A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Anticoagulative and Antihypertensive Activities of *Ecklonia cava*

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2004. 06.

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

2004. 6.

This thesis has been examined and approved.

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2004. 6.
Date

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CONTENTS

국문초록

5종의 당분해효소 (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo)와 단백질분해효소 (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase)를 이용해 가수분해된 7 종의 갈조류 (감태, 패, 모자반, 괭생이모자반, 큰잎모자반, 지충이, 고리매) 가수분해물에 대한 잠재적 항응고 활성과 항고혈압 활성을 평가하였다. 7종의 갈조류 중 AMG (an exol, 4-alpha-D-glucose)로 가수분해된 감태의 샘플에서 항응고 활성이 가장 우수한 것으로 나타났고, Flavourzyme으로 가수분해된 감태의 샘플에서 우수한 항고혈압 활성이 나타났다. AMG로 가수분해된 샘플(>30kD)은 APTT (activated partial thromboplastin time; >1500 sec)와 TT (thrombin time; >2000 sec)에서 유의적인 활성을 나타내었지만, PT (prothrombin time; 15 sec)에서는 유의적 활성을 나타내지 않았다. 항고혈압 활성에서 Captopril의 IC_{50}값이 0.046 μg/ml로 나타낸 것에 비해 Flavourzyme으로 가수분해된 감태의 샘플에서는 0.44 μg/ml (IC_{50})로 나타났다. 분자량별로 분획한 실험의 결과는 두 활성(항응고, 항고혈압)의 주성분들이 30 kDa이상의 분획에 집중되어있다는 것을 설명해 준다. 이러한 결과는 감태의 crude sample이 의약산업에 좋은 원료가 된다는 것을 증명해 주는 것이다. 앞으로 감태의 AMG와 Flavourzyme의 >30 kDa 분획으로부터 활성물질의 분리를 수행해야할 것이다.

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INTRODUCTION

Heparin and Captopril are drugs of the choice in prevention and treatment of cardiovascular disorders. Heparin and its low molecular weight derivatives have been using over 60 years for the prevention of thromboembolic disorders, can not be administrated orally and is therefore difficult to use for extended therapy (Daniel *et al.,* 1999). Captopril has been using as an anthypetensive drug over long time period, but has been known to make several side effects including cough, taste differences and skin rashes (Atkinson and Robertson, 1979).

Due to these limitations of forementioned compounds, the need of developing new anticoagulant and antihypertensive agent is continuous to captive in the field of pharmaceutical industry. Beside industrial applications, polysaccharides and protein compounds of algal origin have emerged as an important source of pharmaceutical industry. This may be due to their special ability to work as natural pharmacological compounds. Specially marine algal polysaccharides have potential ability to prolong blood coagulation time, this is due to their hemi-ester helphate groups in their sugar residues (Pereira *et al*., 1999). Therefore, anticoagulant properties of marine algae have been extensively studied for more than 60 years. This includes the compounds such as fucoidin, fucoidan, ascophyllan, sargassan and glucuronoxylofucan. They compose the family of polydisperse heteromolecules based on L-fucose, D-xylose, D-glucuronic acid, D-mannose and D-galactose. Recently it has been reported that highly purified fucan from *Fucus visiculosis* and *Eisenia bicyclies* showed relatively high activity compared to that of Heparin (Pereira *et al*., 2002). Therefore, it is clear that seaweeds are good alternative source for anticoagulative drug production. Knowledge of potential heparinoids from marine algae is gaining importance in recent years. Recently there was a case study on the changes of the haemorrhage, plasma cholesterol and albumin and clinical effects in 36 children with reftractory nephrosis after treatment with fucans. The result of that study suggest that fucan might be used in the anticoagulant treatment of refractory nephrosis (Shanmugam and Mody, 2000).

Therefore, algal anticoagulants, in future, may add a new dimension in vascular disorders. In addition, to the above special ability, it is well known fact that dietary ingestion of seaweeds have been shown to decrease blood pressure in humans (Suetsuna and Nakano, 2000). Also, in spontaniously hypertensive rats, oral dosage of peptides which were isolated from algae caused significant sustained reduction in blood pressure after 24 hours (Suetsuna, 1998). Hence, scientists have been focused their special attention in marine algae in order to evaluate natural anti-hypertensive compounds. Even if seaweed contain low amount of proteins, the amino acid heterogeneity is high within that proteins. Therefore, it is believed that evaluation of algal species is fruitful way to innovate new anti-hypertensive compounds.

In this study, we introduce five carbohydrases and five proteases to hydrolyze algal species. No research investigations so far which deal with producing enzymatic extracts and investigation their relevant bio-active compounds. Enzymatic exaction make efficient extraction of bio-active compounds from brown algae. Therefore, the objective of this study is to evaluate enzymatic extracts of algae in anticoagulative and antihypertensive assays. Then, try to expand enzymatic extracts for commercial purposes since they are less toxic and highly soluble in water than organic extracts.

MATERIALS AND METHODS

Materials

Marine brown alga, *E. cava*, used in this study was collected from close shores of Jeju Island in Korea during March and October 2004. Salt, sand and epiphytes were removed using tap water. Finally, seaweed samples were rinsed carefully with fresh water and freeze-dried at -20℃ for further experimentation. APPT (ellagic + bovine pospolipid) and $CaCl_2$ solution was obtained from International Reagents Corporation (Japan), PT (rabbit thromboplastion) and TT reagents were purchased from Fisher Scientific Company (USA). Carbohydrases such as Viscozyme L (a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase), Celluclast 1.5 L FG (catalyzing the breakdown of cellulose in to glucose, cellobiose and higher glucose polymers), AMG 300L (an exo1, 4-alpha-d-glucosidase), Termamyl 120L (a heat stable alpha amylase), Ultraflo L (a heat stable multi-active beta-glucanase) and Proteases such as Protamax (hydrolysis of food proteins), Kojizyme 500MG (boosting of the soya sauce fermentation), Neutrase 0.8L (an endoprotease), Flavourzyme 500MG (containing both endopeptidase and exopeptidase activities), Alcalase 2.4L FG (an endoprotease) were obtained from Navo Co. Novozyme Nordisk, Bagsvaed, Denmark). Hippuryl-L-histydyl-L-leucine (HHL) and angiotensin-1 converting enzyme (ACE) were obtained from Sigma Chemicals Co. All other chemicals used in this study were 90 % or grater purity.

Digestion of brown algae

The enzymatic extract preparation was followed the method of Heo *et al.,* (2003) with slight modifications. Dried algae sample was ground (MFC SI mill, Janke and Kunkel Ika-Wreck, Staufen, Germany) and sieved through a 50 standard testing sieve. A hundred gram of alga sample was homogenized with water (2 L), and then 1 g or 1 ml enzyme was mixed. The enzymatic hydrolytic reactions were performed for 12 h to achieve optimum degree of the hydrolysis. Before digestion, homoginate pH was adjusted to relevant pH value to optimize digestion process, thereafter digests were boiled for 10 min at 100℃ to inactive the enzyme. Each sample was purified by centrifugation (3000 rpm, for 20 min at 4℃) to remove residue and stored supernatant in -60℃ for further experiments.

