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Purification and Characterization of Neoagarotetraose from Hydrolyzed Agar

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The whitening effect, tyrosinase inhibition, and cytotoxicity of neoagarotetraose were measured after its purification from hydrolyzed agar by gel filtration chromatography. In melanoma B16F10 cells, the melanin content of neoagarotetraose-treated cells was the same as that treated by kojic acid or arbutin. In addition, tyrosinase of melanoma cells was strongly inhibited by neoagarotetraose at a concentration of 1 μg/ml and similarly inhibited at 10 and 100 μg/ml compared with those by arbutin or kojic acid. The activity of mushroom tyrosinase showed a 38% inhibition by neoagarotetraose at 1 μg/ml, and this inhibitory effect was more efficient than that by kojic acid. Neoagarotetraose revealed a similar IC_{50} (50% inhibition concentration) value for mushroom tyrosinase as that by kojic acid. These data suggest that the neoagarotetraose generated from agar by recombinant β-agarase might be a good candidate as a cosmetic additive for the whitening effect.

Keywords: β-Agarase, mushroom tyrosinase, neoagarohexaose, neoagarooligosaccharides, neoagarotetraose, whitening effect

Melanins are the major pigments for human skin, and are produced by melanomacytes. Melanins are generally thought of as negative with regard to skin-lightening, although they clearly serve a valuable purpose in protecting the skin and underlying tissues against UV-induced skin injury. The reduction of melanin biosynthesis has classically been one of the primary goals in an effort to maintain white skin [4, 8]. Tyrosinase inhibitors and UV avoidance may be the least omnipresent methods for the maintenance of white skin, and tyrosinase inhibitors are increasingly utilized in cosmetics [4] and medications [10].

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Arbutin and kojic acid are very effective tyrosinase inhibitors, but there are some evidences that they possessed cytotoxicity. Many natural tyrosinase inhibitors have been screened in previous studies [7–9], but these inhibitors often revealed low activity, high toxicity, insufficient penetration, or undefined clinical efficiency [1]. Hence, it is important to find tyrosinase inhibitors with high activity and low toxicity in cosmetic and pharmaceutical industries for the treatment of hyperpigmentation.

Kobayashi *et al.* [5] demonstrated the whitening effect of neoagarobiose, and we reported that the whitening effect of agar-hydrolyzed neoagarooligosaccharides was more profound than the report by Kobayashi *et al.* [5, 6]. In the previous report, we suggested that neoagarobiose and neoagarotetraose would be the principal whitening agents, because neoagarooligosaccharides exhibited a whitening effect but neoagarohexaose did not [6]. However, we did not determine which compound was important for the whitening effect.

In this study, we purified neoagarotetraose from hydrolyzed agar by gel filtration, tested it for whitening effect, tyrosinase inhibition, and cytotoxicity, and subsequently compared the results with those of other well-known tyrosinase inhibitors.

A recombinant β-agarase from *Agarivorans* sp. JA-1 was expressed and purified as previously described [6]. Enzymatic agar hydrolysis was conducted at 50°C in 665 ml of 50 mM TAPS (pH 8.0) buffer containing 1 mM NaCl, 1 mM $CaCl_2$, and 35 g of agar with 700 U enzyme for 48 h. Neoagarooligosaccharides from agar were purified *via* gel filtration chromatography on a glass column C26/70 (2.6 cm×70 cm; Amersham Pharmacia Biotech Inc., U.S.A.) containing Bio-Gel P-2 gel (Bio-Lad Laboratories, Inc., U.S.A.) with a bed volume of 360 ml, which had been previously equilibrated with water. Neoagarooligosaccharides were eluted at a flow rate of 0.3 ml/min using water. The fractions (300 μl) were collected and identified *via* thin-layer chromatography (TLC). The melanin content,

tyrosinase activity, and cytotoxicity assay in B16F10 melanoma cells were determined by a spectrophotometeric method, as previously described [6]. Mushroom tyrosinase inhibitory activity was determined by the modified dopachrome method, as described by Ishihara *et al.* [3], using L-tyrosine as the substrate. Twenty ml of test sample solutions (final concentrations: 0.1, 1, 10, and 100 μg/ml) was added to 220 μl of 0.1 M phosphate buffer (pH 6.5), with 20 μl (2,000 U/ml) of mushroom tyrosinase (Sigma), in 40 μl of 1.5 mM L-tyrosine. The assay mixture was incubated at 37°C for 30 min. Instead of a sample in 0.1 M phosphate buffer (pH 6.5), 0.1 M phosphate buffer (pH 6.5) was added to a blank solution. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 490 nm.

