Activity of Naringinase in the Extracts from *Citrus unshiu* waste Treated with Brewer's Yeasts and Their Inhibitory Effects on Angiotensin Converting Enzyme

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Summary

Naringinase activity and inhibitory potency against Angiotensin-converting enzyme were tested in the extract from *Citrus unshiu* waste treated with various brewer's yeasts. Naringinase activity in the yeasts tested was much smaller than that in some fungi. It was, however, proved that if an appropriate incubation method is adopted, analysis method is effective for this system.

Inhibitory effect in the extract, on the contrary, was shown being positive in almost all strains tested. These results suggest that this approach will be a promising method to make a component of functional foods.

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Naringin (4', 5, 7-trihydroxyflavone-7-rhamnoglucoside) is known as the main bitter component of several citrus juices. Its hydrolysis by naringinase results in being bitterless, forming naringenin which has less bitterness. Naringinase is included in some fungi and yeasts. It is composed of two enzymes which are α-rhamnosidase, splitting naringin into rhamnose and prunin (4', 5, 7-trihydroxyflavone-7-glucoside) and β-glucosidase, hydrolyzing prunin into glucose and naringenin (4', 5, 7-trihydroxy-flavone)[1,2] Prunin bitterness is evaluated to be less than one-third of that due to naringin. Various methods of debittering of fruit juice have been reported.[3,4] Immobilized naringinase was used as important method.[2,5-10]

Angiotensin Converting Enzyme (ACE) is a dipeptidyl carboxypeptidase which removes a dipeptide from the biologically inactive decapeptide angiotensin I and produces the potent vasopressor octapeptide angiotensin II. Thus, ACE plays a pivotal role in blood pressure regulation.[11-13] Much current attention

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has been focused on angiotensin I converting enzyme inhibitors in terms of pharmaseutical activity.[14-16] Inhibitory activity against ACE is shown in some kind of foods.[17] It has been reported that some kinds of citrus include substances showing hypotensive activity.[18,19] They are flavonoid glucosides and identified on some of them.[19] Moreover, various phenyl propanoid glucosides have also been demonstrated as vasodepressive substances.[20]

In the course of our screening program, we found that citrus waste treated with brewer's yeasts produced an ACE inhibitor which remains to be identified.

Materials and Methods

Materials *Citrus unshiu* waste, strained lees was cut to as small species with a knife as possible. After that it was crushed with a homoginizer (glass). A small amount of distilled water was added to the small species so prepared sample is of high viscosity.

Naringin was obtained from Fulka AG (Switzerland) and naringenin purchased from Nakarai Chemicals Co. Ltd.

Brewer's yeasts used are IFO 0199, 0213, 0216, 0304, 0396, 0565 and 1457, and NVCL (Nakano Vinegar Co. Ltd. stocked strain) 8005, 8007, 8011, 8014, 8015, 8017, 8019, 8020, 8024, 8028, 8035, 8048, 8065, 8074, 8076, 8081, 8084, 8089 and 8094. Among them the strains used for the assay of inhibitory potency to ACE were as follows : IFO 0396, 0565, NVCL 8005, 8011, 8014, 8017, 8018, 8019, 8028, 8048, 8065, 8081, 8089.

Incubation The sample obtained above was used without autoclaving, and was inoculated with a mass of yeast, which was cultured on an agar for a few days, and incubated in the box at 30°C for several days-several weeks. The incubated sample was stirred with a spatula once a day.

Analysis The cultured sample was centrifuged at *ca* 8000×g for 20 min. The several ml of the upper layer was extracted with ethyl ether. After evaporation of solvent, the equal quantity of methanol as the sample withdrawn was added. The sample was analyzed by gas chromatography (Yanaco G80, Yanagimoto Mfg Co. Ltd., Kyoto, Japan): column, Silicone OV 17 (1.5m); detector, FID; column temperature, 300°C. It was also analyzed by a high performance liquid chromatography (JASCO LPC-150, JAPAN SPECTROSCOPIC Co. Ltd.) at room temperature: column, Unisil Pack F3-100B; detector, JASCO UVIDEC-100-V (210nm).

