Effects of Phytosterols on Anti-complementary Activity

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Several phytosterols, stigmasterol, campesterol and β-sitosterol, were shown to have potent anti-complementary activities. Stigmasterol was the most potent. A marked consumption of C4 was observed to have occurred when serum was incubated with these phytosterols, and β-sitosterol and stigmasterol showed higher C4 consumption than campesterol. After the incubation of serum with these phytosterols in the absence of Ca²⁺ ions, cleavage of C3 in the serum was detected by immunoelectrophoresis. Stigmasterol caused greater C3 cleavage than the other two compounds. Stigmasterol also showed higher consumption of complement than campesterol and β-sitosterol when rabbit erythrocytes were used in the assay system in the absence of Ca²⁺ ions. These results indicate that these phytosterols activate complement via both the alternative and classical pathways.

Keywords—stigmasterol; β-sitosterol; campesterol; anti-complementary activity; complement activation

The complement system plays an important role in host defence, inflammation or allergic reactions, and activation of the complement system occurs via both the classical and alternative pathways.1) The classical pathway is activated by immune complexes containing immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies, acute-phase proteins such as C-reactive protein, and ribonucleic acid (RNA) tumor viruses. The alternative pathway is directly activated by polysaccharides, certain immunoglobulins, viruses, fungi, bacteria, certain animal cells and parasites. Some anti-complementary polysaccharides, for example, lipopolysaccharides,2) β(1→3)glucan,3) 6-branched β(1→3)glucan,3) and inuline4) have already been isolated from bacteria, fungi and plants. Recently, potent anti-complementary activity has also been observed in the extracts of several Chinese herbs,5) and these active principles were characterized as complex arabinogalactans.5b-e,i-1) However, not many low-molecular weight organic substances having anti-complementary activity have been reported. During screening for anti-complementary substances, we have found potent anti-complementary activity of several phytosterols, β-sitosterol, stigmasterol and campesterol, isolated from soybean meal.

In the present paper, we describe the anti-complementary activity of these three phytosterols and their modes of action.

Materials and Methods

Materials—Normal human serum (NHS) was obtained from a healthy adult. β-Sitosterol, stigmasterol and campesterol were purchased from Sigma Co. The chemical structures of these compounds are shown in Fig. 1. The purity of these phytosterols was checked by high performance liquid chromatography (HPLC). Commercial β-sitosterol was a mixture of β-sitosterol and campesterol, and therefore it was purified by HPLC using a YMC-Pak A-
Anti-complementary Activity—Each of the phytosterols was dissolved in isopropanol then diluted with water to a final concentration of 5% isopropanol, and these solutions were used for assay. The anti-complementary activity was measured as described previously. Gelatin–veronal-buffered saline (pH 7.4) containing 500 nM Mg$^{2+}$ and 150 nM Ca$^{2+}$ (GVB$^{2+}$) was prepared as described previously. Various dilutions of sample in 5% isopropanol (50 μl) were incubated with 50 μl of NHS and 50 μl of GVB$^{2+}$. The mixtures were incubated at 37°C for 30 min and the residual total hemolytic complement (TCHSO) was determined by a method using IgM–hemolysin-sensitized sheep erythrocytes (EA) at 1 × 10⁸ cells/ml. NHS was incubated with 5% isopropanol and GVB$^{2+}$ to provide a control. The anti-complementary activity of the phytosterol was expressed as the percentage inhibition with respect to TCHSO of the control.

Determination of Complement Hemolysis through the Alternative Complement Pathway (ACHSO) ACHSO was determined in 10 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetracetic acid (EGTA) containing 2 mM MgCl₂ in GVB$^{2+}$ (Mg$^{2+}$–EGTA–GVB$^{2+}$). A sample was incubated with Mg$^{2+}$–EGTA–GVB$^{2+}$ and NHS at 37°C for 30 min, and the residual complement of the mixtures was measured in terms of the hemolysis of rabbit erythrocytes (5 × 10⁷ cells/ml) incubated with Mg$^{2+}$–EGTA–GVB$^{2+}$.

CROSSED IMMUNOELECTROPHORESIS—NHS was incubated with an equal volume of the solution of the phytosterol with Mg$^{2+}$–EGTA–GVB$^{2+}$ for 30 min at 37°C. The serum was then subjected to crossed immunoelectrophoresis to locate the C3 cleavage products. Shortly after the first run (barbital buffer pH 8.6, ionic strength 0.025, with 1% agarose), the second run was carried out on a gel plate (1.5-mm layer) containing 0.5% rabbit anti-human C3 serum at a potential gradient of 1 mA/cm for 10 h. After the electrophoresis, the plate was fixed and stained with Ponceau 3R.

Determination of C4—Titration of C4 was performed using intermediate cells EAC1gp for C4. EAC1gp cells were prepared from EA (1 × 10⁹ cells/ml) incubated with C1 solution (1 × 10¹² SFU/ml) in the ratio of 28:1 at 4°C for 1 h.

