ฤทธิ์ต้านอนุมูลอิสระของกรดไฮยาลูรอนิกโมเลกุลเล็กที่ได้จากกระบวนการพลาสมาวัฏภาคของเหลว
Antioxidant activity of hyaluronic acid fragments generated by the liquid phase plasma process

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระของกรดไฮยาลูรอนิกที่มีน้ำหนักโมเลกุลที่แตกต่างกัน ได้แก่ 9.5, 19.0, 200 และ 1100 kDa ซึ่งได้จากการย่อยสลายด้วยกระบวนการพลาสมาวัฏภาคของเหลว โดยเมื่อทดสอบโดยใช้วิธี 2,2-diphenyl-1-picrylhydrazyl (DPPH) พบว่ากรดไฮยาลูรอนิกที่มีน้ำหนักโมเลกุล 9.5 และ19.0 kDa แสดงคุณสมบัติต้านอนุมูลอิสระ มีค่า IC50=2.766 และ 5.862 mg/ml ตามลำดับ ซึ่งมีค่ามากกว่าที่ 200 และ 1,100 kDa ดาลตัน (IC50= >10 mg/ml) ในขณะที่เมื่อทดสอบด้วยวิธี 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) พบว่า กรดไฮยาลูรอนิกที่น้ำหนักโมเลกุล 9.5 kDa มีฤทธิ์ต้านอนุมูลอิสระสูงที่สุด โดยมีค่า %inhibition เท่ากับ 88.04% เมื่อเทียบกับที่น้ำหนักโมเลกุล 19.0 (65.86%), 200 (31.901%) , 1,100 (18.87%) kDa ตามลำดับ การทดลองชี้ว่ากรดไฮยาลูรอนิกที่ถูกลดน้ำหนักโมเลกุลด้วยกระบวนการพลาสมาวัฏภาคของเหลวมีฤทธิ์ต้านอนุมูลอิสระได้ดี ซึ่งมีศักยภาพในการประยุกต์ใช้ในอุตสาหกรรมเครื่องสำอางและยา

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Abstract

This study aimed to evaluate anti-oxidant activity of hyaluronic acid (HA) with molecular weight (MW); 9.5, 19.0, 200 and 1,100 kDa obtained from the liquid phase plasma process. The result found that, by using DPPH assay, HA MW 9.5 and 19 kDa exhibited strong antioxidant activities with IC50 2.766 and 5.862 mg/ml respectively that were higher than the result from HA, MW 200 and 1,100 kDa (IC50= >10 mg/ml). On the other hand, Using ABTS

assay, HA MW 9.5 kDa exhibited the highest antioxidant activities with the 88.04% inhibition that was higher than 65.86%, 31.901% and 18.87% obtaining from the HA, MW 19, 200 and 1,100 kDa respectively. The result pointed that HA fragments from liquid phase plasma process occupied antioxidant potential that was able to apply in cosmetic and medical industries.

Keywords: hyaluronic acid, liquid phase plasma process, antioxidant

Introduction

Hyaluronic acid (HA) is a linear natural polysaccharide that found in almost all living organisms. Its chemical structure consists of N-acetylglucosamine and D-glucuronic acid, linked via alternating beta-1,4 and beta-1,3 glycosidic bonds. HA is well known for its benefits which widely applied in cosmetic formulations as moisturizer, antioxidant, reducing fine lines and wrinkles as well as increasing skin elasticity. Moreover, HA is an ideal biomaterial for pharmaceutical, biotechnical and medical usages. For medical usages, it was applied for tissue engineering, dermatological fillers as well as viscosupplementation for osteoarthritis treatment in order to healing pain and lubricating the joint. The sources of hyaluronic acid chemicals are synthesized from the *Streptococcus* strain of bacteria and obtained from rooster combs or from the aqueous humor of the eyes of cows. Hyaluronic acid are commonly high molecular weight biopolymers that was around 50 - 20,000 kDa. This leaded to its high viscous and difficult to dissolve in water and therefore, limit its biological activity. The chance to improve the biological activity may be degraded the long chain polymers to small fragments.

Preparations of hyaluronic acid fragments can be done in many ways including acid hydrolysis, ultrasonic degradation or thermal degradation by microwave and the use of free radical. Anywise, those possess various disadvantages on hyaluronic acid products such as chemical contamination and losing its function due to overheating. The liquid phase plasma process is a new process discovered by Takai O. (2008). This process popularly applied in various fields such as nanoparticles synthesis and organic degradations. The principle of this process is to produce plasma surrounded by liquid phase using electric potentials to stimulate free radicals such as the hydroxyl group ($OH^{\cdot}$) and this free radical subsequently induces organic degradation. In addition, the liquid phase plasma process does not require chemicals and high temperature to operate. Therefore, it doesn't have chemical contamination as well as thermal degradation in the system.

This research aimed to evaluate the antioxidant activity of various molecular weights of hyaluronic acid fragments generated by the liquid phase plasma process comparing to the

native hyaluronic acid. The research may be a guideline for preparation of hyaluronic acid with high antioxidant activity.

