



White Sorghum (*Sorghum bicolor* (L.) Moench) Bran Extracts Suppressed IgE Production by U266 Cells

Takuya SUGAHARA,^{1,2,†} Sogo NISHIMOTO,^{1,3} Yoko MORIOKA,¹ Koichi NAKANO,⁴ and Keiko NAKANO⁴

¹Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan

²South Ehime Fisheries Research Center, Ehime University, 1289-1 Funakoshi, Ainan, Ehime 798-4205, Japan

³Center for Marine Environmental Studies, Ehime University, 2-5 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

⁴Nakano Sangyo Co., Ltd., 33-25 Asahi-shinmachi, Takamatsu, Kagawa 760-0064, Japan

Received April 6, 2009; Accepted June 7, 2009; Online Publication, September 7, 2009

[doi:10.1271/bbb.90245]

Sorghum, *Sorghum bicolor* (L.) Moench, is the fifth most important cereal crop in the world. In this study, we identified the IgE production-suppressing activity of white sorghum bran extracts. White sorghum is one of the genotypes of sorghum. White sorghum bran extracts in 10 mM sodium phosphate buffer (pH 7.4) suppressed IgE production in human myeloma cell line U266. The extracts suppressed IgE production by decreasing mRNA transcription level of IgE, but they did not affect IgA or IgG production of mice splenocytes *in vitro*. Heat treatment and trypsin digestion did not affect IgE production-suppressing activity. The white sorghum bran extracts were fractionated by ultrafiltration, and the molecular weight of the active substance was estimated to be less than 1,000.

Key words: allergy; IgE production-suppressing factor; white sorghum bran

Sorghum, *Sorghum bicolor* (L.) Moench, is the fifth most important cereal crop in the world, after wheat, rice, corn, and barley. It outperforms other cereals under various environmental stresses and is thus generally more economical to produce.¹⁾ The USA is the largest producer and exporter of sorghum. More than 35% of sorghum is grown directly for human consumption, and the rest is used primarily for animal feed and alcohol and industrial products. In addition, sorghum is an important food in developing countries of the semi-arid tropics, and it serves as one of the main sources of energy and protein for these populations.^{1,2)} It is also one of the main coarse cereal crops of India, and the demand for it for human consumption is rising.³⁾ In India, white sorghum is widely used in a variety of foods, but not in other countries such as Japan and the USA.

From a biochemical point of view, sorghum is a rich source of various phytochemicals, including tannins, phenolic acids, anthocyanins, phytosterols, and policosanols.⁴⁾ These phytochemicals have potential to significantly impact human health. This means that sorghum contains many substances possessing biological functions. Especially, the antioxidant properties of sorghum has been well investigated,^{5,6)} but there have

been few studies investigating the immunological functions of sorghum. We have screened the biological functions of white sorghum (WS), one of the genotypes of sorghum.⁷⁾ Endosperm extracts stimulated IgM production in the human hybridoma cell line and human peripheral blood lymphocytes, and the active substance was assumed to be protein (unpublished data). In addition, bran extracts induced apoptotic cell death against a mouse colon cancer cell line (unpublished data). The anti-allergy activity of white sorghum bran extracts was also examined here, because there are few reports concerned with such biological functions of white sorghum. IgE is an immunoglobulin involved in the allergic response. The binding of the allergen to IgE induces release of chemical mediators such as histamine and leukotriene from mast cells and basophiles. In this study, we examined the IgE production-suppressing activity of water-soluble WS bran extracts.

Materials and Methods

Reagents. Anti-human IgE antibody and biotin-conjugated anti-human IgE antibody were purchased from Biosource International (Camarillo, CA). Goat anti-mouse IgG, biotinylated goat anti-mouse IgG, goat anti-mouse IgA, biotinylated goat anti-mouse IgA, and horseradish peroxidase-streptavidin conjugate were from Invitrogen (Calsbad, CA). BALB/c mice were from Japan SLC (Shizuoka, Japan). ERDF medium was from Kyokuto Pharmaceutical (Tokyo).

Sample preparation. WS grains were powdered in a food mill (Iwatani, Tokyo) to obtain a fine powder. The bran powder (25 g) was suspended in 100 ml of 10 mM sodium phosphate buffer, pH 7.4 (NaPB), and gently stirred for 12 h at 4 °C. Insoluble substances were removed by centrifugation at 8,000 × g for 20 min, and the supernatant was collected and sterilized by filtration using a 0.22-μm filter (Millipore, Billerica, MA).

