RESEARCH ARTICLE

Potential protein markers for nutritional health effects on colorectal cancer in the mouse as revealed by proteomics analysis

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It is suggested that colorectal cancer might be prevented by changes in diet, and vegetable consumption has been demonstrated to have a protective effect. Until now, little is known about the effects of vegetable consumption at the proteome level. Therefore, the effect of increased vegetable intake on the protein expression in the colonic mucosa of healthy mice was studied. Aim was to identify the proteins that are differentially expressed by increased vegetable consumption and to discriminate their possible role in the protection against colorectal cancer. Mice were fed four different vegetable diets, which was followed by analysis of total cellular protein from colonic mucosal cells by a combination of 2-DE and MS. We found 30 proteins that were differentially expressed in one or more diets as compared to the control diet. Six could be identified by MALDI-TOF MS: myosin regulatory light chain 2, carbonic anhydrase I, high-mobility group protein 1, pancreatitis-associated protein 3, glyceraldehyde-3-phosphate dehydrogenase and ATP synthase oligomycin sensitivity conferral protein. Alterations in the levels of these proteins agree with a role in the protection against colon cancer. We conclude that these proteins are suitable markers for the health effect of food on cancer. The observed altered protein levels therefore provide support for the protective effects of vegetables against colorectal cancer.

Keywords:
Colorectal cancer / MALDI-TOF mass spectrometry / Two-dimensional gel electrophoresis / Vegetables

1 Introduction

Colorectal cancer is one of the most common types of cancer in the Western world. Although mortality has declined, the incidence is generally increasing and preventive measures are therefore needed [1]. The aetiology of colon cancer is complex and involves both genetic and environmental factors. Known risk factors include a positive family history, age, meat and alcohol consumption and fat intake. It has been suggested that over 70% of colon cancer cases can be prevented by modification of the diet and life style risk factors [2]. Inverse associations are reported with physical activity, vegetable intake and, although less consistent, with fruit consumption [3–5].

Vegetables contain a large number of potentially anti-carcinogenic compounds that may affect the process of carcinogenesis via various mechanisms. Using mice and rats, various studies have been performed investigating the role of individual vegetable-derived compounds in the protection against colon cancer. The majority shows a protective effect of vegetable components against colon cancer [6–9].
Vegetables can exert their protective effect against colon cancer via several mechanisms such as free radical scavenging, modulation of enzyme levels and transcriptional regulation. So far, the effect of vegetable consumption on gene and protein levels in the colon has not yet been extensively studied in vivo and the targets at the genome and proteome levels are largely unknown. Some studies have already described the effect of individual vegetable components on gene expression in mouse models and human cell lines [10, 11]. However, often these effects were studied in relation to, for example simultaneous exposure to carcinogens [11, 12]. These studies present evidence for an anticarcinogenic effect of single vegetable components. In order for genes to exert their effect, changes on the transcriptomic level will have to be translated to the proteome and eventually to the metabolome. A few studies have been performed exploring the effect of consumption of single food components on protein expression or enzyme activity in the colon and colonic cell lines [13–15]. These studies focused in general on the regulation of specific enzymes involved in the metabolism of carcinogens or expression of proteins important for cell cycle regulation and apoptosis. However, little is yet known about the effect of vegetable consumption on the total protein expression pattern.

In a previous study, we described the effect of vegetable consumption on gene expression in colon epithelial cells from mice [16]. mRNA expression levels, however, often do not reflect the protein expression levels and the predictive value of mRNA expression levels is therefore limited for cellular physiology. Post-translational modifications and protein turnover are not reflected in gene expression, and protein analysis is needed to complement the mRNA expression data. In the current study, the effect of increased vegetable levels in the diet on the protein expression in the colon mucosa of healthy mice was studied. The results obtained from our study provide new insights in the biological mechanisms that possibly are related to or even directly involved in the cancer risk reduction properties of vegetables.

