Protective Effects of Melanoidins Derived from Soy Sauce and Soy Paste on NO-Induced DNA Damage

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We determined the protective activity of melanoidins against DNA damage using the comet assay. We used nitric oxide (NO) as a DNA damaging agent and HL60 (human promyelocytic leukemic cell line). Melanoidins were prepared from soy sauce and soy paste (miso). When the cells were incubated with NO (1 mM) and melanoidin (0–0.25%) for 30 min, soy sauce melanoidin strongly inhibited NO-induced DNA damage in a dose dependent manner. Miso melanoidin and melanoidin prepared from a glycine/glucose model system also inhibited the DNA damage. Melanoidins showed the protective effect even when cells were subsequently incubated with NO after the treatment and removal of melanoidins. As melanoidins did not trap NO under the condition used, they seemed to inhibit the DNA damage indirectly through a modification of some cellular function.

Keywords: melanoidin, DNA damage, comet assay, nitric oxide (NO), soy sauce, miso, HL60

Maillard reaction is one of the most important reactions in chemical changes of food components during storage and processing of foods. Melanoidins, advanced glycation end products of Maillard reaction, are brown polymerized pigments that are produced in food systems containing proteins or amino acids and reducing sugars (Ikan *et al.*, 1996). They are contained in many foods such as soy sauce, miso, roasted coffee etc., and play an important role in adding desirable flavor and color to food. In addition to making food more desirable, there have been many reports about their physiological functions (Hayase & Kato, 1994), such as promotion of faecal bacterial growth (Ames *et al.*, 1999), suppression of tumor cell growth (Kamei *et al.*, 1997), inhibition of digestive enzymes (Hirano *et al.*, 1994, 1996), antioxidative activity (Chuyen *et al.*, 1998), and antimutagenic activity (Lee *et al.*, 1994). We examined DNA damage-protecting activities of melanoidins using the comet assay, a single cell gel electrophoresis assay which has been used to detect the genotoxic effect of irradiation of various foods (Cerda *et al.*, 1997; Singh, 1988). Subsequent improvements of the sensitivity and methodology have made it possible to detect single- and double-strand breaks, alkali labile sites, incomplete excision repair sites and genomic structural discontinuities. Recently we have shown that the comet assay is an easy, quick and highly sensitive method to detect the protective effect of food components against DNA damage induced by several chemical mutagens (Miwa & Hongo, 2000; Miwa *et al.*, 1999, 2000; Sugai & Miwa, 1999). In this paper, we evaluated the DNA damage-protecting activity of melanoidins derived from soy sauce, miso paste and glycine-glucose model melanoidin against DNA damage of HL60 cells induced by nitric oxide using the comet assay.

Materials and Methods

Cell preparation The cell line HL60 (human promyelocytic leukemia) was used in this experiment. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a CO_2 incubator (air: 95%, CO_2: 5%). Before the assay, cells were centrifuged at 1000 $\times g$ for 5 min and medium was changed to FBS-free DMEM.

Soy sauce melanoidin Soy sauce melanoidin was prepared as follows. Soy sauce (koi-kuchi, Yamasa Co., Ltd, Choshi) was dialyzed against water for a week at 2°C, then the nondialyzable fraction was freeze-dried. The dried product was solubilized in water and the solution was subjected to Sephadex G-25 column chromatography. The absorption at 440 nm and 470 nm were monitored, and colored fractions were collected. The collected fractions were subjected to Sephadex G-100 column chromatography. Colored fractions were again collected similar to the above procedure, and the freeze-dried product was used as a soy sauce melanoidin sample.

Miso paste melanoidin Miso paste (Hatcho-miso, Hatcho-miso Co. Inc, Okazaki) was suspended in hot water (70°C), and the suspension was centrifuged at 2000 rpm for 10 min. The precipitate was re-extracted with hot water and centrifuged again. The two supernatants were then mixed together and the mixture was filtered. The soluble fraction was dialyzed against 0.9% NaCl for 1 day and then against water for 6 days at 2°C. The nondialyzate was treated the same as soy sauce to prepare the melanoidin fraction.