Preparation of plasma sample

Normal pooled plasma was made from 10 individual healthy donors, without history of bleeding or thrombosis. Nine parts of blood collected by venipuncture were drawn into one part of 3.8 % sodium citrate. Blood was centrifuged for 20 min at 2400 rpm, and then plasma was stored at -60℃ until use.

Activated partial thromboplastin time (APTT)

Citrated normal human plasma (90 ㎕) was mixed with a solution of algal extract (10 ㎕) and incubated for 1 min at 37℃, then APTT reagent (100 ㎕) was added to the mixture and incubated for 5 min at 37℃. Thereafter clotting was induced by adding 0.025 mol/l $CaCl_2$ (100 ㎕) and APTT time was recorded by coagulation machine (dual -channel 1 clot-2, Seac, Italy).

Prothrombin time (PT)

Citrated normal human plasma (90 ㎕) was mixed with a solution of algal extract (10 ㎕) and incubated for 10 min. Then 200 ㎕ pre-incubated (10 min at 37℃) prothrombine time reagent was added and clotting time was recorded.

Thrombin time (TT)

In thrombin time assay, citrated normal human plasma (190 ㎕) was mixed with a solution of algal extract (10 ㎕) and incubated for 2 min. Then pre-incubated TT reagent (10 min, at 37℃) was added (100 ㎕) into the mixture and clotting time was recorded. All algal extracts including heparin were dissolved in water.

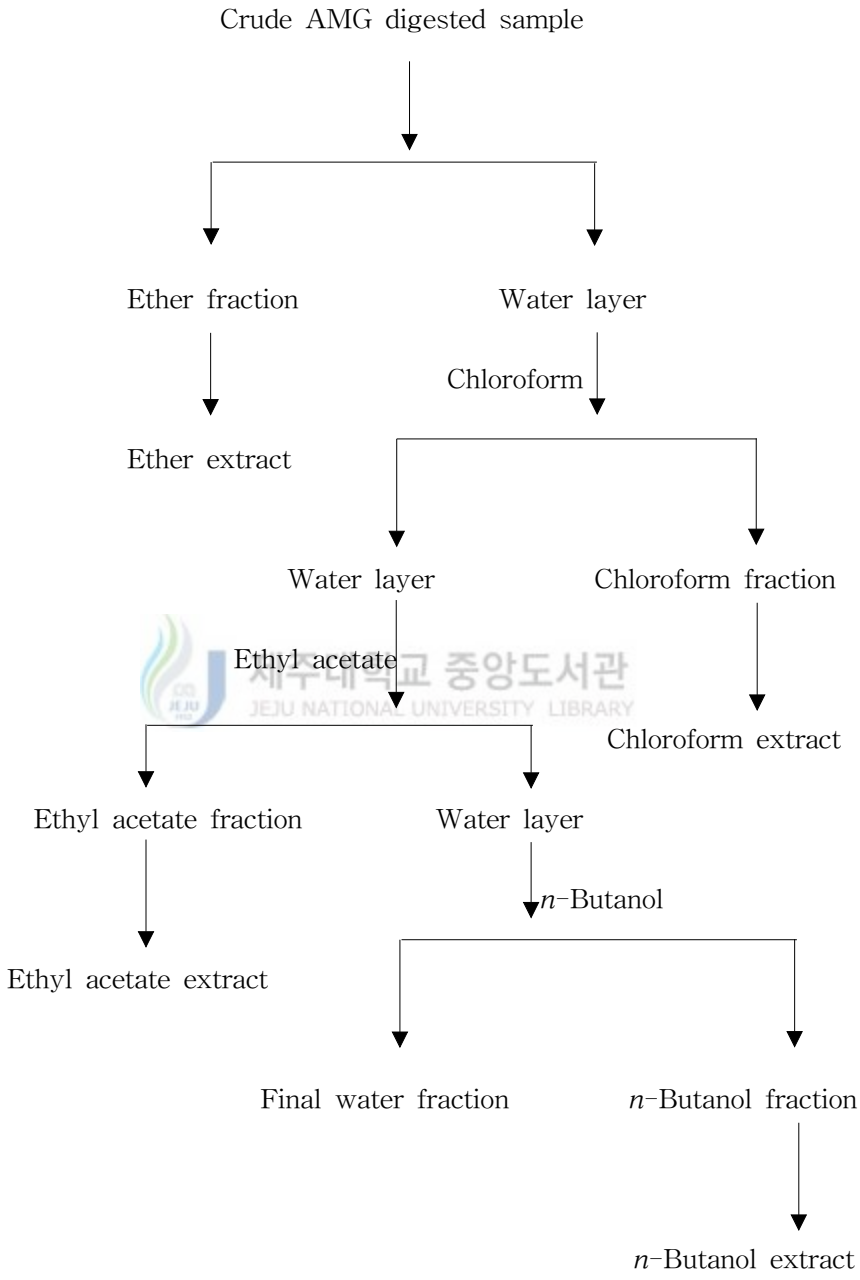

Fig. 1. Organic solvent fractionation of AMG extract of *E. cava*

Molecular weight fractionation

The molecular weight fractionation of this experiment was conducted by molecular weight cut off machine (Millipore Labscale TFF system) with 5, 10 and 30 kD sieves.

Fig. 2. The molecular weight fractionater.
(Millipore Labscale TFF system)

Separation of *E. cava* polysaccharides

The AMG digest of *E. cava* (200 ml) was mixed with 95 % ethanol (400 ml) and kept for 30 min at room temperature. Then precipitated polysaccharides were collected by centrifugation at 10,000 rpm for 20 min at 4℃. The resulted crude polysaccharides were then freeze dried and subjected for further experiments.

Determination of protein and carbohydrate

Crude carbohydrate was determined by phenol-sulfuric acid reaction, using glucose as the calibration standard (AOAC, 1990). The amount of crude protein was determined by Lowry method, absorbance at 540 nm using bovine serum albumin as the calibration standard (Lowry *et al.*, 1951)

ACE inhibitory assay

ACE inhibitory activity was assayed by a method of Cushman and Cheung (1970) with slight modifications. HHL was dissolved in 100 mM sodium borate buffer, pH 8.3, containing 300 mM NaCl. A 200 ㎕ of a 5 mM HHL solution was mixed with 80 ㎕ of a Captopril/sample solution, and then incubated for 3 min at 37℃. The reaction was initiated by adding of 20 ㎕ of an ACE solution in distilled water (100 mU/ml), and the mixture was incubated for 30 min at 37℃. The reaction was stopped by addition of 250 ㎕ of 1 M HCl. The hippuric acid liberated by the ACE reaction was extracted with 1.7 ml ethyl acetate, and the solvent was evaporated in an oven at 120℃. The content was dissolved by addition of 1ml distilled water and its UV spectra density was measured at 228 nm.

The extent of inhibition was calculated as follows:

$$\text{Inhibition } \% = \frac{Ac - (As-Ab)}{Ac}$$

Ac = Absorbance of the control sample at 280nm
As = Absorbance of the inhibiter contain sample at 280nm
Ab = Absorbance of the blank (without added ACE enzyme) at 280nm

Inhibition was expressed as the concentration of the component that inhibits 50 % of the ACE activity (IC_{50}).