2 Materials and methods

2.1 Animals and diets

Eight-week-old female C57BL6 mice (Charles River Laboratories, France) were randomly assigned to one of four dietary groups, each consisting of seven animals. During 7 days of acclimatization, all the animals received the standard control diet. Next, each group was fed one of four different diets, containing a 0 (referred to as control diet), 10, 20 and 40% w/w vegetables mixture, respectively, for a period of 2 weeks. The vegetables mixture used in the present mice study consisted of cauliflower (30% wet weight), carrots (30%), peas (30%) and onions (10%). Diets were refreshed every 2 days and provided as powdered feed. The 20% casein reference diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the four different diets. The vegetables were purchased as a single batch at the supermarket and separately cooked under household conditions. After freezing (−20°C), the vegetables were lyophilized, ground and combined [16]. Before the vegetables mixture was mixed with the basal diet, it was analysed for macronutrient content (Hope Farms). The vegetables mixture added to the basal diet was at the expense of carbohydrates. Diets were adjusted for the amount of carbohydrates with dextrose/cellose and cellulose (dicacel) (Hope Farms), resulting in similar energy densities. No antioxidants or preservatives were added. The composition of the different diets is given in Table 1.

The animals were maintained under controlled environmental conditions (temperature (21 ± 1)°C, relative humidity (50 ± 10)%), 12-h light/dark cycle). Body weights of the mice were recorded weekly. The study was approved by the Institutional Committee of Animal Experimentation of the University of Maastricht.

2.2 Tissue sampling

Mice were sacrificed by bleeding the vena cava inferior under Nembutal (Sanofi Sante, Maassluis, The Netherlands) anesthesia. Nembutal was administered subcutaneously in the neck at a dose of 60 mg/kg body weight. The large intestine was removed and placed on a specially made plastic box.

Table 1. Composition of the four diets in g/kg

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control diet&lt;sup&gt;α&lt;/sup&gt;</th>
<th>10% Vegetable diet&lt;sup&gt;β&lt;/sup&gt;</th>
<th>20% Vegetable diet&lt;sup&gt;β&lt;/sup&gt;</th>
<th>40% Vegetable diet&lt;sup&gt;β&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn starch</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dicacel/cellulose</td>
<td>78</td>
<td>71</td>
<td>64</td>
<td>50</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;·7H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Standard vitamin premix</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Standard micronutrient premix</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Vegetables mixture&lt;sup&gt;θ&lt;/sup&gt;</td>
<td>0</td>
<td>100</td>
<td>200</td>
<td>400</td>
</tr>
</tbody>
</table>

<sup>a</sup> Control diet is the basal 20% casein reference diet.

<sup>b</sup> In the vegetable diets, the basal diet was adjusted for cellose/dextrose and dicacel/cellulose resulting in similar energy densities for all diets. The digestible energy of each diet was 15.59 kJ/g.

<sup>c</sup> Composition (% wet weight): cauliflower (30%), carrots (30%), peas (30%) and onions (10%).
which was kept at 4°C. After removing the rectum, the colon was opened longitudinally with fine scissors, and mucus and faeces were removed. Colonic mucosal cells were incubated in Trizol™ (GIBCO Life Technologies, Breda, The Netherlands) for 3 min and scraped off the muscle layer with the edge of a sterile glass slide. Cells were transferred into 800 µL of Trizol, homogenized by resuspension and stored at −80°C.

2.3 Protein isolation

Protein from mouse colon mucosal cells was isolated from the residuals after the isolation of RNA using Trizol Reagent according to the manufacturer instructions. Total RNA was used for gene expression studies [16]. After precipitation of the RNA, the supernatant was collected and stored at −80°C for isolation of total protein according to the manufacturer instructions, except that two additional wash steps of the protein pellet were performed in 0.3 M guanidine hydrochloride in 95% ethanol and one additional wash in 95% ethanol to reduce contamination. Total protein from two to three mice was pooled obtaining in total three pools per diet similar to the pools in the gene expression studies. Protein concentrations were determined using the DC Protein Assay (BioRad, Veenendaal, The Netherlands).

2.4 2-DE and protein identification

One hundred micrograms of total protein of each pool was loaded on immobilized dry strips (pH 3–10, 24-cm long; Amersham Biosciences, Buckinghamshire, UK) for the first dimension. IEF and the second-dimension run were performed as described in [17]. In gel proteins were silver-stained according to [18]. Protein expression patterns on the 2-DE gels were analysed using the PDQuest 7.1.1 program (BioRad). The average spot density from the three replicate gels of each diet was calculated and the protein spots were analysed for differential protein expression upon consumption of vegetables by comparison of these average values to those of the control diet. Proteins that showed at least two times enhanced/decreased expression were selected for identification. The differentially expressed proteins were excised from the gel and processed on a Massprep digestion robot (Waters, Manchester, UK) according to [17] except that 0.5% H₂O₂ was used for destaining of the silver-stained spots. MALDI-TOF MS analysis and subsequent database search were performed as described in [17].

