

# Partially N-Deacetylated Chitin Elicitor Induces Antimicrobial Flavonoids in Pea Epicotyls

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Pisatin elicitors including fragments of chitosan and partially N-deacetylated chitins (DACs) strongly induced (+)- $\alpha$ ,2',4,4'-tetrahydroxydihydrochalcone, (–)-4',7-dihydroxyflavanone, and 4,4'-dihydroxy-2'-methoxychalcone in *Pisum sativum*. These three flavonoids with moderate or weak antimicrobial activities were effectively induced by the treatment of pea with either the biotic elicitors or an abiotic elicitor  $\text{CuCl}_2$ . The examination of chitosan derivatives with different molecular size and different N-acetylation for the flavonoid-inducing activity in pea epicotyl assay revealed that the structural features required for the flavonoids induction were different from those required for (+)-pisatin induction. (+)-2-Hydroxypisatin, a possible intermediate in pisatin degradation pathway, which has been isolated from pea seedlings treated with the abiotic elicitor or infected by fungi was not induced by the chitosaccharides in pea epicotyl assay. These findings suggest that pea plants could distinguish invading pathogens by the difference of structural features of chitosaccharides derived from chitin, a major constituent of fungal cell walls. Thereby, the plant could accumulate antimicrobial compounds responsible for the chemical defense against invading pathogens.

## Introduction

Elicitor-active oligosaccharide fragments derived from the constituents of fungal cell walls including chitin ( $\beta$ -1,4-linked N-acetyl-D-glucosaminoglycan) and  $\beta$ -glucan strongly induce phytoalexins (Ryan, 1988; Darvill *et al.*, 1992). In *Pisum sativum*, chitin-derived fragments have been shown to induce (+)-pisatin (Kobayashi *et al.*, 1994a; Hadwiger and Beckman, 1980; Kendra and Hadwiger, 1984). (+)-Pisatin, which has been reported to have a broad spectrum of antimicrobial activity, could play an important role in the chemical defense of *Pisum sativum* (Perrin and Bottomley, 1962). The phytoalexin must be degraded rapidly after the chemical defense is no longer required because it is fungitoxic as well as phytotoxic.

We previously reported that (+)-2-hydroxypisatin, which appeared to be an intermediate in the degradation pathway of (+)-pisatin, was isolated

from  $\text{CuCl}_2$ -treated pea seedlings (Kobayashi *et al.*, 1993a), and recently that potent elicitors such as  $\text{NaNO}_2$ -degraded fragments of chitosan ( $\beta$ -1,4-linked D-glucosaminoglycan) and partially N-deacetylated chitins (DACs) induced several unidentified phenolic compounds together with (+)-pisatin in pea epicotyl assay (Kobayashi *et al.*, 1994a). Such elicitor assay system using the chitosan derivatives as elicitors could provide a useful tool for the research on both the regulation of flavonoid phytoalexin production and its degradation pathway.

In this paper, we conducted the characterization of the unidentified compounds and the evaluation of their antimicrobial activities. Further, the chitosan derivatives with different molecular size and different degree of N-acetylation were examined for the inducing activity of the compounds to clarify the structural features of the saccharides required for elicitor activity.

## Materials and Methods

### Chemicals

Chitin was purchased from Wako Pure Chemical Industries, Ltd. Chitosan with the degree of N-

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acetylation 0% was obtained from Funakoshi Co., Ltd. Partially N-deacetylated chitins with the degree of N-acetylation 32% and 56% and their intermediate-sized fragments were prepared as reported previously (Kobayashi *et al.*, 1994a). A mixture of chitosan monomer through nonamer and their 37, 66 and 91% N-acetylated derivatives were prepared as described previously (Kobayashi *et al.*, 1994a).