Assay for ACE Inhibitory Activity[21] The activity was determined by the use of extract from rabbit lung acetone powder, in which the crude ACE was prepared by blending 10g of the powder in 100ml of 50mM potassium phosphate buffer, pH 8.3, and centrifuging for 40 min at 40000×g. The clear supernatant

obtained is of high ACE activity and stable at 4°C. Inhibitory activity was determined as percentage of inhibition of assay sample to activity for HHL (hippuryl-L-histidyl-L-leucine). Each 500μl aliquot contains the following components: 250μl of 100mM potassium phosphate buffer, pH 8.3: 50μl of 3M NaCl; 50μl of 50mM HHL; 50μl of distilled water; 50μl of ACE solution; 50μl of sample solution (or 50% methanol for blank). The sample solution for assay was prepared by centrifugation (3000×g, 15min) of the incubation sample in *Incubation.* The assay mixture was incubated for 30min in a shaker at 37°C. The reaction was terminated by addition of 500μl of 1N HCl. After addition of 3ml of ethylacetate, the mixture was shaked vigorously for *ca* 10 seconds, repeatedly, then was centrifuged at 1000rpm for 3min. One ml of the upper layer was dried at 120°C in an oil bath. After dryness, 3ml of distilled water was added and shaked. The solution was measured at 228 nm.

Results

Naringinase Activity Standard samples of naringin and naringenin were easily detected by both gaschromatography and liquid chromatography in above mentioned method. The spectra of both gas and liquid chromatography are shown in Fig 1. Among the yeasts tested, only NVCL 8011 produced a very slight amount of naringin (Fig 2). Bitterness in the samples treated was weakened relatively, suggesting that the degradation could occur by the action of naringinase in yeast. Moreover, an additional peak was detected in Fig 2 (③). This substance may be speculated as an intermediate, glucose-O-naringenin, during the degradation of naringin to naringenin.

For comparison of naringinase activity of yeast with that of fungi, naringin was used as a substrate against fungi (*Aspergillus niger*). The reuslt is shown in Fig 3. In the figure, an additional peak is also detected. This may be speculated as an intermediate, glucose-O-naringenin as well, though remained to be identified.

ACE Inhibitory Activity ACE inhibitory activity of the yeasts is depicted in Table 1. Negative activity was obtained in two cases. This should be due to that uv absorption was blocked by the curdy samples, which will be due to weaker pectinase activity in the yeasts. Each value in the table is shown as relative one to the reference of untreated sample.

Discussion

The present experiments were achieved without stirring during incubation. Gas and liquid chromatographies, however, proved to be effective for detecting naringinase activity from the viewpoint of quantitative analysis of

