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Inhibitory Effects of Brown Algae Extracts on Histamine Production in Mackerel Muscle *via* Inhibition of Growth and Histidine Decarboxylase Activity of *Morganella morganii*

Dong Hyun Kim[1], Koth Bong Woo Ri Kim[2], Ji Young Cho[3], and Dong Hyun Ahn[4]*

[1]*Hydro colloid Div., MSC Co., Ltd, Yangsan, Kyeongnam 626-280, Republic of Korea*
[2]*Institute of Fisheries Sciences, Pukyong National University, Busan 619-911, Republic of Korea*
[3]*Department of Marine Biotechnology, Soonchunhyang University, Asan 336-900, Republic of Korea*
[4]*Department of Food Science & Technology/Institute of Food Science, Pukyong National University, Busan 608-737, Republic of Korea*

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**Corresponding author*
Phone: +82-51-629-5831;
Fax: +82-51-629-5824;
E-mail: dhahn@pknu.ac.kr

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This study was performed to investigate the inhibitory effects of brown algae extracts on histamine production in mackerel muscle. First, antimicrobial activities of brown algae extracts against *Morganella morganii* were investigated using a disk diffusion method. An ethanol extract of *Ecklonia cava* (ECEE) exhibited strong antimicrobial activity. The minimum inhibitory concentration (MIC) of ECEE was 2 mg/ml. Furthermore, the brown algae extracts were examined for their ability to inhibit crude histidine decarboxylase (HDC) of *M. morganii*. The ethanol extract of *Eisenia bicyclis* (EBEE) and ECEE exhibited significant inhibitory activities (19.82% and 33.79%, respectively) at a concentration of 1 mg/ml. To obtain the phlorotannin dieckol, ECEE and EBEE were subjected to liquid-liquid extraction, silica gel column chromatography, and HPLC. Dieckol exhibited substantial inhibitory activity with an IC_{50} value of 0.61 mg/ml, and exhibited competitive inhibition. These extracts were also tested on mackerel muscle. The viable cell counts and histamine production in mackerel muscle inoculated with *M. morganii* treated with ≥2.5 MIC of ECEE (weight basis) were highly inhibited compared with the untreated sample. Furthermore, treatment of crude HDC-inoculated mackerel muscle with 0.5% ECEE and 0.5% EBEE (weight basis), which exhibited excellent inhibitory activities against crude HDC, reduced the overall histamine production by 46.29% and 56.89%, respectively, compared with the untreated sample. Thus, these inhibitory effects of ECEE and EBEE should be helpful in enhancing the safety of mackerel by suppressing histamine production in this fish species.

Keywords: Brown algae, histamine, histidine decarboxylase, mackerel muscle, *Morganella morganii*

Introduction

Free L-histidine is present at high levels in mackerel muscle. Histamine is formed by decarboxylation of free histidine *via* endogenous or bacterial histidine decarboxylase (HDC) activity [28]. Endogenous production of HDC in fish muscle is insignificant when compared with the bacterial enzyme [24]. The ingestion of spoiled fish containing unusually high levels of histamine produced by bacterial HDC is more likely to cause toxic effects than the ingestion of the same amount of pure histamine by mouth [17]. Histamine poisoning is a type of food poisoning with symptoms that include flushing, sweating, nausea, vomiting, diarrhea, headache, palpitations, dizziness, rash, and occasionally swelling of the face and tongue [22]. In most cases, histamine levels in the implicated fish have been over 200 ppm and often greater than 500 ppm. However, a guidance level of 50 ppm has been set, because the

detection of 50 ppm histamine in one section of a fish or lot suggests the possibility that histamine levels in other sections may exceed 500 ppm [3].

Histamine-producing bacteria have been isolated not only from fish and other seafood products but also from various types of fermented products, such as cheese, fermented sausage, and wine [7]. Histamine in fermented foods is mainly produced by gram-positive lactic acid bacteria, whereas the histamine formed in fish products is primarily produced by gram-negative enteric bacteria [7].

Among all the histamine-producing bacteria in fish, *Morganella morganii* is one of the most prolific histamine formers. Mackerel muscle inoculated with *M. morganii* exhibited a higher histamine content, 461 ppm, after 24 h of incubation at 25°C than the muscles in other fish species, such as albacore (343 ppm), mahi-mahi (334 ppm), and salmon (<50 ppm) [11].

Histamine-producing bacteria naturally exist on the gills, external surfaces, and in the gut of live, saltwater fish, with no harm to the fish. However, upon death, defense mechanisms of the fish no longer inhibit intestinal bacteria, particularly *M. morganii*, which make their way through the intestinal walls and into the flesh of the intestinal cavity. Once the HDC is present in the fish, it can continue to produce histamine in fish even if the bacteria are not active. Furthermore, the HDC remains stable in the frozen state and may be very rapidly reactivated after thawing, and can remain active at near-refrigeration temperatures [10]. It is known that death of mackerels is a frequent occurrence during harvesting, before the mackerels are pulled to the surface of the water, which facilitates microbial contamination, resulting in the production of high levels of histamine in mackerel.

