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Whey Proteins as Functional Food Ingredients?★

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ABSTRACT

Putative anti-cancer activity of whey proteins has been investigated in an animal model to evaluate their potential role in disease prevention, and to contribute to a basis for their inclusion as ingredients in functional foods. Animal feeding trials have compared the efficacy of dietary whey proteins in retarding chemically induced colon cancer in a rat model of the disease. Dairy proteins, in particular whey protein, were found to be efficacious in retardation of colon cancer in young rats compared with other dietary proteins (meat, soy). The influence of dietary whey protein on development of colon cancer in mature rats has also been examined. Results similar to those with younger animals have been demonstrated, a finding that suggests age does not significantly alter the outcome. Efficacy of whey protein fractions has also been assessed. Preliminary results suggest that diets supplemented with lactoferrin or with β-lactoglobulin enhance protection against the development of putative tumor precursors (aberrant crypts) in the hind gut wall. The mechanism behind the apparent anti-cancer activity of dietary whey proteins in these studies may be related to their sulfur amino acid content, for which there is a high requirement in the rat, and hypothesised role in protecting DNA in methylated form. In a parallel study, a number of potential functional foods containing whey protein (flavored milk, pasta, ice cream, dessert pudding, muesli, and savory dip) have been developed in preparation for human clinical trials. The foods containing whey protein were generally highly acceptable in taste trials. These products are expected to be suitable as delivery vehicles for dietary whey protein in studies aimed at substantiating the human health benefits of this protein source.

Keywords: whey protein; functional food ingredients; lactoferrin; bioactive factors; intestinal cancer; dietary protein

INTRODUCTION

Whey is a byproduct of cheese and casein manufacture, and contains approximately 20% of the original milk protein. Volumes of this dairy fluid are on the rise worldwide (Fig. 1), concurrent with a global increase in the production of cheese and, to a lesser extent, casein. Indeed, by the turn of the century volumes of whey produced worldwide are projected to approach 100×10^9 L/yr. Most importantly, such huge volumes of whey represent a massive and growing protein resource available to the food and related industries of about 500×10^6 kg/yr (Fig. 1). Historically, this protein resource has either been considered a waste-product and disposed of in the most cost-effective manner or processed into relatively low-value commodities such as whey powder and various grades of whey protein concentrate. While the latter products have captured some of the value of whey protein as a food ingredient, the full potential of this resource has yet to be realised.

Whey represents a rich and varied mixture of secreted proteins with wide-ranging chemical, physical and functional properties (Smithers *et al*., 1996). These proteins not only play an important role in nutrition as an exceptionally rich and balanced source of amino acids (Regester *et al*., 1996), but in a number of instances also appear to have specific physiological actions, *in vivo*. While it has long been recognised that several whey proteins confer non-immune protection to the neonate against disease, these and other dairy proteins also have putative biological and physiological effects (Table 1). Such proteins include α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, immunoglobulins, glycomacropeptide, and a variety of growth factors. These proteins have been implicated in a number of biological effects observed in human and animal studies, ranging from anti-cancer activity to influence on digestive function (Table 1). Moreover, these same proteins, once partially digested, serve as a source of bioactive peptides with further physiological activity. To the food industry, these putative biological and physiological activities offer several opportunities. First, they provide the rationale for a thorough investigation and rigorous scientific evaluation of potential health benefits elicited by such proteins when included in the diet. Second, they provide the basis, once substantiated in human trials, for development of valuable whey protein-based food ingredients targeted at non-traditional dairy markets, such as the functional food sector. This market may provide, at least in part, the scope for realization of the potential of whey protein and whey protein fractions as important ingredients in health-promoting foods.

Functional foods (also referred to as physiologically functional foods, nutraceuticals, pharmafoods or

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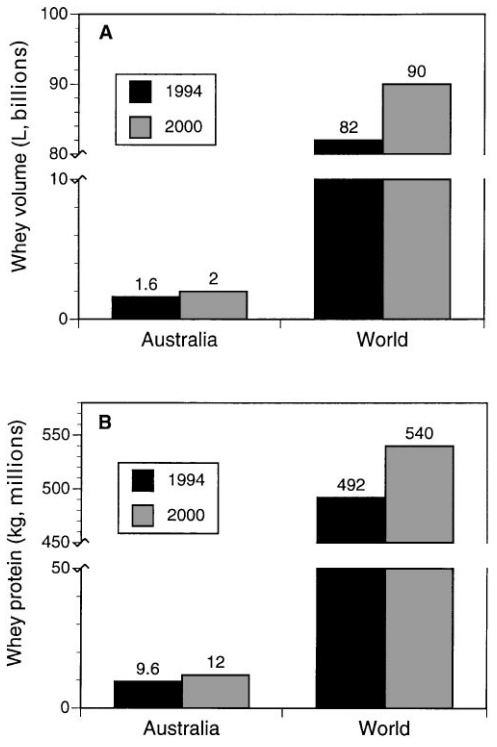

Fig. 1. Volumes of dairy whey (A) and quantities of whey protein (B) produced in Australia and in the world each year. Figures are presented for the mid-1990s, and for the turn of the century (projected). The latter value is based on estimated annual increases in the production of cheese.

designer foods) are those that offer an identified health benefit when consumed. These are foods made from natural ingredients, consumed as part of the normal diet which, in addition to nutritional sustenance, confer a specific health advantage. The functional food market is in a phase of rapid growth, particularly in Asia and North America. The worldwide market size for this food sector is projected to reach in excess of US$ 80,000 million annually by the turn of the century, and thus represents a lucrative opportunity for the food industry (Sloan, 1996). The growing interest in foods that offer a health benefit can be traced to a strong consumer desire for 'health through food', and to the serious social and economic costs of poor or inappropriate diet. The nature of the functional food sector and the design of ingredients for functional foods are also being influenced, particularly in Asia, with changing food consumption patterns, most notably a strong and growing desire for edible protein. For example, over the past 30–40 yrs the Asian diet has progressively included more animal protein, and this increase has often involved dairy-derived proteins. Data presented in Fig. 2 show the growth in protein consumption by Japanese consumers over the past 40 yrs, an increase of some 36% (Department of the Prime Minister and Cabinet, 1994).

