Antioxidant Effects of Protein Hydrolysates in the Reaction with Glucose

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ABSTRACT: Maillard reaction products (MRP) obtained by reaction between glucose and protein hydrolysates from casein and fish were investigated. The influence of parameters such as reaction time and glucose concentration was studied. The antioxidative activity of MRP was determined by using the β-carotene/linoleate model and the 1,1-diphenyl-2-picrylhydrazyl method. All experiments showed that the antioxidative effect was improved by 20–30% when the hydrolysates were reached with glucose. A dramatic increase in antiradical efficiency of the MRP (up to 75%) was also observed. The study of the chromatographic profiles obtained before and after the Maillard reaction found changes in absorbance at 280 nm, indicating molecular rearrangements that could be involved in the improvement of the antioxidative and free radical-scavenging activities.

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The Maillard reaction, or nonenzymic browning, occurs when carbonyl groups, usually from reducing sugars, condense with free amino groups, most commonly from peptides and proteins. It is largely responsible for the color and flavor of many processed foods (1). In addition to its sensory implications, the Maillard reaction can be responsible for a reduction in the nutritional value of foods by involving compounds such as basic amino acids or vitamins that condense with sugars, or by the chelation of metals such as copper and zinc. Besides nutrient loss, foods can be subjected to other chemical changes resulting from Maillard reaction products (MRP), such as the formation of a wide variety of brown melanoidins. Experiments, mainly carried out on simple model systems, have shown that MRP exhibit antioxidant properties, in particular, strong scavenging activity against hydroxyl radical and superoxide anion (2,3). In addition, experiments carried out in different food systems such as tomato derivatives and coffee showed that, although the concentration of natural antioxidants was significantly reduced as a consequence of the thermal treatments, the overall antioxidant properties of the food products were maintained or even enhanced by the developments of MRP (4,5).

Certain protein hydrolysates have previously been reported to be antioxidative *per se*. However, the antioxidant effect of protein hydrolysates was considerably improved when reacting with glucose (6). Lingnert and Eriksson (3) investigated the ability of peptides to form antioxidative MRP and concluded that potent antioxidants were able to form when reacting peptides with sugars, as the antioxidative effect is dependent not only on which amino acids constitute the peptide but also on their sequence. These authors suggested that (i) the basic amino acids were the ones most capable of forming antioxidative products and (ii) it was probable that the N-terminal amino acid was important, since the α-amino group could be used in the Maillard reaction. The purpose of this study was to compare the potential ability of two hydrolysates of casein and fish to form antioxidants by the Maillard reaction in the presence of glucose.

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EXPERIMENTAL PROCEDURES

Materials. Casein peptone N1 (H1) and cod viscera hydrolysate (H2) were supplied, respectively, by Organotechnie S.A. (Courneuve, France) and Biotec-Maczymal (Oslo, Norway).

Determination of protein concentration. Soluble protein contents in the hydrolysates (mg/mL) were determined according to a procedure based on a modification of the micro-Lowry method (7), using BSA as the standard.

Preparation of MRP. The MRP were prepared by reacting glucose (12 or 40 mg/mL) and protein hydrolysates (4 mg/mL), followed by a heating treatment at 100°C. The browning extent of the MRP was determined by measuring the absorbance at 420 nm according to the procedure of Morales and Jimenez-Perez (10).

M.W. distribution profile. The MRP were separated by fast protein liquid chromatography–size exclusion chromatography using a SUPERDEX Peptide HR 10/30 column (Pharmacia, Uppsala, Sweden) according to the procedure described by Guérard *et al.* (8). Absorbances were monitored at 220 and 280 nm.

Measurement of antioxidative activity. Antioxidative properties of the MRP were determined using the β-carotene/linoleate model system according to the procedure of Marco (9). The antiradical activity of the MRP was estimated according to a slight modification of the procedure reported by Morales and Jimenez-Perez (10). An aliquot of sample (200 μL) was added to 1 mL of a solution of 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$), prepared fresh daily, at a concentration of 74 mg/L in ethanol. The mixture was shaken vigorously for 1 h at 25°C. The sample was

centrifuged at 10,000 × *g* for 5 min, then absorption of the supernatant was measured at 520 nm. Several preliminary experiments were performed to determine the kinetics of radical-scavenging activity of samples and the time needed to reach the established steady state. The DPPH• concentration in the reaction medium was calculated from the calibration curve, determined by linear regression: $[DPPH^{\bullet}]_t = 0.0241\ (A_{520\ nm}) + 0.022$ ($r^2 = 0.9995$). The antiradical activity (AA) of the sample was expressed as the percentage disappearance of DPPH•:

$$AA(\%) = (100 - ([DPPH^{\bullet}]_t/[DPPH^{\bullet}]_{H_2O})\cdot 100) \quad [1]$$

where $[DPPH^{\bullet}]_{H_2O}$ is the concentration of DPPH• in the presence of water instead of hydrolysate.