Cells and cell culture. Human myeloma cell line U266 producing IgE was obtained from ATCC. U266 cells were maintained in ERDF medium supplemented with 5% fetal bovine serum (FBS) before assay. Mouse splenocytes were prepared from 6-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan), and cultured in 5% FBS-ERDF medium. All the experiments using mice described herein were approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations.

[†] To whom correspondence should be addressed. Tel/Fax: +81-89-946-9863; E-mail: mars95@agr.ehime-u.ac.jp

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Ig, immunoglobulin; IL-4, interleukin 4; NaPB, sodium phosphate buffer; PBS, phosphate buffered saline; WS, white sorghum

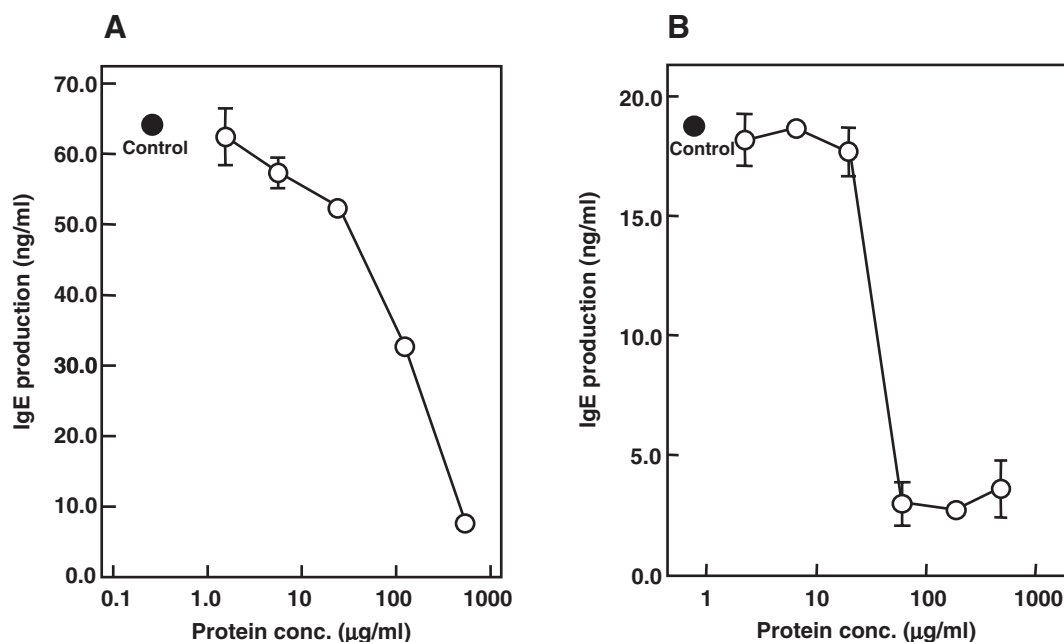


Fig. 1. Effect of WS Grain Extracts on the IgE Production of U266 Cells.

Extracts of WS whole grains and endosperms in 10 mM NaPB were added to ITES-ERDF medium at various concentrations, and U266 cells were inoculated at 1×10^5 cells/ml. U266 cells were cultured for 24 h in a CO₂ incubator and culture medium was collected. The amount of human IgE in the culture medium was measured by ELISA. The amount of the substances in extracts was represented as the protein concentration. Results are expressed as means $\pm$ SD ($n = 3$) for independent experiments. A, WS whole grain extracts; B, WS bran extracts.