However, despite these problems, efforts to inhibit histamine production in fish have been limited, and only a few studies by Shakila *et al*. [25] and Wendakoon and Sakaguchi [32, 33] on the inhibitory effects of spices on amine production have been reported. Furthermore, even in Korea, despite the large amounts of mackerel consumed as food, no information is available on the effects of natural materials mostly used to control histamine formation in mackerel products either *in vitro* or *in situ*. Only Mah *et al*. [20] reported the inhibitory effects of garlic and spices on biogenic amine production by gram-positive bacteria in the Korean salted and fermented anchovy product *myeolchi-jeot*. Marine algae are abundant sources of various structurally different bioactive secondary metabolites, because they have wide thermal, pressure, and nutrient ranges, including both photic and aphotic zones [2, 6].

Among marine algae, brown algae (Phaeophyta) are known to contain bioactive compounds, including polysaccharides [35], polyphloroglucinol phenolic compounds, nonpolar compounds such as phlorotannins [18], nonpolyphenolic compounds such as terpenes [27], carotenoids such as fucoxanthin, volatile halogenated organic compounds [1], and oxylipins [12]. Therefore, the aim of this study was to investigate the inhibitory effects of brown algae on the strong histamine-former *M. morganii*, and crude HDC derived from *M. morganii*, to inhibit histamine production in mackerel muscle and prevent histamine fish poisoning.

Materials and Methods

Reagents and Media

Mueller-Hinton (MH) broth, Trypticase Soy broth (TSB), and Plate Count Agar (PCA) were purchased from Difco (Detroit, MI, USA). L-Histidine monohydrochloride monohydrate, pyridoxal-5-phosphate, polyethylene glycol No. 300, and potassium phosphate monobasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium phosphate dibasic and sodium ethlenediaminetetraacetic acid (EDTA) were purchased from Yakuri (Kyoto, Japan). 1,4-Dithiothreitol was purchased from Roche Applied Science (Indianapolis, IN, USA). All solvents for liquid-liquid extraction, silica gel column chromatography, and HPLC were purchased from Junsei (Tokyo, Japan).

Samples

Six species of brown algae were used in this study. *Ecklonia cava* and *Eisenia bicyclis* were collected from the east coast of Korea, and *Sargassum sagamianum*, *Sargassum thunbergii*, *Myagropsis myagroides*, and *Sargassum horneri* were collected from the coasts of Busan in Korea. Salt, epiphytes, and sand were removed using tap water. The brown algae were dried at room temperature and then ground using a crusher (DA 282; Daesung, Seoul, Korea). The powder was stored at −70°C until use.

Test Microorganism

For this study, *Morganella morganii* IFO 3848, ATCC 25830 [21], was purchased from the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea) and used for the determination of antimicrobial activities and in the crude HDC inhibitory activity test with brown algae extracts. The stock culture was maintained on TSA slants at 4°C.

Preparation of Brown Algae Extracts

Dried and milled algae were extracted with 94% ethanol or water at a ratio of 1:10 (w/v) for 24 h at room temperature. After extraction, the samples were centrifuged (UNION 32R; Hanil Co., Incheon, Korea) for 10 min at 3,000 rpm and the supernatants collected. The residues were re-extracted twice using the same method. The collected supernatants were filtered and evaporated

using a rotary evaporator (RE 200; Yamato Co., Tokyo, Japan). The concentrate was stored at −20°C until use.

Determination of Antimicrobial Activity

The antimicrobial activities of the brown algae extracts were evaluated using the disk diffusion method. Bacteria (10^6 CFU/ml) were inoculated onto the surface of pre-dried MH agar. Sterile 6 mm filter paper disks were placed on the plates and impregnated with 20 µl of algal extracts. The extracts were allowed to diffuse across the disk surface for 1 h at room temperature, and the plates were incubated at 37°C for 24 h. Antimicrobial activity was measured as the width of the clear zone of growth inhibition. Next, the antimicrobial sample with the strongest activity was evaluated using the minimum inhibitory concentration (MIC) test. Sterilized MH agar that had not hardened yet was mixed with various concentrations of the algal extract in sterile test tubes. Then, the test tubes were inoculated with 10^6 CFU/ml *M. morganii.* These mixtures were poured into a plate and then dried. MIC was defined as the lowest concentration of the algal extract that inhibited the growth of *M. morganii* in the plate after incubation.

Preparation of Crude HDC from *M. morganii*

M. morganii HDC was purified as described previously [30]. For enzyme production, the bacterium was inoculated into TSB at a concentration of 10^6 CFU/ml and incubated at 25°C for 24 h without agitation. Cells were harvested from 1 L of culture by centrifugation at 12,000 ×*g* for 30 min at 4°C and washed with enzyme buffer (0.1 M potassium phosphate (pH 6.5), 0.1 mM sodium EDTA, 0.01 mM pyridoxal-5′-phosphate, 0.02 mM dithiothreitol, 1% (v/v) polyethylene glycol No. 300). Approximately 1.5 g of wet cells was obtained. Next, 1 g of the collected washed cells was suspended in 4 ml of the same buffer, cooled in an ice bath, and then broken by 20 sec pulses from an ultrasonic disruptor for a total of 20 min (VCX-130; Sonics & Materials Inc., CT, USA). The suspension was centrifuged at 12,000 ×*g* for 30 min at 4°C; the precipitate was suspended in 3 ml of the same buffer and sonicated again in the same manner. The two supernatant solutions were combined and used as the source of the enzyme for the enzyme assay.