Results

Anticoagulative activity of brown algae

In this study seven brown algal species (*E. cava, I. okamurae, S. fulvellum, S. horneri, S. coreanum, S. thunbergii,* and *S. lomentaria*) were digested with carbohydrases (Viscozyme, Celluclast, AMG, Termamy and Ultraflo) and screened to evaluate their potential anticoagulant activities. All representative results are shown in Table 1. The enzymatic extracts of some species of brown algae showed high anticoagulative activities (such as those from *E. cava*, *S. horneri* and *S. coreanum*) while other species showed less anticoagulant activities. The APTT activity without addition of algal extract was accompanied by a very low APTT value (32 sec). Addition of *E. cava* extracts to the medium showed higher APTT activity than those from other extracts. All *E. cava* samples (digested by five carbohydrases) indicated >300 sec for APTT assay (80 μg extract in assay mixture). Moreover, in this study, *S. horneri* sample also exhibited a good anticoagulative activity. Viscozyme, Celluclast and Ultraflo extracts of *S. horneri* exhibited >300 sec APTT values, in contrast AMG and Termamyl digested samples of this alga exhibited 120 and 165 sec APTT values respectively. Meanwhile at the same experimental conditions, *S. coreanum* showed mild anticoagulative activity. In addition, APTT activity of this species did not varied much according to the digestive enzyme. In this study *E. cava* sample exhibited a good anticoagulative activity. Therefore, extracts of *E. cava* were subjected to further experiments.

Table 1. Anticoagulant activity of enzymatic extracts of brown algae.

Species	Activated partial thromboplastin time (sec)				
	1[a]	2	3	4	5
Ecklonia cava	>300	>300	>300	>300	>300
Ishige okamurae	35	32	38	31	35
Sargassum fullvelum	32	35	32	32	33
Sargassum horneri	>300	>300	120	165	>300
Sargassum coreanum	125	152	114	131	115
Sargassum thunbergii	30	35	32	31	36
Scytosipon lomentaria	45	35	61	45	52

All data points are means of two determinations, sample concentration 80 ㎍/ml in an assay mixture. [a]1, Viscozyme ext; 2, Celluclast ext; 3, AMG ext; 4, Termamyl ext; 5, Ultraflo ext.

Table 2. results indicate the detailed anticoagulative activity of the *E. cava* which was digested by five carbohydrases. All tested digests of *E. cava* were able to prolonge the APTT considerably, the time was >300 sec in all samples, that of control without added extract showed 32 sec. Hence, the presence of *E. cava* extract in the mixture extended coagulation time (APTT) considerably. *E. cava* sample which was digested by AMG showed the highest APTT activity compared to all other extracts. Except the latter sample the other samples showed almost similar APTT activities (around 300 sec). In prothrombin time (PT) assay, almost all extracts of *E. cava* did not show good activities, however AMG digested sample showed 15 sec activity with that of 11 sec in control. Therefore, it can be predicted that *E. cava* sample does not have good activity for prolonging the prothrombin time in normal human citrated plasma. In addition, *E. cava* sample showed a considerable activity towards the thrombin time (TT) assay. As observed from other two assays (APTT and PT) in this assay also AMG extract of *E. cava* showed the best activity (124 sec). Meanwhile, Ultraflo and Viscozyme digests of *E. cava* also indicated a considerable activity (120 and 108 respectively). Celluclast and Termamyl extracts of *E. cava* were not able to prolong the thrombin time, activities were almost similar to that of control (11 sec).

Table 2. Anticoagulant activity of *E. cava* digested by different carbohydrases.

Sample	APTT	PT	TT
Viscozyme ext.	320	11	108
Celluclast ext.	348	12	21
AMG ext.	>1500	15	124
Termamyl ext.	300	11	21
Ultraflo ext.	350	11	120
Control	32	11	11

All data points are means of two determinations. 80 μg/ml of sample amount in an assay mixture.

Organic solvent fractionation results of the AMG enzyme digested *E. cava* sample are shown in Table 3. Ether, ethyl acetate and *n*-butanol fractions of AMG digested sample were unable to show satisfactory APTT activity compared to that of chloroform fraction. Chloroform fraction exhibited 1200 sec in APTT assay, but this activity was also less than that were recorded from original and final water fractions (>1500 sec). In PT assay, original sample, chloroform fraction and final water fraction showed almost two times higher activities (24, 22 and 28 sec) than that of control (11 sec). Ether, ethyl acetate and *n*-butanol fractions activities towards the PT experiment were similar to that of control. Also, for thrombin time (TT) assay the latter three fractions exhibited poor activities, those were almost similar as recorded from control sample. Furthermore, in TT assay, original sample, chloroform fraction and final water fraction indicated clear activities (were higher than 300 sec).

The molecular weight fractionation results of this experiment shows in Table 4. It is interesting to mention that >30 kD fraction of AMG extract of *E. cava* recorded the highest activity for all tested anticoagulant experiments. Especially, it was very effective in prolonging the APTT and TT (over >1500 sec) than PT activity (20 sec). All other fractions (bellow 05, 05-10 and 10-30 kD) were not strong enough to extend anticoagulation time period in all tested assays. However, 05-10 kD fraction of AMG digested *E. cava* showed 30 sec for PT assay. Therefore, >30 kD fraction was subjected to further experiments. Each molecular weight fraction of this experiment contain high polysaccharide amount (>85%) therefore the active compound is supposed to be a polysaccharide.

Table 3. Anticoagulant activity of AMG extract of *E. cava* after organic solvent fractionation.

Sample	APTT	PT	TT
Original sample	>1500	24	>300
Ether fraction	32	11	11
Chloroform fraction	1200	22	>300
Ethyl acetate fraction	65	11	15
n-Butanol fraction	59	12	16
Final water fraction	>1500	28	>300
Control	32	11	11

Results are expressed as means of two determinations. Sample concentration 80 μg/ml per assay mixture.

Table 4. Anticoagulant activity results for the different molecular weight fractions of AMG hydrolyzed *E. cava* sample.

Sample (80* μg/ml)	APTT	PT	TT
Above 30 kD fraction	>1500	20	2000
30-10 kD fraction	35	13	13
10-5 kD fraction	32	30	11
Below 5 kD fraction	32	11	11
Control	32	11	11

Results are expressed as means of two determinations. *Each sample fraction contains >85 % polysaccharide amount.

The dose dependant anticoagulant activity of the polysaccharide fraction, which was separated from AMG digested *E. cava* was compared with that of heparin. The results of this experiment is shown in Fig. 1. It is obvious that crude polysaccharides have clear dose dependant APTT prolonging ability. Since it is a crude extract of polysaccharide the activity reduce slightly at low concentrations, however at higher concentrations there was a distinct activity over APTT test. In this experiment, heparin (at 0.03 μg in assay mixture) showed 800 sec activity in APTT assay, while control showed only 32 sec APTT activity.

