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Anticoagulant activity of marine green and brown algae collected from Jeju Island in Korea

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Abstract

Twenty-two algal species were evaluated for their potential anticoagulant activities. Hot water extracts from selected species, *Codium fragile* and *Sargassum horneri* showed high activated partial thromboplastin time (APTT). Ultraflo extract of *C. fragile* and *S. horneri* exhibited the most potent anticoagulant activity. Furthermore, in both algal species, active compounds were mainly concentrated in >30 kDa faction. The crude polysaccharide fraction (>30 kDa; CpoF) of *C. fragile* composed of ~80% carbohydrate and ~19% of protein; the crude polysaccharide fraction (>30 kDa; CpoF) of *S. horneri* was composed of 97% of carbohydrate and ~2% of protein. Therefore, most probably the active compound, or compounds of the algal species were related to high molecular weight polysaccharide, or a complex form with carbohydrate and protein (proteoglycan).

Keywords: Algae; Enzymatic hydrolysis; Anticoagulant activity; *Codium fragile*; *Sargassum horneri*

1. Introduction

Jeju Island is located in the southwest sea of the Korean peninsula and is highlighted for its uniqueness. Especially, in the coastal area of this Island the seawater level fluctuates rapidly. Therefore, the algal species present along the shoes of Jeju Island may require high endogenous biological protection as an adaptative response to this especial environment. Recently several biologically important seaweed species from Jeju Island have been reported (Athukorala et al., 2003, 2005; Siriwardhana et al., 2003; Heo et al., 2005; Karawita et al., 2005). However, yet there are few or less systematically studied reports regarding the potential anticoagulant activity of Jeju Island seaweeds.

In 1913, scientists investigated blood anticoagulant properties of marine brown algae (Killing, 1913). Even if it is difficult to elucidate the exact structure of the anticoagulant polysaccharides isolated from algae, the research interest to isolate anticoagulant compounds from marine seaweeds is continuously increased in the field of pharmaceutical industry. Heparin is the drug of the choice in prevention of thromboembolic disorders. But recently alternative drugs for heparin are in high demand due to its bad and long-term side effects. Therefore, as an alternative source, seaweed polysaccharides gain much attention in the pharmaceutical industry to develop better and safe drugs with low or less side effects. Recently, there was a case study on the changes of the haemorrhage, plasma cholesterol and albumin and clinical effects in 36 children with refractory nephrosis after treatment with fucans. The results 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. Hence, the aim of this study was to screen Jeju Island seaweeds for their potential anticoagulant activities, which might expand the possibility to find better anticoagulant drug.

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2. Methods

2.1. Materials

Marine green and brown algae used in this study were collected along the shores of Jeju Island in Korea. Salt, sand and epiphytes were removed using tap water. Finally, seaweed samples were rinsed with fresh water and freeze-dried at −20 °C for further experiments. APTT (ellagic + bovine phospholipid) and $CaCl_2$ solution were obtained from International Reagents Corporation (Japan), PT (rabbit thromboplastin) 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 1.5L FG (catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymers), AMG 300L (an exo 1,4-alpha-D-glucosidase), Termamyl 120L (a heat stable alpha-amylases), Ultraflo L (a heat stable multi-active beta-glucanase) and five 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 2.4 L FG (a endoprotease) were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). Heparin was purchased from Sigma and all the other chemicals used in this study had 90%, or grater purity.

2.2. Water extracts from algae

One gram of the ground algal powder was mixed with 50 ml of water and placed in shaking incubator for 12 h at 70 °C. The mixtures were centrifuged at 3500 rpm for 20 min at 4 °C and filtered with Whatman filter paper. Finally, each supernatant was subjected for anticoagulant assay.

2.3. Enzymatic extracts from algae

The preparation of enzymatic extracts was followed as previously reported (Heo et al., 2003). Dried alga 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 algae sample was homogenized with water (2L), 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 the digestion pH of the homogenate was adjusted to its optimal pH value. As soon as the enzymatic reactions complete, the digests were boiled for 10 min at 100 °C to inactivate the enzyme. Each sample was clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. All samples were kept in −20 °C for further experiments.

2.4. Blood coagulation assay

Normal pooled plasma was made from ten individual healthy donors, without history of bleeding or thrombosis. Nine parts of blood collected by venipuncture ware drawn into one part of 3.8% sodium citrate. Blood was centrifuged for 20 min at 2400 × *g*, and the plasma was stored at −60 °C until use. All coagulation assays were performed with four individual replicates using dual-channel clot-2, (SEAC, Italy) and mean values were taken. For activated partial thromboplastin time (APTT) assay, citrated normal human plasma (90 μl) was mixed with a solution of algal extract (10 μl) and incubated for 1 min at 37 °C, then APTT reagent (100 μl) was added to the mixture and incubated for 5 min at 37 °C. Thereafter clotting was induced by adding 0.025 M $CaCl_2$ (100 μl) and clotting time was recorded. In prothrombin time (PT) assay, citrated normal human plasma (90 μl) was mixed with a solution of algal extract (10 μl) and incubated for 10 min. Then, PT (200 μl) pre-incubated for 10 min at 37 °C was added and clotting time was recorded. For thrombin time (TT) measurement, citrated normal human plasma (190 μl) was mixed with a solution of algal extract (10 μl) and incubated for 2 min. Then pre-incubated TT reagent (10 min, at 37 °C) was added (100 μl) into the mixture and clotting time was recorded. All algal extracts including heparin were dissolved in water and in the control group, only saline was used.

2.5. Crude polysaccharide separation

The enzymatic extract (240 ml) was mixed well with 480 ml of 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at a room temperature and then crude polysaccharides were collected by centrifugation at 10,000 × *g* for 20 min at 4 °C (Matsubara et al., 2000; Kuda et al., 2002). Hereafter, the collected precipitate was referred to as crude polysaccharide fraction (CpoF) and the resultant supernatant was referred to as crude phenolic fraction (CphF). CpoF and CphF were concentrated separately under vacuum at 40 °C and removed all ethanol, and then samples were dissolved in water for further experiments.

2.6. Molecular weight fractionation of algal extract

Molecular weight fraction of the enzymatic extracts from algae was conducted by our previous method (Athukorala et al., 2006a,b). Algal extract solution was passed through micro-filtration membranes (5, 10 and 30 kDa) using Millipore's Lab scale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) to obtain different molecular weight fractions. Finally, all the fractions (>30, 30 ~ 10, 10 ~ 5 and <5 kDa) were separately processed to evaluate anticoagulant activity.