Determination of Crude HDC Inhibitory Activity

The crude HDC inhibitory activity assay was performed according to the method of Kanki *et al*. [10], with slight modifications. A 1 ml volume of the enzyme buffer was preincubated with 100 µl of brown algae extracts at 37°C for 5 min. Then, 100 µl of crude HDC was added and the mixture was preincubated for an additional 5 min. The reaction was initiated by adding 200 µl of a 200 mM solution of L-histidine monohydrochloride as a substrate. After 15 min, the reaction was terminated by heating the mixture in boiling water for 5 min. Quantitative analysis of histamine was performed with a Histamine Test kit (Kikkoman Co., Tokyo, Japan) using a UV/visible spectrophotometer (GENESYS10 UV; Rochester, NY, USA) at 470 nm. The inhibitory activity was calculated according to the formula

$$I\ (\%) = (1-[A-B]/C) \times 100$$

where A is the absorbance value with inhibitor and enzyme, B is the absorbance value with inhibitor and without enzyme as negative control, and C is the absorbance value with enzyme and without inhibitor; the IC_{50} value, the concentration of the extract that produced 50% inhibition of maximal activity, was determined.

Isolation, Purification, and Identification of Dieckol

To isolate the anti-crude HDC inhibitory substance from an ethanol extract of *Ecklonia cava* (ECEE) and an ethanol extract of *Eisenia bicyclis* (EBEE), ECEE and EBEE were resuspended in distilled water at a ratio of 1:10 (w/v) and partitioned with an equal volume of ethyl acetate (EA) at 180 rpm for 1 h. The EA fractions from ECEE and EBEE, which exhibited high crude HDC inhibitory activities, were loaded onto a silica gel column (230–400 mesh; Merck Co., Darmstadt, Germany) and subjected to step-wise elution with chloroform-methanol and ethyl acetate-methanol, respectively (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10; 200 ml of each). Fractions of 100 ml were collected and evaporated to dryness. The chloroform-methanol eluate (8:2, EA6 subfraction) from ECEE and ethyl acetate-methanol eluate (10:1, EA2 subfraction) from EBEE were dissolved in methanol and filtered using a 0.45 µm disposable syringe filter (Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan). After filtration, HPLC was performed on a Gilson instrument using a µBondapak C18 reverse phase column (7.8 × 300 mm, 10 µm) (Gilson-France, Viliers-Le-Bel, France; Gilson, Middletown, WI, USA). The flow rate was 1.3 ml/min, and the mobile phase in the HPLC analysis consisted of 36% acetonitrile to isolate dieckol from ECEE. To isolate dieckol from EBEE, the program was set to a linear gradient of 28%–50% acetonitrile in 10 min. The samples were monitored at 254 nm (Fig. 1). The 6 and 5 peaks separated from ECEE and EBEE by HPLC, respectively, were analyzed on LC/ESI-MS (Nanospace SI-2; Shiseido, Tokyo, Japan) with a C18 column (Luna 5 micron-C18, 150 × 1 mm; Phenomenex, Torrance, Canada) at a flow rate of 50 µl/min, and data were obtained from a LCQ Deca XP mass spectrometer (Thermo Finnigan, San Jose, CA, USA). For 1-D NMR, ^1H and ^{13}C spectra were obtained in methanol-d (CD_3OD) using JNM-ECP 400 (500 MHz; JEOL Co., Tokyo, Japan) and Avance II 900 (900 MHz; Bruker, Karlsruhe, Germany) spectrometers, respectively.

Determination of the Type of Crude HDC Inhibition by Dieckol

Different concentrations of dieckol (0, 0.1, and 0.2 mM) were added to each reaction mixture and the enzyme activity was measured at different concentrations of the substrate (5, 8, and 10 mM). The type of crude HDC inhibition in the presence of