2.5 1-D Immunoblotting

Total cellular proteins were separated by SDS-PAGE (12% acrylamide) followed by immunoblotting. Ten micrograms of total protein was dissolved in 1% SDS and 30 mM DTT, 15 mM Tris-HCl buffer (pH 6.8), 10% glycerol and 0.006% bromophenol blue (final concentrations). SDS-polyacrylamide gelelectrophoresis was performed in 12% w/v slab gels using the (mini)Protean II equipment (BioRad). After separation, the proteins were electrophoretically transferred to NC paper (Schleicher & Schuell, Dassel, Germany) in blotting buffer (25 mM Tris, 200 mM glycine, 20% methanol) using the (mini)Trans-blot (BioRad). Subsequently free protein binding sites on the NC filter were blocked with 5% BSA w/v and 0.1% Tween-20 v/v in PBS for 1 h at room temperature (RT) under gentle agitation. The blots were incubated with the first antibody in PBS, 0.05% Tween-20 for 1 h at RT or overnight (o/n) at 4°C. Antibodies used were: anti-HMG1 1:5 000 (United States Biological, Swampscott, USA) and anti-CAH1 1:2 0000 (United States Biological). After rinsing in excess PBS, 0.05% Tween-20, detection was accomplished using an appropriate chemoluminescent secondary antibody (Abcam, Cambridge, UK): rabbit-anti-mouse IgG peroxidase-conjugated 1:5 000 in PBS, 0.05% Tween-20 for HMG-1 and rabbit-anti-goat IgG peroxidase-conjugated 1:6 0000 in PBS, 0.05% Tween-20 for carbonic anhydrase I (CAH-1), for 1 h at RT. After rinsing in excess PBS, 0.05% Tween-20, the blots were incubated for 5 min at RT with ECL plus Western blotting detection reagent (Amersham Biosciences) according to the manufacturer instructions. The signal was recorded using a LAS 3000 camera in combination with the Las-3000 pro program (Fuji, Tilburg, The Netherlands). Protein band densities were calculated and corrected for background using Aïda version 3.5 (Raytest Benelux bv, Tilburg, The Netherlands). Molecular weight values were estimated using standard molecular weight markers.

2.6 2-D immunoblotting

The pooled proteins of either the control group or the 40% diet group were equally mixed to obtain a master-pool of 12 µg of protein per diet. This protein mix was loaded on immobilized dry strips (pH 3–10, 11-cm long) for the first dimension. IEF and the second-dimension run were performed as described in [17]. The second-dimension was run on Criterion Tris-HCl gels (12.5%, BioRad). After separation, the proteins were electrophoretically transferred to NC paper in blotting buffer (25 mM Tris, 200 mM glycine, 20% methanol) using the Criterion blotter (BioRad). Subsequently free protein binding sites on the NC filter were blocked with 5% nonfat dry milk (BioRad) w/v and 0.1% Tween-20 v/v in PBS for 1 h at 4°C under gently agitation. The blots were incubated with the first antibody, anti-HMG-1 1:1 0000 (United States Biological), in 0.5% nonfat dry milk and 0.1% Tween-20 in PBS, o/n at 4°C. Detection was accomplished using the chemoluminescent secondary antibody rabbit-anti-mouse IgG peroxidase-conjugated (Abcam) 1:2 0000 in PBS, 0.1% Tween-20, for 1 h at 4°C. After rinsing in excess PBS, 0.1% Tween-20 the blots were incubated for 5 min at RT with ECL plus Western blotting detection reagent (Amersham Biosciences) according to the manufacturer instructions. The signal was transferred to an autoradiographic film in the dark. Finally the film was
developed in a Kodak x-Omat 2000 processor. Molecular weight values were estimated using standard molecular weight markers.

2.7 Statistical analysis

Statistical analysis of protein expression was performed using the Student’s t-test carried out with SPSS software version 6.1. A p-value <0.05 was considered as statistical significant.