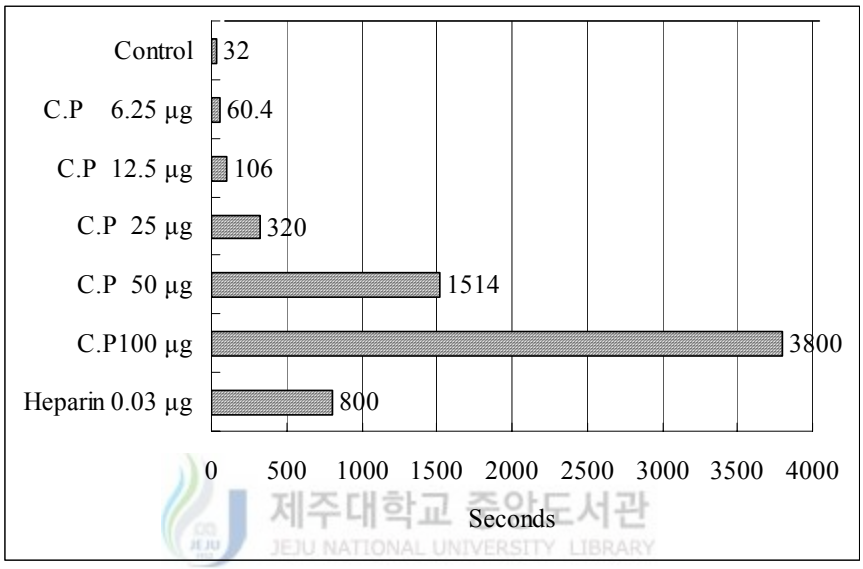

Fig. 3. Comparison of anticoagulant activity (activated partial thromboplastin time) of *E. cava* crude polysaccharide (separated from the >30 kD AMG digest) with that of Heparin. Results are average of two experiments.
*CP-Crude Polysaccharide.

Antihypertensive activity of brown algae

The results of the ACE inhibitory activities of different water extracts of seven brown algal species (*E. cava, I. okamurae, S. fulvellum, S. horneri, S. coreanum, S. thunbergii,* and *S. lomentaria*) are shown in Table 5. The water extract of *E. cava* had higher ACE inhibitory activity (36%) than those from others. Water extracts of *S. fulvellum* and *I. okamurae* also exhibited good ACE inhibitory activities (24 and 22% re- spectively), meanwhile *S. horneri* and *S. thunbergii* water extracts showed a little low but similar activities (18 to 17% respectively). Compared to other algal species, *S. lomentaria* water extract showed the lowest activity (5%) for the ACE inhibitory assay.

Since, all algal extracts constituted with ACE inhibitory compounds, those algal samples were then subjected to protein digestion. After enzymetic digestion (Protamex, Kojizyme, Neutrase, Flavourzyme, and Alcalase) those samples were then subjected to ACE inhibitory activity assay. As shown in Fig. 2. all protease extracts of *E. cava* showed more than 60% inhibition activities. Specially, Flavourzyme and neutrase hydrolyzed *E. cava* samples showed higher activities (80 and 76 % respectively) than that of other counterparts. Protease extract of *I. okamurae* also had considerable activity over ACE inhibition assay (Fig. 3). Except Flavourzyme hydrolyzed sample, other four counterparts showed over 59% ACE inhibition activities. The highest activity (85 %) of this species was recorded from Alcalase hydrolyzed sample. In Figure 4. Kojizyme and Protamax extracts of *S. fulvellum* exhibited very high ACE inhibitory activities (97-74 %), in contrast other hydrolyzed fractions of this species did not show possible activities towards ACE inhibition. According to the results of Fig. 5. *S. horneri* hydrolyzed samples also had good ACE inhibitory activities. Specially, Kojizyme and Neutrase digested *S. horneri* samples showed good ACE inhibitory activities (91 and 86 % respectively), while Protamax extract had moderate ACE inhibitory activity (57 %). Flavourzyme digested *S. horneri* sample indicated low inhibitory activity, while Alcalase digested sample indicated poor inhibitory activity.

Table 5. ACE inhibitory activities of several brown algal water extracts.

Species	ACE Inhibitory activity (%)
Ecklonia cava	36.1
Ishige okamurae	22.9
Sargassum fulvellum	24.3
Sargassum horneri	18.4
Sargassum coreanum	11.2
Sargassum thunbergii	17.3
Scytosiphon lomentaria	5.1

Results are means of three determination.
Sample concentration 1 mg/ml.

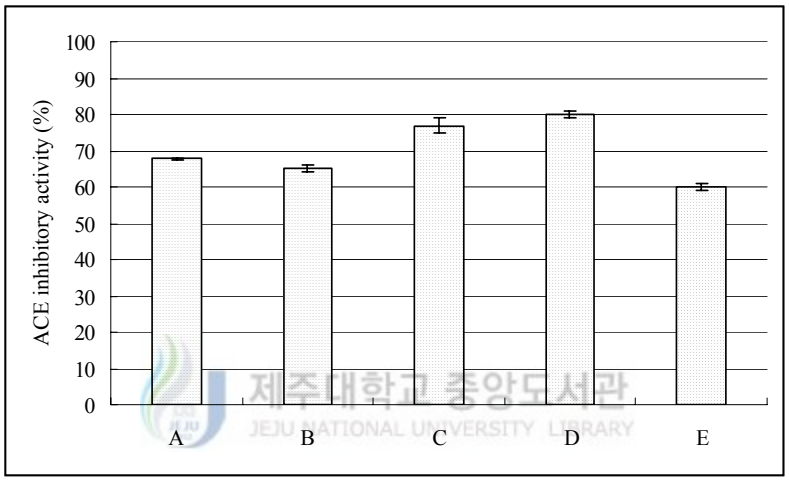

Fig. 4. ACE inhibitory activity of *E. cava* hydrolyzed with different proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext.; (D), Flavourzyme ext.; (E), Alcalase ext. Error bars show the standard deviation of three determinations. Sample concentration 1 mg/ml.

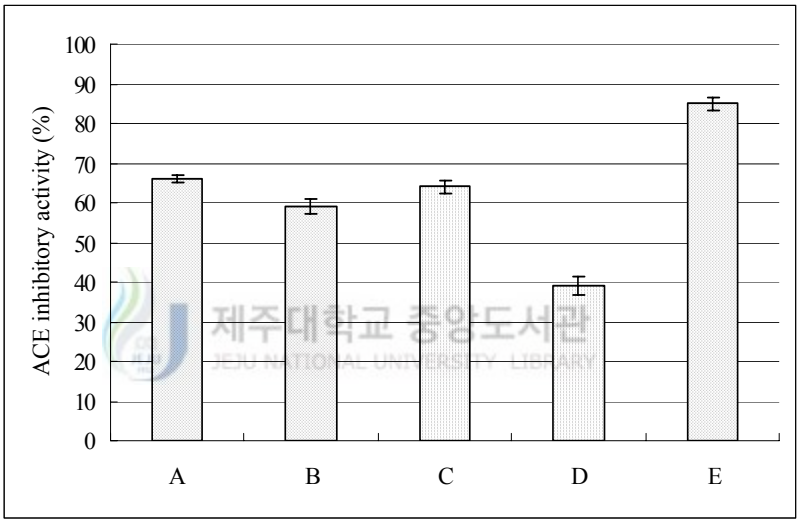

Fig. 5. ACE inhibitory activity of *I. okamurae* hydrolyzed with proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext.; (D), Flavourzyme ext.; (E), Alcalase ext. Error bars show the standard deviation of three determinations.
Sample concentration 1 mg/ml.