Fig. 1 represents confirmation of the purified neoagarotetraose by TLC. Hydrolyzed agar (6 g) was purified using gel filtration, and 52 mg of purified neoagarotetraose was obtained. Previously, we showed that neoagarotetraose was the main product of hydrolyzed agar, but we did not confirm the functional activity of neoagarotetraose [6].

Fig. 2 represents that neoagarotetraose reduced the melanin contents in murine melanoma B16F10 cells from 0.1 μg/ml in a dose-dependent manner, as is also the case with well-known whitening agents, arbutin and kojic acid. However neoagarohexaose evidenced a whitening effect only at concentrations of 100 μg/ml. Kobayashi *et al.* [5] reported that neoagarobiose had a whitening effect at 100 μg/ml in melanoma cells, whereas neoagarotetraose reduced the melanin content from 0.1 μg/ml in this study (Fig. 2).

Neoagarotetraose inhibited tyrosinase activity in melanoma cells (Fig. 3A) and mushroom tyrosinase *in vitro* (Fig. 3B) from 0.1 μg/ml in a dose-dependent fashion. Neoagarotetraose showed more effective inhibition of tyrosinase in melanoma cells at 1 μg/ml than arbutin and kojic acid (Fig. 3A). Neoagarotetraose also revealed a stronger inhibitory effect than kojic acid against mushroom tyrosinase at 1 μg/ml (Fig. 3B). The IC_{50} (50% inhibition concentration) values of kojic acid, arbutin, and neoagarotetraose were measured as 9.5, 1.0, and 12.0 μg/ml, respectively, and the value of neoagarotetraose was within the error range of that of kojic acid (Fig. 3B). Neoagarohexaose also showed an inhibitory effect on the tyrosinase activity of melanoma cells and mushroom, although its levels of activity were significantly lower than those of neoagarotetraose (Figs. 2 and 3). The IC_{50} values of neoagarooligosaccharides and neoagarohexaose were 22.5 and 98.0 μg/ml, respectively. These findings indicate that neoagarotetraose is the important component for the whitening activity of neoagarooligosaccharides (Fig. 2).

Many natural and synthetic tyrosinase inhibitors have already been reported in previous studies [5, 7, 11]. Hera *et al.* [2] isolated epigallocatechin gallate, epigallocatechin, and epigallocatechin from tea leaves and reported 95%, 17%, and 11% inhibitions of monophenolase activity, respectively, at 150 μg/ml. Neoagarotetraose induced a 30% inhibition of tyrosinase activity of melanoma cells at 1 μg/ml (Fig. 3A). Furthermore, neoagarotetraose induced a 38% inhibition of mushroom tyrosinase activity at 1 μg/ml (Fig. 3B). Hence, the observed reduction of melanin contents (Fig. 2) might be due principally to the tyrosinase inhibition effect of neoagarotetraose.

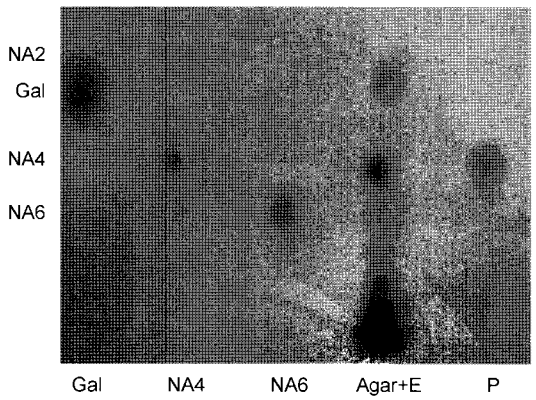

Fig. 1. TLC of the purified neoagarotetraose *via* gel filtration chromatography.
The reactions were conducted at 50°C in 665 ml of 50 mM TAPS (pH 8.0) buffer containing 1 mM NaCl, 1 mM $CaCl_2$, and 35 g agar with 700 U of enzyme for 48 h. The reaction mixture was developed *via* TLC. Gal, D-galactose; NA2, neoagarobiose; NA4, neoagarotetraose; NA6, neoagarohexaose; Agar+E, hydrolyzed product; P, purified neoagaroteraose.