3 Results

3.1 2-DE and protein identification

Mice received one of four different diets, containing a 0, 10, 20 or 40% w/w vegetables mixture, respectively, for a period of 2 wk. Differential protein expression in colonic mucosal cells was analysed by 2-DE. Spot densities were calculated as a measure for protein expression, and the average expression in the diet groups was compared to that of mice receiving the control diet (0% vegetables). Spots that showed at least two times increased or decreased protein expression as compared to control were selected for protein identification using MALDI-TOF MS. Thirty proteins displayed differential expression, of which six could be identified. Figure 1 shows an example of a 2-DE gel in which the identified proteins are indicated. The expression patterns of the identified proteins are shown in Fig. 2 and their properties have been summarized in Table 2. One of the identified spots was the smooth muscle isoform of myosin regulatory light chain 2 (MLRN). MLRN expression was very similar to control in both the 10 and 20% diets, but showed an increased, although not significant, expression for the 40%-diet group. The spot representing CAH-1 was up-regulated in all diets, although this up-regulation was statistically significant only for the 20% diet. High-mobility group protein 1 (HMG-1) expression was decreased upon vegetable consumption, but not to a statistically significant degree. Pancreatitis-associated protein 3 (PAP3) (precursor) was absent in control mice, displayed only low expression in the 10 and 20% diets but was significantly increased in the 40% diet. The spot representing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed reduced protein expression in all diet groups compared to control mice. The peptide fragment mass spectrum obtained from the last protein spot identified was found to match the mitochondrial ATP synthase oligomycin sensitivity conferral protein (precursor) (OSCP), and its protein expression was reduced in all diets compared to control (only statistically significant for the 20% diet).

3.2 1-D immunoblotting

Figure 3 shows the relative protein expression of HMG-1 and CAH-1 in the vegetable-rich diets compared to control, observed upon immunoblotting. HMG-1 and CAH-1 were chosen based on their 2-DE expression patterns, their function and antibody availability. All blots showed a protein band of the correct mass. The 25 kDa band for HMG-1 (Fig. 3A) showed a dose-dependent up-regulation compared to control, which was found to be significant for the 10 and 40% diets but not the 20% diet (Fig. 3B). This is in contradiction to its 2-DE expression pattern where HMG-1 showed a reduced, although not significant, expression. CAH-1 showed a single band at 28 kDa (Fig. 3A) and was significantly up-regulated in all diets compared to control (Fig. 3B).
Figure 2. Expression pattern of the identified spots from the 2-D gels (A) and their relative protein expression compared to control (B). Three spots shown in (A) indicated with C, 10, 20 and 40%, respectively, derive from the three pooled samples of each diet. Average protein level for controls was set at 100%; * : \( p < 0.05 \). Third spot of the control group for HMG-1 was regarded as missing value. C, control diet; 10%, 10%-diet; 20%, 20%-diet; 40%, 40%-diet.

Table 2. Protein spots isolated from 2-D gels identified by MALDI-TOF MS in combination with searches in the Swiss-Prot protein database

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Swiss-Prot abbreviation</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin regulatory light chain 2, smooth muscle isoform (MLRN)</td>
<td>Q9CQ19</td>
<td>MLRN_MOUSE</td>
<td>Regulation of smooth muscle contraction</td>
</tr>
<tr>
<td>Carbonic anhydrase I (CAH-1)</td>
<td>P13634</td>
<td>CAH1_MOUSE</td>
<td>Catalyzation of carbon dioxide hydration, electrolyte transport, maintenance of pH</td>
</tr>
<tr>
<td>High-mobility group protein 1 (HMG-1)</td>
<td>P07155</td>
<td>HMG1_MOUSE</td>
<td>Regulation of transcription and mediator of differentiation</td>
</tr>
<tr>
<td>Pancreatitis-associated protein 3 (precursor) (PAP3)</td>
<td>O09049</td>
<td>PAP3_MOUSE</td>
<td>Growth and regeneration of cells and tissues</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>P16858</td>
<td>G3P_MOUSE</td>
<td>Energy production, membrane transport and fusion, microtubuli assembly, nuclear RNA export, protein phosphotransferase and kinase reactions, translational control, DNA replication and repair and apoptosis</td>
</tr>
<tr>
<td>ATP synthase oligomycin sensitivity conferral protein, mitochondrial (precursor) (OSCP)</td>
<td>Q9DB20</td>
<td>ATPO_MOUSE</td>
<td>Component of ATP synthase which is a key enzyme in cellular energy production</td>
</tr>
</tbody>
</table>
diet on protein expression, we analysed protein expression levels in colonic mucosal cells from mice that were fed four different diets for 2 wk, containing respectively 0, 10, 20 or 40% vegetables (mixture of cauliflower, carrots, peas and onions). Each vegetable represents a subclass of vegetables that is known to interact via different mechanisms with the process of carcinogenesis. Faecal mass and the methods used to prepare the vegetables might induce effects on colon protein expression that cannot solely be attributed to the vegetable components. Resistant starch might be produced during the preparation of the vegetables and is thought to have a protective effect against colon cancer via the production of butyrate in the lumen of the colon [19–21]. However, the effectiveness of resistant starch in preventing colon cancer still remains to be assessed and it is therefore hard to predict the impact of food preparation and the concomitant formation of starch on the protection against colon cancer. Although resistant starch formation cannot be neglected, we have focused in this study on vegetables containing compounds known to interact with the process of carcinogenesis.

