Antimutagenicity of Heat-Denatured Ovalbumin, before and after Digestion, As Compared to Caseinate, BSA, and Soy Protein

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The antimutagenicity of ovalbumin was investigated and compared to that of sodium caseinate, bovine serum albumin and soy protein. Antimutagenicity was measured against *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in the *E. coli* DNA repair liquid suspension assay. Heat-denatured ovalbumin showed a strong increase in antimutagenicity compared to undenatured ovalbumin. The antimutagenicity of heat-denatured ovalbumin was superior to the antimutagenicity found for the other proteins tested. After digestion of native and heat-denatured ovalbumin using the pH-stat method, antimutagenicity remained. Antimutagenicity measurements with samples taken from a gastro-intestinal simulator showed strong comutagenic properties. This turned out to be due to comutagenicity against MNNG caused by bile acids and lipase. It was concluded that food proteins exhibit different antimutagenic properties toward MNNG and that the choice of a particular protein digestion model can influence results to a great extent.

Keywords: *Antimutagenicity; bovine serum albumin; cancer prevention; casein; dietary protein; E. coli; MNNG; ovalbumin; pH-stat; protein digestion; soy protein*

INTRODUCTION

Studies on the carcinogen scavenging capacity of proteins have been performed since the late 40's (Miller and Miller, 1947). It was discovered that *p*-dimethylaminoazobenzene was able to bind to liver proteins in the rat. At first, the binding of carcinogens to proteins was thought to be the major cause of cancer and much research was done on this subject. For example, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was found to react with several proteins and amino acids (Skinner et al., 1960; Sugimura, 1968; McCalla and Reuvers, 1968; Schulz and McCalla, 1969; Nagao et al., 1971). Dietary proteins however, were not investigated. Nowadays, it is known that most carcinogens not only can bind to proteins but also can bind to DNA, the latter being an important step in the development of cancer (Miller and Miller, 1981). Work on the reactivity of mutagens and carcinogens has since focused on the interaction with DNA. Protein adducts were mostly studied in environmental toxicology as reviewed by Meyer and Bechtold (1996) and Hemminki et al. (1995). The adducts are used as a biomarker of exposure to, for example, xenobiotics such as mutagens and carcinogens.

Recently, a lot of attention is focused on the antimutagenic and anticarcinogenic properties of dietary components. In this context, special attention is paid to interactions between mutagens and protein(food)s. Apart from caloric effects, it has been demonstrated that proteins have antimutagenic (Jongen et al., 1987; van Boekel et al., 1993; Hosono et al., 1988) and anticarcinogenic properties (Bounous et al., 1991; Gridley et al., 1983; McIntosh et al., 1995) as well as tumor-inducing properties (Nutter et al., 1983).

Most studies on the antimutagenicity of proteins or protein-containing foods focus on casein or other milk proteins and soy protein. Another interesting dietary protein is ovalbumin (a major egg protein). To our knowledge, there is only one study concerning the antimutagenicity of ovalbumin (Hosono et al., 1988). These authors found ovalbumin to protect against pepper-induced mutagenicity in the Ames test. The present study was performed to investigate the antimutagenicity of the dietary protein ovalbumin toward MNNG, a well-known, highly reactive, model nitrosamide (Lawley and Thatcher, 1970). The effect of ovalbumin was compared to that of sodium caseinate, soy protein, and bovine serum albumin (BSA). The effect of protein digestion on antimutagenicity was also tested. As a test system, the *E. coli* DNA repair assay, earlier described by Mohn et al. (1984b), was chosen because of the histidine tolerance of the *E. coli* assay for testing protein hydrolysate samples. The more often used Ames test is not histidine tolerant and therefore not suited for working with protein hydrolysates.