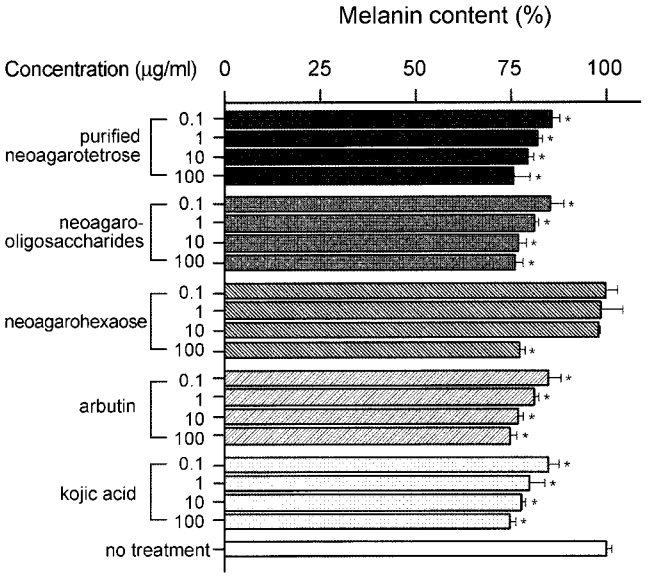

Fig. 2. Whitening effects of the purified neoagarotetraose, neoagarooligosaccharides, neoagarohexaose, arbutin, and kojic acid at various concentrations in B16F10 cells.
Means±SEM for three wells are expressed as the fold-increase as compared with no treatment. Factorial ANOVA with Fisher's PLSD post-hoc test $*p<0.001$ as compared with no treatment. This experiment was repeated at least twice yielding reproducible results.

