Isolation and Purification of Anticoagulant Polysaccharide Compound from Fermented Edible Brown Seaweed, *Laminaria ochotensis*

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**Introduction:**

- Many human diseases
  - Viral
  - Bacterial
  - Cancer
  - Oxidative stress

Treatments and control of diseases

Pharmaceuticals

- Marine organisms are rich sources of bioactive compounds
- Seaweed is major component in marine resource

- Bioactive compounds
- Sulfated polysaccharides
- Antiviral
- Anti bacterial
- Anti cancerous
- Anti mutagenic
- Antioxidant
- Anticoagulant
Blood clotting:

- Natural process in which blood cells & fibrin strands clump together to stop bleeding after a blood vessel has been injured.

Blood clots become dangerous when they block blood flow through an artery or vein.

- When a blood clot blocks blood flow to an artery in the heart or brain, a heart attack or stroke may result.

Medications such as anticoagulants (which help prevent blood clots) and clot busters (which help to dissolve blood clots) are prescribed to prevent and treat blood clots.
WHAT IS HEPARIN !!!

- Heparin is an anticoagulant
- Highly sulfated polysaccharide
- Reduce the risk of abnormal blood clotting

However:

Heparin shows many disadvantages

- Hence new opportunities have been opened up for:
  - anti-thrombosis research for discovering more
  - effective,
  - safer &
  - easier to use novel anticoagulant agents.
Extracting methods of anticoagulant compounds from seaweeds:

- Organic solvents
- Hot water extraction
- Acid and alkaline digestion
- Enzymatic extraction methods

Fermentation

Carbohydrates $\rightarrow$ Ethanol + carbon dioxide

(Maltose & Glucose)
Brown Seaweed - *Laminaria ochotensis*

- One of the major seaweeds used for food & chemical extracts

**Scientific classification**
- **Kingdom:** Protista
- **Division:** Heterokontophyta
- **Class:** Phaeophyceae
- **Order:** Laminariales
- **Family:** Laminariaceae
- **Genus:** Laminaria
- **Species:** *Laminaria ochotensis*

**Used as:**
- Animal Feed
- Fertilizers
- Industrial Applications

Extensively farmed in China, Korea & Japan.
Objectives:

- To isolate & purify anticoagulative polysaccharide compound from fermented *L. ochotensis*.

- To characterize the purified compound with respect to coagulation cascade.

- To isolate and identify bacteria responsible for fermentation.
Methodology:

- **Fermentation process**

  - Sample collection - Around the Jeju coast, South Korea
  - Washed with tap water
  - Freeze dried
  - 1.5 g Freeze dried seaweed + 15% sugar + 300 ml water
  - Incubated at 25°C for 10 weeks
  - Measured biweekly

  - **Anticoagulant activity**
    - (Activated partial thromboplastin time - APTT)
  - **Yield (dry matter)** - mg/ml
purification of anticoagulant compound from fermented *L. ochotensis*

Selected the Sample
Week that had high anticoagulant activity

Ethanol precipitation for 24 h at 4 °C

Centrifuged at 10000 rpm for 30 min at 4 °C

Freeze dried

Applied to DEAE -Ion exchange chromatography

Measured Total polysaccharide concentration
Phenol- Sulfuric test - absorbance @ 490 nm
Selected active fractions by activity tests

Anticoagulant activity - APTT
Heparin like activity - Glycosoaminoglycan test
Absorbance @ 540 nm

Applied to Sepherose-4B-Gel filtration chromatography

Measured total polysaccharide concentration

Selected active fractions by activity tests

High activity fractions pooled, concentrated & freeze dried

Agarose gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE)
Methodology

- **Bacteria identification from fermented *L. ochotensis***

  - Selected the Sample - 6th week (Week that had highest APTT activity)
  - Plated on LB agar
  - Incubated at 30 °C for 14 h
  - Inoculation (LB broth)
  - Extracted Genomic DNA
  - Amplified with 16S ribosomal (rDNA)
  - PCR product purification
  - sequenced the DNA by direct sequencing
Results and Discussion:

Table 1. Anticoagulant activity (APTT) and yield (dry matter) estimation of *L. ochotensis* from 1st-10th week of fermentation period.

Anticoagulant activity was measured at biweekly intervals up to 10th week by activated partial thromboplastin test (APTT). Data were average of three experiments.
Figure 1 (A): Purification of anticoagulant polysaccharide from *L. ochotensis* by anion exchange chromatography on DEAE.