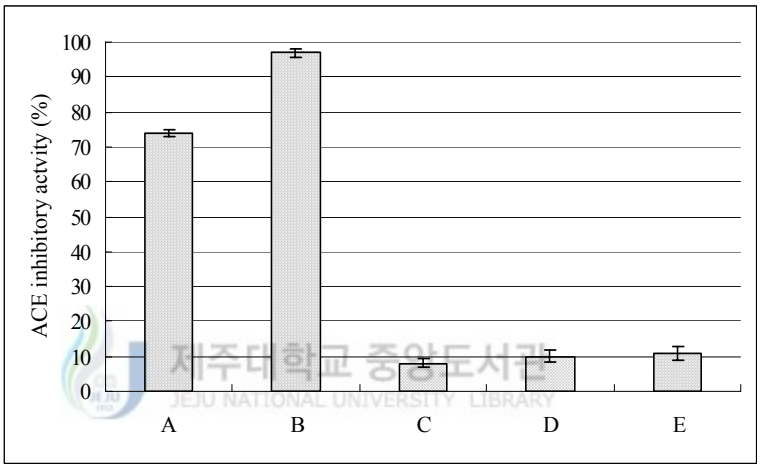

Fig. 6. ACE inhibitory activity of *S. fullvelum* hydrolyzed with proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext.; (D), Flavourzyme ext.; (E), Alcalase ext. Error bars show the standard deviation of three determinations.
Sample concentration 1 mg/ml.

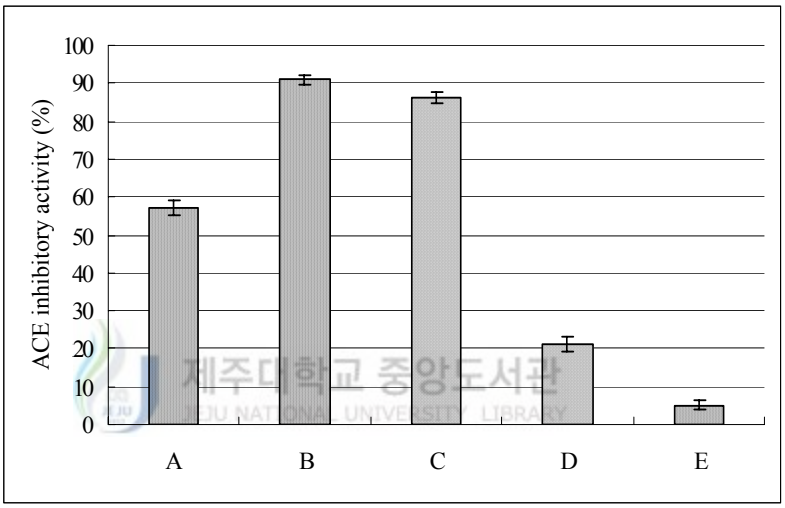

Fig. 7. ACE inhibitory activity of *S. honeri* hydrolyzed with proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext.; (D), Flavourzyme ext.; (E), Alcalase ext. Error bars show the standard deviation of three determinations. Sample concentration 1 mg/ml

S. coreanum ACE inhibitory activity results are shown in Fig. 6.

Protamax, Neutrase and Flavourzyme extracts of *S. coreanum* showed above 65 % inhibitory activities, while the other two samples (Kojizyme, Alcalase) showed >45 % ACE inhibitory activities. *S. thunbergii* extracts indicated low ACE inhibitory activities (Fig. 7) compared to others. Neutrase digested *S. thunbergii* sample showed 52 % ACE inhibition, while Protamax extract of this sample followed by 35 % ACE inhibition. Other three fractions (Alcalase, Kojizyme and Flavourzyme digested samples) indicated a mild ACE inhibitory activities (34, 25 and 21 %). According to the ACE inhibitory results of *S. lomentaria* (Fig. 8) Kojizyme and Neutrase hydrolyzed algal extracts had better activities (62 and 60 % respectively) than obtained from other counterparts. Protamax and Alcalase hydrolyzed *S. lomentaria* samples showed average ACE inhibitory activities (35-39 %).

When we evaluate all results of above study it is clear that *E. cava* composed with relatively high ACE inhibitory compounds. Therefore, dose dependant activity of protease hydrolyzed *E. cava* was evaluated in order to examine precise IC_{50} value of each sample, and resulted IC_{50} values were compared with that of Captopril.

In Fig. 9, the percentage of inhibition is expressed against the Captopril concentration. As we expected ACE inhibitory activity of the Captopril increase with the sample concentration. The activity reached at highest level (98 %) at 5 μg/ml. This means that the complete inhibition was not reached even at higher concentrations. The measured IC_{50} (concentration of compound at which the reaction was inhibited by 50 %) with this spectrometric assay was approximately 0.0465 μg/ml. The dose dependant ACE inhibitory results of Flavourzyme extract of *E. cava* is shown in Fig. 10. The figure shows clearly that ACE inhibitory activity of Flavourzyme digested *E. cava* sample increase with dose dependant manner, but activity tend to become decrease at higher concentrations. The maximum value (81%) of this sample was recorded at 50 μg/ml concentration. The IC_{50} value of this sample was 0.44 μg/ml. The dose dependant activity results of Neutrace hydrolyzed *E. cava* present in the Fig. 11, this hydrozylate reached to its maximum inhibitory activity (64 %) at 50 μg/ml. Addition of this extract to the mixture rended 4.47 μg/ml IC_{50} value.

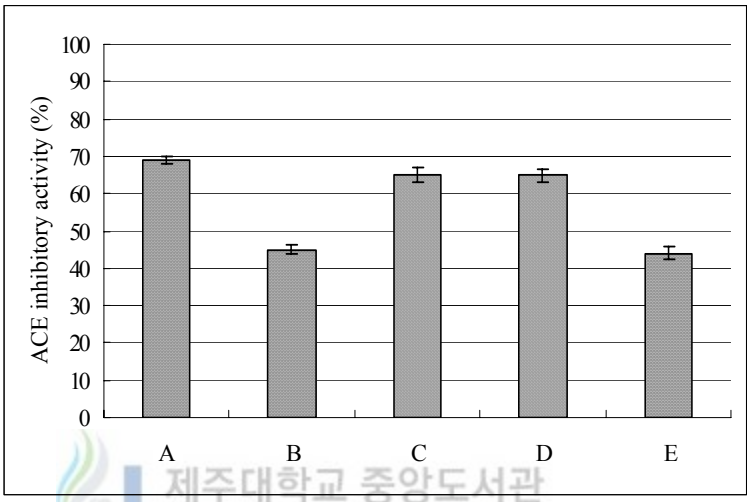

Fig. 8. ACE inhibitory activity of *S. coreanum* hydrolyzed with different proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext.; (D), Flavourzyme ext.; (E), Alcalase ext. Error bars show the standard deviation of three determinations. Sample concentration 1 mg/ml.