Assay of IgE production-suppressing activity. The IgE production-suppressing activity of WS grain extracts was examined by measuring the amount of IgE secreted by U266 cells. An assay of the IgE production-suppressing activity was performed in a 96-well culture plate. U266 cells were inoculated in ERDF medium supplemented with 10 μ g/ml of insulin (Sigma), 20 μ g/ml of transferrin (Sigma), 20 μ M ethanolamine (Sigma), and 25 nM sodium selenite (Sigma) (ITES-ERDF) along with WS grain extracts at a density of 1×10^5 cells/ml, and cultured at 37 °C under humidified 5% CO₂-95% air for 24 h. After cultivation, the amount of IgE secreted in the culture medium was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 1.0 μ g/ml of goat anti-human IgE antibody in carbonate buffer (pH 9.6) was added to a 96-well ELISA plate at 100 μ l/well, and this was incubated for 2 h at 37 °C. After washing with 0.05% Tween20-phosphate buffered saline (T-PBS) 3 times, each well was blocked with 1.0% bovine serum albumin (BSA)-PBS for 2 h at 37 °C. Following washing with T-PBS 3 times, each well was treated with 50 μ l of culture supernatant for 1 h at 37 °C. Then 100 μ l/well of biotinylated anti-human IgE antibody diluted 1,000 times with 1.0% BSA-PBS was added, and this was incubated for 1 h at 37 °C. After washing with T-PBS 3 times, streptavidin-horseradish peroxidase conjugate diluted 1,000 times with 1.0% BSA-PBS was added, and incubated for 1 h at 37 °C.

Following this reaction, 0.6 mg/ml of 2,2'-azino-bis(ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) dissolved in 0.03% H₂O₂-0.05 M citrate buffer (pH 4.0) was added to the wells at 100 μ l/well, and the absorbance at 415 nm was measured after the addition of 100 μ l/well of 1.5% oxalic acid to terminate the coloring reaction. The IgE concentration was quantitatively determined by standard curve using human IgE standard solution.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. U266 cells were cultured in ITES-ERDF medium containing 400 μ g/ml of WS bran extracts for 24 h, harvested by centrifugation, and washed twice with PBS. Total RNA was isolated using Sepasol-RNA I super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. One mg of total RNA was used as a template for a cDNA synthesis reaction performed using MMLV-reverse transcriptase (Promega, Madison, WI) and an oligo-dT20 primer (Toyobo, Osaka, Japan). The PCR reaction was performed using Takara Ex Taq DNA polymerase (Takara, Kyoto, Japan). The

resulting cDNA samples were subjected to 30 cycles of PCR amplification performed under the following conditions: denaturation at 98 °C for 10 s, annealing at 60 °C for 2 s, and DNA synthesis at 74 °C for 30 s, according to the manufacturer's recommendations. Human GAPDH cDNA was amplified as an internal control. The PCR primer sequences were as follows: for human GAPDH sense,⁸⁾ 5'-ACCACAGTCCATGCCATCAC-3', and antisense, 5'-TCCACCACCCTGTTGCTGTA-3'; and for human IgE ϵ chain sense, 5'-GAAGTGTGCAGATTCCAACC-3', and antisense 5'-GCATGAAGTTCTGGATCAGG-3'. The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel, and were stained using SYBR Gold (Molecular Probes, Eugene, OR).

Results and Discussion

Effect of the WS grains on IgE production of U266 cells

WS whole grains and endosperms were ground with a mill, and each powder was suspended in 10 mM NaPB, and water-soluble substances were extracted. U266 cells were inoculated in ITES-ERDF medium supplemented with various concentrations of each extract and cultured for 24 h. The amount of extracts in culture medium was represented by the protein concentration. As shown in Fig. 1A, WS whole grain extracts dose-dependently suppressed IgE production of U266 cells. On the other hand, WS endosperm extracts did not affect IgE production at any dose (data not shown). It is supposed from this result that the IgE production suppressing substance in WS is contained in the bran of the grains. To confirm this hypothesis, an extract was prepared from WS bran powder, and the suppression activity was assessed. WS bran extracts in NaPB strongly suppressed the IgE production of U266 cells (Fig. 1B). The specific activity of the WS bran extracts was more than 10-fold more than that of the whole grain extracts.

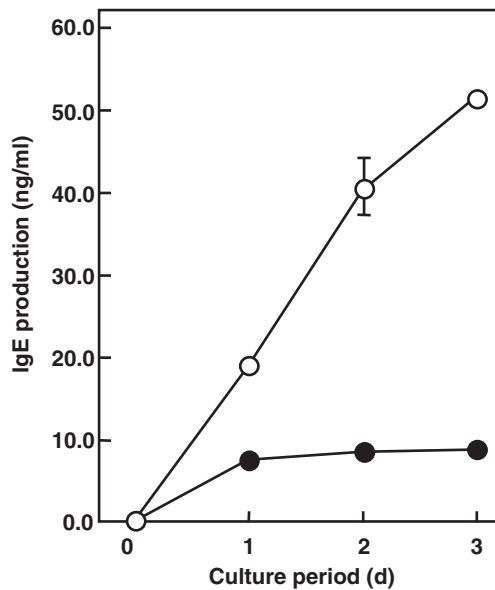


Fig. 2. Time-Dependent Effect of WS Bran Extracts on the IgE Production of U266 Cells.