The putative biological activity of whey proteins and their derived peptides, the rapidly expanding functional food market, and the growing consumer demand for high-quality protein combine to provide an attractive foundation for the development of whey protein and whey protein fractions as functional food ingredients. To strengthen this foundation and to provide evidence for health claims associated with functional foods containing whey proteins, scientific substantiation of the putative physiological activity of these proteins will be essential in both animal and human trials.

For hundreds or perhaps thousands of years, various cultures and societies have used whey in the prevention and treatment of disease as part of folk medicine. Indeed, there are references to various health benefits of whey in the Italian literature dating back to the early 17th century (Baricellus, 1603). During this period of history, whey was often prescribed for the treatment of numerous ailments, including acute septic conditions and gastrointestinal infections. Rather than rely on folk medicine however, we have pursued a careful scientific investigation of the physiological functionality of the whey protein components and their possible role in disease prevention. For these studies we have focused much of our attention on colorectal cancer.

Colorectal or bowel cancer is one of the most common neoplastic diseases affecting men and women in western countries. There is increasing evidence that components of the diet in these countries, particularly red meat and animal fat, are linked in a causative fashion to the high incidence of colon cancer (Giovannucci *et al*., 1994; Willett *et al*., 1990). Dairy foods are usually included in animal-based diets, rich in red meat and animal fat, and are capable of contributing a significant amount of dietary protein and/or fat, although the fat issue is being addressed through the increasing availability of low-fat dairy products. Our own studies (McIntosh *et al*., 1995) and those of others (Papenburg *et al*., 1990; Nutter *et al*., 1990) have established that dietary dairy proteins are protective against the development of colon tumors in a rat model of the disease in which tumors are induced with dimethylhydrazine. Indeed, whey proteins were found to be particularly effective at lowering the incidence and burden of gastrointestinal tumors in the test animals, relative to meat and soybean proteins (McIntosh *et al*., 1995).

In the present study, we have complemented our earlier work with young animals by examining the influence of dietary meat and whey protein on metabolism and chemically induced cancer incidence in a mature age rat model that reflects more closely the stage in life at which greatest susceptibility to colon cancer occurs. We have also extended our initial work with total protein sources to examine the influence of diets supplemented with selected whey protein fractions on development of putative tumor precursors in the hind gut wall. A possible mechanism for the anti-cancer activity of dietary whey proteins has also been proposed based on the sulfur amino acid content of these proteins and their hypothesised ability to protect DNA in methylated form. Preliminary development and sensory evaluation of a number of potential functional food products, in which whey protein has been incorporated as the active ingredient, provides a basis for substantiating the results presented here in human clinical trials.

EXPERIMENTAL PROCEDURES

Materials

Protein products

Whey protein concentrate (80% protein) was supplied by United Milk Tasmania Ltd (Allansford, Victoria).

Table 1. Putative Biological Activity of Whey Proteins and Peptides

Protein or peptide	Putative activity	Reference
Total whey protein	Anti-carcinogenic	Bounous *et al.* (1988a) Bounous *et al.* (1991) McIntosh *et al.* (1995) Kennedy *et al.* (1995)
	Immunostimulatory	Bounous *et al.* (1988b) Bounous *et al.* (1989a) Bounous *et al.* (1993)
	Organism longevity	Bounous *et al.* (1989b)
	Hypocholesterolaemic	Zhang and Beynen (1993)
β-Lactoglobulin	Digestive function	Perez *et al.* (1992)
β-Lactorphin	Opioid agonist	Meisel and Schlimme (1996)
α-Lactalbumin	Anti-carcinogenic	Håkansson *et al.* (1995)
α-Lactorphin	Opioid agonist	Meisel and Schlimme (1996)
Lactoferrin	Antimicrobial	Dionysius *et al.* (1993)
	Iron transport, regulation	Sánchez *et al.* (1992)
	Immunostimulatory	Gahr *et al.* (1991)
	Anti-inflammatory	Hanson *et al.* (1995)
	Cell growth, proliferation	Hagiwara *et al.* (1995)
	Anti-carcinogenic	Bezault *et al.* (1994) Fleet (1995)
Lactoferricin	Antimicrobial	Meisel and Schlimme (1996)
Immunoglobulins	Passive immunity	Kobayashi *et al.* (1991)
Lactoperoxidase	Antibacterial	Kussendrager (1993)
Growth factors	Cell growth, differentiation	Francis *et al.* (1995) Belford *et al.* (1995) Rogers *et al.* (1996)
	Gut cell protection, repair	Howarth *et al.* (1996)
	Wound repair	Belford *et al.* (1997)
Serum albumin		
Serorphin	Opioid agonist	Meisel and Schlimme (1996)
Glycomacropeptide[a]	Digestive function	Stan *et al.* (1983) Yvon *et al.* (1994)

[a] Although the glycomacropeptide is strictly a casein-derived peptide, it has been included here as it appears in the whey after cheesemaking.