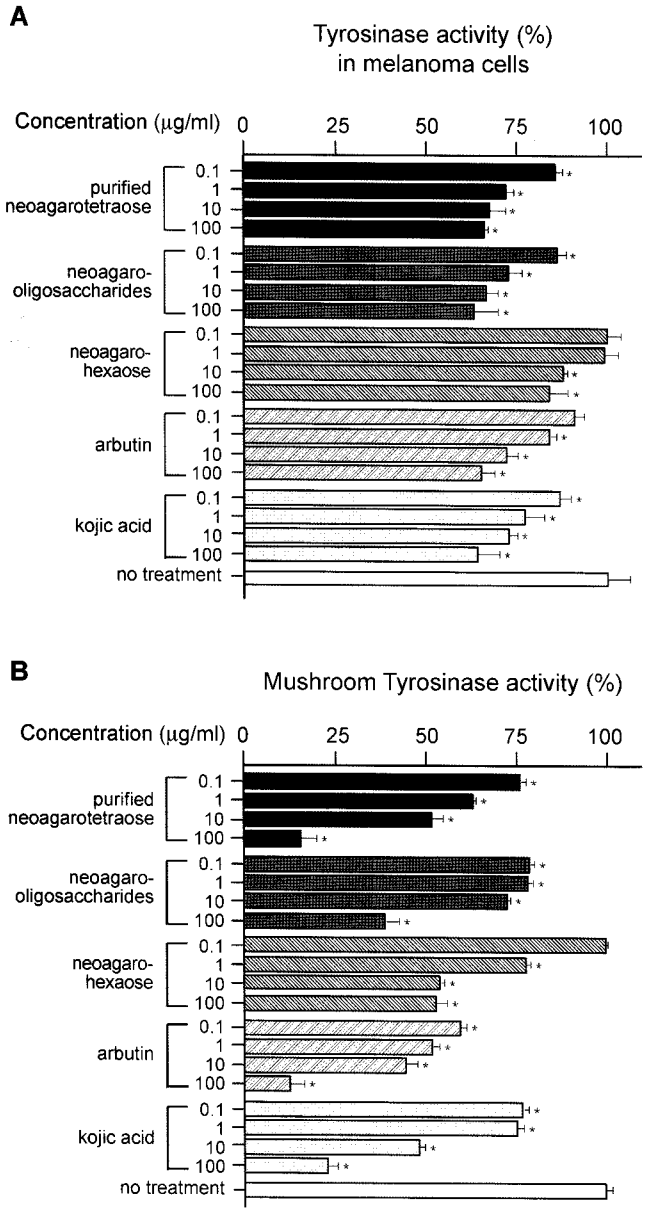

Fig. 3. Tyrosinase activity of B16F10 cells (**A**) and inhibition of mushroom tyrosinase activity (**B**) at various concentrations of the purified neoagarotetraose, neoagarooligosaccharides, neoagarohexaose, arbutin, and kojic acid.

Means±SEM for three wells are expressed as the fold-increase as compared with no treatment. Factorial ANOVA with Fisher's PLSD post-hoc test $*p<0.001$ compared with no treatment. This experiment was repeated at least twice yielding reproducible results.

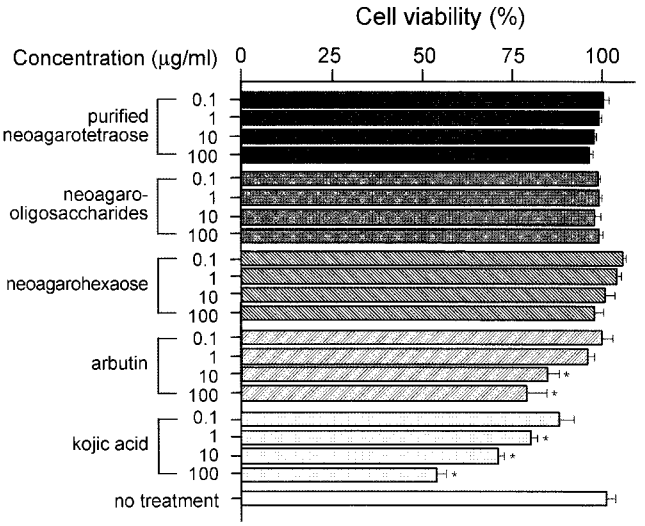

Fig. 4. Cell viability after exposure to the purified neoagarotetraose, neoagarooligosaccharides, neoagarohexaose, arbutin, and kojic acid in B16F10 cells.

Means±SEM for three wells are expressed as the fold-change compared with no treatment. Factorial ANOVA with Fisher's PLSD post-hoc test $*p<0.001$ compared with no treatment. These experiments were repeated at least twice yielding reproducible results.

Because tyrosinase is a crucial enzyme in melanin biosynthesis, tyrosinase inhibitors may prove to be clinically applicable in melanin hyperpigmentation, cosmetics for whitening, and depigmentation after sunburn. A number of tyrosinase inhibitors have been reported, but only a few of these have been utilized as skin-whitening agents, principally as the consequence of various safety concerns [8]. For example, linoleic acid, hinokitol, kojic acid, naturally occurring hydroquinones, catechin, and catechols have all been reported to inhibit enzyme activity, but they also exert deleterious side effects [1, 10]. Fig. 4 represents the cytotoxicity of arbutin and kojic acid as well as the less profound or noncytotoxicity of neoagarooligosaccharides, neoagarohexaose, and neoagarotetraose. We previously reported that neoagarooligosaccharides and neoagarohexaose were not cytotoxic to B16F10 melanoma cells and normal spleen cells at concentrations of up to 100 μg/ml, whereas arbutin and kojic acid evidenced dose-dependent cytotoxicity [6]. Neoagarotetraose revealed no cytotoxicity against B16F10 melanoma cells, as was the case with the neoagarooligosaccharides (Fig. 4).

The inhibitory effect of the purified neoagarotetraose was evaluated by assessing the degree of tyrosinase activity and hydroxylation of L-tyrosine, followed by comparison with neoagarooligosaccharides, neoagarohexaose, arbutin, and kojic acid (Fig. 3). These results suggest that the neoagarotetraose generated from agar by recombinant β-agarase might prove a good candidate for a whitening agent without toxicity (Fig. 4), and might also be utilized in the cosmetic and pharmaceutical industries.

Acknowledgment

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