Model melanoidin One molar D-glucose and 1 molar glycine were dissolved in 500 ml of 0.2 M sodium bicarbonate (pH 6.8). The mixture was refluxed at 95°C for 7 h. The brown solution was dialyzed against water for 2 weeks at 2°C. The nondialyzate was freeze-dried, and the dried product was used as nondialyzable model melanoidin (ND-MEL). A part of the brown

solution was dialyzed using a Spectra/por CE M.W.500 cut tube for 1 week , and nondialyzate was then dialyzed using a Spectra/por CE M.W. 1000 cut tube for 1 week. The dialyzate was freeze-dried and the dried product was used as dialyzable low molecular weight melanoidin (LM-MEL).
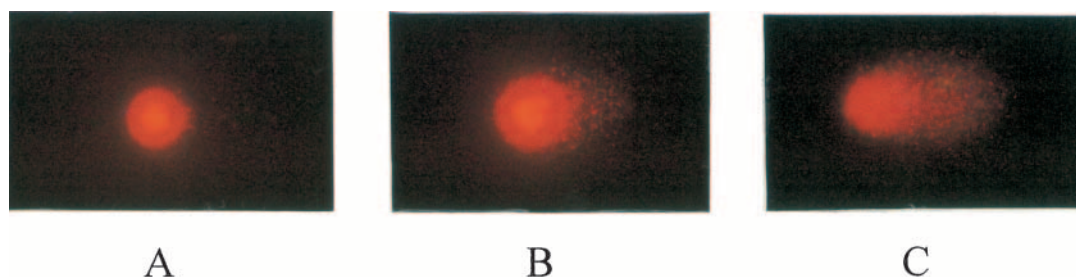
DNA damage treatment Nitric oxide donor (NOC7, DOJINDO LAB., Kumamoto) was used as a DNA-damaging agent. One hundred millimol of NOC7 in 0.1 N NaOH was stocked in a –30°C freezer before use. Melanoidins were dissolved in DMEM to make 0–0.5% solution. One hundred microliters of HL60 cell suspension (2×10^6 cells/ml) and 100 μl of melanoidin solution were placed in each well of 96 well plates. Co-incubation treatment with melanoidins: NOC7 solution was added to each well containing cells and melanoidins, and quickly mixed. Then the plate was incubated for 30 min in a CO_2 incubator. Pre-incubation treatment with melanoidins: Cells and melanoidins were incubated in a CO_2 incubator. After 30 min of incubation, each plate was centrifuged and the supernatant was removed. Two hundred microliters of fresh DMEM was added to each well, and cells were suspended by pipetting. Then 2 μl of NOC7 stock solution was added to each well and the plate was incubated for another 30 min.

