Note

Structure of Antibacterial Substance in Extract of Cocklebur Leaves Used for Preparation of Wheat-Koji (*qu*) Described in the Chinese Classic “*Chi-min-yao-shu*” and Its Effect on Growth of Bacteria and Yeasts

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In our previous publications, we have reported that extract of cocklebur contains an antibacterial substance. This substance was purified using silica gel column chromatography and TLC; it was dissolved in acetone and successively crystallized by adding hexane. The crystal appeared needle-like and white, with a melting point of 60°C and molecular weight of 246. IR, UV and ¹H-NMR spectra revealed that the compound was xanthatin. Minimum inhibitory concentrations (MICs) of xanthatin obtained were 12.5–100 µg/ml against *Bacillus* sp. which usually contaminates koji (*qu*) used in the preparation of alcoholic beverages. MICs of xanthatin were 25–100 µg/ml against *Candida* sp., *Pichia* sp., *Saccharomycopsis* sp. and *Torulaspora* sp.. Therefore, the inhibitory effect exhibited by xanthatin from cocklebur leaf extract against *Bacillus* sp. and some film-forming yeasts would be very useful to prevent contamination in koji and during production of alcoholic beverages.

Keywords: cocklebur, MIC, *Chi-min-yao-shu*, koji (*qu*)

The Chinese literature classic *Chi-Min-Yao-Shu* (Kumashiro & Nishiyama, 1957; Chia, 1961), describes the methods for preparation of nine kinds of wheat-koji (*qu*; Chinese wheat koji) used for the production of alcoholic beverages.

We reported that *Shen-qu* (1), *Shen-qu* (2), *He-dong-shen-qu* and *Ben-qu* were experimentally prepared according to *Chi-Min-Yao-Shu* (Kanauchi *et al.*, 1998a). *Shen-qu* (1) was prepared by kneading roasted wheat, steamed wheat and nonheated wheat with water, while *Shen-qu* (2) was prepared by kneading roasted wheat, steamed wheat and nonheated wheat with extract of cocklebur (*Xanthium strumarium*) leaves. *He-dong-shen-qu* was prepared by kneading roasted wheat, steamed wheat and nonheated wheat with extract of four kinds of leaves (white mulberry, *Gosyuyu*, cocklebur and mugwort leaves), whereas *Ben-qu* was prepared by kneading roasted wheat with water.

We found *Aspergillus oryzae* and *Rhizopus oryzae* to be the dominant microorganisms in all four kinds of koji (*qu*), and learned that the koji had enough enzymatic activities for the production of alcoholic beverages, (Kanauchi *et al.*, 1998a). Moreover, the number of bacteria isolated from *Shen-qu* (2) and *He-dong-shen-qu* was the order of 10⁸, while *Shen-qu* (1) made by kneading wheat with water had the order of 10¹¹ (Kanauchi *et al.*, 1998b). The extract of cocklebur leaves exhibited antibacterial property against some *Bacillus* sp., but not against lactic acid bacteria, fermentative yeasts or molds, which are important components in alcoholic beverage production.

This paper deals with the purification and identification of

the antibacterial substance from the cocklebur leaf extract. The minimum inhibitory concentrations (MICs) of antibacterial substance obtained were measured against *Bacillus* sp. and some strains of film-forming yeasts and fermentative yeasts.

Materials and Method

Preparation of the extract from cocklebur leaves The extract from cocklebur leaves was prepared according to the method described in *Chi-Min-Yao-Shu*: 1 kg of fresh cocklebur leaves (*Xanthium strumarium*) was boiled in 5 l of hot water for about 5 min. The extract was cooled at room temperature and the leaves were removed.

Purification of the antibacterial substance Prior to large-scale extraction, 1 ml of extract from cocklebur leaves was treated with 1 ml each of petroleum ether, ethyl acetate or *n*-butanol. Each fraction was then measured for its antibacterial activity by the paper disk method followed the item. Results obtained showed that petroleum ether effectively extracted the antibacterial substance from the extraction of cocklebur leaves. The procedure for the purification of the antibacterial substance is shown in Fig. 1.

Five liters of petroleum ether was mixed with 5 l of cocklebur leaf extract and agitated for 60 min. The antibacterial fraction was subsequently separated by silica gel column chromatography. One hundred grams of silica gel (C-200, Wako Pure Chemical Industries, Ltd., Osaka) was packed in a glass column (6.9 mm×85 mm) equipped with a filter (Otake *et al.*, 1976). Eluents were 50 ml of a mixture which intermittently varied the ratio of ethanol and petroleum ether. The antibacterial activity was measured by a paper disk

Fig. 1. Fractionation of antibacterial substance from cocklebur.

