Enhancement of Plant Growth Activity of Irradiated Chitosan by Molecular Weight Fractionation

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Received August 15, 2005

The chitosan products irradiated at 25 – 200 kGy in 10% solution showed a positive effect on the growth of barley, while the unirradiated chitosan inhibited the growth of this plant. The 100 kGy irradiated chitosan product with average molecular weight (Mw) approx. 16 kDa was found as optimal to plant growth activity. Separation of the degraded samples was performed using ultrafiltration membranes and was found that the fraction F2 with Mw in range of 1 – 3 kDa not only showed a remarkable effect on the growth of barley and soybean, but also significantly increased the activity of phytoalexin enzymes, namely phenylalanine ammonia lyase (87%) and chitinase (186%). This fraction also increased 15.8% seed yield of soybean after three month cultivation. The results suggested that the irradiated chitosan fraction F2 with Mw in range of 1 – 3 kDa was a trigger for plant growth activity.

Key Words: chitosan, radiation degradation, plant growth promotion, phytoalexin, molecular weight separation

1. Introduction

Chitosan is a copolymer of β-D-glucosamine and β-N-acetyl-D-glucosamine. This polymer has been applied in agriculture as an antimicrobial and phytoalexin-inducting product[1]-[4]. Oligomers of chitosan were reported as promising products for plant growth activity such as plant growth promotion, enhancement of enzyme activity and phytoalexin induction[2],[3].

To produce oligochitosan, the conventional methods using chemical[5]-[7] or enzymatic[8]-[10] degradation have been used. For latter method, more than 30 kinds of enzymes (chitosanase, hemicellulase, lysozyme, etc.) have been used for degradation of chitosan, but there are still some difficulties for large-scale industrial processings[8]. The hydrolysis of chitosan by chemicals was investigated[5],[7] and found to be more convenient than enzymatic method. However, some problems were shown, in purification process as well as in causing environmental pollution[5],[7]. Radiation treatment has been used to degrade chitosan and it was expected to be a useful and efficient tool for degradation of polysaccharides. In previous paper,
we found that the irradiated chitosan promoted
the growth of shoot and plantlets of chrysanthemum, lisanthus and limonium in tissue cul-
ture. In this study we performed the separa-
tion of irradiated chitosan products and studied
the biological activity of the fraction for in vitro
plant.

2. Experimental

2.1 Materials

Plants used in the experiments are barley
(Hordeum vulgare cv. minorimugi) and soybean
(Glycine max cv. beer friend). Chitosan 8B was
supplied by Funakoshi Co. Ltd., Tokyo, Japan.

2.2 Preparation of degraded chitosan

To prepare sample for degradation, 10 g chi-
tosan was kept overnight in 100 mL of 0.4 M
acetic acid solution, at room temperature for
swelling and then stirred for 5 h. The prepared
solution was then irradiated by γ-rays from a
60Co source with dose rate of 10 kGy/h at room
temperature.

2.3 Growth promotion test

The irradiated chitosan was used for growth
promotion test on barley and soybean. In the
case of a barley plant, 10 seeds germinated in
48 h at 30 °C were cultivated in 500 mL solution
containing 0.1% hyponex and irradiated chito-
san. The test on soybean was carried out as fol-
low : 3 seeds germinated in 48 h at 30 °C were
cultivated in 500 mL of 1/5 Stainberg nutrient
solution supplemented with irradiated chitosan.
Samples were grown in a phytotron at 25 ±
1 °C with a photoperiod of 12 h per day, and
the fresh weight was measured after 10 days.

2.4 Protein assay

Protein content in samples is analyzed by us-
}ing protein assay rapid kit of Wako (Wako Co.
Ltd., Osaka, Japan).