With respect to the diets used, cauliflower belongs to the family of cruciferous vegetables. They contain isothiocyanates and indoles, breakdown products of glucosinolates that are present in these vegetables. These compounds affect the levels of detoxification enzymes and modulate the levels of phase I and II metabolizing enzymes [22–24]. Carrots contain antioxidants that protect the cell from free radical damage and suppress cell proliferation [10, 25, 26]. Peas are known to contain large amounts of fiber and are therefore added to the vegetable diets. Dietary fibers dilute and bind carcinogens in the digestive tract, decrease transit time of faecal bulk and inhibit cell proliferation. Onion is a diallyl vegetable, containing organosulphur compounds that can modify carcinogen activation by changing biotransformation and detoxification enzyme levels [25, 27, 28]. Total cellular protein was isolated from the mouse colonic mucosal cells, and the proteins were separated by means of 2-DE and identified by MALDI-TOF MS. The use of 2-DE, coupled to MS, is a widely used technical approach in proteomics research, although there are still some limitations in this technology. Silver staining has a relatively low specificity [29] and is restricted to a ten-fold linear dynamic range. Since it is a rather complex method, the reproducibility is limited and depends largely on the skills of the investigator [30–33]. One method to overcome these limitations is the use of fluorescent dyes that can be used to label or stain proteins either before or after electrophoresis. Fluorescence-based difference gel electrophoresis allows up to three individually labelled samples to be run on a single gel, thereby overcoming the methodological variations in protein migration in 2-DE and thus increasing reproducibility. However, fluorescence labelling is less sensitive than silver staining and requires costly detection systems [30]. Furthermore, pre-electrophoretic labelling may modify protein size and/or charge affecting the protein’s 2-DE pattern [33]. Another method to improve the reproducibility in 2-DE is to increase

### 3.3 2-D immunoblotting

We observed a discrepancy between the expression pattern of HMG-1 as observed in the 2-DE gelelectrophoresis experiments and in the 1-D immunoblotting expression pattern. To further examine the expression pattern of HMG-1, we performed 2-D immunoblotting. With the control diet we observed two spots of the correct mass and expected pI (Fig. 4A; spots 1 and 2). Compared to the control diet, the 40% diet resulted in a decreased intensity of spot 1 and an increased intensity of spot 2 (Fig. 4B).

### 4 Discussion

Diet plays an important role in colorectal cancer. Meat consumption and high fat intake, for example, are known risk factors. On the other hand, vegetable consumption is thought to protect against colorectal cancer. So far, little is known about the changes at the genome and proteome levels that may be related to or involved in this protective mechanism. To study the effect of consumption of a vegetable-rich...
the number of gels per sample or to increase the number of animals per diet group [30]. This requires more animal material that may be hampered by ethical commissions and, in our case, this would require a complete new study. Despite this, with our method we observed 30 differentially expressed proteins in the mouse colon caused by dietary intervention. Six of these proteins were identified, namely HMG-1, GAPDH, CAH-1, PAP3, OSCP and MLRN. All six identified proteins will be discussed in detail below.