#### Induction and isolation of compound **1**

[(+)- $\alpha$ ,2',4,4'-tetrahydroxydihydrochalcone], compound **2** [(-)-4',7-dihydroxyflavanone], and compound **3** [4,4'-dihydroxy-2'-methoxychalcone]

Compounds **1** and **3** were induced by CuCl<sub>2</sub> treatment of pea seedlings (Kobayashi *et al.*, 1993a). The two compounds were also induced by the treatment of pea seedlings with elicitor-active chitosan derivatives. Elicitor-treated pea seedlings were transferred to a large flask containing MeOH. After being allowed to stand for 48 h, the MeOH extract was evaporated to give a thick aq. solution, and was then partitioned with EtOAc. The organic phase was evaporated to dryness, providing 3.45 g of a crude syrup. The syrup was chromatographed on a silica gel [Wakogel C-100 (Wako Pure Chemical Industries, Ltd.)] column eluted stepwise with solvents of increasing polarity from *n*-hexane through EtOAc. The fractions eluted with 80% EtOAc-*n*-hexane (307.5 mg) were rechromatographed on an ODS [YMC·GEL ODS-AQ 120-50 S (YMC Co., Ltd.)] column employing gradient elution from H<sub>2</sub>O through MeOH. The fractions eluted with 50% H<sub>2</sub>O-MeOH, 5.84 mg of **1** was obtained. Ninety-five percent methanol eluates were further purified by a silica gel [Wakogel C-200 (Wako Pure Chemical Industries, Ltd.)] column eluted stepwise with solvents of increasing polarity from benzene through acetone, and finally by a preparative TLC [Kiesel-gel 60 F<sub>254</sub> (Merck)] with benzene-acetone (9:1), 1.27 mg of **3** was obtained.

Compound **2** was induced by the similar procedure as pea epicotyl elicitor assay (Kobayashi *et al.*, 1994a). Five-mm sections of epicotyls were prepared from *ca.* 200 of 10-day-old pea seedlings grown under an aseptic condition and transferred into 300 ml Erlenmeyer flasks containing 100 ml of 100  $\mu$ g/ml aqueous NaNO<sub>2</sub>-degraded chitosan

solution sterilized by autoclaving for 5 min. The flasks were incubated in the dark at 24 °C on a gyratory shaker at 120 rpm. After 24 h incubation, the flasks were filled with MeOH. After being allowed to stand for 24 h, the MeOH extract was evaporated to give a thick aq. solution, and was then partitioned with EtOAc. The organic phase was evaporated to dryness, providing 78.8 mg of a crude syrup. The syrup was chromatographed on a silica gel [Wakogel C-300 (Wako Pure Chemical Industries, Ltd.)] column eluted stepwise with solvents of increasing polarity from benzene through acetone. The fraction eluted with 20% acetone-benzene (8.58 mg) were purified by preparative HPLC using DAISOPAK SP-120-5-ODS column ( $\varnothing$  10 $\times$ 250 mm, DAISO Co., Ltd.) employing gradient elution from 30% MeOH-H<sub>2</sub>O through 90% MeOH-H<sub>2</sub>O at a flow rate of 3.0 ml/min. The gradient was achieved within 35 min. Absorbance at 360 nm was monitored. A peak with *Rt* 26.7 min was collected, and 1.10 mg of **2** was obtained.

#### Compound **1**

[(+)- $\alpha$ ,2',4,4'-tetrahydroxydihydrochalcone]