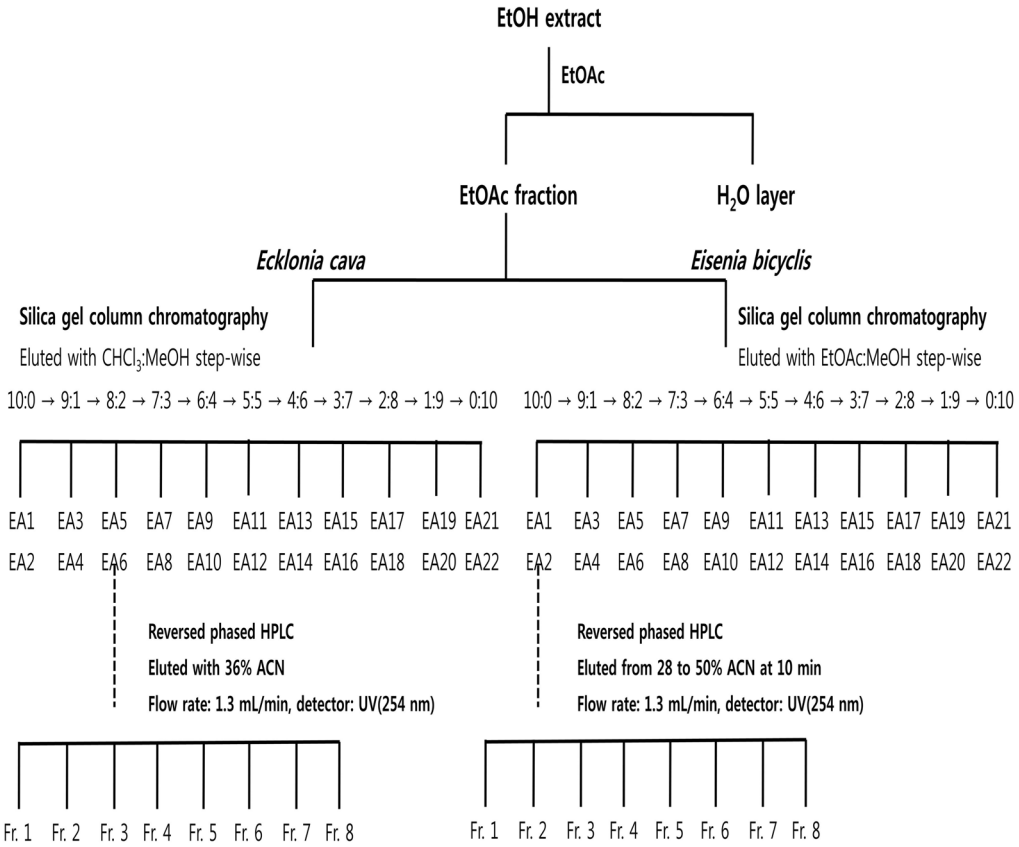

Fig. 1. Flowchart for the extraction and fractionation of *Ecklonia cava* and *Eisenia bicyclis*.

dieckol was determined using Lineweaver-Burk (LB) plots. LB plots of 1/[S] against 1/v, where [S] is the substrate concentration and v is the enzyme activity, in the absence and presence of the different inhibitors were constructed.

Preparation of Mackerel Muscle

Live mackerel were purchased from a local wholesale fish distributor in Busan, Korea. The live fish were transported in seawater to Pukyong National University-Food Resource Development Laboratory, Busan, Korea, and were allowed to rest for over 30 min. The skin, gill, and intestines of the mackerel were aseptically removed under a clean bench (DW-CB-511; Dongwon Science Co., Busan, Korea). The mackerel muscle was rinsed with sterile distilled water. The muscle was then placed in a sterile blender and ground to a mince at 1,000 rpm for 30 sec in an icebox. To determine the histamine inhibitory activity of the antimicrobial substances (with activity against *M. morganii*) in mackerel, the bacterial culture were inoculated into ground muscle at 10^4 CFU/g. Additionally, for histamine inhibition by HDC inhibitory substances, 0.5 ml of crude HDC from *M. morganii* was added to the ground muscle. Then, various concentrations of brown algae extracts (dissolved in 70% fermented alcohol) were added to each sample. Eight grams of each sample was aseptically dispensed into sterile petri dishes and stored at 12°C. For the analyses, samples were taken at 0, 24, 72, and 120 h of storage.

Viable Cell Count Measurements in Mackerel

Two-gram portions of mackerel muscle were aseptically removed from the petri dishes and blended with sterile phosphate-buffered saline (pH 7.4) at a ratio of 1:10 (w/v), followed by serial dilution in the same solution. One milliliter of each diluted sample was dispensed into plates and mixed with PCA. The number of colony was counted after the plates were incubated at 37°C for 24–48 h.

Determination of Histamine Contents in Mackerel Muscle

One-gram portions of mackerel muscle were added to 24 ml of 0.1 M EDTA buffer (pH 8.0) and mixed by shaking for 1 min, and then boiled for 20 min. After cooling, the mixture was centrifuged at 3,000 rpm for 10 min at 4°C, and a 1 ml portion was used for histamine detection using the Histamine Test kit.

Statistical Analysis

Data were analyzed for significance by ANOVA using SAS software (SAS Institute Inc., Cary, NC, USA). The means were

compared using the Duncan's multiple range test to determine significant differences between samples. $P < 0.05$ was considered significant.

Results and Discussion

Antimicrobial Activity of Brown Algae Extracts Against *M. morganii*

To examine the antibacterial activities of brown algae against *M. morganii*, ethanol extracts of three brown algae species, *Ecklonia cava*, *Sargassum sagamianum*, and *Sargassum thunbergii*, were selected based on our previous study on antimicrobial activity of brown algae extracts against microorganisms related to food contamination and food poisoning disease [16]. The antimicrobial activities of the ethanol extracts of brown algae are summarized in Table 1. Only the ethanolic extract of *Ecklonia cava* (ECEE) showed a significant growth inhibitory effect at 10% concentration. Furthermore, to determine the lowest inhibitory concentration of the antimicrobial substance, the MIC test was performed (Table 1). The MIC value of ECEE against *M. morganii* was 2 mg/ml. Until recently, information on the inhibition of histamine production in fish by *M. morganii* using the antimicrobial effects of natural materials was limited, except for the study on the antimicrobial activities of spices against *Enterobacter aerogenes*, which is one of the dominant histamine formers.

Ecklonia cava is known to contain many types of phlorotannins, which are polyphenolic secondary metabolites and consist of polymers of phloroglucinol (1,3,5-trihydroxybenzene) units, such as eckol, dieckol, fucodiphloroethol G, 6,6-bieckol, 7-phloroeckol, and phlorofucofuroeckol [23,34]. Wendakoon and Sakaguchi [32] reported that phenolic compounds of the essential oil from clove ethanol extract may affect the cell membrane of *Enterobacter aerogenes*, resulting in growth inhibition. Specifically, strong interactions between algal phlorotannins and proteins have been reported by Stern *et al*. [29]. Therefore, the bactericidal activity of *Ecklonia cava* against *M. morganii* may interact with bacterial membrane proteins and intracellular enzymes that catalyze the synthesis of the outer membrane of gram-negative bacteria.