MATERIALS AND METHODS

Chemicals. Chemicals were from the following sources: bacto-agar, bacto-peptone, bacto-tryptone (Difco, Detroit, MI); BSA, ovalbumin (Sigma); MNNG (Aldrich); sodium caseinate (DMV, Veghel, The Netherlands); pepsin (2000 FIP-U/g) and pancreatin (1400 FIP-U/g) (Merck); soy protein isolate (Loders Crocklaan, The Netherlands). Chemicals used for the gastro-intestinal simulator were used as specified by Minekus et al. (1995).

***E. coli* LSA.** The *E. coli* liquid suspension assay (LSA) was performed as described earlier by Mohn et al. (1984b,c) with minor adjustments. In short, two bacterial strains were used, basically differing only in DNA-repair capacity and ability to ferment lactose (Mohn, 1984a). *E. coli* K12 strain 343/113/

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753 (-/753) is DNA repair deficient. The -/753 strain ferments lactose from the agar medium, which produces an acidic environment. Colonies of this strain are recognizable as red colonies on an agar plate with neutral red as a pH indicator. *E. coli* K12 strain 343/113/765 (-/765) is DNA repair proficient and does not ferment lactose. It appears as a white colony on agar plates. When a mixture of -/753 and -/765 bacteria is exposed to a mutagen, the number of viable DNA repair deficient bacteria will decrease. After spreading an appropriate dilution out on suitable agar plates, and incubation of the plates at 37 °C for 2 days, this decrease is quantified by a low ratio of (753/765) bacteria.

Heat-denatured ovalbumin was prepared by heating 3 mL of 5.4% (w/v) ovalbumin in phosphate-buffered saline (PBS) in a waterbath at 100 °C for 60 s in test tubes. After heating, the gel formed was disrupted by sonification (Vibracell, Sonics & Materials Inc.) 3 times for 20 s to make it homogeneous. The liquid suspension assays were performed in PBS at pH 7.2 without a metabolic activation system. MNNG (0.7 μg) was preincubated with 900 μL of protein (hydrolysate) solution for 60 min at 37 °C in a shaking waterbath in the dark. After adding 100 μL of bacteria mixture to the preincubation mixture, it was incubated for 120 min under the same conditions as the preincubation. To stop exposure of the bacteria, the mixture was diluted in PBS and spread over agar plates. The plates were incubated at 37 °C in a ventilated stove in the dark for 2 days, and colonies were counted.

The ratio of the -/753 to the -/765 strain was taken as a measure of (anti)mutagenicity. Although the LSA is a DNA damage test, MNNG is known to induce point mutations (IARC, 1974).

All samples (blank, positive control, etc.) were tested with nine plate incubations per experiment. All experiments were at least done twice.

Protection by a protein (hydrolysate) against MNNG-induced mutagenicity was calculated according to the following formula (eq 1). Corrected samples (s_{corr}) were calculated to compensate for influences of, e.g., protein on blank ratios:

$$P_R = \left(\frac{s_{corr} - pc}{bl - pc}\right) \times 100, \text{ with } s_{corr} = \frac{bl}{bl_t} \times s \qquad (1)$$

where P_R is the protection given by the tested substance (e.g., protein), s is the ratio of sample (bacteria + tested substance + mutagen), s_{corr} is the ratio of corrected sample (bacteria + tested substance + mutagen), pc is the ratio in positive control (bacteria + mutagen), bl is the ratio in blank (only bacteria), and bl_t is the ratio in blank of tested substance, e.g., protein (bacteria + tested substance).

In some experiments, MNNG-induced mutagenicity was enhanced by substances other than protein. In those cases, the following equation was used:

$$CM = \frac{s_{corr} - pc}{pc} \times 100, \text{ with } s_{corr} = \frac{bl}{bl_t} \times s \qquad (2)$$

where CM is the comutagenicity of the tested substance.