EIMS (direct inlet) 70 eV *m/z* (rel. int.): 274 [M]<sup>+</sup> (2), 256 [M-H<sub>2</sub>O]<sup>+</sup> (27), 168 [M-106]<sup>+</sup> (24), 137 [M-137]<sup>+</sup> (100), 107 [M-167]<sup>+</sup> (88). [ $\alpha$ ]<sub>D</sub><sup>22</sup> +109.8° (MeOH; *c* 0.1).  $\lambda_{\max}^{\text{MeOH}}$  nm (log  $\epsilon$ ): 227 sh (4.07), 279 (4.08), 316 (3.81).  $\lambda_{\max}^{\text{MeOH}+\text{NaOH}}$  nm (log  $\epsilon$ ): 243 (4.02), 335 (4.33).  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 3429, 1633, 1515, 1454, 1446, 1329, 1246, 1175, 1106, 1079, 823, 548. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$ : 2.88 (1H, dd, *J* = 7.6, 14.0 Hz, H- $\beta$ ), 3.06 (1H, dd, *J* = 4.8, 14.0 Hz, H- $\beta'$ ), 5.19 (1H, dd, *J* = 4.8, 7.6 Hz, H- $\alpha$ ), 6.32 (1H, d, *J* = 2.4 Hz, H-3'), 6.38 (1H, dd, *J* = 2.4, 8.9 Hz, H-5'), 6.71 (2H, d, *J* = 8.5 Hz, H-3, 5), 7.06 (2H, d, *J* = 8.5 Hz, H-2, 6), 7.74 (1H, d, *J* = 8.9 Hz, H-6'). NOESY;  $\delta$  6.38- $\delta$  7.74;  $\delta$  7.74- $\delta$  5.19;  $\delta$  5.19- $\delta$  7.74,  $\delta$  3.06;  $\delta$  3.06- $\delta$  5.19,  $\delta$  2.88;  $\delta$  2.88- $\delta$  3.06,  $\delta$  7.06;  $\delta$  7.06- $\delta$  2.88,  $\delta$  6.71;  $\delta$  6.71- $\delta$  7.06.

#### Compound **2** [(-)-4',7-dihydroxyflavanone]

EIMS (direct inlet) 70 eV *m/z* (rel. int.): 256 [M]<sup>+</sup> (100), 137 (58), 120 (43). [ $\alpha$ ]<sub>D</sub><sup>24</sup> -34.5° (MeOH; *c* 0.1).  $\lambda_{\max}^{\text{MeOH}}$  nm (log  $\epsilon$ ): 275 (4.04), 310 (3.73).  $\lambda_{\max}^{\text{MeOH}+\text{NaOH}}$  nm (log  $\epsilon$ ): 249 (4.12), 326 sh

(4.26), 336 (4.30).  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3 + \text{CD}_3\text{OD}$ )  $\delta$ : 2.73 (1H, dd,  $J = 3.1, 17.0$  Hz, H-3), 3.07 (1H, dd,  $J = 13.1, 17.0$  Hz, H-3'), 5.39 (1H, dd,  $J = 3.1, 13.1$  Hz, H-2), 6.40 (1H, d,  $J = 2.3$  Hz, H-8), 6.52 (1H, dd,  $J = 2.3, 8.7$  Hz, H-6), 6.87 (2H, d,  $J = 8.6$  Hz, H-3', 5'), 7.34 (2H, d,  $J = 8.6$  Hz, H-2', 6'), 7.77 (1H, d,  $J = 8.7$  Hz, H-5).

#### Compound **3** [4,4'-dihydroxy-2'-methoxychalcone]

EIMS (direct inlet) 70 eV  $m/z$  (rel. int.): 270  $[\text{M}]^+$  (71), 255  $[\text{M}-\text{CH}_3]^+$  (36), 164 (79), 151 (100).  $\lambda_{\text{max}}^{\text{MeOH}}$  nm (log  $\epsilon$ ): 236 (3.74), 348 (4.05).  $\lambda_{\text{max}}^{\text{MeOH}+\text{NaOH}}$  nm (log  $\epsilon$ ): 253 (3.71), 417 (4.19).  $^1\text{H}$  NMR (500 MHz, acetone- $d_6$ )  $\delta$ : 3.91 (3H, s, 2'-O-Me), 6.51 (1H, dd,  $J = 2.3, 8.4$  Hz, H-5'), 6.57 (1H, d,  $J = 2.3$  Hz, H-3'), 6.89 (2H, d,  $J = 8.7$  Hz, H-3, 5), 7.47 (1H, d,  $J = 15.0$  Hz, H- $\alpha$ ), 7.55 (1H, d,  $J = 15.0$  Hz, H- $\beta$ ), 7.57 (2H, d,  $J = 8.7$  Hz, H-2, 6), 7.59 (1H, d,  $J = 8.4$  Hz, H-6').