Table 1. Antimicrobial activities of ethanol extracts of various brown algae against *Morganella morganii*.

Samples	Clear zone	MIC (mg/ml)
Ecklonia cava	++[1)]	2.0
Sargassum sagamianum	-	=[2)]
Sargassum thunbergii	-	=

[1)]Growth inhibition size of clearzone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.

[2)]=: not done.

*Concentration: 10% concentrate.

Inhibitory Effect of Brown Algae Extracts on Crude HDC from *M. morganii*

The inhibitory activities of five brown algae species, *Ecklonia cava, Eisenia bicyclis, Sargassum thunbergii, Sargassum horneri*, and *Myagropsis myagroides*, from our previous study on screening of enzyme inhibitors from seaweeds (data not shown), against crude HDC prepared from *M. morganii* were examined (Table 2). Except for *Sargassum horneri*, the ethanol extracts of brown algae showed stronger inhibitory activities than those of water extracts or no significant differences. Among the ethanol extracts, ECEE exhibited the highest inhibitory activity (33.79%) at a concentration of 1 mg/ml, followed by the extracts of *Eisenia bicyclis* and *Sargassum thunbergii* (19.82% and 12.91%, respectively). However, the ethanol extracts of *Sargassum horneri* and *Myagropsis myagroides* did not show any inhibitory activity (<5%). These results are consistent with a previous study showing that the ethanol extracts of several spices had stronger inhibitory activities against crude HDC from *Enterobacter aerogenes* than water extracts [33]. Furthermore, Shakila *et al*. [25] reported that the crude HDC activity of *M. morganii* was reduced by ethanol extracts of spices.

Among the phlorotannins, dieckol is known to inhibit various enzymes, such as matrix metalloproteinase [8], acetylcholinesterase [23], α-glucosidase, α-amylase [13, 14], tyrosinase [9], and angiotensin I-converting enzyme [34]. Therefore, dieckol was selected for further investigation to determine the constituent responsible for the crude HDC inhibitory activity. The extracts were suspended in water

Table 2. Inhibitory activities of brown algae extracts against crude histidine decarboxylase of *M. morganii*.

Samples	Inhibitory activity (%)	
	Ethanol	Water
Ecklonia cava	$33.79 \pm 1.72^{Aa1)}$	13.93 ± 0.19^{Ba}
Eisenia bicyclis	19.82 ± 2.56^{Ab}	5.96 ± 0.95^{Bbc}
Sargassum thunbergii	12.91 ± 3.50^{Ac}	8.22 ± 2.73^{Ab}
Sargassum horneri	0.56 ± 0.79^{Bd}	5.77 ± 2.50^{Abc}
Myagropsis myagroides	0.20 ± 0.17^{Ad}	1.93 ± 1.78^{Ac}

[1)]Means in the same row ($^{A-B}$) and same column ($^{a-d}$) bearing different superscript in samples are significantly different by Duncan's multiple range test ($p < 0.05$).

*Concentration: 1 mg/ml.

with EA to isolate dieckol from *Ecklonia cava* and *Eisenia bicyclis*, because phlorotannins are the predominant EA-soluble compounds in brown algae, according to a previous study on the biological activities of phlorotannins [19]. After partitioning the extracts, the EA-soluble fractions of *Ecklonia cava* (ECEA) and *Eisenia bicyclis* (EBEA) showed higher inhibitory activities (45.29% and 39.84%, respectively) than the ethanol extracts. Furthermore, the subfractions ECEA6 and EBEA2 separated by silica gel column chromatography from ECEA and EBEA, respectively, also showed stronger inhibitory activities (52.58% and 42.44%, respectively) at a concentration of 1 mg/ml (Table 3). The eight fractions were obtained from EBEA2 using HPLC, and a fraction 5 (dieckol) showed the highest HDC inhibitory activity (data not shown). Thus, the HDC inhibitory activities of dieckol from *E. cava* and *E. bicyclis* were measured. Finally, dieckol was isolated from ECEA6 and EBEA2 using HPLC, and was identified by comparing the NMR and MS data with existing literature [13]. The ^{1}H NMR spectrum of dieckol showed signals at 6.14 (1H, s), 6.12 (1H, s), 6.07 (2H, s), 6.06 (1H, d), 6.03 (1H, d), 5.97 (1H, d), 5.94 (1H, d), and 5.91 (3H, m). The ^{13}C NMR duplicated signals at 162.03, 160.30, 157.96, 156.16, 154.69, 152.55, 147.50, 147.45, 147.28, 147.06, 144.46, 144.30, 143.56, 143.46, 138.80, 138.62, 126.61, 126.34, 125.81, 125.75, 125.02, 124.79, 124.73, 100.01, 99.89, 99.64, 99.52, 97.81, 96.36, 95.99, 95.91, and 95.50. The molecular composition of dieckol was

Table 3. Inhibitory activities of *Ecklonia cava* and *Eisenia bicyclis* extracts against crude histidine decarboxylase of *M. morganii*.

	Inhibitory activity (%)	
	Ecklonia cava	*Eisenia bicyclis*
Ethanol extract	33.79 ± 1.72[d2)]	19.82 ± 2.56[c]
Ethyl acetate fraction	45.29 ± 1.22[c]	39.84 ± 1.06[b]
Ethyl acetate subfraction[1)]	52.58 ± 0.46[b]	42.44 ± 0.56[b]
HPLC fraction	60.87 ± 1.89[a, NS3)]	68.24 ± 1.62[a]

[1)]The 2 and 6 subfractions from the ethyl acetate fraction of *E. cava* and *E. bicyclis* by silica gel column chromatography, respectively.