Variances of P_R and CM were calculated with the bootstrapping technique (Efron, 1983). Results are expressed as $P_R \pm$ SEM and $CM \pm$ SEM. Bootstrapping ($n = 200$) was performed using SAS/STAT version 6.

pH-Stat Protein Digestion. Protein digestion was basically performed according to the pH-stat technique first described by Jacobsen et al. (1957). A 665 Dosimat and 614 Impulsomat (Metrohm, Swiss) were used. In short, 50 mL of a 5.4% protein solution was brought at 37 °C and pH 2.00 (6 M HCl), while stirring. After a 30 min pepsin (E:S = 0.013:1) digestion at pH 2.00, the reaction was stopped by raising the pH of the solution to 8.00 with 6 M NaOH. Next, a 90 min pancreatin (E:S = 0.25:1) digestion was performed using the same solution. The pH was kept constant at all times. After digestion, samples were immediately frozen in liquid nitrogen and stored at −20 °C. From the amount of H^+ and OH^- equivalents added to keep the pH constant, the degree of hydrolysis (DH) was calculated (Adler-Nissen et al., 1986).

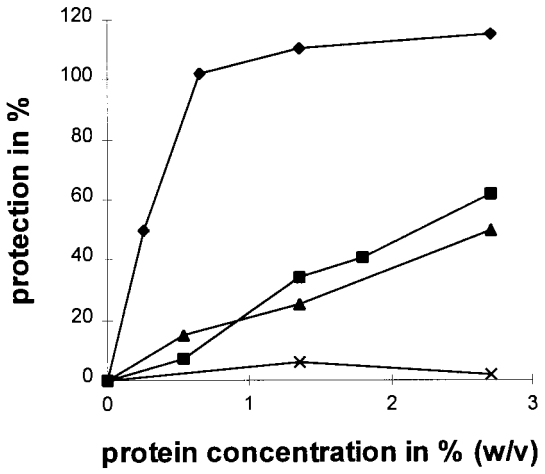

Figure 1. Dose−response curves of the antimutagenic capacity of unheated (×) and heat-denatured ovalbumin (◆) in comparison to bovine serum albumin (■) and sodium caseinate (▲). Antimutagenicity was measured against MNNG in the *E. coli* liquid suspension assay. Data are shown as percentage of protection (P_R). SEM never exceeded 12.8% and was 6% on average.

Table 1. Parameters Used for the Gastro-intestinal Simulator

parameter	value
feeding time	10 min
feeding size	200 mL
pepsin secretion	0.25 mL/min (0.2 mg/mL)
pancreatin secretion	0.25 mL/min (7% solution extracted with 0.1 M bicarbonate)
stomach lipase secretion	0.25 mL/min (0.25 mg/mL)
bile secretion	0.50 mL/min (4% solution)
pH duodenum	6.5
pH curve stomach	3.5 at $t = 0$, 3.2 at $t = 20$, 2.8 at $t = 40$, 2.1 at $t = 60$, 1.8 at $t = 90$, 1.7 at $t = 120$, 1.7 at $t = 360$

Gastro-intestinal Simulator Protein Digestion. The gastro-intestinal simulator as described by Minekus et al. (1995) was used. Parameters were defined as listed in Table 1. Values were chosen to mimic the adult human situation. Samples for antimutagenicity testing were taken from within the intestine after 100 min of digestion, frozen in liquid nitrogen, and stored at −20 °C until further use.

RESULTS AND DISCUSSION

Comparison of Antimutagenicity between Proteins. The antimutagenic capacity of ovalbumin and some other proteins toward MNNG is shown in Figure 1, in which various experiments are summarized. Unheated ovalbumin did not show any relevant protection against MNNG. On the contrary, when ovalbumin was heat-denatured, the protection of a 1% ovalbumin solution under the given conditions was 100%. This clearly exceeded the protection given by the other proteins tested. A clear dose dependency was found for heat-denatured ovalbumin, BSA, and sodium caseinate. The effect of heat-denatured ovalbumin was strong compared to earlier reports on the antimutagenic properties of proteins (Van Boekel et al., 1993; Hosono et al., 1988) although comparison is difficult because other test systems and conditions were used.

