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Effect of Maillard Reaction Products on Protein Digestion. In Vitro Studies

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The low molecular weight fraction of a glucose–lysine reaction mixture, previously shown to affect the in vivo uptake of proteins in the rat, was tested for its effect on a number of gastrointestinal proteases and peptidases in vitro. Of these, carboxypeptidase A was significantly inhibited by 0.5 mg/mL of the Maillard products, and aminopeptidase N (a key enzyme in the brush border hydrolysis of peptides) was strongly inhibited by 0.25 mg/mL. The inhibition of aminopeptidase N was of the mixed type while the inhibition of carboxypeptidase A could not be described by means of classical enzyme kinetics. Gel filtration chromatography indicated that a number of different compounds in the Maillard reaction mixture may inhibit the two enzymes.

INTRODUCTION

Because the ϵ-amino group of lysine reacts in the Maillard reaction, heating proteins with carbohydrates may lead to a reduced nutritional value of the protein. The degree of reduction is dependent on a number of factors, including water activity, type and amount of reducing sugar, and type of protein as well as temperature and time of the heat treatment (Dworschak, 1980; Mauron, 1982). The loss of protein quality may include both a reduced biological value and digestibility. When the heat treatment is mild, a loss in the BV often corresponds to the loss of biologically available lysine caused by the Maillard reaction, provided lysine is the limiting amino acid in the protein (Burvall et al., 1978; Mauron, 1982; Björck et al., 1983). When the heating is more intense, the reduction of protein quality may be greater than the apparent loss of available lysine (measured in vitro) and there may also be a reduction in digestibility (Boctor and Harper, 1968; Adrian, 1982; Björck et al., 1983; Öste and Sjödin, 1984).

There are some reports on possible mechanisms behind these effects of severe heat treatment based on studies of rats. Adrian (1982) has shown that water-soluble premelanoidines from a glucose–glycine reaction mixture reduce the protein digestibility and also affect the utilization of absorbed amino acids. Valle-Riestra and Barnes (1970) suggest that the reduced uptake of a severely heated glucose–egg albumin mixture is the effect of a decreased pancreatic enzyme secretion. Percival and Schneeman (1979) suggest that the enhanced fecal excretion of nitrogen following intake of heated casein is partly due to undigested endogenous protein. The observed decrease in biological value (i.e., enhanced urinary nitrogen excretion) may also be explained by the uptake of nonmetabolizable nitrogen-containing compounds from the gut (Valle-Riestra and Barnes, 1970; Ford and Shorrock, 1971; Pronczuk et al., 1973).

A previous study on the effects of Maillard reaction compounds on the digestion of dietary protein has indicated that a low molecular weight (LMW) fraction of a glucose–lysine reaction mixture affects the utilization of dietary protein in the rat (Öste and Sjödin, 1984). The present paper reports effects of this fraction on the in vitro activities of gastrointestinal proteolytic enzymes.

EXPERIMENTAL SECTION

Glucose–Lysine Reaction Mixture. A low molecular weight (LMW) fraction from a reflux-boiled mixture of equimolar amounts of D-glucose and L-lysine was prepared as described by Öste and Sjödin (1984).

Gastric and Pancreatic Enzymes. Pepsin (EC 3.4.23.2; 2× crystallized, from porcine stomach mucosa), trypsin (EC 3.4.21.4; 2× crystallized, from bovine pancreas), chymotrypsin (EC 3.4.21.1; 3× crystallized, from bovine pancreas), carboxypeptidase A (EC 3.4.17.1; 2× crystallized, from bovine pancreas), and carboxypeptidase B (EC 3.4.17.2; chromatographically pure, from porcine

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pancreas) were purchased from Sigma Chemical Co., St Louis, MO. The activities of pepsin with hemoglobin and trypsin with *N*-benzoylarginine ethyl ester (BAEE) as substrates were measured as described by Bergmeyer (1970). Assays were performed with a pepsin concentration of 2 μg/mL. After 5 min of incubation, the TCA solution (5% w/v) was added and the nonperceptable absorbance at 280 nm was recorded. In increase in absorbance per minute was considered as initial reaction velocity and was plotted vs. the substrate concentration. The assays of trypsin activities were performed with 6 μg/mL of the enzyme and 0–0.9 mM BAEE.