[2)]Means in the same row ([A–B]) and same column ([a–d]) bearing different superscript in samples are significantly different by Duncan's multiple range test ($p < 0.05$).

[3)]NS: Not significantly different between two samples.

*Concentration: 1 mg/ml.

$C_{36}H_{22}O_{18}$ based on LC/ESI-MS (negative mode, $[M-H]^+$ at *m/z* 741.07) (Fig. 2). The HPLC fraction containing dieckol from *Ecklonia cava* and *Eisenia bicyclis* showed the highest inhibitory activities (60.87% and 68.24%, respectively) at a concentration of 1 mg/ml. The inhibitory activities of dieckol from the two sources were not significantly different ($p < 0.05$), and dieckol exhibited an IC_{50} value of 0.8 mM (Table 4). The mode of inhibition by dieckol exhibited competitive inhibition against the crude HDC of *M. morganii* (Fig. 3). A competitive inhibitor binds to the free

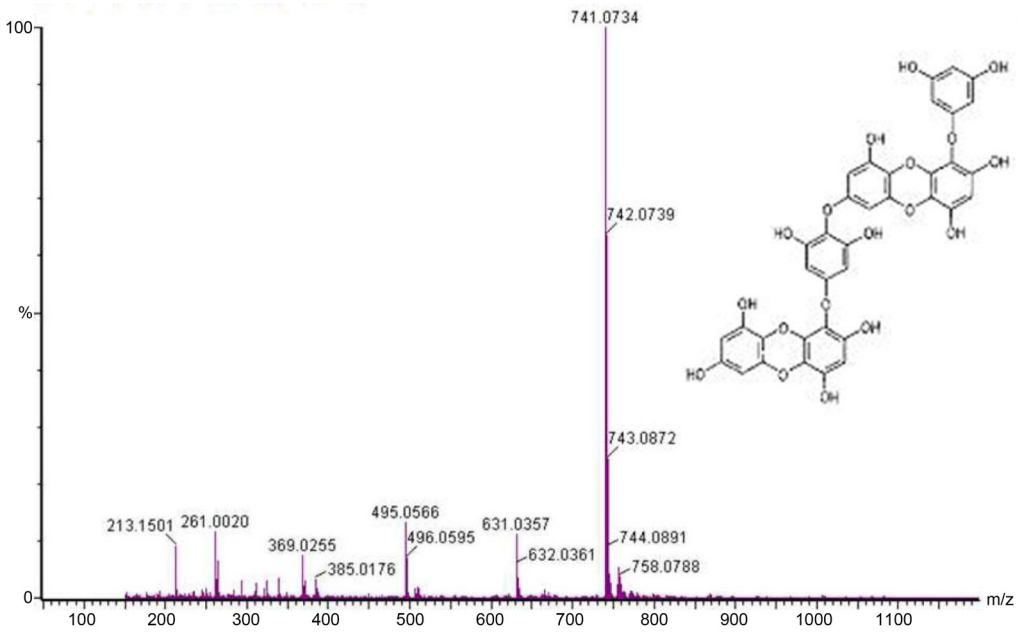

Fig. 2. Negative LC/ESI-MS and structure of dieckol from *Eisenia bicyclis*.

Table 4. Inhibitory activity of dieckol against crude HDC from *M. morganii*.

	Inhibitory activity (%)		IC_{50} (mM)
	1 mg/ml	0.5 mg/ml	
Dieckol	64.93 ± 4.09	40.81 ± 1.51	0.8 ± 0.02

enzyme to prevent substrate binding at the active site. That is, the inhibitor and the substrate are mutually exclusive, often because of true competition for the same site. This result is consistent with the findings of Shibata *et al.* [26], who reported that dieckol is a competitive inhibitor of α-fucosidase from the viscera of turban shell. Additionally, Shibata *et al.* [26] found that the molecular size of phlorotannins is important for strong interaction with enzyme molecules, and that pentamers or hexamers of phloroglucinols act as better inhibitors. In the present study, dieckol is a hexamer. Therefore, it is likely that dieckol competitively binds to the active site of HDC, inhibiting enzyme activity.

Inhibitory Effects of ECEE on Bacterial Growth and Histamine Production in Mackerel Muscle Inoculated with *M. morganii*

Mackerel muscle was inoculated with *M. morganii* (10^4 CFU/g) treated with ECEE at 2.5, 5, and 10 MIC (weight basis), and the bacterial growth and histamine production during the storage of mackerel muscle at 12°C ± 1°C for 120 h were measured.

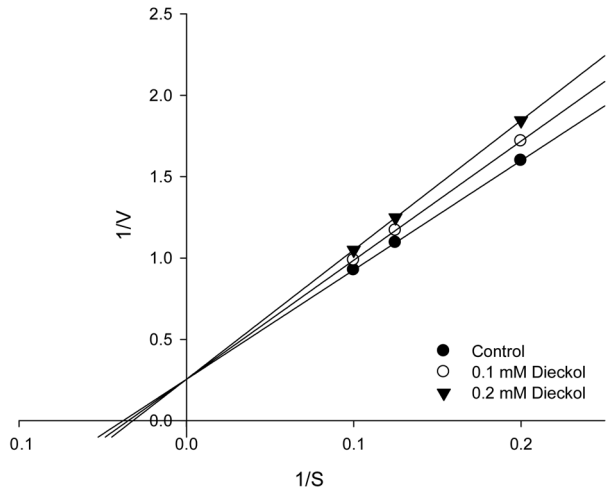

Fig. 3. Lineweaver-Burk plots of crude HDC activity from *Morganella morganii* in the presence of dieckol at various concentrations.

