Improvement of the Antitumor Activity of Black Currant Polysaccharide by an Enzymatic Treatment

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A polysaccharide-rich substance isolated from black currant, named cassis polysaccharide (CAPS), was partially digested with β-galactosidase from Aspergillus oryzae and its immunostimulatory activity was investigated. The in vitro cytokine-inducing effect of CAPS on RAW264 cells was gradually decreased along with lowering of the average MW of CAPS. In vivo, partially digested CAPS with a mean MW of approximately 20,000 showed the most potent antitumor activity against Ehrlich carcinoma in mice.

Key words: black currant; polysaccharide; β-galactosidase; macrophage; antitumor

Black currant (Ribes nigrum L.), originating from Northern Asia and Europe, is a low deciduous shrub with edible fruit purplish-black in color. The fruit of black currant has been used in Chinese folk medicine for over one thousand years,1) and it is well known as a raw material of cassis liqueur, a traditional alcoholic drink in France. We have previously reported that a polysaccharide-rich substance isolated from black currant fruit, designated cassis polysaccharide (CAPS), had powerful immunostimulatory and antitumor activities.2) We have subsequently tried to develop methods to enhance the immunostimulatory (antitumor) activity of CAPS. In order to improve the antitumor activity of polysaccharides, different chemical and physical approaches have been performed.3–6) With regard to practical applications, a mild treatment may be most suitable, especially for food processing. We show in this paper a novel enzymatic digestion method to enhance the immunostimulatory (antitumor) activity of CAPS. In order to improve the antitumor activity of polysaccharides, different chemical and physical approaches have been performed.3–6) With regard to practical applications, a mild treatment may be most suitable, especially for food processing. We show in this paper a novel enzymatic digestion method to enhance the immunostimulatory (antitumor) activity of CAPS.

Enzymatic digestion. The phenol-sulphuric acid method was used to determine the carbohydrate content of CAPS, with glucose used as a standard.8) CAPS was dissolved in a 0.1 M phosphate buffer (pH 5.5) at a polysaccharide concentration of 4 mg/ml, and incubated at 50°C with 10 units/ml of β-galactosidase from Aspergillus oryzae (Sigma-Aldrich). At regular intervals, a portion of the reaction mixture was successively recovered, boiled for 5 min, and centrifuged at 9,000 x g for 10 min. The supernatant, containing partially or completely digested CAPS, was analyzed by gel-filtration chromatography with an HPLC instrument (Shimadzu) equipped with a Shodex OHpak SB-804 HQ column (Showa Denko, Tokyo) that had been equilibrated with PBS (4°C) at a flow rate of 1 ml/min. Detection was done with a refractive index detector (Shimadzu RID-10A). Figure 1 shows the elution profiles of non-digested (0h), partially digested (3 and 8h), and completely digested (35h) CAPS, indicating mean MWs of 230,000, 55,000, 19,000, and 2,400 as each major peak, respectively. It was mainly due to the boiling treatment before the HPLC analysis that the average MW of non-digested CAPS was lower than that which we had previously reported.2) As already known well, β-galactosidase is originally the enzyme involved in the degradation of β-galactosides.
in the hydrolysis of lactose to galactose and glucose. However, as described in our previous paper, \(^2\) CAPS is in the hydrolysis of lactose to galactose and glucose.

MW Change of CAPS during the Enzymatic Digestion.

Fig. 1. Retention time (min)

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>MW of digested CAPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not digested</td>
</tr>
<tr>
<td>3</td>
<td>Digested for 3 h</td>
</tr>
<tr>
<td>8</td>
<td>Digested for 8 h</td>
</tr>
<tr>
<td>10</td>
<td>Digested for 35 h</td>
</tr>
</tbody>
</table>

Aspergillus oryzae galactosidase from Kluyveromyces lactis or Escherichia coli scarcely hydrolyzed CAPS (data not shown). Hence, the enzyme was incubated with pectin or polygalacturonic acid from citrus fruit (Sigma-Aldrich) as a substrate. The enzyme from Aspergillus oryzae was partially investigated. Briefly, the enzyme was incubated with pectin or polygalacturonic acid from citrus fruit (Sigma-Aldrich) as a substrate. The enzyme from Aspergillus oryzae was also found to be able to hydrolyze both substrates, slowly to some extent (data not shown). It is likely that this property of the enzyme can lead to lowering the average MW of CAPS.

Macrophage stimulation. The effect of CAPS on macrophage activation was assessed by measuring the amount of tumor necrosis factor (TNF)-\(\alpha\) produced from RAW264 murine macrophage-like cells. The cells were cultured in an RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 \(\mu\)g/ml of streptomycin at 37°C in air containing 5% CO\(_2\). Each test sample (20 \(\mu\)l/well) was incubated with RAW264 cells (180 \(\mu\)l/well, \(1 \times 10^5\) cells/ml) in a 96-well tissue culture plate. After 16–20 h, the TNF-\(\alpha\) production level was measured by ELISA, using a commercially available kit from R&D Systems (Minneapolis, MN). As shown in Fig. 2, the TNF-\(\alpha\)-inducing activity of CAPS was gradually but not significantly decreased during the progress of enzymatic digestion, that is, along with lowering of the mean MW of CAPS. Although CAPS digestion with hemicellulase has resulted in a significant decrease of macrophage-stimulating activity, \(^3\) \(\beta\)-galactosidase-digested CAPS maintained relatively high activity. In CAPS digestion, \(\beta\)-galactosidase from Aspergillus oryzae may therefore hydrolyze CAPS without appreciably affecting the key active structures probably existing in the CAPS constituent.

Antitumor activity. The antitumor activity of CAPS against Ehrlich carcinoma was examined. A series of enzymatically digested CAPS was dissolved in PBS at a polysaccharide concentration of 8 \(\mu\)g/ml and orally administered (10 ml/kg body wt.) to ICR female mice (5 weeks old) daily for 21 d. Control mice were orally given PBS in a similar manner. Seven days after the first administration, i.e., on day 0, Ehrlich ascites tumor cells (\(2 \times 10^6\)) were subcutaneously inoculated into the inguinal region of the mice. The growth of the tumor cells with time was observed by measuring the size of the carcinoma. On day 14, the tumors were removed and weighed. As can be seen from Fig. 3, in contrast with the in vitro result, the antitumor activity of CAPS had been increased as its digestion proceeded and peaked at its mean MW of 19,000. However, when completely digested (mean MW 2,400), CAPS showed no antitumor action at the administration dosage employed in this study. These results suggest that there may exist an optimum mean MW of CAPS for demonstrating antitumor activity in vivo, at least in mice. The mechanisms of action by CAPS in vivo are now under study.

Commercially available fruit beverages are often made from juice concentrates as raw materials which are clarified with food-processing enzymes such as...
pectinase before their concentration. The clarification step is important for increasing the yield of juice, thus facilitating juice concentration process and preventing the development of haze during the storage of such commodities. However, from the viewpoint of the polysaccharide function, such a clarification step may be undesirable. Exhaustive digestion with food-processing enzymes probably results in a drastic decrease in the polysaccharide function such as an immunostimulatory action. This may also be the case with black currant fruit juice.

Taken together, we have established a novel method for enhancing the in vivo antitumor activity of CAPS by using β-galactosidase from Aspergillus oryzae. This method may be useful not only for black currant, but also for other pectic substance-rich fruits.

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References