The activities of trypsin and of chymotrypsin with casein as substrate were also measured as described by Bergmeyer (1970), but with an incubation temperature of 37 °C. The trypsin concentration in the assay mixture was 3 μg/mL and the substrate concentration 0–5 mg/mL. The chymotrypsin concentration in the assay mixture was 5 μg/mL and the substrate concentration 0–5 mg/mL. The TCA solution (5% w/v) was added after 20 min of incubation, and the nonprecipitable absorbance at 280 nm was recorded and plotted vs. substrate concentration.

The activity of carboxypeptidase A, 5 μg/mL, was measured with 0–0.7 mM *N*-hippuryl-L-phenylalanine as substrate in 0.1 M sodium borate buffer, pH 7.6, 0.3 M in NaCl at a temperature of 30 °C. The hydrolysis was monitored by recording the increase in absorbance at 254 nm. The activity of carboxypeptidase B, 6 μg/mL, was measured with 0–0.7 mM *N*-hippuryl-L-arginine as substrate in 0.1 M sodium borate buffer, pH 7.6 and at a temperature of 30 °C. Also, the hydrolysis was monitored by recording the increase in absorbance at 254 nm.

Inhibition was recorded by assays of enzyme activities in the presence of the LMW fraction.

Enzymes of the Intestinal Mucosa. Preliminary experiments with brush border peptidases were performed with enzymes solubilized from the microvillus membrane vesicle fraction. Microvillus membranes were prepared from pig mucosa as described by Sjöström et al. (1978). The enzymes were solubilized by treatment with 1% Triton X-100 for 1 h and the solution centrifuged at 15.000 rpm in a Sorvall SS 34 rotor for 1 h. The resulting supernatant was used as the enzyme solution.

Preliminary experiments with cytosolic peptidases were performed with a preparation obtained as follows: A piece of pig intestine (about 5 cm) was gently stirred at 4 °C in 20 mL of a 50 mM Tris-HCl buffer, pH 8.0, for 1 h, after which the solution was centrifuged at 15.000 rpm in a Sorvall SS 34 rotor for 30 min. The supernatant was used as an enzyme solution.

The activities of aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), and dipeptidylpeptidase IV were analyzed as described by Sjöström et al. (1978), using L-alanyl-*p*-nitroanilide, L-glutamyl-*p*-nitroanilide, and glycyl-L-prolyl-*p*-nitroanilide as substrates, respectively.

Activity of glycylleucine dipeptidase (EC 3.4.13.11) and alanylproline dipeptidase (EC 3.4.13.9) were assayed by measuring the decrease in absorption in the low ultraviolet range, using glycyl-L-leucine and L-alanyl-L-proline as substrates, respectively, with details as described (Josefsson and Lindberg, 1965).

During the preliminary experiments, the inhibition was recorded by assays of the enzyme activities of the preparations described above in the presence of the LMW fraction.

Kinetic experiments with aminopeptidase N were performed on the pure enzyme prepared as described by Sjöström et al. (1978).

Column Chromatography. A 2.6 × 90 cm column was packed with Sephadex G-50 (superfine grade, swelled in water). A 200-μL portion (38 mg of dry substance) of a LMW fraction of the glucose–lysine reaction mixture, radiolabeled through addition of 5 mCi [U-^{14}C]glucose to the reactants, was placed on the column and eluted with water (equilibrated to 4 °C; flow rate 16.6 mL/h). The UV absorbance of the eluate was recorded. Fractions of 8.3 mL were collected, lyophilized, and redissolved in 1.5 mL of water. The radioactivity of each fraction was assessed by liquid scintillation counting (instrument Mark III, Searle Analytic Inc., Ill). The effects of aliquots of fractions on the activities of carboxypeptidase A and aminopeptidase N were determined. ^{1}H NMR spectra were recorded at 89.60 MHz (^{1}H) with a Jeol FX-90 Q instrument.

RESULTS

Effect of the LMW Fraction on Enzyme Activities. The LMW fraction was tested at concentrations between 0.25 and 1 mg/mL in the assay mixtures. In the enzyme assays with a protein as substrate, inhibitory effects were elucidated by comparing the initial reaction velocities at various substrate concentrations with or without the addition of the LMW fraction. No effect on the pepsin or chymotrypsin activities was observed. When casein was the trypsin substrate, 1.0 mg/mL of the LMW fraction caused a weak decrease in the initial reaction velocity, however only at low substrate concentration. The reduction of initial reaction velocity was never more than about 15%.