Comet assay Electrophoresis of whole cell nuclei was carried out according to the procedure originally developed by Singh *et al.* (1988). NOC7 treated cells were suspended in pre-warmed agarose (0.5% in PBS), and the suspension was put onto a slide glass that had been pre-coated with agarose (0.75% in water). After gelling at 0°C, the slides were treated with sodium sarcosinate (pH 10.0, 4°C, 1 h) for cell lysis. They were immersed in an electrophoresis buffer (pH 13.0) in a flat-bed apparatus for 20 min to unwind DNA in the cells. Electrophoresis was then carried out at 20 V for 30 min. The slides were washed in a 0.4 M Tris buffer (pH 7.5) for 15 min (5 min×3), and then stained with 50 μg/ml of propidium iodide in PBS for 10 min. After being washed with water, a cover glass was attached to the center of each slide glass. The slides were examined under an Olympus microscope equipped with a fluorescent filter.
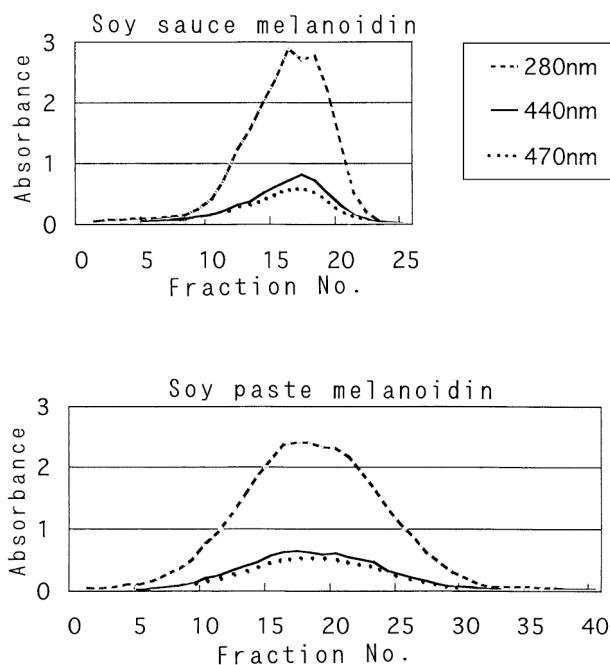
DNA damage evaluation The shape of stained cells was observed and classified into three categories: normal round shape (–), slight-comet shape (±), and clear-comet shape (+) as shown in Fig. 1. More than 100 cells were observed for each treatment and the number of clear-comet shapes were expressed by the comet index as follows.

$$\text{Comet index} = (s-n/p-n)\times100$$

s, *n* and *p* are the percentage of clear-comet shape cells treated for the sample(s), negative control (without NOC7 treatment),

Fig. 2. Sephadex G-100 gel filtration chromatogram of soy sauce and soy paste melanoidins. Soy sauce: Fraction of number from 10 to 21 were collected and lyophilized. Soy paste: Fraction of number from 6 to 30 were collected and lyophilized.

and positive control (with NOC7 treatment without the addition of samples).

Detection of NO trapping activity NOC7 (1 mM) was incubated with melanoidins (0–2.5%) and NO trapping reagent, DAF-2 (diaminofluorecene-2, 20 μM, Daiichi Pure Chemicals Co., Tokyo) for 30 min. Then the fluorescence intensity of each solution was detected using a fluorescence microwell plate reader.

Results and Discussion

Results of Sephadex G-100 gel filtration chromatography of prepared melanoidins are shown in Fig. 2. Using the absorbance at 470, 440 and 280 nm as the index, we collected the colored fractions shown and used these as melanoidin samples.

To determine the suitable concentration of NOC7, various concentrations of NOC7 (0, 0.5, 0.75, 1, 1.5 and 2 mM) were added to the cells and, after 30 min of incubation, the cells were subjected to the comet assay. We chose 1 mM of NOC7 as it yielded approximately 70% of the comet-shaped cells (data not
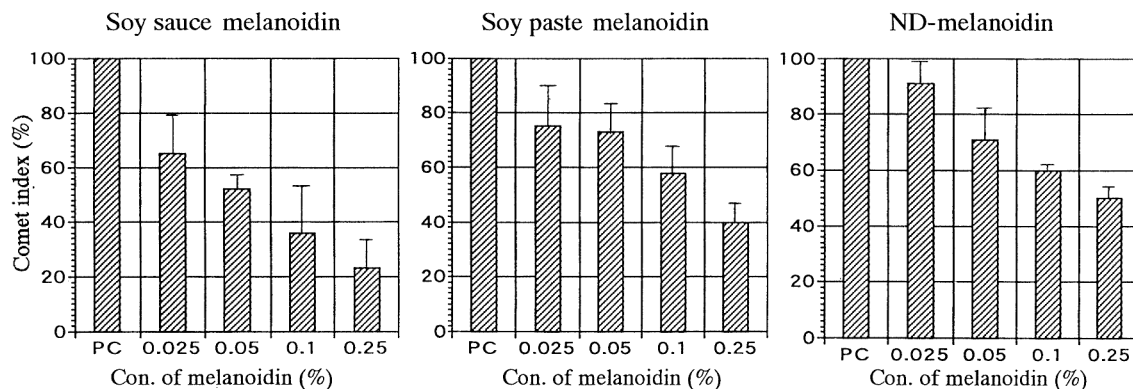

Fig. 1. Evaluation of DNA damage by comet shape under fluorescence microscope. A: Type 1 (normal round shape), B: Type 2 (slight-comet shape), C: Type 3 (clear-comet shape).