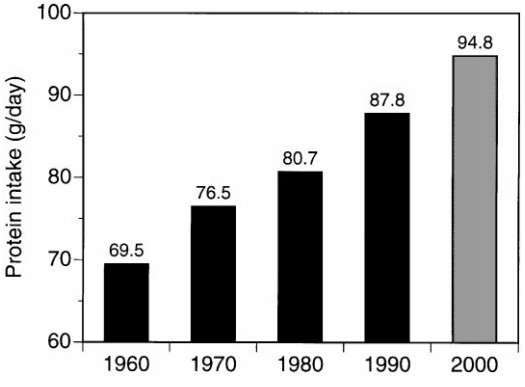

Fig. 2. Growth in the consumption of protein in Japan since 1960. Figures represent the average total quantity of protein consumed each day per capita. The figure for the year 2000 is projected and is based on continued average growth of about 8% each decade. This growth reflects primarily an increase in consumption of meat and dairy protein.

Defatted soybean meal (approx. 50% protein) and casein (>90% protein) were purchased from Milling Industries (Adelaide, South Australia) and from Bonlac Foods Ltd (Melbourne, Victoria), respectively. Beef, used as the meat protein source in some feeding trials, was barbequed to mimic a frequently used method for cooking beef in Australia. The meat was cooked on a hotplate (150°C), dried at 45°C overnight, and finally milled to provide a product containing 70% protein.

Whey protein fractions were prepared from fresh Cheddar cheese whey in the pilot processing facility of the CSIRO Division of Food Science and Technology. Lactoferrin was isolated from whey by adsorption chromatography. Whey was microfiltered (0.8 μm ceramic membrane) and then passed over a strong cation exchange resin bed (SP-Sepharose) pre-equilibrated in 50 mM sodium citrate, pH 6.5. After loading, the resin was washed with water and then eluted stepwise, first with 0.5 M NaCl to eliminate unwanted proteins, followed by 1.5 M NaCl to recover lactoferrin. The latter eluate was concentrated by ultrafiltration, diafiltered and spray dried. The resultant powder contained >90% protein, of which lactoferrin represented >95%. β-Lactoglobulin was isolated from whey using the procedure of Pearce (1987). Fractionation involved acidification of the whey in combination with mild heating. The process resulted in the formation of a reversible α-lactalbumin-enriched coagulum and a soluble phase substantially enriched in β-lactoglobulin. The mixture was separated using a clarifier, and the β-lactoglobulin-enriched fraction was then concentrated by ultrafiltration and spray dried. The resultant powder contained >90% protein, of which β-lactoglobulin represented >80%.

Diets

Diets were based on modified versions of the AIN76A (reformulated as AIN93) or AIN89 formulas using semi-purified ingredients (American Institute of Nutrition, 1977; Reeves *et al*., 1993). Protein powders were added to the basal AIN diet to provide a final protein content equal to 16–20%. Where indicated, whey protein fractions (lactoferrin, β-lactoglobulin) were supplemented into selected diets at 20–25% of the total protein content (3.2–5% of the total diet). The contents of remaining dietary components were adjusted to take account of other constituents in the protein powders, such as fat, carbohydrate or fibre, and thus provide diets of comparable composition apart from the protein source (McIntosh *et al*., 1995). Diets, prepared every 4–6 weeks to avoid staling or loss of nutrient value, were mixed, pelletised, dried to a constant moisture level of 8–10% at 35°C, and stored cool (4°C) until used.

Chemicals

Laboratory chemicals and solvents were analytical reagent grade obtained from local suppliers. Food product ingredients were obtained commercially from local retailers. 1,2-Dimethylhydrazine·(dihydrochloride (dimethylhydrazine) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Methods

Animal feeding trials

The ability of dietary protein to prevent or retard the development of colon cancer has been compared *in vivo* using an established animal model of the disease. The model used was based on the chemical induction of tumors in the colon of Sprague-Dawley rats through subcutaneous injection of the carcinogen dimethylhydrazine (Fig. 3). All animal studies were approved by the Animal Experimentation Ethics Committee (CSIRO Division of Human Nutrition) prior to commencement.

Experimental design was essentially as described previously (McIntosh *et al*., 1995). Male Sprague-Dawley rats were obtained from the CSIRO Division of Human Nutrition small animal colony and were divided into groups (15–22 animals/group) destined to receive diets containing different protein sources. The animals were housed in suspended wire cages, and freely supplied with food and water in a clean air-conditioned environment (23–25°C). With the exception of the mature age rat study, where the animals were 6 months old, rats commenced on the specific diets under study at 4–5 weeks of age. The chemical carcinogen (dimethylhydrazine) was first introduced (15 mg/kg body weight) 3–5 weeks after the animals commenced on the experimental diets, followed by 2 further doses at one week intervals. In the mature age rat study, prior to receiving the experimental diets these animals were fed a standard AIN89 diet for 6 months and were housed under identical conditions to those described above. Rats were maintained on the specified diets for 5–7 months. Weight gain, daily food consumption, and fecal and urine output were measured over the course of the trial. Typically, the animals showed the first signs of colon cancer (blood in the feces) 16–24 weeks after the final dimethylhydrazine injection. Animals were sacrificed about 30 weeks after commencement on the experimental diets. The gastrointestinal tract was then removed and the small and large intestines emptied,