The HMG-1 protein is both a nuclear factor and a secreted protein. In the nucleus HMG-1 binds to DNA and interacts with several transcription factors to regulate transcription [34], while outside the cell it is thought to be a mediator of differentiation [35, 36]. Analysis of HMG-1 protein expression using 2-DE showed a decrease in protein expression for all diets compared to control, although this was not statistically significant. This is in contradiction with the expression pattern observed in the 1-D immunoblotting experiments, where we found that HMG-1 expression was (significantly) up-regulated upon increased vegetable consumption. This difference in protein expression pattern between 2-DE and 1-D immunoblotting might be explained by post-translational modification of the protein. HMG-1 is thought to be regulated by phosphorylation [37]. Hypophosphorylation enhances transport to the nucleus and subsequent binding to DNA [37]. The same protein but with different phosphorylation status will appear on different locations on the gel. To examine the discrepancy between the 2-DE and the 1-D immunoblot expression pattern of HMG-1, we performed a 2-D immunoblot analysis. We identified two spots as HMG-1. Spot 1 showed a small decrease in expression upon vegetable consumption. This is in agreement with the decrease in expression as observed in the 2-DE experiments. Spot 2, on the other hand, displays a significant increase in expression. This coincides with the increase observed in the 1-D immunoblot experiments. Since the increase of spot 2 is much stronger than the decrease of spot 1, this might explain the observed increased expression of total HMG-1 protein in the 1-D immunoblotting experiments. It is known that hypophosphorylation of a protein causes a shift of the protein to a higher pI in the 2-DE pattern [38, 39]. This may be an explanation for the observed changes of the spots on the 2-D-blot representing HMG-1 protein. Taken together, our results suggest that upon consumption of the 40% diet the HMG-1 protein shifts from a phosphorylated state to a hypophosphorylated state. Hypophosphorylated HMG-1 plays a role in the regulation of gene transcription, one of these genes being the tumour suppressor gene P53 [40]. In addition, it is known that HMG-1 plays a role in DNA repair [35, 36]. This indicates that the increased expression of hypophosphorylated protein might reduce the risk for colon cancer. In addition, the results obtained here for HMG-1 using different protein expression analysis methods show the value of 2-D immunoblotting. Experiments based solely on immunoblotting of total protein would have given misleading information on the expression pattern of HMG-1.

GAPDH is a cytoplasmic protein that has long been considered as a classical glycolytic enzyme, playing an important role in energy production [41]. The active glycolytic enzyme consists of a tetramer of four identical subunits. However, mammalian GAPDH displays a wide functional diversity including membrane transport and fusion, microtubulin assembly, nuclear RNA export, protein phosphotransferase and kinase reactions, translational control of gene expression, DNA replication and repair and apoptosis [42]. Our 2-DE experiments indicated a substantially decreased protein expression in all diets compared to control, although this decrease was not statistically significant. For the six proteins that were identified by MALDI-TOF MS, GAPDH was the only protein the gene of which was present in the pool of genes that were analysed in the microarray study [16]. There, only a small, statistically not significant, increase in GAPDH gene expression for the 40% diet was observed, without affecting the expression in the 10%- and 20%-diet groups. The outcome of these results was confirmed by RT-PCR (results not shown). This pattern differs from that found in the 2-DE experiments. Although Piechaczyk et al. [43] observed similar changes in expression for GAPDH protein and mRNA levels in several rat organs under normal conditions, no such relation was found in our experiments. The difference between the mRNA levels and protein levels may be due to different subforms of GAPDH. To exert the wide variety of GAPDH functions, the different subforms of the protein need to be distinguished by the cell. This might be regulated by alternate splicing or post-translational modification and give protein products that differ in pI and/or molecular weight [42, 44]. GAPDH protein expression has been found to be increased in human colorectal cancers [45]. Since GAPDH plays a role in DNA replication and apoptosis [42] GAPDH might be considered as an interesting candidate for protection against colon cancer. However, the evidence so far is too premature to draw definitive conclusions.