2.5 Chitinase assay

To measure chitinase activity, barley root
(about 5 g) was washed three times with 25
mM imidazole/HCl buffer, pH 6.8, containing
1 mM ethylenediamine tetraacetate (EDTA),
and homogenized in the same buffer (10 mL)
at 4 °C. After centrifugation at 20 000 x g for 20
min, the supernatant obtained was used as
crude enzyme. The filtrate obtained was used
for enzyme activity assay. Chitinase was assayed
using 2 mL of enzyme solution contain-
ing 20 mg N-acetyl-chitosan and 30 mM
NaHPO4/35 mM citric acid buffer (pH 3.5).
The reaction was carried out at 37 °C for 60
min with shaking and ceased by adding 1 mL
of 67 mg/mL Na2WO4 in 0.33 N H2SO4. After
centrifugation (15 000 x g, 5 min) to remove the
insoluble substrate, reduced sugar in the super-
natant was measured by Schales’s modification
method. One unit was defined as an amount
of chitinase which produced 1 μmol of reducing
sugars as N-acetyl-D-glucosamine per min.

2.6 L-phenylalanine ammonia-lyase (PAL)
assay

To measure PAL activity, root tissue (about
5 g) was frozen in liquid N2, immediately
ground to a fine powder in the presence of
4 mL of 100 mM sodium borate buffer, pH 8.7,
containing 10 mM 2-mercaptoethanol, at 4 °C,
and the supernatant obtained by centrifugation
(20 000 x g, 20 min) was used as a crude en-
zeyme solution. The activity was measured by
method of Inui, et al. One unit is defined as
the amount of enzyme that converts 1 μmol of
L-phenylalanine to trans-cinnamic in 1 min.
2.7 Fractionation of degraded chitosan

A stirred ultrafiltration cell (model 8400, Millipore Co., USA) was employed for fractionation of degraded chitosan. A series of cellulose ultrafiltration membranes, YM (Mw cut-off 1000 (YM1), 3000 (YM3), 10000 (YM10) and 30000 (YM30)) (Millipore Co., USA) were used with 0.1 M acetic acid solution. The content of oligochitosan fractions was determined by ninhydrin method\(^\text{(14)}\). Each fraction was further purified through precipitation with ethanol.

3. Results and Discussion

3.1 Effect of irradiated chitosan on crop plant

Previously, we reported that when chitosan in 10\% solution was irradiated at 100 kGy and added to the culture media, the highest plant growth activity was shown on flower plants. Therefore in the present study, the effect of irradiated chitosan was studied further using crop plant, namely barley. The effect of irradiation dose for chitosan on the growth of barley was investigated at first and the results were shown in Fig. 1. The unirradiated chitosan inhibited the growth of barley, while the degraded products of chitosan irradiated from 25 to 200 kGy showed a positive effect on the plant growth. The highest growth promotion effect was found in the sample irradiated at 100 kGy (Mw approx. 16 kDa). Since the effect of degraded polysaccharides on plants has been reported to be highly dependent on the size of molecular weight\(^\text{(4,11)}\), the results suggested that the chitosan product irradiated at 100 kGy contained a high yield of active fraction for plant growth activity.

3.2 Fractionation

Using four kinds of UF membranes, the irradiated chitosan products at each dose was separated into 5 fractions, F\(_1\) : with Mw less than 1 kDa, F\(_2\) : with Mw of 1-3 kDa, F\(_3\) : with Mw of 3-10 kDa, F\(_4\) : with Mw of 10-30 kDa and F\(_5\) : with Mw more than 30 kDa. Figure 2 showed that the fraction ratio of five separated fractions in each product showed different profiles in the composition of molecular weight distribution. The contents of high Mw fraction (F\(_4\) and F\(_5\)) were decreased along with the increase of the irradiation dose. The contents of fraction F\(_2\) and F\(_3\) were the highest in the product irradiated at 100 kGy. The 100 kGy
irradiated product, without separation showed higher promotion activity for plant growth (Fig. 1 and our previous paper[1]) and in this product, the content of fraction F2 with Mw in range of 1 - 3 kDa was the highest (25.9%). Therefore, we applied these fractions separated from 100 kGy irradiated product to study further activity for growth promotion on barley and soybean plants.