The viable cell counts of *M. morganii* are shown in Fig. 4. During the storage of all the samples, bacterial colonization was homogeneous; thus, the viable cells in the agar plates are the inoculum, *M. morganii.* Initially, the viable cell counts of all the samples were 10^4 CFU/g, regardless of the presence of ECEE. Then, the viable cell counts of the untreated sample continuously increased from 10^6 CFU/g at 72 h to 10^8 CFU/g at 120 h. However, growth of *M. morganii* was highly inhibited by ECEE at concentrations >2.5 MIC during storage. That is, in the 2.5 MIC-treated sample, the viable cell counts decreased to 10^3 CFU/g after 24 h, and remained constant up to 72 h, and increased to

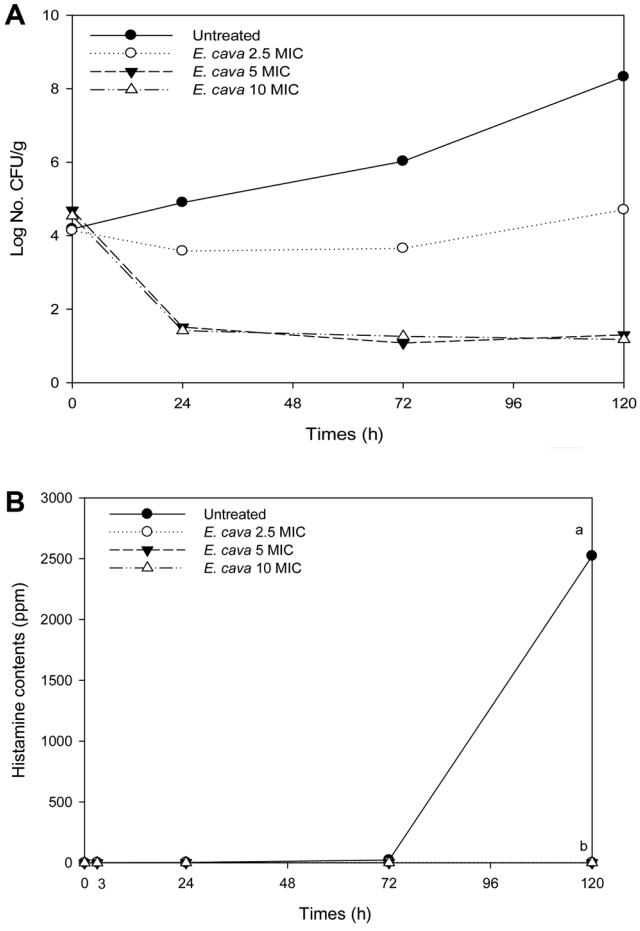

Fig. 4. Changes in viable cell counts (**A**) and histamine content (**B**) in mackerel muscle inoculated with *M. morganii* treated with the ethanol extract of *Ecklonia cava* at 12 ± 1°C for 0–120 h.

The MIC of ECEE against *M. morganii* was 2 mg/ml. The final concentrations of ECEE in mackerel were 2.5–10 MIC. Values with different alphabets are significantly different ($p < 0.05$, Duncan's multiple range test).

about 10^4 CFU/g thereafter. Specifically, in the >10 MIC-treated samples, the viable cell counts decreased to 10^1 CFU/g after 24 h, and remained constant up to 120 h.

Histamine content in the untreated sample reached 22.43 ppm at 72 h, and rapidly accumulated, increasing to 2,523.08 ppm at 120 h of storage (Fig. 4). However, histamine production was highly inhibited in the ECEE-treated samples, and histamine levels did not exceed 3.20 ppm during overall storage. In the study on the patterns of amine production during the growth of the histamine formers *M. morganii*, *Klebsiella planticola*, *Enterobacter aerogenes*, and *Hafnia alvei*, Wendakoon and Sakaguchi [31] reported that the production of histamine begins after a lag phase, and maximum production occurs during the stationary phase. In the presence of a high level of histidine, the HDC of the bacteria was induced because bacterial HDCs are known to be adaptive enzymes, which are produced only when required or whose formation is dependent upon adaptation of the organism to a specific substrate [4, 5]. Wendakoon and Sakaguchi [31] suggested that enzyme synthesis was induced in the early phase of the growth of *M. morganii*. Furthermore, we found that whereas histamine was gradually produced in the untreated sample, the rate of histamine production in the untreated sample increased during the logarithmic phase (120 h) in this study.

According to a previous study on the combined effect of NaCl and clove on the growth and biogenic amine formation of *Enterobacter aerogenes* in mackerel muscle extract [32], although the addition of 0.5% clove to mackerel muscle extract delayed the growth of *Enterobacter aerogenes* and the amine formation because of the antibacterial activity of the phenolic group in the essential oils, amine formation continued as the bacterial growth gradually increased during incubation at 30°C for 30 h. However, the presence of 2% NaCl in combination with 0.5% clove completely inhibited the growth, resulting in no amine production. Lee *et al*. [15] reported that a subfraction of *Myagropsis myagroides*, separated by silica gel column chromatography, had strong antimicrobial activity against *Staphylococcus aureus*, and in the time-kill curve test, regrowth of *Staphylococcus aureus* was observed at 1 MIC and 2 MIC. However, the addition of >4 MIC resulted in mortality within 72 h. Thus, the reason why histamine production was inhibited in the ECEE-treated samples is that the dieckol in ECEE affects bacterial proteins, and HDC synthesis is inhibited in the early phase of the growth of *M. morganii*.

