Isolation and determination of antidotes for botulinum neurotoxin from black tea extract

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Summary

The botulinum neurotoxin produced by Clostridium botulinum exhibits the strongest neurotoxicity, and causes botulism in mammals. In this study, we have isolated and identified the inactivators from black tea extract. The inhibitory activity against the neuromuscular blocking action of botulinus neurotoxin type A was examined using mouse phrenic nerve diaphram preparation. Searching for new antidotes from black tea, lyophilized aqueous acetone extract was suspended in water, and it was extracted with n-hexane, chloroform, ethyl acetate, n-butanol and water, successively. The antidotic activity was shown in the n-butanol extract, and a two-step reversed phase HPLC was carried out for the purification of the fraction. Six flavonoids were isolated as the major active components, and the structures were deduced from their spectral data as kaempferol-3-O-[glc-(6-1)-rha-(3-1)-glc]; kaempfetrin, kaempferol-3-O-[glc-(6-1)-rha]; nicotiflorin, quercetin glycoside and myricetin-3-O-galactoside, myricetin-3-O-glucoside.

Key word
botulinum neurotoxins, Black tea, antidote, flavonoids

Introduction

Botulism is known to be caused by the strongest neurotoxin, botulinum neurotoxin produced by Clostridium botulinum, with a high fatality rate against mammals. This neurotoxin can be classified serologically into 7 types (botulinum neurotoxin:BoNT/A-G). The functional expression of this neurotoxin undergoes 3 stages: 1) attachment of toxic proteins to the surfaces of presynaptic nerve terminals, 2) transfer into cells, and 3) decomposition of target proteins with endopeptidase, a protein-decomposing domain. Botulism is generally observed after eating processed foods such as hams, vacuum-packed foods, and bottled foods, etc. The current therapy for botulism is to administer antiserum, after suffering, and it is the only way to cure it. On the other hand, heat sterilizations foods are effective to prevent botulism by keeping the bacteria away from its growing and multiplying. However, as for the above mentioned foods, it is difficult to heat them enough for the bacteria sterilization. Although antitoxicants against this neurotoxin have been long awaited,
any effective substance have not yet been found. We attempted finding neutralizing substances against botulinum neurotoxin from the “thearubigin fraction” of black tea extract, since it was reported that there is no antitoxic activity in tannic acid, catechins, and theaflavins of black tea\(^1\). Thearubigin is a general term of brown acid pigments contained in the black tea extract, and some were identified as its constituents. In addition, this thearubigin fraction was reported protect from neurotoxin produced by \textit{Clostridium tetani}, the same genus as \textit{Clostridium botulinum} \(^3\) by blocking neurotoxin from attaching to cell surfaces. A sensory evaluation states that the tannin’s function to astringe proteins induces “awkward taste”, “astringency”, and “bitter taste”, when the thearubigin fraction taken from black tea is used as an additive in prosciutto hams and the like.\(^4\) The objectives of this study are isolation and identification of inhibitors against botulinum neurotoxin type A.

**Materials and Methods**

**Reagent and method**

The botulinum neurotoxin type A was purchased from Wako Pure Chemical Industries, Ltd. We used class ddy bisexual mice at the ages of from 3 to 8 months for this experiment.

The specimen of phrenic neuromuscle was prepared based on the Bluelbring’s method for rats\(^5\). The activity against the neuromuscular blocking action of botulinus neurotoxin type A was examined in mouse phrenic nerve diaphragm preparation. The botulinum neurotoxin type A (15 μg / 15 μl) and the specimens (1.5 μg / 20 μl) were mixed and added into Krebs-Ringer solution. The concentration of toxin was fixed at 1.5 nM.

**Separation and Purification**

Five hundred grams of black tea was extracted twice with 80% aqueous actone at room temperature for 12 hours. The extract were combined and concentrated under reduced pressure and freeze dried to yield 330g of crude extract. This extract was suspended in distilled water and partitioned with n-hexane, chloroform, ethyl acetate, and n-butanol, successively and each fractions were evaporated off for dryness to give five fractions, respectively. Twich of neuromuscle was inhibited following treatment with the n-butanol (60.3 g) layer, after which the n-butanollayer was chromatographed by HPLC using a reversed phase ODS column (Shiseido Capcell Pak, inside diameter: 50 mm, length: 100 mm) and fractionated using a stepped gradient of water / MeOH (100:0, 80:20, 60:40, 40:60, 20:80, 0:100, each 3 L). As a result, strong inhibitory activities were observed in 40% methanol fraction (22 g) and 60% methanol fraction (4.1 g). And these fractions were purified with the reversed phase HPLC column using 30% methanol-1% acetic acid as eluent to obtain 2 compounds from 40% methanol fraction and 4 compounds from 60% methanol fraction, respectively. The structures were determined mainly based on NMR spectra, and the inhibitory activity of each component were measured.
Results

There was no antitoxic activity of the thearubigin fraction against botulinum toxin when it was used solely as an additive from the amplitude of twitch of phrenic neuromuscle. The fractions that showed the highest activity, from the n-butanol solution (thearubigin fraction) were 40 % and 60 % methanol eluates, and they were followed by 20 %, 80 %, 0 %, and 100 % methanol fractions in this order. In addition, out of 6 components separated from the 40 %, 60 % methanol fractions, and these components were showed the high antitoxin activity.

The structures of the active components were determined from NMR spectra (α-400, JEOL Co., Ltd.). As a result, these components were identified as quercetin glycoside, kaempfenol-3-O-[glc-(6-1)-rha-(3-1)-glc]; kaempfetin, kaempferol-3-O-[glc-(6-1)-rha]; nicotiflorin, myricetin-3-O-galactoside and myricetin-3-O-glucoside.

Discussion

With the purpose of isolating and purifying inhibitors against botulinum neurotoxin from the black tea extract (thearubigin fraction), we succeeded in isolating 6 components through HPLC using methanol-water and methanol-acetic acid as eluting solvents from the thearubigin fraction. The eluting order of components was almost the same as the study of Finger et al.\(^6\). The components that showed high antitoxin activity were identified as quercetin glycosides, kaempferol glycosides and myricetin glycolsides by comparison of their spectroscopic data with reported values from the literature. (Fig.1)

*Clostridium botulinum* is known to cause the outbreak of mass food poisoning after the ingestion of “Izushi” and “Karashi-Renkon” (fried lotus root with mustard), which occurred in Japan in the past. Botulism has the highest fatality rate among bacterial food poisoning. It is not an infectious disease, but toxicosis due to the oral ingestion of toxin generated by the bacterium in food. From this study, (the active fractions isolated from) black tea can be expected as additives which will pave the way to the preservation of food poisoning.

![Chemical Structure of Active Components](#)
Reference