Difference in protection at the highest protein concentration (2.7%) was tested for significance with the

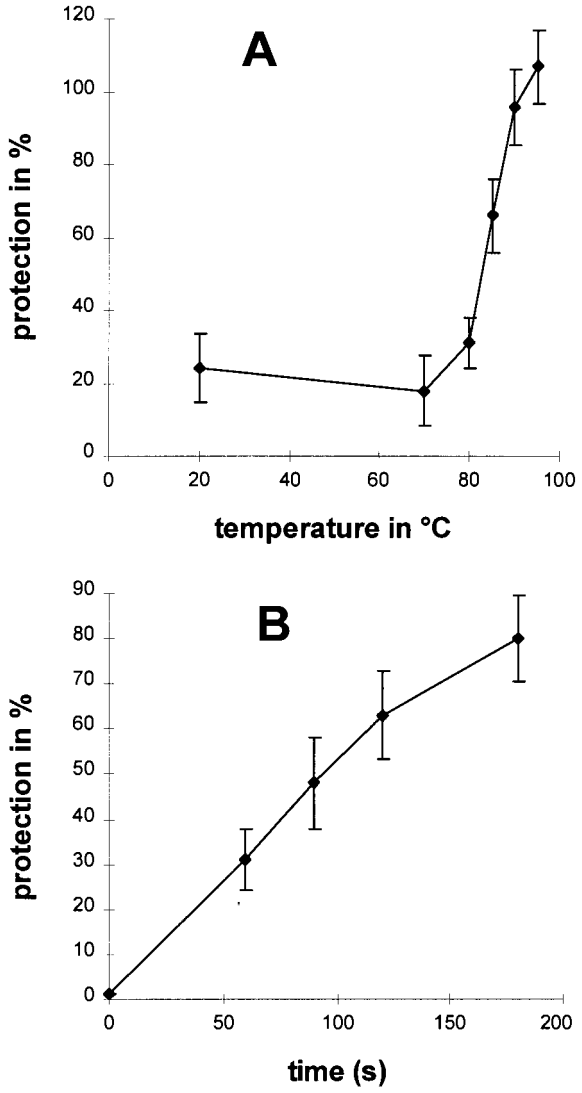

Figure 2. Protection by ovalbumin (A) heated at various temperatures for 60 s (B) as a function of heating time at 80 °C, against MNNG in the *E. coli* liquid suspension assay. Data are shown as percentage of protection (P_R) ± SEM.

Student's *t*-test. All data points differed significantly ($p < 0.05$) except for the difference between BSA and sodium caseinate protein samples.

Effect of Heating on the Antimutagenicity of Ovalbumin. Figure 2 shows that the antimutagenic capacity of ovalbumin was dependent on both the temperature and time of heating. Until 70 °C, no effect of heating was detectable on the antimutagenic capacity of ovalbumin. The effect started at a temperature between 70 and 85 °C. This corresponds to the denaturation temperature of ovalbumin (about 71 °C) (Johnson and Zabik, 1981) as was also indicated by turbidity measurements of the heat-denatured ovalbumin solutions (data not shown). Heating resulted in denaturation and gelation of ovalbumin. Denaturation of globular protein exposes inner protein groups. It is therefore possible that the antimutagenic capacity of ovalbumin is due to hydrophobic sites or specific residues burried in the native protein. It is in this respect of interest to note that heating of casein has no effect on its antimutagenic potential (Van Boekel et al., 1993). Casein has almost no secondary or tertiary structure and cannot be denatured by heating (Walstra and Jenness, 1984).