3.3 Biological activity of irradiated chitosan fractions

The activity of separated chitosan fractions was tested on barley and soybean and the obtained data was shown in Fig. 3 and 4. The results indicated that the treatments with fractions F3 (Mw in range of 3 - 10 kDa) and F4 (Mw in range of 10 - 30 kDa) did not show any significant effect compared to that of the untreated control. The fraction F5 (Mw > 30 kDa) inhibited the growth of both tested plants. The fraction F1 (Mw < 1 kDa) showed a positive effect, and the fraction F2 with Mw of 1 - 3 kDa showed the highest effect in comparison to the other fractions as well as to the unseparated product. Therefore, using fraction F2, we studied an optimum concentration of this fraction for growth promotion activity for barley.

The effect of concentration of fraction F2 and unseparated chitosan products on the growth of barley were tested and the results were shown in Fig. 5. It was shown that the optimum concentration of the effective fraction was obtained at 20 mg/L and was much lower than that of unseparated sample (50 mg/L).

The oligomers of chitosan obtained by enzymatically degradation have been reported to elicit defense responses in various plants. In particularly, oligochitosan activated plant defensive genes through the octadecanoid pathway. Oligochitosan also reported to elicit peroxidase, chitinase and PAL in plants[15,16]. It was reported that PAL and chitinase are phytoalexin enzymes, which help plants to prevent the infection of microbial diseases. In present
study, the activity of phytoalexin enzymes, namely PAL and chitinase, was investigated for irradiated chitosan fraction F2 and the data was shown in Table 1. Fraction F2 showed a significant effect on the increase of PAL activity (87%) and chitinase activity (168%) compared to that of the untreated control as well as of the unseparated product. That irradiated chitosan oligomers showed not only plant growth promotion effect but also enhanced the activity of phytoalexin enzymes. The results are in agreement with those in previous paper\textsuperscript{2,3,11,15,16). In addition, degraded chitosan was also reported to increase the seed germination rate (6%), dry weight (8%) and the crop yield (10 – 12%) of soybean over the control\textsuperscript{6). Therefore, we applied the optimum fraction F2 (Mw of 1 – 3 kDa) of irradiated chitosan for soybean plant to study crop enhancement effect. Table 2 indicated that the treatment with F2 showed a significant increase in crop yield of soybean (15.8%) compared to that of the untreated one.

In conclusion, it was shown that irradiated chitosan was a promising product to increase the growth of crop plants. Especially, the irradiated chitosan fraction F2 with Mw in range of 1 – 3 kGy was presumed to contain effective compounds for the growth promotion, to enhance activity of phytoalexin enzymes, and to increase crop yield of plants.

**Acknowledgments**

We would like to express our sincere and special gratitude to Mr. K. Ikushima, Musashi Engineering, Co, who was kind enough to provide us a research funding to perform this study. The authors also like to thank Dr. Fumio Yoshii, Dr. Tamikazu Kume and Mr. Toshiaki Yagi of Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute for valuable discussions.
References


要 旨

放射線分解したキトサンの植物生育促進効果

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キトサンを 60Co からの γ 線で照射し、植物生育促進効果を示した 100 kGy の放射線分解産物を
分離し、分子量が 1 〜 3 kDa のフラクションがダイズ、オオムギに高い植物生育促進効果を示す
ことがわかった。このフラクションの生育活性は分離前（50 mg/L）よりも低い濃度（20 mg/L）
で生育を促進することが示された。また、ファイトアレキシンに関与するフィニルアラニンアンモ
ニアリアーゼ（PAL）とキチナーゼ活性を増加させた。放射線分解産物で得られた本フラクショ
ンは植物生育のみならず、防護剤としての作用も示唆された。またこのフラクションはダイズの収
量を増加させる効果も示した。