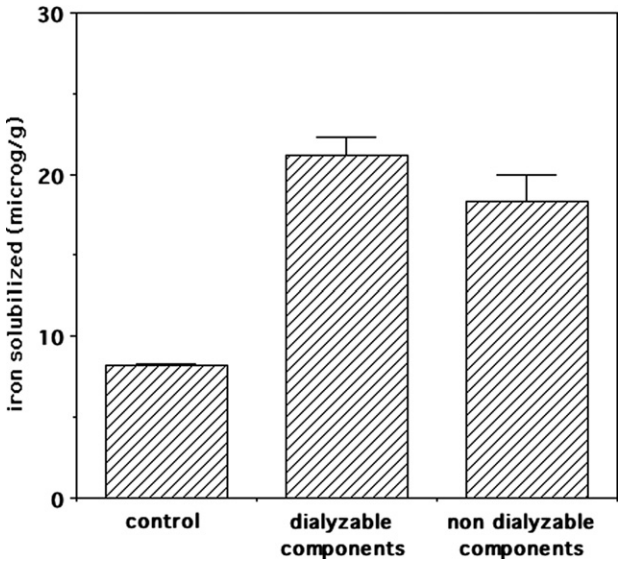

Fig. 5. Production of soluble iron by dialyzable and non-dialyzable components of an extraction using the digestion conditions. Values are means of two experiments ± half the range.

the extraction procedure removed components of mycoprotein which produced dialyzable iron.

Analysis of the 1 KDa dialyzable acid extract showed that it contained siderophores at a level of 176 ± 3 μmoles desferrioxime equivalent/g mycoprotein.

4. Discussion

Mycoprotein is a rich source of dietary protein. Many other food protein sources have some effect on the bioavailability of iron but the effects are widely varied (Fairbanks, 1999). So far animal tissues (meat/fish/poultry) are the only protein sources which have been shown to increase iron absorption (Lynch, 1984). Proteins from milk.egg and plant foods generally depress iron bioavailability because they produce peptides which form insoluble complexes or else do not release the bound iron to mucosal receptors (Berner & Miller, 1985). Chicken muscle, along with other muscle foods, is a known enhancer of iron uptake due to the so called "meat factor". The origin of the factor is generally believed to be peptides resulting from digestion of muscle proteins (Hurrell, Reddy, Juillerat, & Cook, 2006) although there is some evidence for the role of a carbohydrate fraction (Huh, Hotchkiss, Brouillette, & Glahn, 2004). The peptides can chelate and/or reduce ferric iron thereby maintaining its solubility in the intestine and promoting absorption.

The results of the study show that digestion of mycoprotein produced as much dialyzable iron as digestion of chicken muscle. Dialyzable iron formation following in-vitro digestion has been widely used as an in-vitro indicator of iron bioavailability. With some exceptions, correlation of dialyzable iron with human absorption is quite high (Sandberg, 2005). Wolfgor, Drago, Rodriguez, Pellegrino, and Valencia (2002) reported that the best

correlation is achieved when the final pH of the digest is between 6 and 7 and in this study we used a final pH of 6.5 in line with that conclusion. There is a general consensus that dialyzability can be used to compare and rank iron fortification compounds in food matrices although the magnitude of the response may not be similar to that in humans. The usefulness, precision and limitations of iron dialyzability as a predictor of human absorption has recently been reviewed by Lynch (2005) and Sandberg (2005).

While digestion of mycoprotein produced as much dialyzable iron as digestion of chicken muscle, there were several experimental results that indicated that the origin of the dialyzable iron was not the same. For mycoprotein, omission of the enzymes produced as much dialyzable iron as the digested sample, indicating that the components responsible were extracted from mycoprotein and not derived from proteolytic digestion.

Size analysis of the dialyzable iron indicated that molecular weight was smaller than 1 kDa. In this respect, mycoprotein differs from chicken muscle where most of the dialyzable iron was in the range 2–3.5 kDa (Vattem & Mahoney, 2005) and presumed bound to peptides resulting from digestion. Any iron-binding peptides are present in mycoprotein would have to be quite small (<6 amino acid residues) to produce bound dialyzable iron smaller than 1 kDa.

Essentially all of the dialyzable iron from mycoprotein samples, whether digested or not, was ferric, unlike the chicken sample where about one-third was ferrous. This may be of significance for bioavailability since it is thought that iron must be reduced to be absorbed (Woolenberg & Rummel, 1987) either in the intestinal lumen or at the absorption sites. Furthermore Kapsokefalou and Miller (1991) found that dialyzable ferrous iron was a better predictor of iron bioavailability than total dialyzable iron for a range of proteins and muscle foods. The near absence of dialyzable ferrous iron from mycoprotein samples could therefore indicate low bioavailability.

Acid extraction of mycoprotein effectively released components which bound ferric iron whereas water extraction did not. However it is possible that water extracted components which bound iron and then precipitated, which could explain why the iron solubilized by water extracts was significantly lower than in the control (no extract). Extraction using the digestion conditions (acid and then neutral pH) produced both dialyzable and non-dialyzable components which solubilized ferric iron. Accordingly, mycoprotein must contain both low and high molecular weight iron chelators.

Analysis of the low molecular weight fraction from both digests and extractions, which contained peptide material, showed that they contained siderophores. These are small peptides (600–1000 Da) secreted by most microbes and some plants, which chelate poorly soluble ferric iron and permit uptake of the mineral (Dreschel & Jung, 1998) The majority of the filamentous fungi produce siderophores, which chelate ferric iron very tightly via a hydroxamic acid functional group (Winkleman, 1990). Siderophore formation has been reported when *F. venenatum* was grown in iron-restricted cultures (Wiebe, 2002). It is therefore clear that formation of dialyzable iron by extraction or digestion of mycoprotein is due, in part at least and perhaps entirely, to chelation by siderophores bound to or within the cellular material. We found a higher level of siderophores in the digests than in the acid extract. This could be due to the breakdown of the cell mass during digestion or to the longer extraction period, both of which could promote extraction. Our results do not rule out the possibility that other small compounds, which chelate iron, are extracted from the cell mass, e.g. peptides other than siderophores or organic acids. In this regard analysis of mycoprotein extract for citric acid, by HPLC, proved negative.

The binding constants for ferric iron by hydroxamate siderophores are in the range 10^{23}–10^{30} (Dreschel & Jung, 1998) which provides for a very strong complex and may make absorption difficult. In this respect siderophores may be classed with small compounds such as phytate, organic acids and phenolics, which provide high dialyzable iron but which bind iron too tightly to permit absorption (Sandberg, 2005). Alternatively they might be classed with chelators such as EDTA which also bind iron tightly, but which can enhance absorption, especially in the presence of competitive inhibitors such as phytate (Hurrell, 2002). However, there is no evidence of an specific uptake pathway for siderophore-bound iron in humans.

In conclusion the production of dialyzable iron by mycoprotein is due in part or even entirely to binding by siderophores. Since these do not reduce the iron but instead bind tightly to the ferric form the bioavailability of iron is likely to be low compared to chicken muscle. However, this can only be confirmed by uptake studies, preferably with human subjects.

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