#### Elicitor bioassay

Pea epicotyl elicitor assay and HPLC analysis of induced compounds were performed as described previously (Kobayashi *et al.*, 1994a). Retention times of (+)- $\alpha,2',4,4'$ -tetrahydroxydihydrochalcone, (-)-4',7-dihydroxyflavanone, 4,4'-dihydroxy-2'-methoxychalcone, and (+)-2-hydroxypisatin are 26.2, 28.8, 31.8, and 28.7 min, respectively.

#### Antimicrobial assay

The antimicrobial assay was previously described (Kobayashi *et al.*, 1994b).

## Results

#### Identification of three flavonoids induced by the treatment with elicitor-active fragments of chitosan and DACs

Three unidentified phenolic compounds, **1**, **2**, and **3** were induced in pea epicotyls by the treatment with potent inducers of (+)-pisatin such as  $\text{NaNO}_2$ -degraded fragments of chitosan and DACs as shown in Fig. 1. The compounds, **1** and **3** were also induced by the treatment of pea seedlings grown under not an aseptic condition with either aq.  $\text{CuCl}_2$  solution (5 mM) or  $\text{NaNO}_2$ -degraded chitosan and DACs (100  $\mu\text{g}/\text{ml}$ ), and identified respectively as (+)- $\alpha,2',4,4'$ -tetrahydroxydihydrochalcone (Ferrari *et al.*, 1983) and 4,4'-dihydroxy-

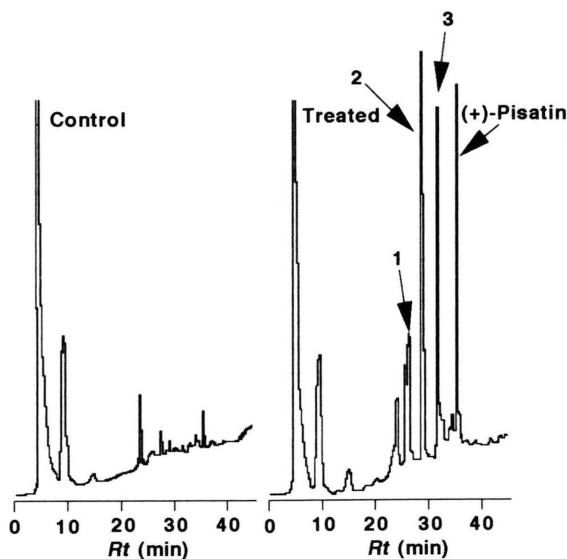


Fig. 1. HPLC profiles of flavonoids and (+)-pisatin accumulated in pea epicotyl sections treated with  $\text{NaNO}_2$  degraded chitosan (100  $\mu\text{g}/\text{ml}$ ) for 48 h.  $R_t$ , retention time.

2'-methoxychalcone (Carlson and Dolphin, 1982; Fig. 2). The substitution of the alcoholic hydroxyl group at C- $\alpha$  in **1** was confirmed by NOEs observed between H- $\beta$  ( $\delta$  2.88) and H-2, 6 ( $\delta$  7.06), H- $\alpha$  ( $\delta$  5.19) and H-6' ( $\delta$  7.74), together with the fragments with  $m/z$  168 in EIMS spectrum (Fig. 2). The isolation of (+)- $\alpha,2',4,4'$ -tetrahydroxydihydrochalcone is the first example in *Pisum sativum*. (+)-2-Hydroxypisatin was also isolated from the elicitor-treated pea seedlings as reported previously (Kobayashi *et al.*, 1993a). The compounds **1** and **3** were effectively induced in the pea seedlings treated by the elicitors. However, compound **2** was not found in this method, but found in the higher level in the pea epicotyl system. The identity of this compound, (-)-4',7-dihydroxyflavanone [(-)-liquiritigenin] was established by the comparison of spectral data reported (Mabry *et al.*, 1970). The antimicrobial potentialities of these compounds were evaluated by the assay using *Bacillus subtilis* and *Aspergillus candidus*. MICs (the minimum inhibitory concentrations) of these compounds are 250, 500, and 125  $\mu\text{g}/\text{ml}$  against *Bacillus subtilis* and 500, >500, and 500  $\mu\text{g}/\text{ml}$  against *Aspergillus candidus*, respectively. (+)-Pisatin totally inhibited spore germination of the