In all experiments, samples were analyzed in triplicate, and mean values ± SD were recorded.

RESULTS AND DISCUSSION

Hydrolysates and antioxidant efficiency. The protein contents were 32.5 ± 0.02 and 18.1 ± 0.08% for H1 and H2, respectively. The antioxidant efficiencies of the two hydrolysates tested at a 4 mg/mL protein concentration were dissimilar, as the oxidation of β-carotene in the presence of H1 was lower than in the presence of H2 (23.8 ± 0.46 and 46.4 ± 0.23% respectively).

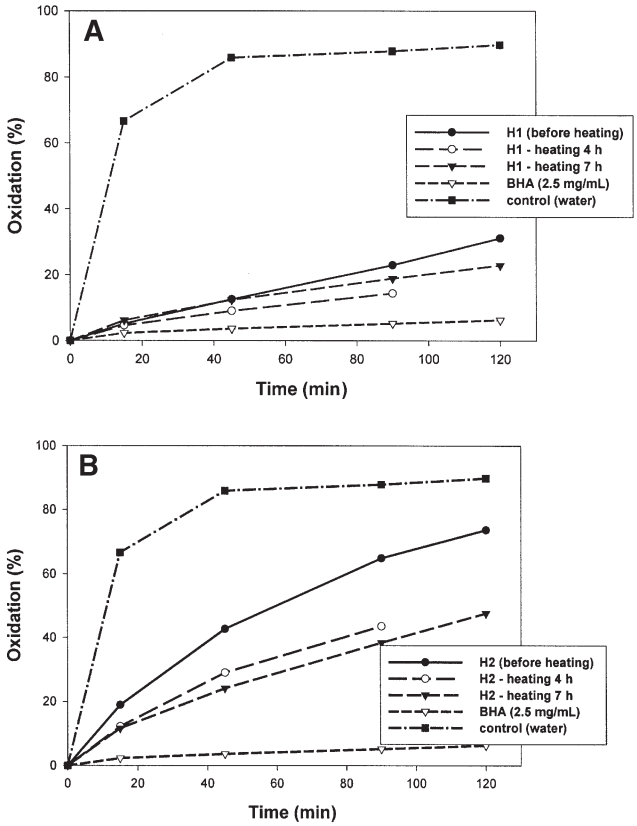

FIG. 1. Effect of Maillard reaction products (MRP) of (A) casein hydrolysate (H1) and (B) cod viscera hydrolysate (H2) on the oxidation of β-carotene. The glucose concentration was 12 mg/mL.

Antioxidant activity. The antioxidative effect of MRP prepared from H1 and H2 is shown in Figure 1. Under the conditions adopted for the Maillard reaction, an increase in antioxidant efficiency was developed, but this activity always remained lower than the BHA efficiency. After 7 h of heating, the protection against the bleaching of β-carotene was twice as high in the presence of MRP from H1 as in the presence of MRP from H2. Similar results were obtained with a 40 mg/mL glucose concentration.

Free radical-scavenging efficiency of MRP. The reduction of the free radical DPPH• in the presence of H1 and H2 before heating and in the presence of MRP is shown in Figure 2. The residual DPPH• in the presence of MRP prepared from H1 decreased from 95 to 20–25% (after 4–7 h heating) for the two tested glucose concentrations (12 and 40 mg/mL). These values were dramatically increased in the case of MRP prepared from H2. After 4 h heating at a 40 mg/mL glucose concentration (Fig. 2B), the free DPPH• was nearly fully scavenged by the MRP, suggesting the high antiradical efficiency of the new compounds formed.

Chromatographic profiles of MRP. The MRP were analyzed using size exclusion chromatography. After a 4-h incubation in the presence of glucose, a small increase in total areas [+18% for H1 (Fig. 3A) and +14.5% for H2 (Fig. 4A)] was observed at 220 nm. The greatest increase in total area was noticed at 280 nm, mainly for H1 (+285%, Fig. 3B), as the total area was increased by 85% for H2. These results suggested that the formation of the MRP was strongly dependent on the nature of the hydrolysate tested (composition of amino acids, free amino acids available, M.W. of peptides, etc.). The Maillard reaction induced the formation of a high proportion of phenolic compounds, mainly for the casein peptone H1, which may be related to the increase in antioxidant/free radical scavenging properties.