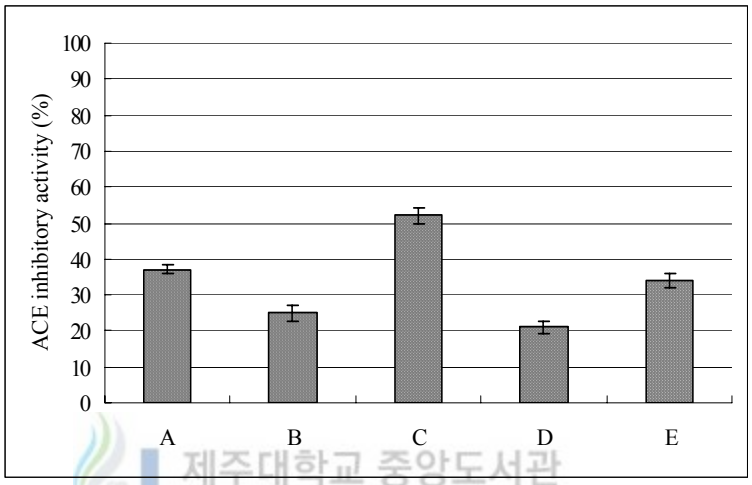

Fig. 9. ACE inhibitory activity of *S. thunbergii* hydrolyzed with different proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext.; (D), Flavourzyme ext.; (E), Alcalase ext. Error bars show the standard deviation of three determinations. Sample concentration 1 mg/ml.

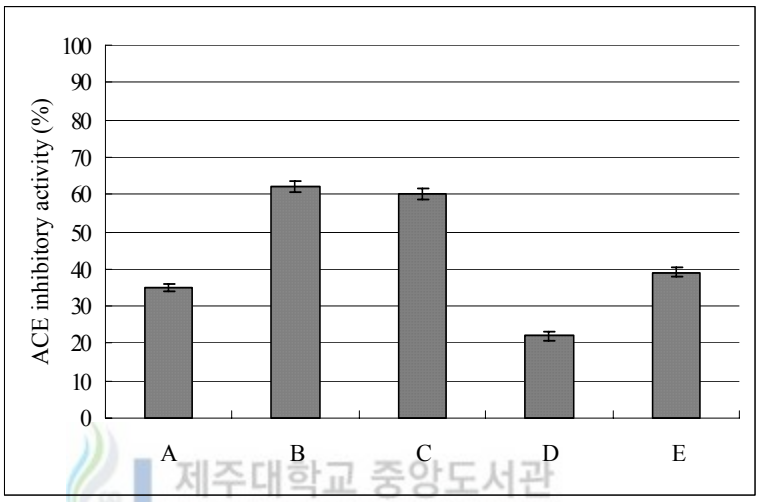

Fig. 10. ACE inhibitory activity of *S. lomentaria* hydrolyzed with different proteases. (A), Protamax ext.; (B), Kojizyme ext.; (C), Neutrase ext; (D), Flavourzyme ext; (E), Alcalase ext. Error bars show the standard deviation of three determinations.

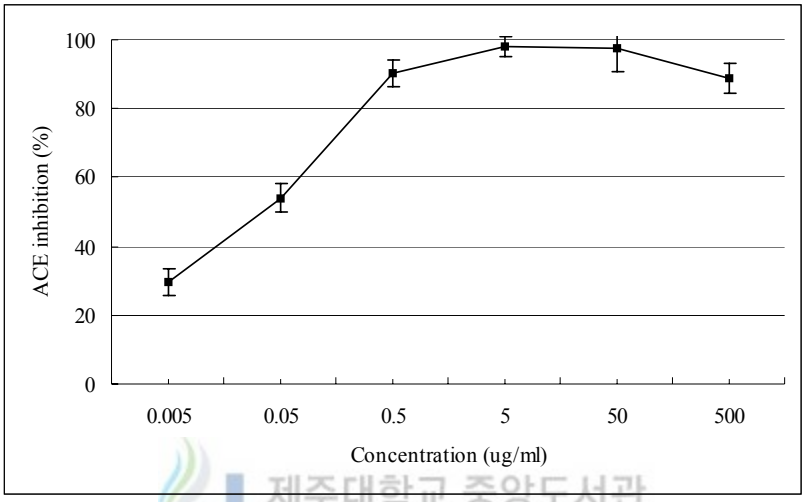

Fig. 11. ACE inhibitory activity of Captopril. Values are mean of three determinations. IC_{50} value of the sample is 0.046 ㎍/ml.

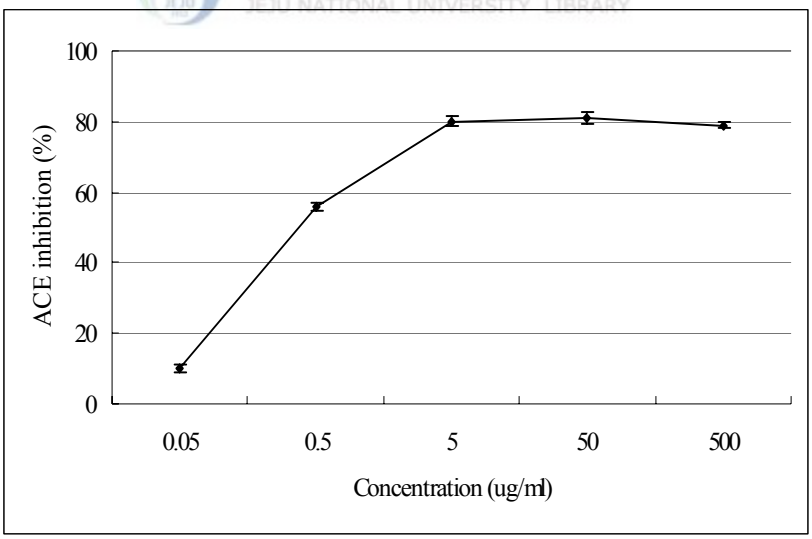

Fig. 12. ACE inhibitory activity of Flavourzyme digested *E. cava* sample. Values are mean of three determinations. IC_{50} value of the sample is 0.44 ㎍/ml

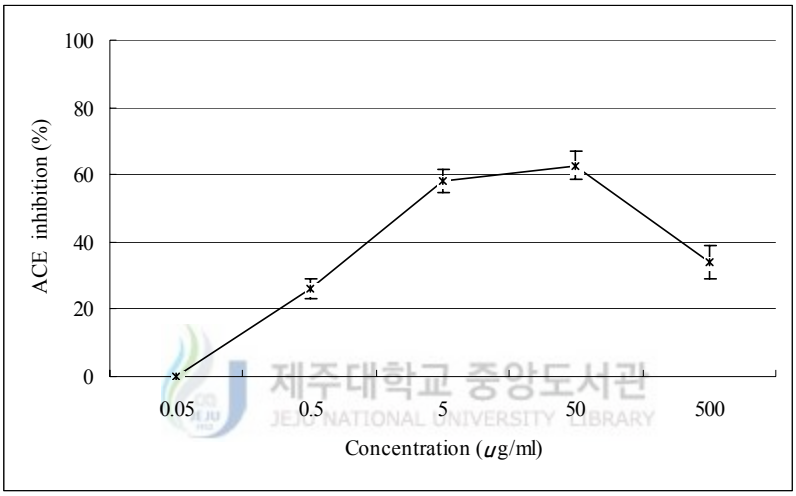

Fig. 13. ACE inhibitory activity of Neutrase digested *E. cava* sample. Values are mean of three determinations. IC_{50} value of the sample is 4.47 ㎍/ml.