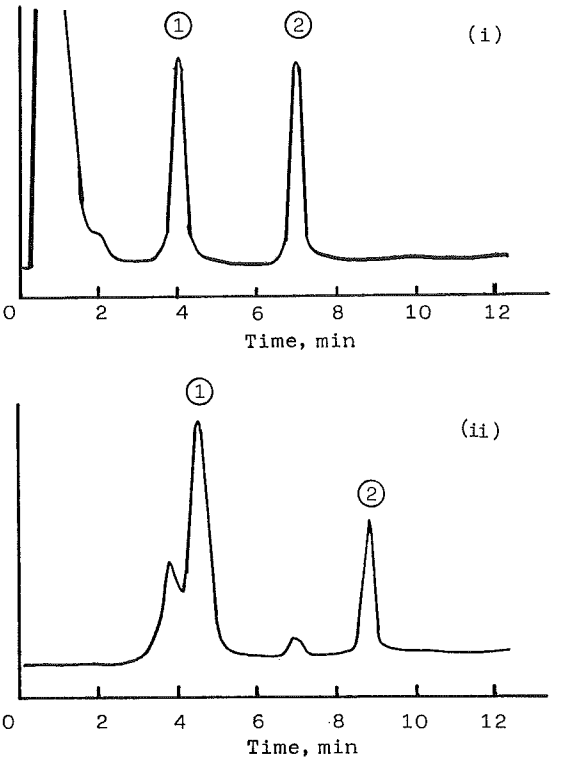

Fig. 1 GLC ((i)) and LC ((ii)) spectra of standard naringin and naringenin
(i) ① : Naringenin, ② : Naringin
Carrier, N_2 gas; flow rate, 50ml/min; H_2 pressure, 0.6kg/cm^2
(ii) ① : Naringin, ② : Naringenin
Eluent, 60% (v/v) mehtanol (0.15% perchloric acid); flow rate, 0.5ml/min; room temperature

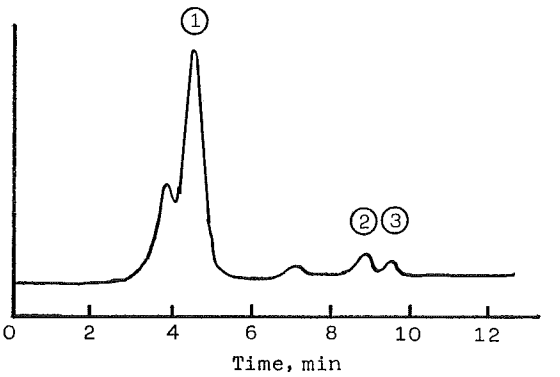

Fig. 2 LC spectrum of yeast-treated sample
① : Naringin, ② : Naringenin, ③ : Unidentified
Conditions is the same as that noted in Fig 1, (ii).

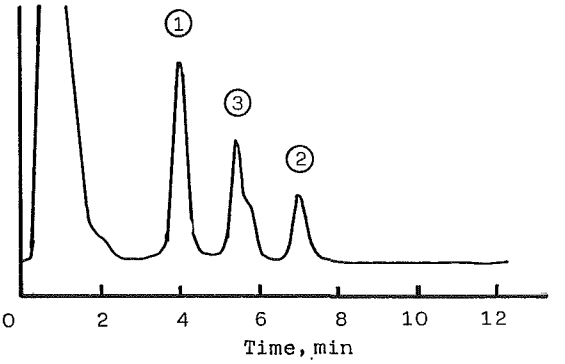

Fig. 3 GLC spectrum of yeast-treated sample
① : Naringenin, ② : Naringin, ③ : Unidentified
Conditions is the same as that noted in Fig. 1, (i).

Table 1. ACE inhibitory test

strain		activity(%)	strain	activity(%)
IFO	0396	39.59	8019	58.90
	0565	−42.47	8028	72.84
NVCL	8005	26.48	8048	−33.33
	8011	75.72	8065	54.31
	8014	19.18	8081	24.92
	8017	32.88	8089	66.77
	8018	22.04		

Each value is relative one to the untreated sample. Value of 70.44% was obtained as an absolute one of the untreated sample to distilled water.

naringin and naringenin. Thus, among the brewer's yeasts, NVCL 8011 had relative naringinase activity.

Interestingly, concerning the ACE inhibitory activity, when citrus waste was treated with brewer's yeasts the substance having potent ACE inhibitory property was produced. Isolation and structure elucidation of the substance will be published in separated paper.

Acknowledgement

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醸造酵母で処理した温州ミカン粕抽出物中のナリンギナーゼ活性とアンジオテンシン変換酵素阻害活性について

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温州ミカンの絞り粕をホモジナイズして，醸造酵母で処理した際のナリンギン由来の苦みの除去（ナリンギナーゼ活性）と，処理抽出液中に含まれるアンジオテンシン変換酵素に対する阻害効果について調べた。ガスクロマトグラフや液体クロマトグラフで定量可能なナリンギナーゼ活性は，供用した酵母中一株のみにしか見られなかったが，ナリンギナーゼ活性の比較的強い株の存在も予知され，用いた定量法が有効であることを確認した。一方，アンジオテンシン変換酵素阻害活性の方は，テストしたほとんどの酵母においてみられ，機能性食品開発の一方法として期待のもてる方法であることが認められた。