A weak noncompetitive inhibition of trypsin was obtained with BAEE as substrate. This was indicated by a reduction of maximum reaction velocity (V_{max}) from 7×10^{-2} to 5×10^{-2} mM min^{-1}, upon the addition of 0.5 mg/mL of the LMW fraction, with no change in the Michaelis constant ($K_m = 5.1 \times 10^{-2}$ mM).

Carboxypeptidase A and, to a smaller extent, carboxypeptidase B were inhibited by 0.5 mg/mL of the LMW fraction. The inhibition of the carboxypeptidases did not obey classical enzyme kinetics, as indicated by the Lineweaver–Burk plot of carboxypeptidase A activities shown in Figure 1, and values of apparent reduction of V_{max} or K_m could not be calculated. The enzymatic activities were completely restored after dialysis of the reaction mixture.

The preliminary experiments with the preparations of brush border membrane and cytosolic enzymes revealed that the activities of two enzymes were affected by the LMW fraction. A strong inhibition of aminopeptidase N was observed with 0.25 mg/mL of the LMW fraction. Kinetic experiments with the purified enzyme showed a mixed type of inhibition with a strong noncompetitive element (Figure 1). A comparatively weaker inhibition was obtained with the cytosol enzyme glycylleucine dipeptidase. At a substrate concentration of approximately $5M_m$ the reduction of initial velocity was 50% with 0.7 mg/mL of the LMW fraction in the assay mixture. The nature of this inhibition was not further investigated.

Thus, no apparent effect was observed on the activities of brush border membrane enzymes aminopeptidase A and dipeptidylpeptidase IV or the cytosol enzyme alanylproline dipeptidase.

Studies on Fractions from Column Chromatography. The elution profile of the LMW fraction from the glucose–lysine reaction mixture, as monitored by UV absorbance, is shown in Figure 2. The distribution of the label from reactant glucose is also shown. The chromatography was performed on Sephadex G-50, which has a

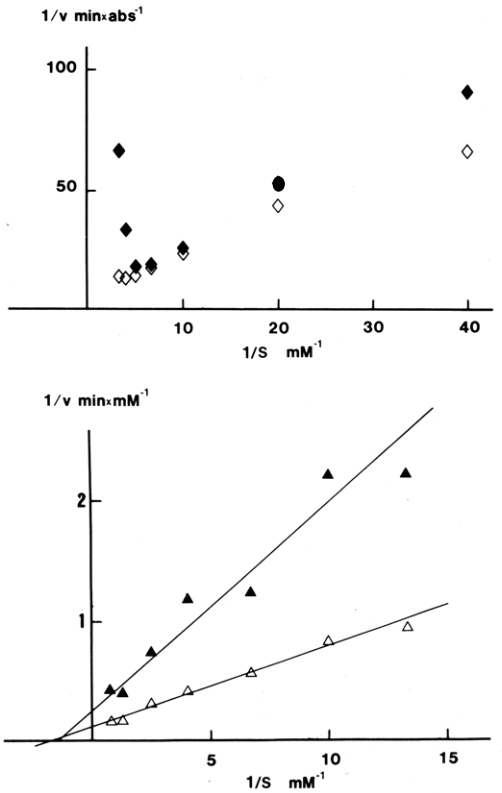

Figure 1. Effect of the low molecular weight (LMW) fraction of the glucose–lysine reaction mixture on enzyme kinetics, reciprocal plot of velocity (v) vs. substrate concentration (S): upper figure, hydrolysis of *N*-hippuryl-L-phenylalanine by carboxypeptidase A (in 0.1 M sodium borate, 0.3 M NaCl, pH 7.6 at 30 °C); filled symbols, addition of 0.5 mg/mL of the LMW fraction; open symbols, no addition; lower figure, hydrolysis of L-alanyl-*p*-nitroanilide by aminopeptidase N (in 0.05 M Tris-HCl, pH 7.3 at 37 °C); filled symbols, addition of 0.25 mg/mL of the LMW fraction (K_m 0.7 mM, V_{max} 40 mM min^{-1}); open symbols, no addition (K_m 0.5 mM, V_{max} 80 mM min^{-1}).

separation range of 1500–30 000 Da for proteins and 500–10 000 Da for dextrans. The shape of the profiles of both absorbance and radioactivity indicates that the majority of compounds elute late and have molecular weights in the lower range. The maximum of UV absorbance did not coincide with the maximum of radioactivity.