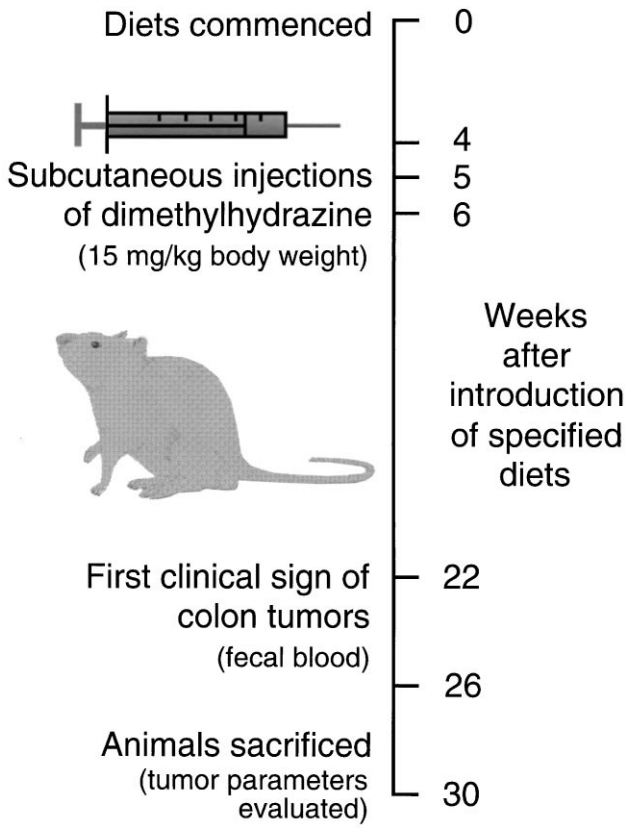

Fig. 3. Schematic representation of the animal feeding trials designed to assess the impact of dietary protein source on colon tumorigenesis in rats. For experiments with young animals the rats commenced on specified diets at 4 weeks of age, and for the feeding experiment with older animals the rats commenced on specified diets at 6 months of age.

opened longitudinally and examined in detail for tumors both visually and by histological assessment. Tumors were categorised into adenoma or adenocarcinoma, and tissue samples were also taken for biochemical and histological analysis.

Chemical and histological analysis

Amino acid analysis. Sulfur amino acid (methionine, cysteine) content of the dietary protein sources was determined by HPLC following acid hydrolysis and pre-column derivatisation using the Waters Pico-Tag® technology (Bidlingmeyer *et al*., 1984).

Urinary metabolites. Urine samples collected in metabolic cages were analysed for phenol and *p*-cresol using the method of Yoshikawa *et al.* (1986).

Meat mutagens. Samples of barbecued beef were sent to the Lawrence Livermore National Laboratory, University of California, for examination of meat mutagens. The following mutagens were identified: 2-amino-3,8-dimethylimidazo{4,5-f}quinoxaline, 0.8 ng/g; and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 7.0 ng/g. An Ames test on the barbecued beef returned a weak positive for mutagenicity.

Aberrant crypts. Histology of the gut wall was examined in order to assess the impact of dietary protein on

the development of aberrant crypt foci, which are putative neoplastic markers of future colon cancer events (McLellan and Bird, 1988; Tudek *et al*., 1989). Cleaned colons were backed onto filter paper strips and fixed in buffered formalin solution overnight (Tudek *et al*., 1989). The tissue was then stained with 0.2% methylene blue in phosphate-buffered saline for 20 min. The number, size and location of aberrant crypt foci, and the multiplicity of aberrant crypts per focus were determined by examination of the fixed tissue samples using a light microscope. Aberrant crypt foci were categorised into groups containing 4 or 5 aberrant crypts per focus. These groupings are more predictive of cancer endpoints than foci containing 3 aberrant crypts (Magnuson *et al*., 1993).

Statistical analysis

Results were statistically compared using the paired-difference Student *t*-test and Pearson Chi-squared analysis. Results from the aberrant crypt study were analysed using one-way ANOVA and the Tukey Kramer method for multiple comparisons. Statistical significance was accepted for $P < 0.05$.

Whey protein food product development

Prototype foods and beverages, supplemented with whey protein concentrate (80%), have been prepared and subjected to preliminary organoleptic evaluation. Products included muesli bar, ice cream, chocolate-flavored low-fat (1.5%) milk, french onion dip, instant lychee pudding, and pasta. Product variations were prepared in the absence (control) and in the presence of various amounts of whey protein concentrate powder using modified in-house recipes. The content of incorporated protein powder varied for each product as follows: muesli bar (12.6%), ice cream (4.4%), chocolate milk (3.0%), onion dip (6.4%), lychee pudding (5.0%) and pasta (9.4%). Products were assessed by a laboratory taste panel (20–25 volunteers) using an hedonic scale for selected product characteristics, and statistically analysed using one-way ANOVA.

RESULTS AND DISCUSSION

Role of whey protein in colon carcinogenesis: Animal feeding studies

Total protein sources

Previous studies, both from our group (McIntosh *et al*., 1995) and others (Papenburg *et al*., 1990; Nutter *et al*., 1990; Bounous *et al*., 1991), have provided strong evidence for the role of dietary dairy proteins in retarding the development of colon cancer in animal models of the disease. While results from these studies have been compelling, all have involved young animals. Similar studies of the impact of dietary protein source on colon tumorigenesis in a mature age animal model have been lacking. This shortcoming needs to be addressed as risk of cancer increases with age in all animal species. In an endeavour to correct this shortcoming and to examine the effects of a common source of animal protein, the influence of barbequed meat and whey protein on colon cancer etiology has been studied using a mature age (6–11 months) rat model of the disease. In addition to standard measures of tumorigenesis, several measures of protein and fat metabolism have been used to identify the influence of barbequed red meat relative to whey protein on colon fermentation and metabolism.