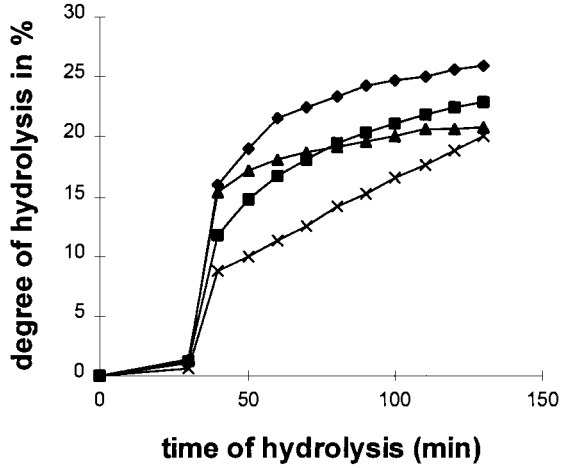

Figure 3. Digestion of soy protein (◆), heat-denatured ovalbumin (■), sodium caseinate (▲), and unheated ovalbumin (×) using the pH-stat technique. The proteins were first digested with pepsin (1:0.013) for 30 min at 37 °C at pH 2.00, followed by pancreatin digestion at pH 8.00 (1:0.25) for 90 min.

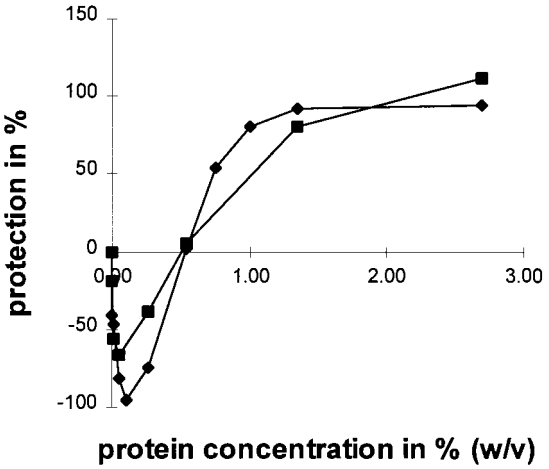

Figure 4. Dose−effect curves for antimutagenicity of protein hydrolysates of unheated (◆) and heat-denatured ovalbumin (■) against MNNG in the *E. coli* liquid suspension assay. Data are shown as percentage of protection (P_R). A negative protection indicates enhancement of the mutagenicity of MNNG. SEMs (not shown) were comparable to other experiments.

Antimutagenicity of Digested Proteins. To investigate the effect of digestion of proteins, hydrolysates were prepared using the pH-stat technique. Figure 3 shows the relation between time of hydrolysis and DH. After 30 min of pepsin digestion and 90 min of pancreatin digestion, the protein hydrolysates had approximately the same DH although there were differences in reaction kinetics.

Ovalbumin hydrolysate showed a dose-dependent antimutagenicity from 0.5 until 3.0 mg/mL protein (Figure 4). Hydrolyzed, unheated ovalbumin showed the same antimutagenic capacity as hydrolyzed denatured ovalbumin. So, after hydrolysis to a degree of about 22%, heating of ovalbumin before digestion did not seem to be a factor of importance any more. This can be explained by the above-described hypothesis that exposure of inner protein groups increases the antimutagenic capacity of ovalbumin. This means that the route via which the inner protein groups are exposed is unimportant. This hypothesis was investigated for other proteins by testing protein hydrolysates of soy and sodium caseinate (2.7%), also digested by the pH-stat method (Figure 3). Results are shown in Figure 5.

Table 2. Comutagenic Effects of Bile Acids and Lipase against MNNG in the *E. coli* Liquid Suspension Assay[a]

	blank	positive control (MNNG)	only lipase or bile	sample (lipase/bile and MNNG)	calculated comutagenicity (%)
lipase, 20 °C (0.025%)	0.69 ± 0.10	0.14 ± 0.03	1.28 ± 0.17	0.01 ± 0.01	100
lipase, 100 °C (0.025%)	0.69 ± 0.10	0.14 ± 0.03	1.12 ± 0.34	0.01 ± 0.01	97
bile, 0.05%	0.69 ± 0.10	0.14 ± 0.03	0.63 ± 0.08	0.10 ± 0.03	23
bile, 0.25%	0.95 ± 0.09	0.24 ± 0.07	1.59 ± 0.18	0.03 ± 0.01	93
bile, 0.50%	0.95 ± 0.09	0.24 ± 0.07	1.62 ± 0.17	0.05 ± 0.02	88
bile, 1.00%	0.95 ± 0.09	0.24 ± 0.07	1.67 ± 0.15	0.04 ± 0.01	91