In Figure 2 is also shown the relative degree of inhibition of carboxypeptidase A and of aminopeptidase N by aliquots of fractions of the elute. The inhibition of carboxypeptidase A seemed roughly to coincide with the UV absorbance. The inhibition of aminopeptidase N varied considerably from fraction to fraction.

Both carboxypeptidase A and aminopeptidase N were strongly inhibited by a common fraction. This fraction corresponded to a single peak in the UV absorbance curve (indicated by an arrow in the figure). ^1H NMR analysis indicated that it mainly contained 2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine, a compound isolated from a glucose–lysine reaction mixture by Miller et al. (1984) and Nakayama et al. (1980).

DISCUSSION

The LMW fraction from the glucose–lysine reaction mixture affected the activity of some, but not all, of the enzymes involved in protein digestion in vivo, at least to a concentration as low as 0.5 mg/mL. The question is whether this partial inhibition might explain the earlier observed (Öste and Sjödin, 1984) reduced plasma level of amino acids derived from dietary egg white protein in rats that were orally fed a diet containing 1.5% (w/w) of the

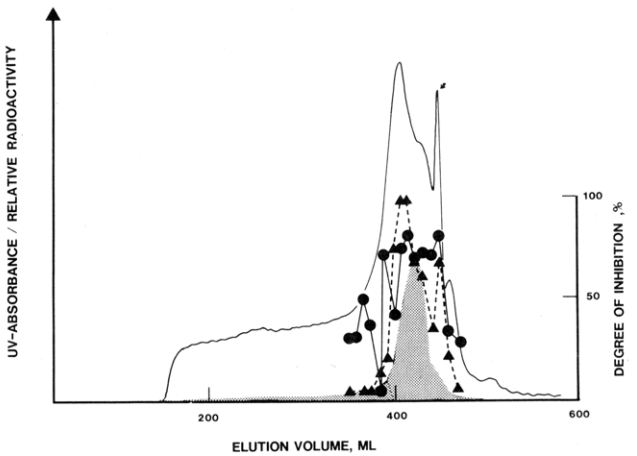

Figure 2. Elution profile of a Sephadex G-50 (superfine grade) gel permeation column of low molecular weight glucose–lysine reaction products (the LMW fraction). UV absorbance was recorded as the top solid line. Shaded area shows relative distribution of a ^{14}C label originating from the reactant glucose. Inhibition of aminopeptidase N (●) and carboxypeptidase A (▲) by aliquots of eluent fractions was measured as the reduction of reaction velocity at a 1.0 mM concentration of substrate for both enzymes.

LMW fraction. In that study, each rat consumed 22.5 mg of the LMW fraction. Assuming a total volume of digestive juices of 5–10 mL, the concentration in the rat intestine will most likely be sufficient to bring about an enzyme inhibition, even with a high degree of absorption of the compounds to nonspecific proteins. The inhibitory effects observed in vitro were of the noncompetitive or the mixed type. This means that even under the conditions of excess substrate concentration, likely to exist at least initially in the lumen of the intestine, an effect on the substrate turnover would be expected. If the enzyme activities thereby become rate limiting for the overall digestion and absorption, a reduced blood uptake of amino acids initially bound to protein might result. However, it is unclear to what extent a partially reduced enzyme activity in vivo will actually lead to an impaired amino acid uptake. The reduced (50–70%) level of pancreatic enzymes in aged rats does not reduce protein digestibility (Snook, 1973). Total exclusion of pancreatic secretion, however, leads to a markedly reduced protein digestibility (Snook, 1973). As for the inhibition of specific enzymes, it has been reported that, during in vitro conditions, the inhibition of only one of the enzymes leads to a notable reduction of proteolytic capacity of the mixture of pancreatic enzymes (Gertler et al., 1980). This was observed also when the exopeptidases were inhibited.

Of the pancreatic enzymes investigated in the present study, carboxypeptidase A, carboxypeptidase B, and trypsin were affected by the Maillard reaction mixture. However, the degree of inhibition was in no case substantial at 0.5 mg/mL, and the slight effect on trypsin digestion of casein at 1.0 mg/mL of the reaction mixture could only be observed at low substrate concentrations. It seemed thus that these effects were not sufficient to bring about a notable effect in vivo.