Fig. 2. Thin layer chromatogram of antibacterial substance from extract of cocklebur leaves. I: *Xanthium strumarium* leaves extracted by hot water, II: Extracted by petroleum ether, III: Fractionated with silica gel chromatography. Detection was made by spraying conc. H_2SO_4 .

method. The antibacterial fraction obtained was further purified by TLC (silica gel 60, 20×20 cm, Merck, Darmstadt, Germany) using a mixture of ethyl acetate, toluene and acetic acid (1: 1: 0.1) as solvent. The detected spot was scraped off

Fig. 3. Chemical structure of the antibacterial substance isolated from the extract of cocklebur leaves.

from the plate and extracted with 50% ethanol in water. The extract was evaporated to remove solvent. The antibacterial substance was dissolved in acetone and was successively crystallized by adding hexane.

Paper disk test The test strain, *Bacillus subtilis* NRIC 1016 was obtained from the Culture Collection Center of Tokyo University of Agriculture and inoculated in the medium containing 3% bouillon (Nissui Seiyaku Co., Ltd., Tokyo) and 1.5% agar. Seventy microliters of sample solution was added to a paper disk, and the disk was placed on the plate medium. After cultivation at 37°C for 24 h, the diameter of growth inhibition zone was measured.

Melting point and molecular weight Melting point of the crystals was measured by MP-S3 (Yanagimoto Co., Ltd., Kyoto) melting point apparatus. The crystals were dissolved in 3-nitrobenzyl alcohol, and the molecular weight was measured by a JJMS-AX505 (JEOL, Ltd., Tokyo) mass-spectrometer.

Table 1. Minimum inhibitory concentrations (MICs) of Xanthatin against the growth of some *Bacillo* sp.

Test strains	MIC ($\mu\text{g/ml}$)
<i>Bacillus cereus</i> NRIC 1003	50
<i>Bacillus coagulans</i> NRIC 1004	100
<i>Bacillus circulans</i> NRIC 1004	100
<i>Bacillus firmus</i> NRIC 1006	100
<i>Bacillus licheniformis</i> NRIC 1007	100
<i>Bacillus pumilus</i> NRIC 1010	100
<i>Bacillus sphaericus</i> NRIC 1013	50
<i>Bacillus subtilis</i> NRIC 1016 (IFO3009)	12.5
<i>Bacillus subtilis</i> NRIC 1521	12.5

Bacillus strains were inoculated in 3% bouillon medium (Nissui Seiyaku Co., Ltd.). The crystal sample was added at the rate of 100, 50, 25, 12.5, 6.25 $\mu\text{g/ml}$ in the medium, in which the tested strains inoculated were incubated at 37°C for 24 h.

UV, IR and $^1\text{H-NMR}$ spectra The UV spectrum was measured in 99.5% ethanol by a UV-1600 (Shimadzu, Kyoto) spectrophotometer and the IR spectrum by an IR-470 (Shimadzu) IR-spectrometer. The $^1\text{H-NMR}$ spectrum was measured in CDCl_3 by a JNM-A400 (JEOL, Ltd.) NMR-spectrometer.

MICs of the antibacterial substance The microorganisms tested were 9 strains of *Bacillus* sp., 9 strains of *Candida* sp., 4 strains of *Pichia* sp., 4 strains of *Saccharomyces* sp., 3 strains of *Zygosaccharomyces* sp. and 6 strains of other genera. *Bacillus* strains were grown in 3% bouillon medium (Nissui Seiyaku Co., Ltd), while the yeasts were cultivated on a GYP medium containing 1% glucose, 0.5% yeast extract, 0.5% polypeptone. The crystal sample was added to the medium at a rate of 100, 50, 25, 12.5, 6.25 $\mu\text{g/ml}$. *Bacillus* sp. were incubated at 37°C for 24 h and some yeasts were incubated at 30°C for 24 h.

Results and Discussion

The antibacterial fraction was obtained on the silica gel column by eluting with 40% ethanol in petroleum ether. Antibacterial activity was measured by a paper disk method, and the diameter of the growth inhibition zone was observed to be 19 mm. Figure 2 shows the results of TLC analysis of the active fraction. Lane I shows the separation of the crude extract of cocklebur leaves, and lanes II and III show those of the petroleum ether extract and the purified extract, respectively. The R_f value of the antibacterial spots was 0.7. This substance was crystallized by adding hexane. The crystals were needle-like, with a melting point of 59–60°C, which was lower than that reported by Ginesta-Peris *et al.* (1994).

Maximum absorption in the UV spectrum of the crystal was at 270 nm, which is characteristic of the lactone structure (Geissman *et al.*, 1954). The peak at 3000 cm^{-1} in the IR spectrum represents C-H bond. It also shows a well-defined absorption at 1700 cm^{-1} , characteristic for a lactone. The sharp peaks at 1670 and 1600 cm^{-1} represent the unsaturated carbonyl system, while that at 1150 cm^{-1} represents methyl group, and the peak at about 960 cm^{-1} corresponds to vinylene. These data agreed with those reported by Geissman *et al.* (1954). The $^1\text{H-NMR}$ spectrum showed the following characteristic signals: 1.20 ppm (3H, d, $J=14.0$ Hz); 2.29 ppm (3H, s); 4.65 ppm (H, 3d, $J=2.7, 9.9, 12.5$ Hz); 5.50 ppm (H, d,

Table 2. Minimum inhibitory concentrations (MICs) of xanthatin against the growth of yeasts.