Rats maintained consistent growth throughout the 5-month study period. At completion of the experiment no significant difference was detected in mean body weights between the two dietary groups (1010 $\pm$ 34 g, barbecued beef; 943 $\pm$ 21 g, whey protein concentrate).

Influence of the experimental diets on tumor numbers and distribution within the gastrointestinal tract is shown in Fig. 4. Tumor incidence in the rats showed a trend increasing from 33% in the whey protein-fed group to 59% in those animals receiving the barbecued beef diet, although this difference was not significant ($P = 0.09$) (Pearson Chi-squared test). Two additional tumors (fibrous pleimorphic histiocytomas) were noted in rats receiving the barbecued beef diet, a type which has not been observed in any of our previous studies using younger animals. Histological examination of the gastrointestinal tumors showed the proportion of adenomas to adenocarcinomas was in the ratio 1.2 to 1. A study of the influence of dietary protein on colon tumorigenesis, induced by dimethylhydrazine in young male rats, has previously established the importance of protein type on cancer development (McIntosh *et al*., 1995). In this earlier study, young rats fed a whey protein-based diet showed only a 30% incidence of tumors at the completion of the study compared with 55% incidence for the group receiving a meat-based diet. The present study has demonstrated a similar trend, although not statistically significant, and has shown that age of the rat does not alter the outcome. In examination of meat intake and its

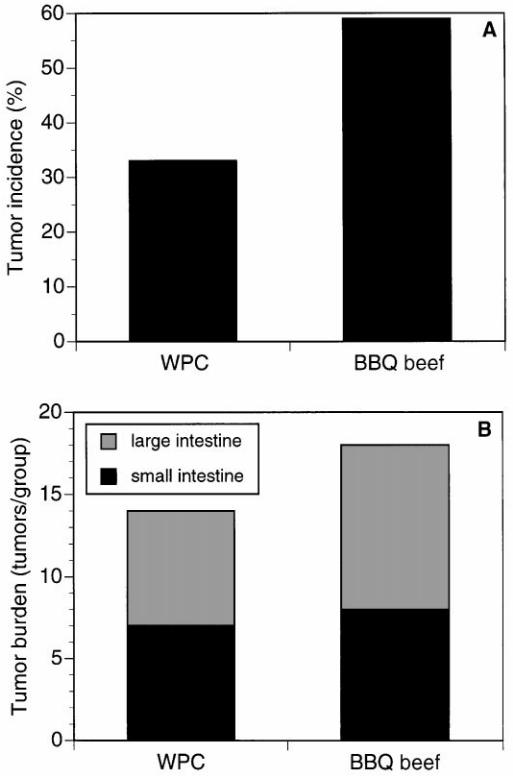

Fig. 4. Tumor parameters from rats receiving whey protein concentrate (WPC) and barbecued beef (BBQ beef) diets. (A) Tumor incidence (%) among animals in each dietary group. (B) Tumor burden (total tumors/group) for animals in each dietary group. $n = 21$ (WPC) and 22 (BBQ beef).

link to colon carcinogenesis, other workers in the field have repeatedly represented fat as the most important dietary factor influencing cancer development (Kinlen, 1982; Goldbohm *et al*., 1994). However, in the present study total fat was kept constant (20%), with a small qualitative difference in fatty acid composition being presented by the greater amount of saturated fat contributed from the barbequed meat. Apart from barbequing, the main difference between the dietary regimes was the protein source. The two sources were both of animal origin and were added to provide a very similar final protein content, which did however vary significantly in amino acid composition. The cooking method may also have contributed to the impact of the meat diet on cancer risk as pyrolysis products (putative meat mutagens) were shown to be present in the barbequed meat.

The content of *p*-cresol and phenol was determined in the urine of rats collected in metabolic cages over a 24 h period. Results are presented in Fig. 5. There was a significantly higher excretion of both metabolites in the urine of the barbecued beef-fed rats, approximately 3-fold for phenol and 5-fold for *p*-cresol ($P < 0.001$). Urinary volumes were comparable for the two dietary groups (13.9 ± 5.2 mL/d, whey protein concentrate; 14.2 ± 4.7 mL/d, barbecued beef). Bacteria can play a major role in metabolism and resultant initiation of cancer by their ability to convert bile acids, steroids and fatty acids to toxic compounds which may be carcinogenic. While no enzymatic work has been undertaken in the present study, the appearance of high urinary concentrations of *p*-cresol and phenol, known promoters of cancer, in the meat-fed rats, provides some evidence of microbial-derived toxic metabolites. In this regard, the whey protein-fed rats were clearly less subject to the influence of these metabolites which may be construed as beneficial.

When considered with our earlier study (McIntosh *et al*., 1995), the present results suggest that dietary protein source continues to influence susceptibility to colon carcinogenesis at an age when growth has slowed. In particular, dietary whey protein continues to demonstrate benefit in retarding development of colon tumors in the animal model. This study also suggests that whey proteins favourably influence colonic metabolic processes relative to barbequed meat by significantly reducing levels of potentially toxic by-products (phenol, *p*-cresols) derived from colonic protein metabolism. This conclusion supports the findings of other research groups (Clinton *et al*., 1992; Govers *et al*., 1993) that dairy proteins promote a bacterial fermentative outcome 'more protective' to the host organism.