[a] Lipase was tested before and after heating (5 min in boiling water). Results are from two different experiments. Ratios of -/753 to -/765 are shown ± SD.

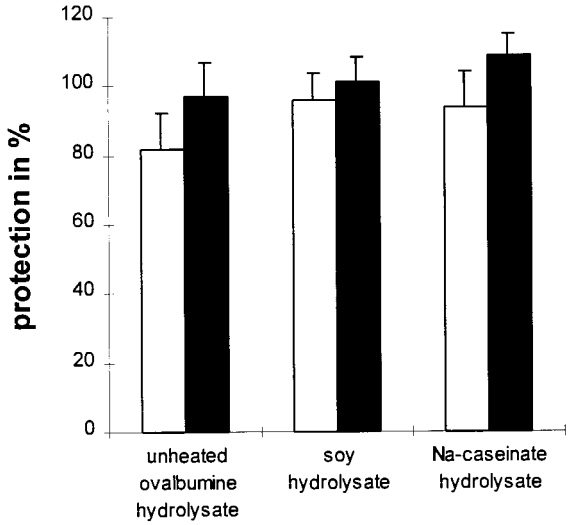

Figure 5. Comparison between protection of three dietary proteins (2.7%) after pH-stat hydrolysis (degree of hydrolysis: about 22%) against MNNG in the *E. coli* DNA repair assay. Data are shown as a percentage of protection (P_R) ± SEM. Both samples before enzyme inactivation (□) and after enzyme inactivation (■) were tested.

Because hydrolysates still contained proteolytic enzymes, samples were tested before and after heat inactivation (5 min, 100 °C) of these enzymes. When combined with Figure 1, Figure 5 shows that the antimutagenicity of sodium caseinate, soy, and ovalbumin (all unheated) were all increased after digestion. Undigested soy protein (2.7%) showed no antimutagenicity (Goeptar et al., 1997). Undigested sodium caseinate (2.7%) protected about 45%, and 2.7% undigested unheated ovalbumin protected only for 15% (Figure 1). So the antimutagenicity increased in all cases after digestion. There was no significant ($p < 0.05$) increase in protection found from the enzyme inactivation treatment.

pH-stat hydrolysates exhibited comutagenicity at the lower concentrations (Figure 4). Therefore, the protection of undigested heat-denatured ovalbumin (Figure 1) cannot be compared with both heated and unheated digested ovalbumins. At higher protein concentrations, the comutagenic effect was apparently overruled by the presence of a high protein concentration. It was hypothesized that the comutagenicity found was due to the presence of impurities in pancreatin. The nature of the impurities followed from the experiments discussed below.

The Gastro-intestinal Simulator. Because pH-stat digestion is only a rough estimate of the physiological situation, we used a model that comes closer to the in vivo situation. The gastro-intestinal simulator described by Minekus et al. (1995) is an example of such a model. Pilot experiments were performed by using protein digests from the stomach, duodenum, jejunum, and ileum compartment for antimutagenicity testing. However, MNNG mutagenicity turned out to be highly affected by blank digestions (just water) (Figure 6). After testing all possible substances used in the gastro-intestinal simulator, bile acids and lipase turned out to be strongly comutagenic to MNNG in the *E. coli* assay used (Table 2) although samples with only lipase or bile