Figure 1 (B): APTT activity of the fractions giving high absorbance for both phenol-$\text{H}_2\text{SO}_4$ assay and glycosaminoglycan assay after anion exchange chromatography.

**Results and Discussion**

A - Fractions were collected and assayed total polysaccharide content (□), heparin like activity (♦) and APTT activity was reported by seconds (sec). The fractions showing high anticoagulant activity (F81-F83) indicated by bold bar were pooled, dialyzed against distilled water and concentrated.
Figure 2 (A)
Gel filtration chromatography of the anticoagulant polysaccharide from *L. ochotensis*.

**Results and Discussion**

**Figure 2 (B)**
APTT activity of the fractions giving high absorbance for both phenol-H$_2$SO$_4$ assay and glycosaminoglycan assay after gel filtration chromatography.

A- Fractions were collected and assayed total polysaccharide content (○), heparin like activity (♦) and activity is reported by seconds (sec). The fractions showing high anticoagulant activity (F8-F11) indicated by bold bar were collected, pooled, concentrated and used as purified anticoagulant.

B- Anticoagulant activity was measured by APTT assay (♦) and activity is reported by seconds (sec).
Results and Discussion

Figure 3: Agarose (I) & polyacrylamide gel electrophoresis (II) of the purified polysaccharide obtained from *L. ochotensis*.

Lane A - Purified polysaccharide, 60-500 kD
Lane B - Dextran sulfate sodium salt from *Leuconostoc* sp, 8 kD
Lane C - Chondroitin 6 sulfate sodium salt from shark cartilage, 60 kD
Lane D - Chondroitin sulfate B sodium salt, 20 kD
Table 1. Comparison of anticoagulant activities of purified *L. ochotensis* by different anticoagulant assays APTT, PT (prothrombin time) and TT (thrombin time) tests.

<table>
<thead>
<tr>
<th>Clotting time (sec)</th>
<th>CT ratio</th>
<th>Control (Water)</th>
<th><em>L. Ochotensis</em> anticoagulant 31.0 ug/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT 41.7±1.7</td>
<td>9.25</td>
<td>386.0±4.58</td>
<td>L. Ochotensis anticoagulant 31.0 ug/ml</td>
</tr>
<tr>
<td>TT 11.4±0.9</td>
<td>2.85</td>
<td>32.6 ±4.13</td>
<td>L. Ochotensis anticoagulant 31.0 ug/ml</td>
</tr>
<tr>
<td>PT 12.3±1.7</td>
<td>1.83</td>
<td>22.6 ±6.55</td>
<td>L. Ochotensis anticoagulant 31.0 ug/ml</td>
</tr>
</tbody>
</table>

◆ The clotting time ratio was determined by dividing the clotting time obtained with algal polysaccharide by the time achieved under similar conditions with control (water).

◆ Data were average of three experiments. Each value is expressed as mean ± standard deviation (n=3).
Table 2: APTT activity values of commercial heparin and purified anticoagulant compound after sepherose-4B chromatography at different concentrations.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Anticoagulant activity</th>
<th>APTT (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Commercial heparin</td>
<td>Purified compound</td>
</tr>
<tr>
<td>7.5</td>
<td>59.5±6.26</td>
<td>61.3±7.1</td>
</tr>
<tr>
<td>15.0</td>
<td>72.2±3.67</td>
<td>133.4±6.02</td>
</tr>
<tr>
<td>31.0</td>
<td>186.5±14.18</td>
<td>386.0±13.53</td>
</tr>
</tbody>
</table>

Data were average of three experiments. Each value is expressed as mean ± standard deviation (n=3).
Bacteria Identification from fermented *L. ochotensis*

The amplified 16 rRNA sequence of isolated colony was compared with published 16 rRNA sequences available in public database using NCBI Blast search.

It was found that the sequences was similar to that of *Bacillus subtilis* sp.
**Conclusion:**

- The purified anticoagulant polysaccharide concentration - 31.0 μg/ml
- Sulfate concentration - 15.9 μg/ml

- Characterization of purified sulfated polysaccharide
  - Molecular size - between 60-500 kDa
  - Stronger anticoagulant activity than the commercial heparin
  - Inhibit the both extrinsic, intrinsic path ways as well as thrombin & fibrin polymerization activity

- Bacteria responsible for the fermentation was identified as *Bacillus subtilis* sp.
THANK YOU.