In Fig. 12, Alcalase hydrolyzed *E. cava* results for the ACE inhibition is presented. Alcalase extract of *E. cava* showed dose dependant activity over ACE inhibition, however at higher concentrations (500 ug/ml) activity became to decrease. The highest inhibition activity (91 %) of this sample was recorded at 50 μg/ml concentration and the IC_{50} of this sample was 4.1 μg/ml. In Fig. 13. ACE inhibitory activity of protamax hydrolyzed *E. cava* increased gradually with the increased concentrations and were 0, 26, 58, 62 and 34 % respectively. From 0.05 to 5 μg/ml sample concentration inhibition activity increased rapidly, but there after activity tend to become decrease. The IC_{50} of this fraction was 4.3 ug/ml. In Fig. 14. Kojizyme digested *E. cava* results is presented, according to the results of this study the ACE inhibition activity increased slowly with the sample concentration. The maximum inhibition of this digest was 68 % at 50 μg/ml concentration.

Among these algal extracts, *E. cava* sample which was digested by flavourzyme remained the most effective and gave the lower IC_{50} in ACE inhibition assay. Therefore, further experiments was conducted to separate its active compound. In order to get an idea about the molecular weight of the active fraction this digest was passed thorough molecular sieve and relevant molecular cut off fractions we evaluated on ACE inhibition assay. The result of this study is shown in Table 6. In this experiment we separated the original sample into four molecular weight cut offs, above 30 kD fraction showed highest ACE inhibition activity (76 %). Bellow 5 and 5-10 kD molecular cut off fractions had 18 and 12 % ACE inhibition activities respectively, however 10-30 fraction had poor ACE inhibition activity.

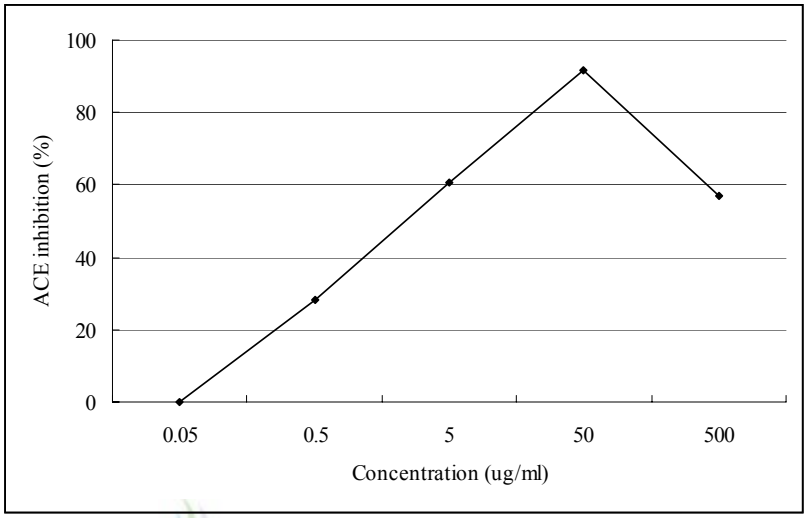

Fig. 14. ACE inhibitory activity of Alcalase digested *E. cava* sample. Values are mean of three determinations. IC_{50} value of the sample is 4.1 ㎍/ml.

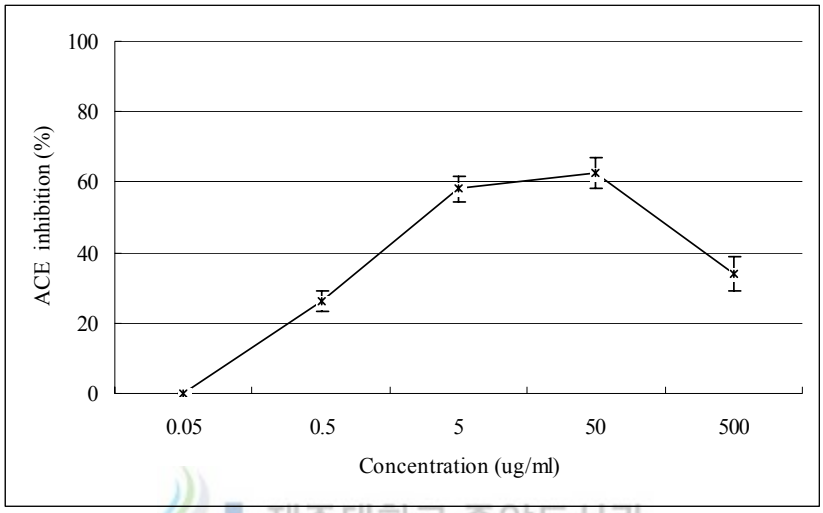

Fig. 15. ACE inhibitory activity of Protamex digested *E. cava* sample. Values are mean of three determinations. IC_{50} value of the sample is 4.3 ㎍/ml.

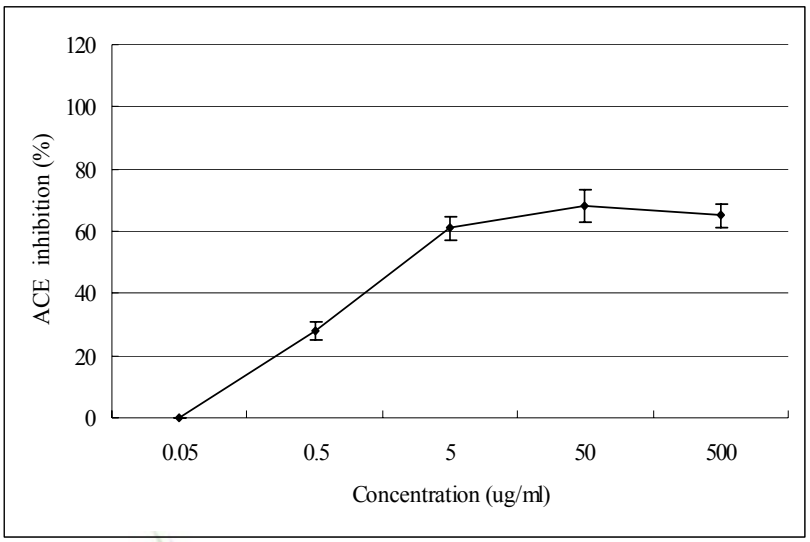

Fig. 16. ACE inhibitory activity of Kojizyme digested *E. cava* sample. Values are mean of three determinations. IC_{50} value of the sample is 4.47 ㎍/ml.

Table 6. Molecular weight fractionation results of the Flavourazyme hydrolyzed *E. cava* for ACE inhibition assay and relevant protein amount in each fraction.

Fraction	ACE inhibition (%)	Protein (%)
Above 30 kD fraction	76.6	27
30-10 kD fraction	-	13
10-5 kD fraction	12.1	13
Below 5 kD fraction	18.3	13

All activities are means of three determinations.
Sample concentration 80 ㎍/ml per reaction mixture.

Discussion

The anticoagulant activities of several brown algal species were compared and evaluated in several *in-vitro* assays. There are grater incidence of anticoagulant activity in extracts from brown algae compared to red and green algae (Shanmugan and Mody, 2000).