Selected whey protein fractions

The protective effect of dietary whey protein against the development of dimethylhydrazine-induced colon cancer in laboratory animals has been related to its ability to deliver, in a biologically-available form, amino acid and peptide precursors (cysteine, glutamyl-cysteine) of glutathione, a ubiquitous cellular protective agent (Bounous and Gold, 1991; McIntosh *et al*., 1995). This hypothesis suggests that whey protein fractions rich in sulphur amino acids should manifest potentially greater activity against the development of colon cancer than the total whey protein product. In order to test this hypothesis, two such fractions—lactoferrin and β-lactoglobulin—have been studied in the rat model of colon cancer.

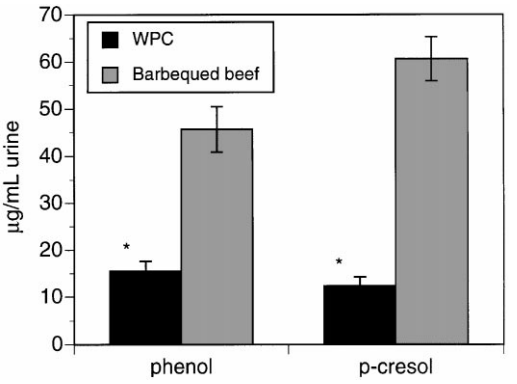

Fig. 5. Content of phenol and *p*-cresol excreted in the urine of rats fed a standard diet (based on AIN89) in which the protein source was either whey protein concentrate (WPC) or barbequed beef. The animal treatment groups comprised 21 (WPC) and 22 (barbequed beef) rats. Values represent the mean ± SEM. The contents of urinary phenol and *p*-cresol for animals receiving the WPC diet (*) are significantly different ($P < 0.001$) from those for animals receiving the beef diet, based on Student's *t*-test statistical analysis.

Thus, the ability of lactoferrin and of β-lactoglobulin, when supplemented into a diet otherwise associated with poor protection in the colon cancer model, to reduce the number of putative tumor precursor cells (aberrant crypts) in the distal colon of rats has been assessed. The proteins were added as supplements in a base diet containing soybean meal as the principle protein source, and compared with a diet containing whey protein concentrate alone. Formulation allowed for the preparation of diets containing a gradation of total sulphur amino acid content in the dietary protein source as follows: whey protein concentrate (4.4%), soybean meal + lactoferrin (4.2%), soybean meal + β-lactoglobulin (3.6%), and soybean meal (2.1%).

Rats showed similar rates of growth during the study period. At termination of the trial there was no significant difference in mean body weights between the four treatment groups (726 ± 16 g, whey protein concentrate; 739 ± 13 g, soy + lactoferrin; 757 ± 21 g, soy + β-lactoglobulin; 736 ± 25 g, soy).

Influence of dietary protein source on development of aberrant crypt foci is shown in Fig. 6. While total foci counts between the four dietary groups failed to show significance (data not shown), foci containing 5 aberrant crypts showed significant variation. These foci were found in 3-fold greater abundance in distal colon tissue taken from animals in the soy-fed group compared to those in the other dietary groups. Tukey Kramer multiple comparisons analysis revealed a significant difference between the number of foci containing 5 aberrant crypts for the soy-fed animals, and the number of these foci found in animals receiving the whey protein and the soy + whey protein fractions diets ($P < 0.05$) (Fig. 6). Of particular note, lactoferrin and β-lactoglobulin, when supplemented into a diet associated with apparent poor protection against development of colon tumor precursors (soybean meal), showed the ability to significantly improve the outcome. Both fractions reduced the number of foci containing 5 aberrant crypts to approximately that observed for whey protein concentrate (Fig. 6). Aberrant crypt foci (5 aberrant crypts/focus) appear to be

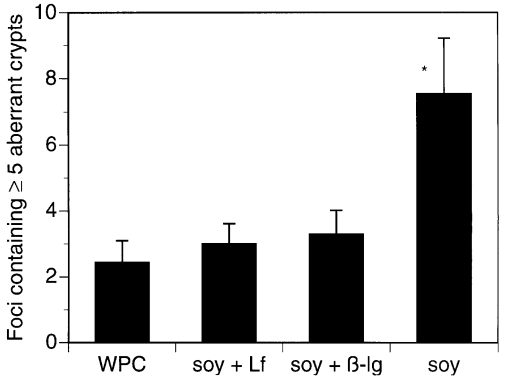

Fig. 6. Influence of dietary protein on the occurrence of aberrant crypts in intestinal foci (≥ 5 aberrant crypts/focus) for rats treated with dimethylhydrazine (20 weeks post-initiation). The four treatment groups (15 animals/group) received diets based on a modified AIN93 formula containing 15% protein from the following sources: whey protein concentrate (WPC), soybean meal + lactoferrin (soy + Lf), soybean meal + β-lactoglobulin (soy + β-lg) or soybean meal. Lactoferrin and β-lactoglobulin were supplemented at 5% of the total diet. Values represent the mean $\pm$ SEM. The value for soybean meal alone (*) is significantly different ($P < 0.05$) from values for the other treatment groups, based on ANOVA and Tukey–Kramer multiple comparisons analysis.

early and putative markers of future colon cancer events (Magnuson *et al*., 1993). The impact of dietary protein on this predictor of tumorigenesis follows a trend similar to that of protein on standard tumor markers (McIntosh *et al*., 1995). In general terms, whey protein appears to be a protective protein source, while soy protein is relatively less protective. Whey protein fractions rich in sulphur amino acids (e.g., lactoferrin, β-lactoglobulin) would appear to have the ability to significantly improve the protective efficacy of a dietary protein source otherwise associated with low protection against colon tumorigenesis in the animal model (Fig. 6) (McIntosh *et al*., 1995).