Test strains	MIC ($\mu\text{g/ml}$)
<i>Candida apicola</i> NRIC 1802	100
<i>Candida diddensiae</i> NRIC 1804	100
<i>Candida halophila</i> NRIC 1786	100
<i>Candida kefyr</i> NRIC 1290	50
<i>Candida milleri</i> NRIC 1796	100
<i>Candida mogii</i> NRIC 1805	25
<i>Candida nodaensis</i> NRIC 1788	50
<i>Candida steatolytica</i> NRIC 1902	100
<i>Candida versatilis</i> NRIC 1790	100
<i>Debaryomyces hansenii</i> NRIC 1690	—
<i>Kluyveromyces drosophilum</i> NRIC 1319	—
<i>Kluyveromyces fragilis</i> NRIC 1324	—
<i>Pichia anomala</i> NRIC 1312	100
<i>Pichia etchellsii</i> NRIC 1795	100
<i>Pichia muscicola</i> NRIC 1314	25
<i>Pichia ohmeri</i> NRIC 1799	—
<i>Saccharomyces cerevisiae</i> NRIC 1560	—
<i>Saccharomyces cerevisiae</i> (Kyokai No. 7)	—
<i>Saccharomyces diastaticus</i> NRIC 1396	—
<i>Saccharomyces fragilis</i> NRIC 1567	—
<i>Saccharomycopsis lipolytica</i> NRIC 1291	—
<i>Schizosaccharomyces malidevorans</i> NRIC 1430	12.5
<i>Torulaspora delbrueckii</i> NRIC 1437	100
<i>Zygosaccharomyces bisporus</i> NRIC 1801	25
<i>Zygosaccharomyces naniwaensis</i> NRIC 1446	100
<i>Zygosaccharomyces rouxii</i> NRIC 1812	—

—; not inhibited by xanthatin.

Yeasts were inoculated in GYP medium consisting of 1% glucose, 0.5% yeast extract, 0.5% polypeptone. The crystal sample was added at the rate of 100, 50, 25, 12.5, 6.25 $\mu\text{g/ml}$ in the medium, in which the test strains inoculated were incubated at 30°C for 24 h.

$J=4.0$ Hz); 6.20 ppm (H, d, $J=1.0$ Hz); 6.31 ppm (H, 2d, $J=3.2$ Hz); 6.96 ppm (H, d, $J=16.0$ Hz). These data agreed with the reported data (Ginesta-Peris *et al.*, 1994). Mass-spectrum showed the main peak was at 247 m/z. Therefore the molecular weight of the compound was determined to be 246. From these results, the compound was figured to be xanthatin (Little *et al.*, 1950; Geissman *et al.*, 1954; Gupta & Gupta, 1975), and the structure is shown in Fig. 3.

Table 1 shows the MICs of the purified xanthatin against *Bacillus* strains: 25 $\mu\text{g/ml}$ against *B. subtilis*, 50 $\mu\text{g/ml}$ against *B. sphaericus* and *B. cereus* and 100 $\mu\text{g/ml}$ against the other *Bacillus* strains. Among the *Bacillus* sp., the most sensitive strain was *B. subtilis*, known to be a common contaminant in koji (Sato & Takahashi, 1960; Sato, 1960). Little *et al.* (1950) reported that xanthatin inhibited the growth of Gram-positive bacteria, which supports our data. Table 2 shows the MICs against the yeasts examined. MICs of xanthatin were 25 $\mu\text{g/ml}$ against *Candida mogii* and 50–100 $\mu\text{g/ml}$ against other *Candida* strains. The compound did not inhibit the growth of *Saccharomyces* sp., however, except for *S. fragilis* (MIC: 12.5 $\mu\text{g/ml}$). MICs of xanthatin were 25–100 $\mu\text{g/ml}$ against *Pichia anomala* (*Hansenula anomala*).

It was reported that xanthatin inhibited the growth of some Gram-positive bacteria and fungi (Little *et al.*, 1950; Ginesta-Peris *et al.*, 1994; Sethi *et al.*, 1971), but no report mentioned that xanthatin was used for inhibition of microbial growth during production of food and alcoholic beverages. Many scholars reported that the leaves of cocklebur or their extract

were added to Chinese koji as a preventive against certain diseases and for nourishment, a so-called herbal medicine (Yamazaki, 1945; Oya, 1990; Otani, 1973). In this work, however, we found that the extract of cocklebur leaves contained an antibacterial substance which inhibited the growth of bacteria and many yeasts which contaminate alcoholic beverages during production.

We therefore suggest that xanthatin from the extract of cocklebur leaves is very useful to prevent from contamination of microorganism spoiled alcoholic beverages on the production of them.

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