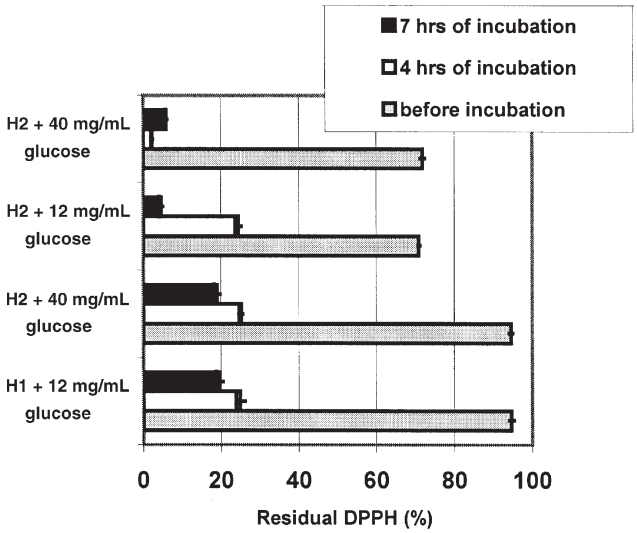

FIG. 2. Effect of MRP on the scavenging of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH•). The residual DPPH• was measured after 60 min. For abbreviation see Figure 1.

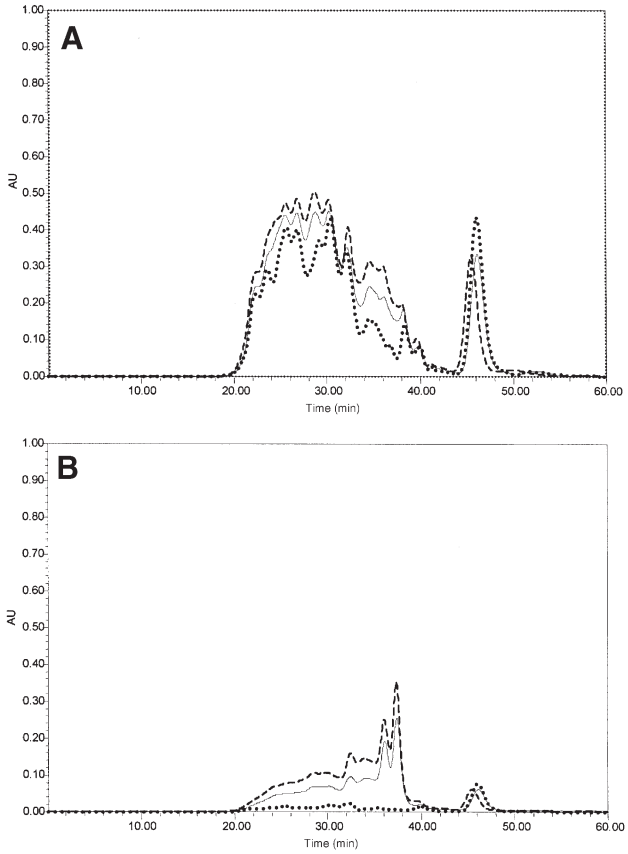

FIG. 3. Elution of MRP prepared from casein hydrolysate (H1) (4 mg/mL) + glucose (40 mg/mL). The fractionation range of the Superdex HR 10/30 column (Pharmacia, Uppsala, Sweden) was 7000 to 100 Da. Absorbance was monitored at (A) 220 nm and (B) 280 nm. (· · · · before incubation; — after 4 h incubation; ---- after 7 h incubation.) For abbreviations see Figure 1.