The relationship between sulfur amino acid content of the dietary protein source and the aberrant crypt data reported in Fig. 6 was statistically evaluated. Result of this analysis is shown in Fig. 7. A significant inverse relationship was found between the number of foci containing 5 aberrant crypts and the sulphur amino acid content of the dietary protein source ($r^2 = -0.96$, $P = 0.03$). Similarly, an inverse relationship was found for foci containing 4 aberrant crypts and the sulphur amino acid content of the dietary protein source, although the data did not reach significance ($r^2 = -0.94$, $P = 0.06$) (data not shown). Thus, a dietary protein source rich in sulphur amino acids appears to lead to a reduced number of putative tumor precursors. A similar inverse trend was determined when the tumor incidence and burden data reported in our earlier publication (McIntosh *et al*., 1995) were analysed with respect to sulphur amino acid content of the dietary protein source (data not shown). Again, tumor incidence and tumor burden were lowest when the animals received a diet containing a protein source richest in sulphur amino acids, although the data did not reach significance ($r^2 = -0.93$, $P = 0.07$).

These findings complement our earlier report (McIntosh *et al*., 1995) and lend further support to the suggestion that dietary whey protein is protective against colon tumorigenesis in the rat. Further, this study has extended the work detailed in our earlier report to show that individual proteins from whey such as β-lactoglobulin, and particularly lactoferrin, offer considerable promise as protective agents in the diet. This and other research lends support to an intriguing functional role for lactoferrin in cancer protection deserving of further investigation (Bezault *et al*., 1994; Fleet, 1995). Whether this potential relates to the sulphur amino acid content of the protein or other aspects (e.g., iron transport or regulation, immunostimulation) (Table 1) must await further research.

Role of whey protein in colon carcinogenesis: mechanism of action?

Nutritionists are showing an increasing interest in the sulphur amino acid content of dietary proteins as these amino acids are often limiting for growth and maturation. When common dietary proteins are classified according to their adequacy for growth in the rat, dairy proteins, and in particular whey protein, rank very highly (Sarwar *et al*., 1985). Indeed, the rank order of proteins in this report equates closely with their sulphur amino acid content. The conclusion that a link exists between the nutritional value of a protein source and its sulphur amino acid content is hard to avoid, although such a link in humans is only speculative at present. Proteins rich in sulphur amino acids may provide another benefit through their ability to influence the methylation status of DNA and thereby influence susceptibility to tumorigenesis, and this association provides the basis for a possible mechanism of action.

Methionine and cysteine (which can be converted into methionine with a 60–80% efficiency when need demands) influence cellular methylation status, and this influence may extend to an important positive impact on the stability of DNA in the methylated form (Rogers, 1993). This stabilised form of DNA is projected to result in a lowered rate of carcinogenesis. Thus, nutritional factors that lead to a more favourable methylation status would be expected to lower susceptibility to cancer development. We propose that sulphur amino acid-rich proteins, such as whole whey protein and the whey protein fractions lactoferrin and β-lactoglobulin, may retard the development of colon tumors (McIntosh *et al*., 1995) (Fig. 4) and tumor precursors (Fig. 6) through provision of biologically-available methionine and cysteine. These amino acids would in turn have a positive influence on cellular methylation status and a stabilising effect on DNA. The significant reverse association between aberrant crypts (putative tumor precursors) and sulphur amino acid content of dietary protein (Fig. 7) is suggestive of such a link, although other mechanisms are possible (e.g. influence on glutathione status) (Bounous *et al*., 1989a). This proposed influence of dietary whey proteins on colon cancer etiology will require further investigation and confirmation, particularly in humans where the requirement for sulphur amino acids is lower than that for the rat.

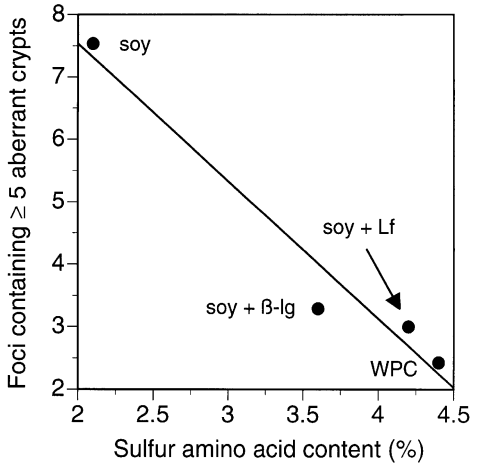

Fig. 7. Relationship between the sulphur amino acid (methionine + cysteine) content of dietary protein sources used in rat feeding trials and the aberrant crypt data reported in Fig. 6. A significant inverse linear relationship was observed between the number of intestinal foci containing ≥ 5 aberrant crypts and the sulphur amino acid content of the dietary protein source being consumed by the animals ($r^2 = -0.96$, $P = 0.03$).

Whey protein food product development: basis for projected clinical trials

In order to provide a basis for development of whey protein-enriched functional foods, any health benefits observed for dietary whey proteins in the animal model will need to be substantiated in human clinical trials. Thus, the requirement to prepare acceptable whey protein-enriched food and beverage vehicles for use in trials using human subjects represented an important parallel development in the current study. Previous work has led to development of processing conditions for the manufacture of a high-protein whey-based drink, either standalone or fermented with yogurt cultures, based on ultrafiltered cheese whey retentate (Johnson *et al*., 1996). When sweetened and flavored, this drink provided a consumer-acceptable vehicle for delivery of whey protein into the diet.