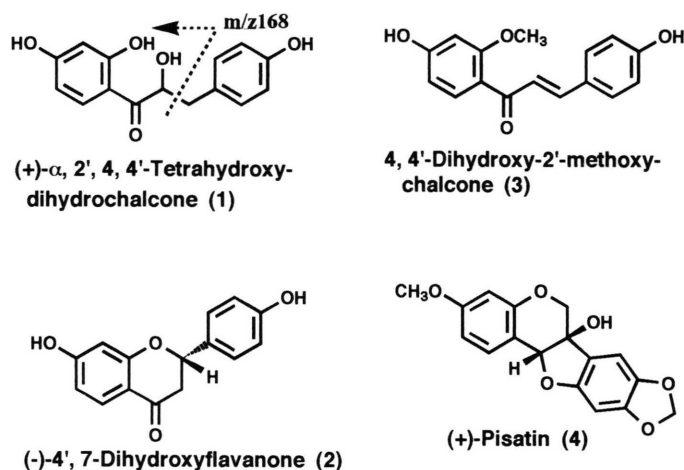


Fig. 2. Flavonoids induced by the treatment with elicitor-active fragments of chitosan and DACs.

fungus at 7.8  $\mu\text{g/ml}$  and the growth of the bacterium at 250  $\mu\text{g/ml}$ .

#### *Time course for the accumulation of the three flavonoids after treatment with elicitors*

Time courses for the accumulation of the (+)- $\alpha$ , 2', 4, 4'-tetrahydroxydihydrochalcone, (-)-4', 7-dihydroxyflavanone, and 4, 4'-dihydroxy-2'-methoxychalcone after treatment of pea epicotyls with elicitors were determined (Fig. 3). The  $\text{NaNO}_2$ -degraded chitosan (100  $\mu\text{g/ml}$ ) was employed as the elicitor. These three compounds were maximally accumulated at 72, 24, and 48 h, respectively. The amounts at the accumulation maxima of (-)-4', 7-dihydroxyflavanone and 4, 4'-dihydroxy-2'-methoxychalcone were comparable to that of (+)-pisatin, while (+)- $\alpha$ , 2', 4, 4'-tetrahydroxydihydrochalcone was detected in very low level (*ca.* 20  $\mu\text{g/g fr. wt.}$ ). A trace amount of these three compounds was also induced in the control by the wound stress. (+)-2-Hydroxypisatin was not found in this time course experiments.

#### *Flavonoid-inducing activity of chitosan, DACs, and chitin*

Polysaccharides including chitosan (degree of N-acetylation 0%), DAC 32%, DAC 56%, and chitin were tested for the flavonoid-inducing activity in 2 and 4 mg/ml suspensions (Fig. 4).

The most prominent induction of the three compounds was seen in the two DACs. These polysaccharides induced 4, 4'-dihydroxy-2'-methoxychalcone

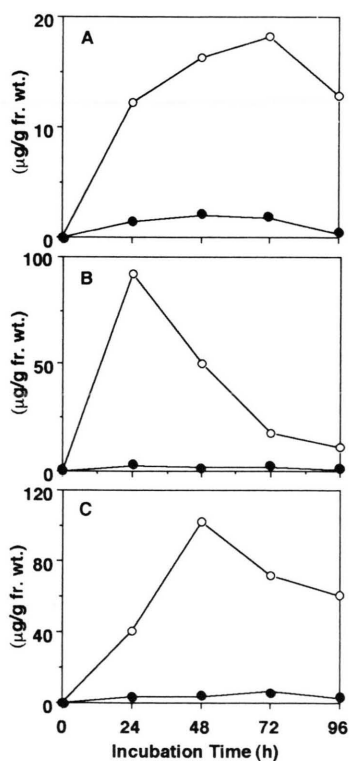


Fig. 3. Time course of the accumulation of (+)- $\alpha$ , 2', 4, 4'-tetrahydroxydihydrochalcone (A), (-)-4', 7-dihydroxyflavanone (B), and 4, 4'-dihydroxy-2'-methoxychalcone (C) in pea epicotyl sections treated by  $\text{NaNO}_2$ -degraded chitosan (100  $\mu\text{g/ml}$ ).  $\text{NaNO}_2$ -degraded chitosan 100  $\mu\text{g/ml}$  (○), control (●).