The activated partial thromboplastin time of this study clearly indicate that some of them (specially *E. cava*) have good anticoagulative activities. According to previous findings (Nishino, *et al*, 1989 and Pereira, *et al*, 1999) this anticoagulative activity is supposed to be due to polysaccharide compounds (Yang *et al*., 2002). Therefore, in this study we used five carbohydrases to digest seaweeds samples. The AMG (an exo1, 4 -alpha-d-glucosidase) digested *E. cava* sample showed clear distinct activity over other digests. This may be due to the special target breaking ability of this enzyme. This enzyme can hydrolyze 1, 4 as well as 1, 6 alpha linkages from the substrate solution. Further, 1, 4-alpha linkages was hydrolyzes more readily than 1, 6 alpha linkages. According to previous findings, the branch structure of polysaccharides can be effect on the blood coagulation activities (Yoon *et al*., 2002). Synthetic sulfated dextran with mannose branches showed higher anticoagulant activity than sulfated dextran without branches (Yang *et al*., 2002). Therefore, it is suggested that AMG digested sample can raise the anticoagulative activity of *E. cava* than other enzymes. After AMG digestion, parental polysaccharides may change into special structures and then interact with the coagulation factors more strongly to lead anticoagulant activity. The organic solvent fractionation of AMG digested *E. cava*, shows active compounds can be partly separated by chloroform. This ability may be due to special organic solvent ability of chloroform. However, most of the active compounds were concentrated on final water fraction. The highest dry matter content also was observed in the last water fraction, therefore this results explain the inability of organic solvents to separate active compounds. This is also a good clue about active compound, fats and proteins can be easily dissolved in organic solvent, but major polysaccharides are unable to dissolve in organic solvents.

Hence, it can be predict that active compound is belonging to the family of polysaccharides. Molecular weight fractionate results of the polysaccharides of *E. cava* indicate that all active compounds are concentrated in >30 KD fraction. According to most of the seaweed extracted anticoagulant experiments, the size of the active compound is around 50-850,000 Da. (Shanmugam and Mody, 2000). Yang *et al.*, (2002) sugested that the anticoagulant activity improve with the increased molecular weights.

In this study it is obvious that the active compound of the extract does not have a clear inhibition ability over PT assay. However there was a clear activity over APTT and TT assays. Since this sample have good activity for APTT assay the active compounds of this sample is able to inhibit the intrinsic and common pathways of the coagulation cascade of human body. As well as prolongation of TT indicates inhibition of thrombin activity or fibrin polymerization. Therefore, it can be conclude that the active polysaccharide fraction of this alga inhibit both intrinsic and or common pathways of coagulation cascade and the thrombin activity or conversion of fibrinogen to fibrin. No effect on the PT assay indicates that this active compound do not have effect on extrinsic pathway of the blood coagulation. According to previous studies, eisenine, biotin, and laminine are known compounds of *E. cava* for its biological activities (Ahn *et al.*, 2004). Also, ekol, 8-8-biekol and pholorofucofuroeckol have been isolated from *E. kurome* as anticoagulants agents. The potential anticoagulant activity of *E. cava* also may be due to similar kind of compounds as in *E. kurome*. However, further purification steps should be tack place in order to reveal the active compound.

ACE plays an important physiological role in regulation of blood pressure and electrolyte homeostasis. It cleaves angiotensin I to angiotensin II, which is a powerful vasoconstructor and salt retaining octapeptide. Moreover, it catalyzs the inactivation of bradykinin, which is a vasodialaror and natrituretic nonapeptide. Recently, many ACE inhibitors have been isolated from enzymatic digests of various food materials including marine algae. To obtain the active substances for ACE inhibition the screening was performed on 7 brown algal species.

The inhibition activity for the water extracts of the samples shows moderate activity towards ACE inhibition. This may due to low affinity of water to extract the active principle. After enzyme digestion the activity of each fraction increased dramatically. Enzyme digestion make active compound more available in the reaction mixture, hence the activity increase rapidly. *E. cava* sample have good ACE inhibition activity than observed from others this may be due to its high protein (10 %) concentration (Heo *et al*., 2003). Specially, Flavourazyme hydrolyzed sample of *E. cava* showed very low IC_{50} value for ACE inhibition, this indicate it as a potential source for antihypertensive drug discovery. The active compound is mainly concentrate in >30 kD fraction, therefore, the molecular weight of the active fraction should be high.

Thus, it can be assume that present antihypertensive compound of *E. cava* may be a kind of bio-active complex rather than an separate peptide. Although ACE inhibitory peptides derived from algal species are less active than captopril, they are much important since some of then can be used as dilatory food supplement. Therefore, the demand for edible alga continuously captive in the field of food processing industry.

SUMMARY

Enzyme hydrolyzed seven brown algal species (*Ecklonia cava, Ishige okamurae, Sargassum fulvellum, Sargassum horneri, Sargassum coreanum, Sargassum thunbergii,* and *Scytosiphon lomentaria*) using five carbohydrases (Viscozyme, Celluclast, AMG, Termamy and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) were screened in order to evaluate their potential anticoagulant and antihypertensive activities. Among the alga, AMG (an exo1, 4-alpha-d-glucosidase) digested sample of *E. cava* showed excellent activity in anticoagulant assay and Flavourzyme digested sample showed good activity in ACE inhibitory assay. AMG extract (>30 kD) of *E. cava* (80 μg in assay mixture) showed a remarkable activity in activated partial thromboplastin time (>1500 sec) and thrombin time (2000 sec) assays but not in prothrombin time (15 sec) assay. Flavourzyme digested sample of *E. cava* showed 0.44 μg/ml of IC_{50} value compared to that of Captopril 0.046 μg/ml in ACE enzyme inhibition assay. Molecular weight fractionation result explained that the both active principles (anticoagulative and antihypertensive) were concentrated in >30 kD fraction. These results prove the fact that crude sample of *E. cava* is a good source for pharmaceutical industry. Further experiments will be carried out to isolate the active compound from both AMG and Flavourzyme (>30 kD) fractions of *E. cava*.

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ACKNOWLEDGEMENT

I am deeply indebted to my supervisor, Dr. You-Jin Jeon, Department of Marine Biotechnology, Cheju National University, South Korea, whose guidance, stimulating suggestions and encouragement helped me in all the time of research and writing of this thesis.

I am also grateful to Dr. Ki-Wan Lee, thesis director, Cheju National University, Marine Biology Department, for providing laboratory facilities and directing me to make this research a successful. His continues suggestions and advices helped me to achieve my goal.

Also I wish to offer my humble gratitude to Dr. Choon-Bok Song, Dr. Kwang-Sik Choi, Dr. Je-Hee Lee, Dr. In-Kyu Yeo, Dr. Geun-Tae Park, Dr. Soo-Hyun Kim and Dr. Jin-Hwan Ha, Cheju National University, for providing me their laboratory facilities during my study period.

Also I wish to express my deep thank to Dr. Udaya Wanasundara and Mrs. Dammika Rathnasiri for guiding me to study in South Korea.

I am also thankful to my laboratory members (Soo-Jin Heo, Kil-Nam Kim, Seon-Heui Cha, In-Sun Kim, Jin-Hee Park, Seung-Hong Lee, Ha-Na Chung, In-Shik Shin, Hyun-Pi Yang, Won-Suk Kim and Young-Bin Oh), all Ocean Science Collage students, and to Chul-Hong Oh, Kyung-Il Park, Ho-Jin Park, Kang-Hyun Sil, who helped me in different ways through out this study and made this study a pleasure.

Also, I greatly acknowledge to all of my Sri Lankan friends who study in Cheju National University (Nalin, Rohan, Mahinda, Herath, Prashani and Helani) for their valuable support which was much needed for the completion of this research.

Last but not the least, I extend my loving and deep gratitude to my cherished parents and loving brothers for their love and patience.