U266 cells were cultured in ITES-ERDF medium with (●) and without (○) 100 µg of protein/ml of the WS bran extracts for 3 d. Culture medium was collected every 24 h, and the amount of IgE in the culture medium was measured by ELISA. Results are expressed as means $\pm$ SD ($n = 3$) of independent experiments.

Time-dependent effect of the WS bran extracts on the IgE production of U266 cells

The time-dependent effect of sorghum bran extracts on U266 cells was examined. U266 cells were cultured in ITES-ERDF medium containing 100 µg protein/ml of WS bran extracts for 3 d. As shown in Fig. 2, the WS bran extracts completely suppressed the IgE production of the U266 cells within 1 d after inoculation.

Effect of the WS bran extracts on Ig production of mouse splenocytes

The effect of the immunoglobulin production of the WS bran extracts on mouse splenocytes was investigated. Splenocytes from BALB/c mice were cultured in ITES-ERDF medium supplemented with 400 µg protein/ml of WS bran extracts for 24 h, and the amounts of IgA and IgG in culture medium were measured. As summarized in Table 1, IgG production of splenocytes was slightly decreased by the WS barn extracts, but there was no statistical difference between WS bran extract-supplemented medium and the control medium. In addition, IgA production was not affected by the WS bran extracts.

Mode of action of WS bran extracts

The correlation between the IgE production level and cell viability was examined to confirm that the effect of WS bran extracts on U266 cells was suppression of IgE production in the cells or cytotoxicity. U266 cells were cultured in ITES-ERDF medium containing 100 µg protein/ml of WS bran extracts for 24 h, and the IgE concentration in culture medium and cell viability were measured. As indicated in Table 2, IgE production was significantly suppressed by the WS bran extracts, but a trypan blue dye exclusion test revealed that cell viability was almost same as the control level. This suggests that

Table 1. Effect of the WS Bran Extracts on Ig Production of Mice Splenocytes

	Protein conc. (µg/ml)	Ig production (ng/ml)
IgG	0	60.2 $\pm$ 0.9
	400	49.5 $\pm$ 4.3
IgA	0	72.5 $\pm$ 3.7
	400	64.9 $\pm$ 0.6

Mouse splenocytes were prepared from 6-week-old female BALB/c mice. Splenocytes were cultured in ITES-ERDF medium supplemented with 400 µg protein/ml of WS bran extracts for 24 h, and the amounts of IgA and IgG in culture medium were measured. Results are expressed as means $\pm$ SD ($n = 4$).

Table 2. IgE Production-Suppressing Effect and Cytotoxicity of the WS Bran Extracts

Protein conc. (µg/ml)	Ig production (ng/ml)	Viability (%)
0	90.7 $\pm$ 2.5	82.0 $\pm$ 7.9
100	17.0 $\pm$ 0.3*	79.0 $\pm$ 6.8

U266 cells were cultured in ITES-ERDF along with and without 100 µg of protein/ml of WS bran extracts for 24 h. The IgE concentration in culture medium was measured by ELISA, and cell viability was measured by the trypan blue dye exclusion test. Results are expressed as means $\pm$ SD ($n = 3$).

* $p < 0.001$ vs. solvent control.

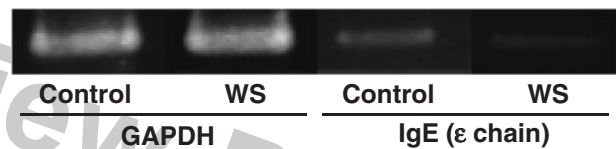


Fig. 3. Effect of the WS Bran Extracts on the Transcription Activity of U266 Cells.

U266 cells were cultured in ITES-ERDF medium with and without 100 µg/ml of WS bran extracts at 1×10^5 cells/ml and collected 24 h after inoculation. Total RNA was prepared and analyzed for ϵ chain in U266 cells (A) by RT-PCR. GAPDH mRNA was analyzed as an internal control.

the effect of WS bran extracts is due to suppression of IgE production.