Results

The anti-complementary activities of the phytosterols are shown in Fig. 2. Stigmasterol, campesterol and β-sitosterol all had potent activities, and stigmasterol was the most potent. Under the same conditions, anti-complementary arabinogalactan mixture (1000 μg/ml) showed about 80% of the anti-complementary activity (data not shown). The activation of the classical pathway is initiated by C1 which exists in NHS as a Ca$^{2+}$ dependent complex of three subunits. Activated C1 then activates C4 and C2. Therefore we measured the C4 content in NHS after the incubation with phytosterols to determine their involvement in the activation of the classical pathway. NHS was incubated with stigmasterol, campesterol and β-sitosterol in GVB$^{2+}$ at 37°C for 30 min, and the residual activity of C4 was estimated by hemolytic assay (Fig. 3). The three phytosterols decreased the C4 content of NHS dose dependently. When NHS incubated with 100 μg/ml of stigmasterol or β-sitosterol was used for C4 titration, 90% of the hemolytic titer of C4 was consumed. Campesterol also decreased the C4 content of NHS significantly. These results showed that the classical pathway was activated by these phytosterols. Because Ca$^{2+}$ is required for the activation of complement via the classical pathway but not the alternative pathway, the activation through the alternative pathway was
measured by the use of Ca²⁺ depleted NHS in the presence of EGTA. When these phytosterols were incubated with NHS in Mg²⁺–EGTA–GVB²⁻ at 37 °C for 30 min and a hemolytic assay (ACH₅₀) was carried out using rabbit erythrocytes, stigmasterol, campesterol and β-sitosterol showed dose-dependent anti-complementary activities on ACH₅₀ (ACP activity) (Fig. 4). In the case of stigmasterol, 100% ACP activity was observed when a concentration of 50 μg/ml was used for the assay. The order of ACP activities of these phytosterols was stigmasterol > campesterol > β-sitosterol. The activation of the alternative pathway causes C3 cleavage due to the activation of C3 but does not require the activation of C1, C4 or C2, or the presence of Ca²⁺. Therefore, crossed immunoelectrophoresis was carried out after the incubation of NHS with these phytosterols in Mg²⁺–EGTA–GVB²⁻ to determine whether C3 activation had occurred. When crossed immunoelectrophoresis was carried out after the incubation of NHS with 5% isopropanol as the solvent in Mg²⁺–EGTA–GVB²⁻, a slight cleavage of C3 was apparent, but more significant cleavage of C3 was obtained in the serum treated with these phytosterols (Fig. 5). Potent ACP-active stigmasterol caused the greatest C3 cleavage. These results indicated that the alternative pathway was also activated by these phytosterols. It should be noted that 5% isopropanol showed about 20% ACP activity when water was used as the control.

**Discussion**

The present investigation demonstrated that the phytosterols β-sitosterol, stigmasterol
and campesterol showed potent anti-complementary activities. During this study, Ebihara et al. reported that 3β-hydroxystigmast-5-ene-7-one obtained from the root of Dichroa febrifuga LOUR also has potent anti-complementary activity, as did several other sterols, but they did not report the mode of action of this activity. Phytosterols used in this study were not soluble in water, so several organic solvents (5% in water) were tested for ability to solubilize the phytosterols and for effect on the anti-complementary activity. Isopropanol was shown to have rather lower activity than others such as dimethylsulfoxide, tetrahydrofuran, ethanol and methanol (unpublished results). In the present experiment, stigmasterol was shown to have the most potent anti-complementary activity among the phytosterols tested. However, it is not known whether these activities depend on the specific structure of these phytosterols, because stigmasterol showed the highest solubility in 5% isopropanol, whereas some insoluble precipitates appeared in the case of the other two phytosterols after the addition of water to

Fig. 5. C3 Activation by Phytosterols

NHS was incubated with an equal volume of (1) 5% isopropanol, (2) stigmasterol, (3) campesterol, or (4) β-sitosterol (0.1 mg/ml) with GVB²⁺ (A) or Mg²⁺-EGTA-GVB²⁻ (B) at 37°C for 30 min. The sera were then subjected to crossed immunoelectrophoresis, to locate C3 cleavage products. The anode is to the left.
make 5% isopropanol solution.

We found this activity in soybean meal extract, which contains stigmasterol and campesterol as the major constituents. It is known that these phytosterols are contained widely in many plants, including several Chinese herbs. These phytosterols activated the complement system via not only the classical but also the alternative pathway. The alternative pathway does not require antibodies and is directly activated by bacteria, viruses, fungi, helminth and protozoan parasites, and lymphoblastoid cells. Thus, in general, the alternative pathway constitutes the natural defence mechanism of the non-immune host. Therefore these phytosterols are suggested to have potent non-specific immunopotentiating activities.

It has been reported that some Chinese herbs and their prescriptions (Kampo hozai) have various immunomodulating activities such as interferon-producing activity, anti-complementary activity, mitogenic activity, anti-complementary activity, mitogenic activity, host-mediated anti-tumor activity, reticuloendothelial system activation etc. These activities have been generally observed in the high-molecular weight fractions of Chinese herbal extract, but low-molecular weight substances having these activities have been reported. We have not yet examined whether these phytosterols have other immunomodulating activities, but the present results suggested that these phytosterols may be responsible, at least in part (together with high-molecular-weight substances such as polysaccharide), for the immunomodulating activities of Chinese herbal extract.

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References