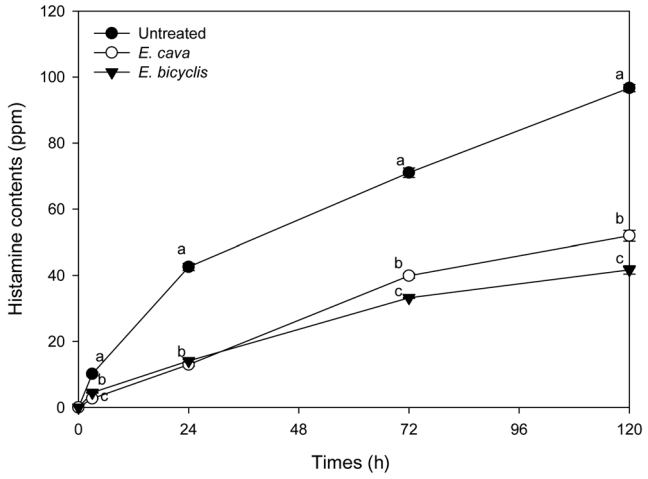

Fig. 5. Changes in histamine content in mackerel muscle inoculated with crude HDC from *M. morganii* treated with ethanol extracts of *Ecklonia cava* and *Eisenia bicyclis* at 12 ± 1°C for 0–120 h.

The final concentrations of the two samples in mackerel were 5 mg/ml. Values with different alphabets are significantly different ($p < 0.05$, Duncan's multiple range test).

Inhibitory Effect of ECEE and EBEE on Histamine Production in Mackerel Muscle Inoculated with Crude HDC from *M. morganii*

To determine the inhibitory effect of the HDC inhibitory substances on histamine production *in situ*, crude HDC from *M. morganii* was added to mackerel muscle, followed by ECEE and EBEE, which possess superior crude HDC inhibitory activities, at a concentration of 0.5% each (weight basis), and histamine production was analyzed during storage of mackerel muscle at 12°C ±1°C for 120 h.

Fig. 5 shows the quantitative changes in histamine production in mackerel muscle. Histamine levels in all the samples increased steadily during storage. However, compared with the untreated sample, histamine formation was considerably delayed in ECEE- and EBEE-treated mackerel muscle during storage. In particular, after 120 h, the histamine content of the EBEE-treated sample was 41.67 ppm, showing the highest reduction of 56.89% in histamine content, compared with the untreated sample with 96.67 ppm ($p < 0.05$). The histamine content of the ECEE-treated sample was 51.92 ppm, which corresponded to a significant reduction of 46.29%, compared with the untreated sample. These results are consistent with the above observation that ECEE and EBEE effectively inhibited histamine formation by crude HDC from *M. morganii*. However, unlike the observations made by *in vitro* studies on the inhibitory activity against crude HDC from

M. morganii, EBEE showed a slightly higher inhibitory effect on histamine production than ECEE *in situ*. The biological activities of natural materials can be affected by certain food components and metabolism when applied to the subjects of an experiment, resulting in reduced biological effects *in situ*, compared with that of *in vitro* studies. For example, Lee *et al*. [15] reported that the antibacterial activity of *Myagropsis myagroides* diminished in the presence of high levels of fat and/or protein in foodstuffs. Furthermore, Shakila *et al*. [25] reported that among the several commercially available spices, clove and cinnamon showed a strong inhibitory activity against crude HDC from *M. morganii in vitro*, and histamine production in mackerel decreased *in situ*.

Because histamine is produced not by the addition of histamine-forming bacteria, but by the addition of HDC to sterile mackerel muscle, bacterial HDC is an independent factor in histamine formation, and, as a result, in histamine fish-poisoning outbreaks. As mentioned above, mackerel death and decomposition are frequent occurrences. Not only is raw mackerel generally consumed, but processed foods such as canned and salted products are also widely used to extend the shelf life of mackerel in Korea. However, this process requires butchering or filleting, which results in direct contact between HDC-positive bacteria and mackerel muscle. If the mackerel is contaminated by HDC-positive microorganisms, particularly before the process, HDCs produce histamine in fish, which is heat-stable and, therefore, does not disappear.

We hypothesize that the action of the phlorotannin dieckol in ECEE and EBEE prevents the activation of HDC originated from *M. morganii* and can be used to inhibit histamine production. Furthermore, ECEE and EBEE may prevent histamine poisoning when applied to processed mackerel products.

In conclusion, the ECEE and EBEE inhibited the growth of *M. morganii* and the HDC activity originated from *M. morganii*, and the dieckol isolated from ECEE and EBEE was the highest HDC inhibitory activity. When ECEE and EBEE were subjected to mackerel muscle inoculated with *M. morganii* and HDC, the histamine production of mackerel muscle was significantly inhibited by both extracts. Therefore, these results suggest that ECEE and EBEE can be used to inhibit the histamine production of mackerel.

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