The effect of WS bran extracts on the transcription level of mRNA for the IgE ϵ chain in U266 cells was examined by RT-PCR. U266 cells were treated with 100 µg/ml of WS bran extracts for 24 h, and the mRNA level for the ϵ chain of IgE was examined. As indicated in Fig. 3, the ϵ chain-mRNA expression level in the U266 cells was obviously depressed by treatment with WS bran extracts. It is supposed from this result that the decrease in IgE production is due to suppression of the expression level of ϵ chain mRNA in U266 cells.

Effect of wheat bran extracts on the IgE production of U266 cells

The IgE production suppressing activity of WS bran extracts was compared with other typical cereal bran extracts. Wheat bran extracts was prepared by the same procedure as WS bran extracts, and the activity was compared. Each extract was added to the ITES-ERDF medium at 100 µg protein/ml, and U266 cells were cultured for 24 h. The wheat bran extracts slightly suppressed IgE production (Fig. 4), but the specific activity was obviously lower than that of the WS bran

extracts. It is supposed from this result that wheat bran also contains active substances, but the amount is lower than for WS bran. Identification of the active substance in WS bran is an important subject.

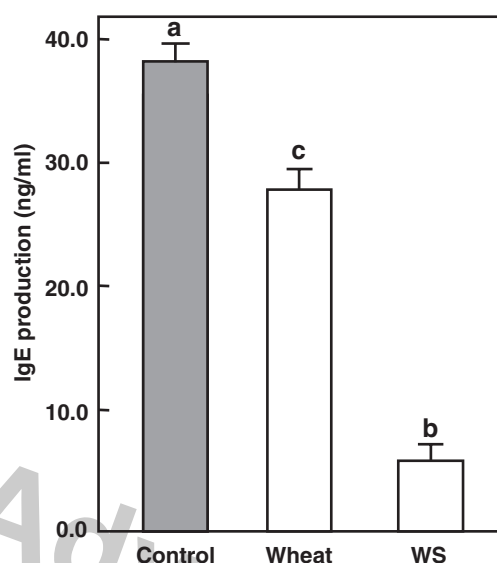


Fig. 4. Effect of the Wheat Bran Extracts on IgE Production of U266 Cells.

Wheat bran extracts and WS bran extracts in 10 mM NaPB were added to ITES-ERDF medium at 100 µg of protein/ml, and U266 cells were inoculated at 1×10^5 cells/ml. U266 cells were cultured for 24 h in a CO₂ incubator, and culture medium was collected. The amount of human IgE in the culture medium was measured by ELISA. The results are expressed as means $\pm$ SD (n = 3) of independent experiments. Letters that are different from each other denote significant differences at $p < 0.01$.

Fractionation of active substances in the WS bran extracts

WS bran extracts in 10 mM NaPB were fractionated by ultrafiltration. Crude extracts were filtrated with an ultrafiltration membrane (MW cut 5 kDa), and IgE production suppressing activity was assayed. As indicated in Fig. 5A, the activity of the filtrate was higher than that of the crude extracts. The specific activity was more than 10-fold that of the crude extracts. On the other hand, the specific activity of concentrated extracts mainly containing substances with molecular weights above 5 kDa decreased. This suggests that the molecular weight of the active substance is less than 5 kDa.

The filtrate was fractionated by ultrafiltration with a membrane of exclusion size 1 kDa. As shown in Fig. 5B, the filtrates suppressed the IgE production of U266 cells, and this means the molecular weight of the active substance was less than 1 kDa. However, the concentrate also suppressed IgE production as much as the filtrate did. It is supposed from this result that several substances participate in suppression of the IgE production of U266 cells.

Characterization of the active substances in the WS bran

The WS bran extracts were ultrafiltrated, and the filtrate (MW < 1 kDa) was collected. The filtrate was heated at various temperatures for 15 min, and residual activity was assessed. As shown in Fig. 6A, the activity was stimulated by heat treatment. The suppression activity was maximal at 100 °C treatment. In addition, the filtrate was trypsinized by 750 µg/ml of trypsin for 30 min, and residual activity was assessed. As shown in Fig. 6B, the activity was not affected by trypsin treat-