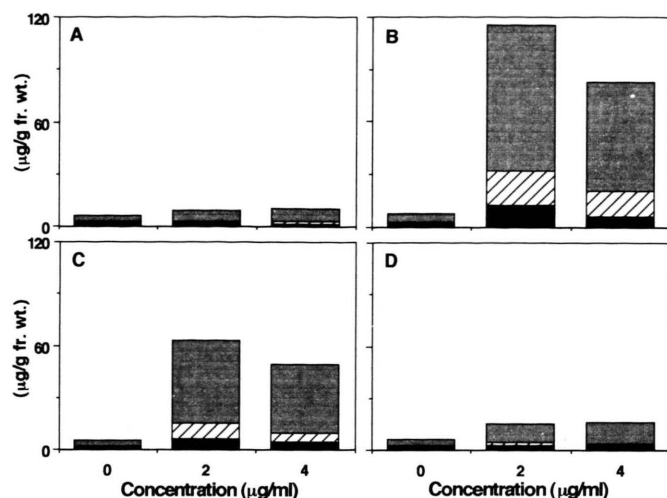


Fig. 4. Flavonoid-inducing activity of chitosan (degree of N-acetylation 0%, A), DAC 32% (partially N-deacetylated chitin with degree of N-acetylation 32%, B), DAC 56% (partially N-deacetylated chitin with degree of N-acetylation 56%, C), and chitin (D). (+)- $\alpha,2',4,4'$ -Tetrahydroxydihydrochalcone (■), (-)-4',7-dihydroxyflavanone (▨), 4',4'-dihydroxy-2'-methoxychalcone (▩).

cone in an especially high level. The DAC 32% with a lower degree of N-acetylation was more active than DAC 56%, especially at the lower concentration. Chitosan and chitin suspensions showed little or no activities.

#### Flavonoid-inducing activity of $\text{NaNO}_2$ -degraded chitosan and DACs

Intermediate-sized fragments obtained by limited  $\text{NaNO}_2$  degradation of the chitosan, DAC 32%, and DAC 56% were examined for the inducing activities of the three flavonoids at concentrations ranging from 7.8 to 250  $\mu\text{g/ml}$  (Fig. 5).

The  $\text{NaNO}_2$ -degraded DAC 32% was the most potent elicitor among these saccharides. The fragment markedly induced the flavonoids at 62.5, 125, and 250  $\mu\text{g/ml}$ . Elicitor activity of the  $\text{NaNO}_2$ -degraded chitosan was comparable to that of  $\text{NaNO}_2$ -degraded DAC 32%. Only at the concentration of 250  $\mu\text{g/ml}$ , the  $\text{NaNO}_2$ -degraded DAC 56% fragments showed a strong activity.

#### Flavonoid-inducing activity of chitosan oligomers with different degrees of N-acetylation

The chitosan oligomer fraction containing monomer through nonamer as well as their 37, 66 and 91% N-acetylated derivatives were assayed for the flavonoid-inducing activities at concentrations ranging from 7.8 to 250  $\mu\text{g/ml}$  (Fig. 6). Both the original chitosan oligomer mixture and the 37% N-acetylated derivative showed a moder-