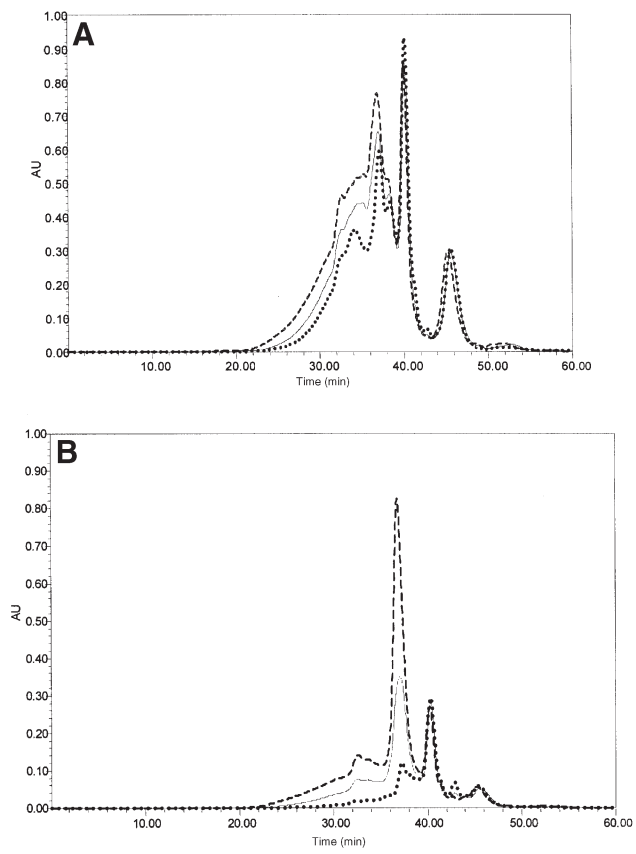

FIG. 4. Elution of MRP prepared from cod viscera hydrolysate (H2) (4 mg/mL) + glucose (40 mg/mL). The fractionation range of the Superdex HR 10/30 column was 7000 to 100 Da. Absorbance was monitored at (A) 220 nm and (B) 280 nm. (· · · · before incubation; — after 4 h incubation; ---- after 7 h incubation.) For abbreviations see Figure 1, and for manufacturer see Figure 3.

The two hydrolysates from casein and cod viscera possessed antioxidant activity *per se*, but the overall antioxidant properties varied according to the hydrolysate tested. H1 was more efficient against the bleaching of β-carotene than H2, which demonstrated a better free radical DPPH$^{\bullet}$ scavenging activity. From the chromatographic data, we observed that the proportion of small M.W. peptides was higher in H2 than in H1, suggesting that the cod viscera preparation was more extensively hydrolyzed than the casein peptone. However, this difference in proportion cannot fully explain the increase or decrease in antioxidant activities. Chen *et al.* (11) have explored the residue–activity relationship of a synthetic peptide, Leu-Leu-Pro-His-His (LLPHH), originally isolated from a hydrolysate of soybean β-conglycinin and of 28 structurally related peptides. The authors showed shown that, among the most important factors for antioxidant efficiency, the size of peptides was not significant. The antioxidative activity of the peptides mainly depended on their amino acid sequences as well as the constituent amino acids. For example, the segment His-His in LLPHH was necessary for the antioxidative activity, as the small peptide Pro-Pro-His is twice as potent as the reference peptide LLPHH.

The antioxidant efficiency was considerably improved when reacting the hydrolysates with glucose. Fractionation of MRP by gel filtration showed antioxidative activity to be evenly distributed over the colored fractions (12). Comparisons at the same color intensity (A_{490}) showed that colored fractions in the lower M.W. range had a stronger antioxidative effect than fractions in the higher M.W. range. The antioxidant properties of the Maillard browning products are widely documented but poorly understood. They may be due to the formation of phenolic-type structure and/or the metal-chelating properties of melanoidins. From the chromatographic data, the increase in absorbance at 280 nm suggested some molecular rearrangements with phenolic structure. Ames (1) showed that the formation of Maillard browning products with antioxidant effects increased with heating time at 100°C, and also at acidic pH values, because the use of a low pH favored the hydrolysis of sucrose to reducing sugars and hence browning. Darker products showed a greater antioxidant effect than lighter ones. In addition, Hayase *et al.* (13) showed that melanoidins were strongly active in scavenging active oxygen species. Nondialyzable melanoidin (HM melanoidin) and low M.W. melanoidin (LM melanoidin)

prepared from a D-glucose–glycine system effectively scavenged superoxides to a similar degree as in the case of superoxide dismutase. Hydrogen peroxides were also effectively scavenged with an increase in the concentration of HM and LM melanoidins. In addition, the melanoidins had a desmutagenic effect against heat-induced mutagens such as Trp-P-2 (3-amino-1-methyl-5H-pyrido(4,3-b)indole (14). The antioxidant properties of MRP could also exert an anticarcinogenic activity by inactivating reactive oxygen species that were involved in the process of cancer. Feasible mechanisms were the inhibition of nitrosamine formation, direct scavenging of ultimate carcinogenic species, and antipromotor activity (15). Therefore, it was not clear whether melanoidins were carcinogenic or anticarcinogenic, but any anticarcinogenic activity was likely to be associated with their antioxidative properties.

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