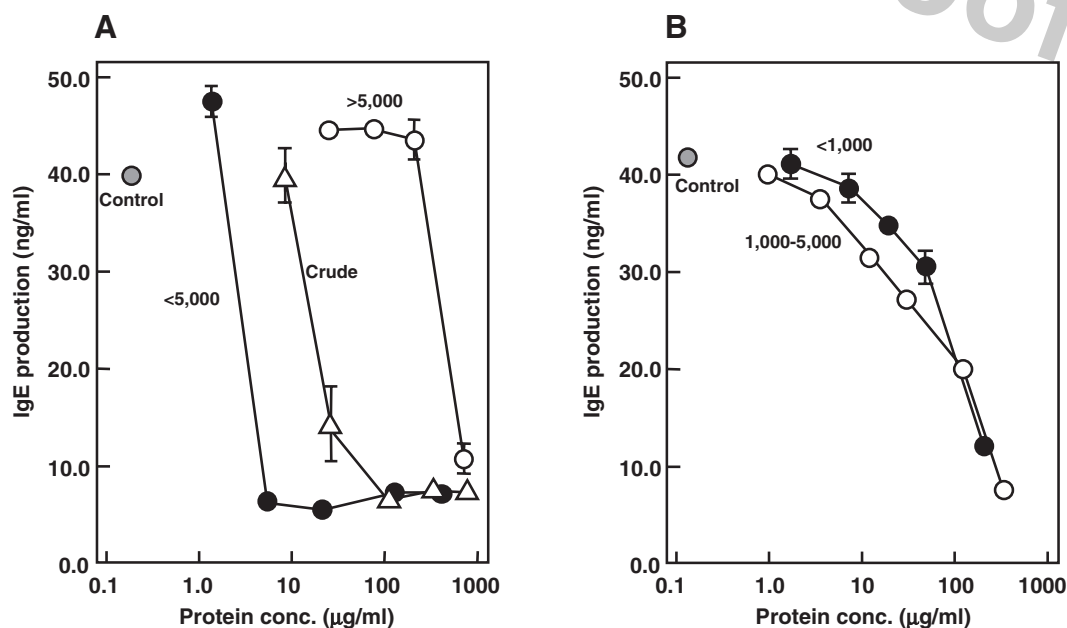


Fig. 5. Fractionation of the WS Bran Extracts by Ultrafiltration.

A, WS bran extract solution was ultrafiltrated using a 5-kDa cut membrane. The IgE production-suppressing activities of the filtrate (●), the residue (○), and crude WS bran extracts (△) were assessed. Each fraction was added to ITES-ERDF medium at various concentrations, and U266 cells were inoculated at 1×10^5 cells/ml. U266 cells were cultured for 24 h in a CO₂ incubator, and culture medium was collected. The amount of human IgE in the culture medium was measured by ELISA. B, The filtrate (less than 5 kDa) was ultrafiltrated using 1-kDa cut membrane. The IgE production-suppressing activities of the filtrate (●) and the residue (○) were assessed as described above. The amounts of the substances in extracts and filtrates were represented as protein concentration. The results are expressed as means $\pm$ SD (n = 3) of independent experiments.