Fig. 3. DNA damage protecting effects of soy sauce, soy paste melanoidins, and ND (non-dialyzable model) melanoidin (co-incubation).

Table 1. NO radical trapping activity of melanoidins.

Concentration of melanoidins (%)	Nitrite (mM)	
	Soy sauce	Soy paste
0	1.0	1.0
0.025	1.4	1.1
0.05	1.4	1.0
0.1	1.4	1.1
0.25	1.1	0.8

Fig. 4. DNA damage protecting effect of soy sauce melanoidin (pre-incubation).

shown). One millimol N,N'-dimethyl-1,3-propanediamine, the by-product which was produced in NO-release reaction from NOC7, had no DNA-damage effect on this assay.

Under the above-described conditions, we performed the co-incubation experiment. As shown in Fig. 3, soy sauce melanoidin strongly inhibited the DNA damage induced by NOC7 in a dose dependent manner. The inhibitory effect of miso melanoidin was a little weaker but essentially the same as that of soy sauce melanoidin. We then determined the effect of model melanoidin prepared from glucose and glycine as described in Materials and Methods. Both non-dialyzable (ND-MEL) (Fig. 3) and dialyzed low molecular weight (LM-MEL) melanoidins (data not shown) had the activity, although that of ND-MEL seemed to be a little stronger. Melanoidins had no toxicity against the HL60 cells under the conditions used in this experiment.

As melanoidins were reported to have oxygen radical scavenging activity (Hayase 1996; 1997; Chuyen *et al.*, 1998), and there are several reports of NO radical trapping activity of some antioxidant compounds such as flavonoids, we were interested in whether the inhibitory effects of both melanoidins were due to the results of direct inhibition by their NO radical trapping activity. Each melanoidin was incubated with NOC7. After 30 min of incubation, released NO was determined using DAF-2 as described in Materials and Methods. No reduction of NO concentration was observed with 0.25% or less melanoidin (Table 1). This suggested that most of the DNA damage suppressing activity of melanoidins shown in Fig. 3 was not due to a direct inhibition of NOC7 through NO trapping activity.

Pre-incubation experiments were then performed. Figure 4 shows the result of the pre-incubation with soy sauce melanoidin. Compared with the co-incubation, soy sauce melanoidin gave slightly less but significant DNA damage protection. Miso mel-
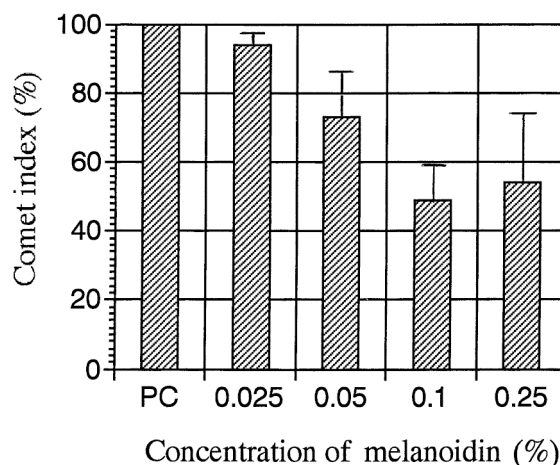
anoidin showed similar activity as that of soy sauce (data not shown). Again, it is suggested that melanoidins do not work through direct inhibition of NO radical but through some events in the cell, such as modification of a certain cellular function like the repair system or something to protect DNA damage.