The residue from pancreatic digestion consists of, in addition to free amino acids and dipeptides, a considerable amount of small oligopeptides (Adibi and Mercer, 1973). The epithelial cells of the intestine have been shown to actively absorb amino acids, dipeptides, and possibly tripeptides. The extent to which higher oligopeptides may be absorbed had not been clarified (Adibi, 1980; Silk, 1980). Studies by Adibi and Morse (1977) show that the brush

border hydrolysis is the rate-limiting step in the absorption of a tetrapeptide, tetraglycine, indicating that the higher oligopeptides are not absorbed but have to be further hydrolyzed prior to uptake. The membrane peptidases aminopeptidase N, aminopeptidase A, and dipeptidylpeptidase IV are probably those responsible for this final digestion of luminal oligopeptides and thus the complete uptake of a dietary protein. Aminopeptidase N constitutes 5–15% of the total membrane protein and should thus be the dominant one (Sjöström et al., 1978). Studies by Friedrich et al. (1980a,b) indicate that the enzyme in fact plays a central role in the brush border digestion of peptides in vitro. This enzyme was strongly inhibited by the LMW fraction.

About 40% of the LMW fraction is absorbed from the small intestine in the rat (unpublished results). A part of the fraction may thus be present in the epithelial cells at the time of intracellular peptide hydrolysis and may affect the final digestion of (di)peptides, since an effect on an intracellular dipeptidase (Gly-Leu dipeptidase) was observed as well.

The results from the studies on chromatographically separated fractions of the Maillard reaction mixture revealed that no single compound could account for the inhibition of aminopeptidase N or carboxypeptidase A. In addition, the profiles of inhibition of the enzymes by the various fractions were different (see Figure 2). This indicates that the compounds may affect the two enzymes to a different extent. The radioactivity in each fraction may roughly be considered proportional to the total amount of Maillard reaction compounds. The profiles of inhibition did not coincide with radioactivity; hence, the inhibitory effect was not a feature common to the majority of compounds in the mixture. The nature of some of the specific compounds that in fact have inhibitory effects will be reported.

The present study supports the assumption that low molecular weight glucose–lysine reaction compounds influence the uptake of protein in rats (Öste and Sjödin, 1984) due to an inhibition of intestinal proteolytic enzymes. It seems most likely that this effect is mainly the result of specific inhibitions of enzymes of the small intestinal mucosa, rather than an interference with the pancreatic enzymes. In particular, the inhibition of aminopeptidase N might be crucial.

Registry No. Carboxypeptidase A, 11075-17-5; aminopeptidase N, 9054-63-1; pepsin, 9001-75-6; chymotrypsin, 9004-07-3; aminopeptidase A, 9074-83-3; dipeptidylpeptidase IV, 9032-67-1; glycylleucine dipeptidase, 9025-31-4; alanylproline dipeptidase, 60831-96-1; trypsin, 9002-07-7; carboxylpeptidase B, 9025-24-5.

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Determination of Energy from Moisture Content in Foods Containing Small Amounts of Fat and Dietary Fiber

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It is shown that for a range of 46 foods of all types that contain small amounts (≤3%) of fat or dietary fiber, or approximately equal amounts of fat and dietary fiber, the available energy E (kJ/100 g) is inversely correlated with the percent moisture (M) by the equation $E = -17.38M + 1699$, $r = -0.998$, over the range $M = 0$–96%. The underlying reason for the empirical correlation is given. Comparison of the calculated energies with nearly 200 energies obtained by standard methods for cereals and root crops of moisture content 38–90% shows that the agreement is within about 5–10%. The method is very simple and rapid and should be useful for routine determinations of energies of cereals, bread, root crops, fruit, and vegetables, particularly in simple laboratories in developing countries and elsewhere.

Although there are many different nutrients that are required in the human diet, the two most important are adequate intakes of energy and protein (FAO/WHO, 1973). The energy intake of a particular diet or of a single food source is normally calculated from the amount of carbohydrate (starch, sugar), protein, fat, and (if present) alcohol that it contains, by the use of suitable factors originally due to Atwater and accepted today with only slight

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