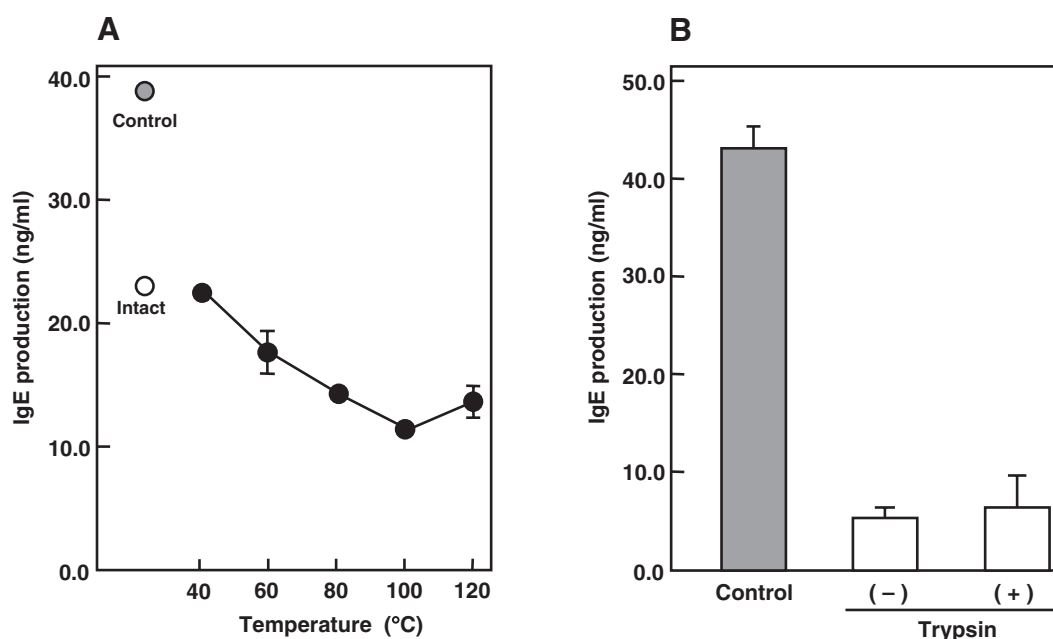


Fig. 6. Characterization of the Active Substances in the WS Bran Extracts.

A, WS bran extract solution ultrafiltrated with a 1-kDa cut membrane was heated at 40, 60, 80, 100, and 120 °C for 15 min. Following heat treatment, the effects on the IgE production of U266 cells were assessed. An open circle (○) indicates the activity of intact sample, and solid circles (●) indicate the activities of heated samples. The gray circle represents the solvent control. B, WS bran extract solution ultrafiltrated with a 1-kDa cut membrane was trypsinized by 750 µg/ml of trypsin for 30 min. Following trypsin treatment, the effects on the IgE production of U266 cells were assessed. Gray bar represents solvent control.

ment. These results suggest that the active substance is heat-stable and trypsin-insensitive. The molecular weight of the substance is estimated as less than 1 kDa. It is supposed from these results that the active substance consists of peptides or water-soluble substances.

WS bran contains substances suppressing IgE production of human myeloma U266 cells without cell death. WS bran extracts suppressed IgE production by depressing the mRNA transcription level for the ϵ chain of IgE. On the other hand, the extracts did not affect the IgG or the IgA production level of mouse splenocytes *in vitro*. This indicates that the WS bran extracts specifically suppress IgE production. Our preliminary study revealed that oral administration of WS bran extracts obviously suppressed the serum IgE level in OVA-challenged mice. In addition, the IL-4 level was also suppressed by WS bran extract *in vivo* (unpublished data). Therefore, WS bran extracts might suppress the allergic response not only by depression of IgE production in B cells, but also by suppression of Th2 activity *in vivo*. We are currently pursuing investigations of the allergy-suppression effects of WS bran extracts *in vivo*.

We are also investigating the active substance in the WS bran extracts. The active substance in WS bran is water-soluble, heat-stable, and trypsin-resistant. As indicated in Fig. 6, activity was enhanced about 2-fold by heat treatment. This means that the molecular structure of the active substance can be modified by

heating, and that activity is enhanced by conformational modification induced by heat-treatment. In other words, the molecular structure of the active substance is not heat-stable. In addition, the substance is contained in much greater amounts in WS bran than in wheat bran. At this point, we cannot determine conclusively what substances in WS bran contribute to the suppression of IgE production. Identification of the active substance in WS bran is now under study.

References

- 1) Hulse JH, Learing EM, and Pearson OE, "Sorghum and the Millets: Their Composition and Nutritive Value," Academic Press, New York (1980).
- 2) Oria MP, Hamaker BR, Axtell JD, and Huong CP, *Proc. Natl. Acad. Sci. USA*, **97**, 5065–5075 (2000).
- 3) Sheorain V, Banka R, and Chavan M, "Technical and Institutional Options for Sorghum Grain Mold Management," eds. Chandrashekar A, Bandyopadhyay R, and Hall AJ, ICRISAT, India, pp. 228–239 (2000).
- 4) Awika JM and Rooney LW, *Phytochemistry*, **64**, 1199–1221 (2004).
- 5) Awika JM, Rooney LW, Wu X, Prior RL, and Cisneros-Zevallos L, *J. Agric. Food Chem.*, **51**, 6657–6662 (2003).
- 6) Kamath VG, Chandrashekar A, and Rajini PS, *J. Cereal Sci.*, **40**, 283–288 (2004).
- 7) Ratnavathi CV and Sashidhar RB, *J. Sci. Food Agric.*, **84**, 1521–1527 (2004).
- 8) Lee YB, Nagai A, and Kim SU, *J. Neurosci. Res.*, **69**, 94–103 (2002).