Objective

1. To determine the effects of molecular weight of hyaluronic acid fragments generated by the liquid phase plasma process on antioxidant activities.

Research Scope

1. Native hyaluronic acid was subjected to depolymerization by liquid phase plasma process. The HA fragments with molecular weight 9.5, 19, 200 and 1100 kDa were selected for antioxidant activity assay by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).

2. Research Duration: Duration of 4 months from May 2018 – August 2018

Research Methodology

1. Chemicals and reagents

This study used hyaluronic acid at the molecular weight of 9.5, 19.0, 200, 1,100 kDa generated by the liquid phase plasma process from the faculty of engineering Naresuan University.

2. Antioxidant activity assay

2.1). DPPH radical scavenging assay

HA solutions were prepared by dissolving HA powders in methanol at various concentrations. The positive control, L-ascorbic acid was prepared in the same method. Each sample or L-ascorbic acid 100 µl were added in 96-well plate. After that, DPPH solution 30 µl was added in each well and left standing in the dark for 30 min at room temperature. The remaining of DPPH was measured the absorbance by spectrophotometer at wavelength of 517 nm. The % Inhibition was calculated by following equation.

$$\% \text{ Inhibition of DPPH radical} = \frac{[A517 \text{ of control} - A517 \text{ of sample}] \times 100}{A517 \text{ of control}}$$

2.2). Antioxidant test by ABTS assay

For antioxidant assay by ABTS method, the ABTS radical cation ($ABTS^{\bullet+}$) was produced by mixing 7.4 mM ABTS and 2.6 mM potassium persulfate in dark condition at room temperature for 12 hours. This reagent solution was diluted by dilution of 1 ml ABTS solution with 60 ml ethanol in order to obtain the absorbance of 1.170±0.02 units at 734 nm. An aliquot of 150 µl of sample was mixed with 2850 µl ABTS solution and the reaction

was kept in the dark condition for 5 min. The absorbance was measured at 734 nm by using a UV-VIS spectrophotometer. The positive control, Trolox was prepared in the same method. The % Inhibition was calculated by following equation.

$$\% \text{ Inhibition of ABTS radical cation decolorization} = \frac{[A734 \text{ of control} - A734 \text{ of sample}] \times 100}{A734 \text{ of control}}$$

3. Statistical analysis

Data were analyzed by one-way ANOVA using the SPSS version 17.0 software package. Significant differences between multiple means were determined by Tukey's Honestly Significant Difference test. The $p < 0.05$ was considered to be statistically significant.

Results and discussions

1. Antioxidant activity by DPPH assay

The DPPH method was selected in this study because it is a fast, easy and reliable method and it does not require a special reaction and device. The antioxidant potential was carried on HA in the range of 9.5 – 1,100 kDa at the concentration of 10 mg/ml. The antioxidant activity tended to increase when the molecular weight of the polymers decreased (Fig.1). At MW 200 and 1,100 kDa, HA exhibited the antioxidant activity only 16.89 and 11.76% since these only calculated as around 5 folds lower than the observation in the HA MW 9.5 kDa ($p < 0.05$). Interestingly, HA MW 19 kDa exhibited the activity of 82.36% approximately to the observation in positive control as ascorbic acid (88.82%). Moreover, at MW 9.5 kDa, HA exhibited the strongest antioxidant activity of 92.05%. The results were very clear that the molecular weight of HA had a great impact on antioxidant activity.

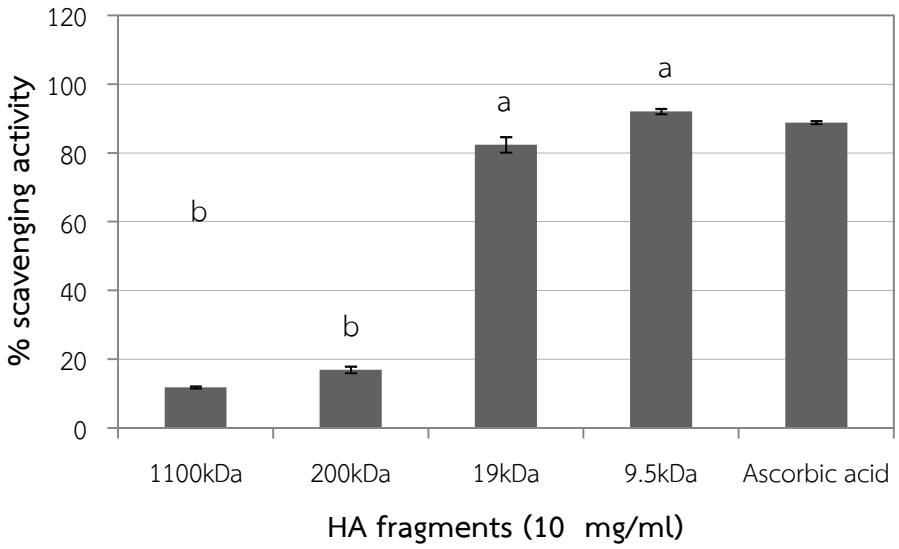

Fig.1 Effect of various molecular weights of hyaluronic acid fragments on antioxidant activity by DPPH assay

The inhibitory concentration, IC50 was shown in Table 1. The hyaluronic acid MW 9.5 and 19.0 kDa exhibited IC50 of 2.7 and 5.8 mg/ml respectively. While, The hyaluronic acid MW 200 and 1,100 kDa exhibited the IC50 more than 10 mg/ml. The result guaranteed that low molecular weight HA had high antioxidant potential comparing to the high molecular weight HA.