CAH-1 is a member of a family of isoenzymes that catalyse the reversible hydration of carbon dioxide and participate in various biological processes like electrolyte transport and maintenance of pH. The isoenzymes differ in kinetic properties, tissue distribution and subcellular localization. CAH-1 is a cytoplasmic isoenzyme that is highly expressed in large intestinal mucosa and takes care of the maintenance of the intracolonic and cellular pH. In colorectal tumours CAH-1 is significantly less expressed than in normal mucosa [46]. Reduced expression of CAH-1 might lead to cellular acidic pH, thereby promoting cellular motility, and contribute to tumour growth and metastasis [47]. Our 2-DE results already indicated an increase in protein expression upon vegetable consumption, although this is only significant for the 20% diet. The immunoblot experiments showed that CAH-1 protein expression is significantly up-regulated in all vegetable diets compared to control. We therefore suggest that consumption of a vegetable-rich diet contributes to maintenance of the intracolonic and cellular pH, which thereby might protect against colon cancer development.
PAP3 belongs to the regenerating gene (Reg) family that constitutes the calcium-dependent lectin (C-type lectin) gene superfamily [48]. The Reg family represents a group of small secretory proteins that can function as acute phase reactants, lectins, antiapoptotic factors and growth factors. The members of the Reg family can be grouped into three subclasses: type I, II and III [48]. There are at least three distinct type III Reg genes in the mouse genome: Reg III alpha (RegIIIα), beta (RegIIIβ) and gamma (RegIIIγ, i.e. PAP3) [49]. The subclasses share considerable amino acid homologies including a conserved trypsin cleavage site. It is thought that the secreted forms represent the precursors and the cleaved forms the activated molecules [50]. Mouse type III Reg genes are expressed strongly in intestinal tracts and PAP3 in particular is expressed strongly in small intestine, moderately in colon and weakly in pancreas [48, 49]. The type III genes are thought to be involved in the growth and regeneration of cells and tissues. In our 2-D experiments we identified the PAP3 precursor protein. It was up-regulated in the 40% diet while hardly any effect was observed from the 10 and 20% diets. Our results suggest that only a high intake of vegetables (40%) stimulates the expression of PAP3 precursor protein. Considering the role of PAP3 in growth and regeneration of cells and tissues, we suggest that activation of the precursor might lead to increased amounts of active PAP3 protein, stimulating the proliferation of healthy cells and/or regeneration of damaged tissue and thus contribute to healthy colon tissue. However, to allow regeneration, it might be necessary to switch off apoptosis, thereby increasing the chance of survival of cells exhibiting genetic mutations in growth-promoting or metastasis-inducing genes [51]. Thus, up-regulation of PAP3 precursor protein and thereby possibly of PAP3 might contribute to proliferation and differentiation of healthy colon tissue, although it also involves a risk for colon cancer. Up-regulation of the precursor protein of PAP3, however, may not necessarily lead to a change in active protein levels. Additional experiments are therefore needed to unravel the regulation of PAP3 protein expression.

ATP production is one of the major chemical reactions in living organisms. ATP synthase is a key enzyme in cellular energy production. During ATP synthesis it uses a proton gradient and the associated membrane potential to synthesize ATP. The ATP synthases are rotary motor complexes that are composed of two discrete sectors (F1 and F0) which are considered to be two separate rotary motors working cooperatively. OSCP is a component of the F0 sector and is considered to serve as a link between the F1 and F0 sectors [52]. OSCP also has been implicated in the binding of F1 to the membrane [53, 54]. In our 2-DE experiments we observed a down-regulation of the OSCP precursor protein upon increased vegetable consumption that was only statistically significant for the 20% diet. Considering the fact that OSCP precursor protein and the active OSCP protein are very likely to be located at different locations on the 2-D gel, reduced expression of OSCP precursor protein might indicate that the amount of precursor is decreased in favour of active, processed OSCP. The processed OSCP might then be used for assembly of ATP synthase and contribute to ATP production. At this moment there are no indications that OSCP might play a role in colorectal cancer.

Myosin is a major component of the contractile elements of smooth muscle and is composed of two identical heavy chains (200 kDa) and two sets of light chains of 20 and 17 kDa [55]. The 20 kDa chain, called regulatory light chain (MLRN), plays a central role in the regulation of smooth muscle contraction [56, 57]. In our 2-DE experiments we found an increase, although not significant, of MLRN protein expression with increasing vegetable intake, most evident for the 40% diet. This might indicate that vegetable consumption increases smooth muscle activity. Vegetables contain high amounts of dietary fibers and are known to stimulate bowel movements, decreasing the passage time through the gut [58, 59]. Mutagenic agents might be present in faeces, and decreased passage time reduces the risk of interaction with the colon tissue and protects against genetic damage. Increased MLRN protein expression might contribute to enhanced bowel movement and as a consequence reduce the passage time of mutagenic agents, lowering the risk for genetic damage and colon cancer.

In the current study, several proteins were identified that displayed differential expression levels in colon mucosa of mice following consumption of diets with different vegetable contents. These proteins play a role in relevant processes like growth, differentiation and apoptosis. Disturbance of these processes plays an important role in carcinogenesis. We think that these proteins can be used as suitable markers to monitor the health effect of food in relation to colorectal cancer. This implies that food inducing the observed changes in protein expression might have a protective effect against colorectal cancer. We conclude that based on the function of the identified proteins our study supports the idea that increased vegetable intake has protective effects against colon cancer.

5 References