In order to expand the range of available product vehicles for delivery of whey protein into the diet, a number of prototype products have been prepared and compared with control variants without added whey protein. The products included muesli bar, ice cream, chocolate-flavoured milk, french onion dip, lychee pudding, and pasta. Consumer evaluation of the product variants is presented in Table 2. This data represents a summary of the responses of a laboratory taste panel to a number of product characteristics. With the exception of the muesli bar product, all whey protein-containing food variants were statistically indistinguishable from the respective controls over a range of product traits, including odor, color, flavor, texture, aftertaste, and overall acceptability. The control and whey protein variants of the muesli bar product showed statistical difference for the characteristics of color and texture, although in overall acceptability the two variants were indistinguishable (Table 2). Indeed, for all products developed color and texture appeared to be the food characteristics most affected by the whey protein supplementation. Adjustment of other components (e.g. water, colouring) was found to compensate for any detrimental influence of the whey protein on these traits, as evidenced by the consumer-acceptable prototype products (Table 2). This work, while still at a preliminary stage, indicates that development of a range of foods enriched with whey protein should not present undue difficulty. Commercial development of functional foods containing whey protein should follow once the human health benefits of these products have been established.

CONCLUSIONS

Whey represents a rich source of proteins with varied chemical, physical and biological properties, but has often been overlooked as a source of physiologically functional protein. As far back as the middle ages, cheese whey has been acknowledged for its therapeutic and

Table 2. Organoleptic Analysis of Prototype Foods Prepared with Whey Protein Concentrate

Product	Variant[a]	Product characteristic (hedonic scale score/15)						Statistical analysis[b]
		Odor	Colour	Flavor	Texture/ mouthfeel	After taste	Overall acceptability	
Muesli bar	Control	11.7	7.0	9.5	9.0	3.1	10.6	$P = 0.002$ (colour) $P = 0.04$ (texture)
	WPC (12.6%)	11.4	5.0	9.3	7.6	3.5	9.5	
Ice cream	Control	3.8	6.9	8.0	9.4	3.5	9.4	NS
	WPC (4.4%)	2.8	6.8	7.7	8.7	5.1	8.5	
Chcolate milk	Control	2.5	7.0	7.9	6.0	4.6	8.5	NS
	WPC (3.0%)	3.6	7.3	6.8	7.3	4.6	8.9	
French onion dip	Control	2.6	6.6	8.6	11.6	4.0	10.8	NS
	WPC (6.4%)	2.7	5.9	9.4	10.6	3.2	9.1	
Lychee pudding	Control	3.3	6.3	8.9	10.2	5.0	8.3	NS
	WPC (5.0%)	3.5	7.2	9.2	8.9	5.9	7.5	
Pasta	Control	11.7	7.0	9.6	7.5	2.8	8.0	NS
	WPC (9.4%)	11.4	6.7	9.5	8.4	3.1	7.0	

[a] Whey protein concentrate (WPC) supplementation as a percentage of the product formulation is shown.
[b] Not significant (NS) by one-way ANOVA unless stated otherwise.

prophylactic value in folk medicine. More recently, it has been recognised that several of the whey proteins confer antibacterial and immune-associated protection to the neonate against disease and that these and other whey proteins also have putative biological effects when ingested, including an anti-cancer action. This putative activity has provided the rationale for further investigation of therapeutic benefits elicited by whey proteins when included in the diet, and the basis, if substantiated in human trials, for development of whey protein-based foods for the expanding functional food market.

To date, scientific studies have focussed primarily on the roles of dietary fat, fibre and carbohydrate in promotion of carcinogenesis, with less consideration of the role of dietary proteins in cancer etiology. This and other studies have investigated the anti-cancer activity of dietary whey proteins in an attempt to evaluate their role in disease prevention. In previous work from our group, total dietary whey protein was demonstrated to have a protective effect against the development of colon cancer in the young rat when compared with other common proteins, including casein, meat and soy. In an extension of this work, the influence of whey and meat protein on the development of colon cancer in mature rats has also been examined. Results similar to those with younger animals have been demonstrated, a finding that suggests the outcome is not influenced by age. The whey protein diet also appeared to reduce the production of potentially carcinogenic metabolites in the gut. Several whey protein fractions have been considered as dietary ingredients with potentially greater activity against the development of colon cancer in rats than the total whey protein product. Preliminary results suggest that diets supplemented with lactoferrin or with β-lactoglobulin reduce the number of aberrant crypt cells (putative tumor precursors) in the colon of animals consuming protein associated with poor protection in the colon cancer model. A possible mechanism for the protective influence of whey proteins has been proposed for the animal model and centers on the ability of these sulphur amino acid-rich proteins to protect DNA by enhancing its methylation status.

Preliminary food product development and evaluation has been undertaken in anticipation of human clinical trials requiring foodstuffs capable of supplying quantities of protective protein into the diet. The most promising of these foods included ice cream, flavoured low-fat milk, onion dip, and pasta. These prototype products, capable of delivering from 5–30 g of whey protein per serving, could form a basis for experimental investigation of these and similar functional foods in human trials.

Results of this study provide the dairy industry with information about potential applications and product development for new whey protein-based foods with the potential to enter the lucrative functional food market, once human health benefits can be established. The findings could also have implications for the perception and ultimately the consumption of all dairy products.

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