Table1. The inhibitory concentration (IC50) of the Hyaluronic acid needed to inhibit 50% of the DPPH radicals (mg/ml)

Hyaluronic acid fragments (kDa)	IC_{50} (mg/ml)
9.5	2.766
19.0	5.862
200	>10
1,100	>10
Ascorbic acid	0.0073

2. **Antioxidant test by ABTS assay**

ABTS•+ is a stable radical not found in the human body. The antioxidant reduces ABTS•+ to ABTS and decolorize it. The antioxidant activity profile from ABTS assay exhibited almost the same character with the DPPH assay. Decreasing the molecular weight of HA leaded to an increasing of antioxidant activity (Fig.2). The highest activity, 88.04% was found in HA with 9.5 kDa while the lowest antioxidant activity, 18.81% was found in HA with 1,100 kDa. This result had confirmed the effect of molecular weight of HA on antioxidant activity.

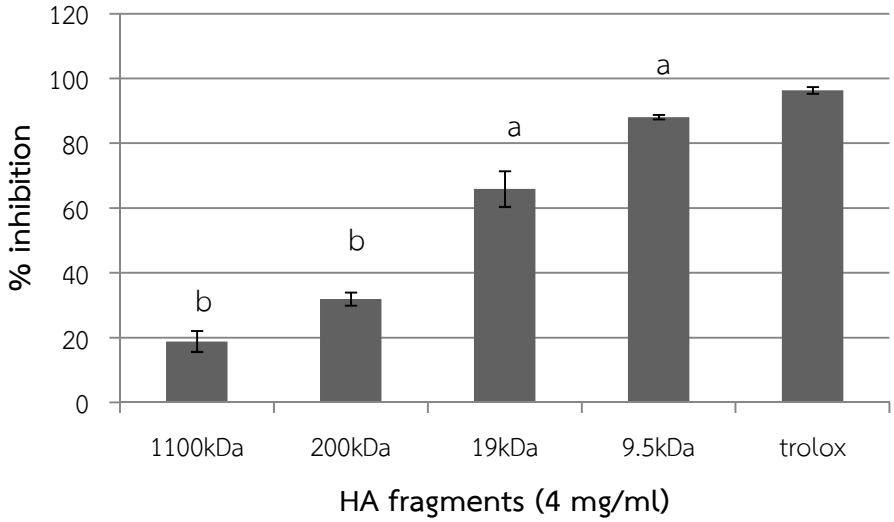

Fig.2 Effect of various molecular weights of hyaluronic acid fragments on antioxidant activity by ABTS assay

The inhibitory concentration, IC50 of the Hyaluronic acid needed to inhibit 50% of the ABTS radicals was shown in Table 2. The hyaluronic acid MW 9.5 and 19.0 kDa exhibited IC50 of 0.402 and 1.190 mg/ml respectively. While, The hyaluronic acid MW 200 and 1,100 kDa exhibited the IC50 more than 4 mg/ml. The result guaranteed that low molecular weight HA had high antioxidant potential comparing to the high molecular weight HA.

Table2. The inhibitory concentration (IC50) of the Hyaluronic acid needed to inhibit 50% of the ABTS radicals (mg/ml)

Hyaluronic acid fragments (kDa)	IC_{50} (mg/ml)
9.5	0.402
19.0	1.190
200	>4
1,100	>4
Trolox	0.0105

From the result, Low molecular weight hyaluronic acid was found to possess high scavenging activity to DPPH and ABTS radicals, which show that the molecular weight is important in antioxidant activity. This result may explain in term of the solubility of HA. HA composed alternatively of β(1-3)-D-glucuronic acid and β(1-4)-N-Acetyl-Glucosamine. This polymer was able to solute in water but its solubility decreased when the molecular weight increased. Short chain HA completely soluted and dispersed in the solution while long chain HA maybe formed inter chain interaction. This leaded to the prevention of the functional groups of HA to react with free radicals and therefore exhibited low antioxidant activity. This result corresponded with the previous report.

Conclusion

The molecular weight of hyaluronic acid had strongly affected on antioxidant properties of the polymer by using in vitro assay; DPPH and ABTS assay. Low molecular weight HA showed highly antioxidant activity comparing to the high molecular weight HA. This characteristic behavior of the polysaccharide offered an opportunity for hyaluronic acid preparation in order to obtain a high antioxidant property.

Suggestions for the future research

1. There should be studied on other methods of antioxidant testing such as superoxide anion scavenging activity, hydroxyl radical scavenging activity, lipid peroxidation

2. There should be studied on other bioactivities of hyaluronic acid fragments generated by the liquid phase plasma process such as pro-collagen type I synthesis, anti-glycation.

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