Despite our data, melanoidins are known to have strong radical scavenging activity. They absorb or inactivate various radicals like·OH , O_2^-, H_2O_2 (Hayase 1996; 1997) and chemical compounds like Trp-P-2, a mutagen (Lee *et al.*, 1994). Peroxynitrite, a reaction product of NO and superoxide, is known to be a stronger DNA damaging agent than NO itself. If something like peroxynitrite had contributed to the DNA damage induced by the addition of NOC7 in the present study, and if melanoidins added in the culture medium could work to scavenge endogenous superoxide produced in the cell to prevent the formation of the peroxynitrite, this could be a possible explanation of the mechanism of the effect of melanoidins on the DNA damage. This should be elucidated further in the future.

References

Ames, J.M., Wynne, A., Hofmann, A., Plos, Saskia and Gibson, G.R. (1999). The effect of a model melanoidin mixture on faecal bacterial populations *in vitro*. *Br. J. Nutr.*, **82**, 489–495.

Cerda, H., Delincee, H. and Rupp, H. (1997). The DNA 'comet assay' as a rapid screening technique to control irradiated food. *Mutat. Res.*, **375**, 167–181.

Chuyen, N.V., Ijichi, K., Umetsu, H. and Moteki, K. (1998). Antioxidative properties of products from amino acids or peptides in the reaction with glucose. *Adv. Exp. Med. Biol.,* **434,** 201–212.

Hayase, F. and Kato, H. (1994). Maillard reaction products: safety and physiologic effects. *Comm. Agric. Food Chem.*, **3**, 111–128.

Hayase, F. (1996). Scavenging of active oxygen by melanoidins. In "The Maillard Reaction," ed. by R. Ikan. John Wiley and Sons, Ltd., England, pp. 89–104.

Hayase, F. (1997). Production and scavenging of active oxygen species by Maillard Reaction. *Nippon Yukagakukaisi*, **46**, 1137–1145 (in Japanese).

Hirano, M., Miura, M. and Gomyo, T. (1994). Melanoidin as a novel trypsin inhibitor. *Biosci. Biotechnol. Biochem.*, **58**, 940–941.

Hirano, M., Miura, M. and Gomyo, T. (1996). Kinetic analysis of the in-hibition by melanoidin of Trypsin. *Biosci. Biotechnol. Biochem.*, **60**, 458–462.

Ikan, R., Rubinsztain, Y., Nissenbaum, A. and Kaplanm, I.R. (1996). "The Maillard Reaction." John Wiley & Sons, England, p. 1

Kamei, H., Hashimoto, Y. Koide, T., Kojima, T., Hasegawa, M. and Umeda, T. (1997). Direct tumor growth suppressive effect of melanoidin extracted from immunomodulator-PSK. *Cancer Biother & Radiopharm.*, **12**, 341–344.

Lee, I.N., Chuyen, N.G., Hayase, F. and Kato, H. (1994). Desmutagenicity of melanoidins against various kinds of mutagens and activated mutagens. *Biosci. Biotechnol. Biochem.,* **58**, 18–23.

Miwa, M. and Hongo, Y. (2000). Application of a single-cell gel electrophoresis (comet assay to screen the antimutagenic activity in foods. *Biosci. Biotechnol. Biochem.*, **64**, 1292–1294.

Miwa, M., Watanabe, T., Kawasumi, T. and Hayase, F. (2000). Protective effects of melanoidins on DNA damage. 5th Japanese Society for Food Factors, Tokyo, p. 41.

Miwa, M. Sumio, Y. and Kim, K. (1999). Protective effects of meats and offals on DNA damage. 45th International Congress of Meat Science and Technology, Yokohama, pp.718–719.

Singh, N.P. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.

Sugai, E. and Miwa, M. (1999). Effects of quercetin on DNA damage evaluated by comet assay. 2nd International Conference on Food Factors, Kyoto. p. 111.