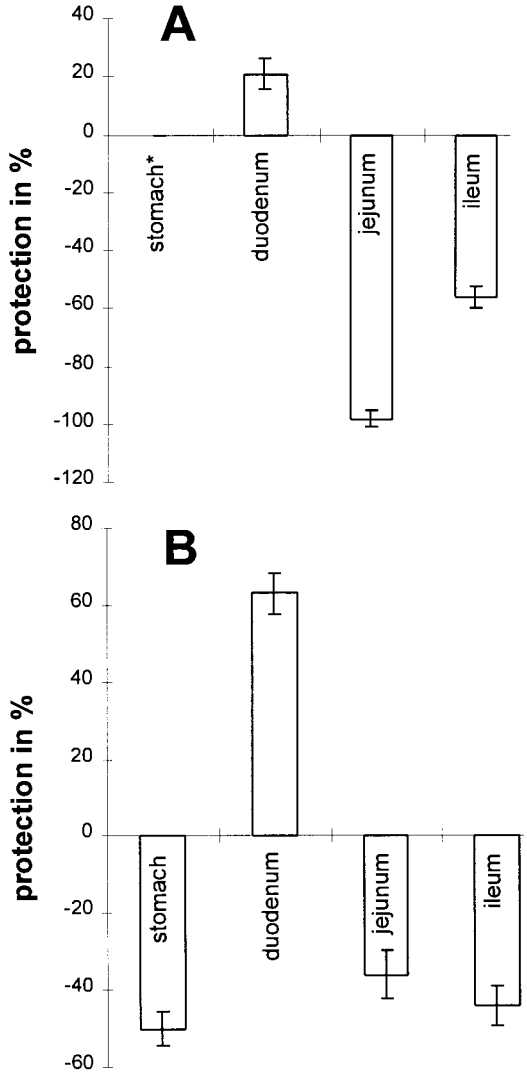

Figure 6. Influence of intestinal contents of the gastro-intestinal simulator on antimutagenicity against MNNG measured in the *E. coli* liquid suspension assay (A) influence of intestinal contents after blank (=water) digestion; (B) influence of intestinal contents after digestion of 5.4% sodium caseinate solution. Data are shown as percentage of protection (P_R) ± SEM. Samples were taken after 100 min of digestion (* = not determined).

showed no negative effect on the growth of -/765 and even a colony stimulating effect on -/753 (only ratios are shown).

The comutagenic capacity of bile acids confirmed previous observations by Wilpart and Roberfroid (1986). No published data could be found on the comutagenicity of lipase although mutagenicity tests have been performed on lipase G from *Penicillium camembertii*. From this research, it was stated that "no evidence of mutagenic potential was found" (Kondo et al., 1994). Our results can explain the comutagenicity found in the protein hydrolysates as shown in Figure 4 because pancreatin contains lipase.

The experiments done so far show that it is useful to test protein hydrolysates from systems more complicated and physiologically relevant than the pH-stat method. Besides a more realistic protein digestion, several intestinal secretions influence the antimutagenicity of the intestinal contents.

CONCLUSION

Heat-denatured ovalbumin was found to be strongly antimutagenic against MNNG in the *E. coli* DNA repair assay compared to sodium caseinate, BSA, and unheated ovalbumin. Heating of intact ovalbumin is necessary to induce antimutagenicity, which suggests that the unfolded molecule is the active species. The antimutagenicity is still present after hydrolysis (DH = about 22%) of both heated and unheated ovalbumin, so heat denaturation is then no longer required.

Several constituents of the intestine influence the (anti)mutagenicity of a diet as shown by gastrointestinal simulator experiments. Therefore, questions concerning the physiological relevance of the antimutagenicity of heat-denatured ovalbumin, are still to be answered.

ABBREVIATIONS USED

-/753, *E. coli* K-12 343/113/753; -/765, *E. coli* K-12 343/113/765; BSA, bovine serum albumin; DH, degree of hydrolysis; E:S, enzyme:substrate ratio; LSA, liquid suspension assay; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; PBS, phosphate buffered saline.

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