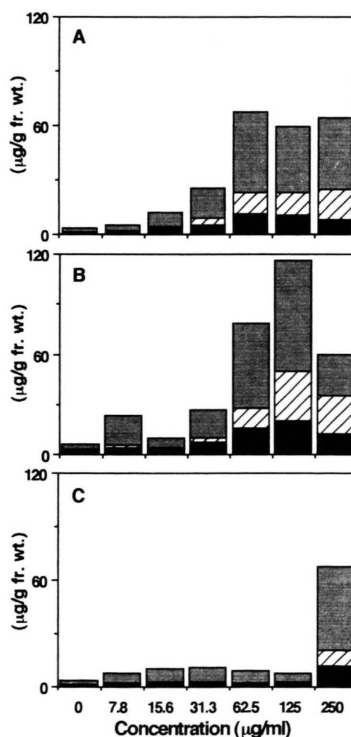


Fig. 5. Flavonoid-inducing activity of  $\text{NaNO}_2$ -degraded fragments derived from chitosan (degree of N-acetylation 0%, A), DAC 32% (partially N-deacetylated chitin with degree of N-acetylation 32%, B), and DAC 56% (partially N-deacetylated chitin with degree of N-acetylation 56%, C). (+)- $\alpha,2',4,4'$ -tetrahydroxydihydrochalcone (■), (-)-4',7-dihydroxyflavanone (▨), 4',4'-dihydroxy-2'-methoxychalcone (▩).



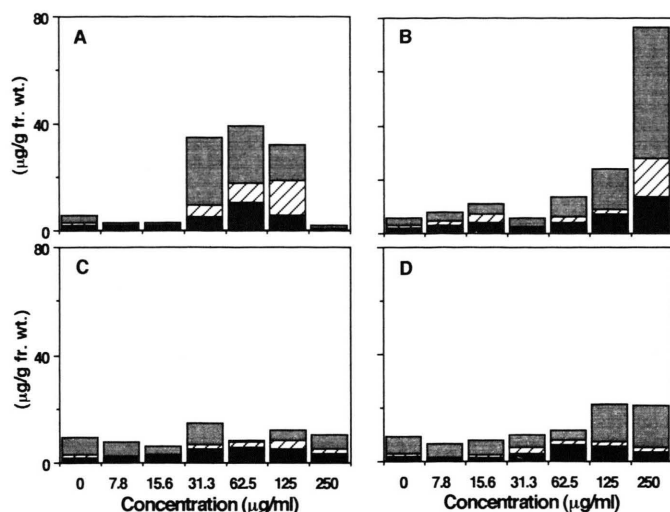


Fig. 6. Flavonoid-inducing activity of chitosan oligomers with degree of N-acetylation 5% (A), 37% (B), 66% (C), and 91% (D). (+)- $\alpha$ ,2',4,4'-tetrahydroxydihydrochalcone (■), (-)-4',7-dihydroxyflavanone (▨), 4,4'-dihydroxy-2'-methoxychalcone (□).

ate activity among the samples tested. The former exhibited activities at 31.3, 62.5, and 125  $\mu\text{g/ml}$ , the latter only at 250  $\mu\text{g/ml}$ . The amounts of the flavonoids induced by the treatment with the original chitosan oligomer mixture and the 37% N-acetylated derivative were  $\frac{1}{2}$  to  $\frac{1}{3}$  of those induced by the DACs and  $\text{NaNO}_2$ -degraded fragments. Neither the 61% N-acetylated derivative nor the 91% N-acetylated derivative exhibited elicitor activities.

## Discussion

Phytoalexin accumulation is one of the most important defense system in higher plants. A pterocarpanoid phytoalexin, (+)-pisatin, is accumulated as a major phytoalexin in *Pisum sativum* upon either fungal invasions or elicitor treatments (Kobayashi *et al.*, 1993a). In addition to (+)-pisatin, several phytoalexins and stress metabolites have been characterized in *Pisum sativum*. Three pterocarpanoid phytoalexins have been identified from pea epicotyls infected with the fungus *Fusarium solani* f. sp. *pisi* (Pueppke and VanEtten, 1975). HPLC analysis of isoflavonoid accumulation in pea seedlings treated with an abiotic elicitor  $\text{CuCl}_2$  revealed the induction of intermediates in (+)-pisatin biosynthesis together with 4,4'-dihydroxy-2'-methoxychalcone, obtustyrene and xenognosin as stress metabolites (Carlson and Dolphin, 1981, 1982). These compounds might

play an important role in the chemical defense of pea plants. However, their significance in disease resistance and induction mechanisms are not thoroughly investigated. We recently reported that pisatin elicitors including fragments of both chitosan and DACs strongly induced several unidentified phenolic compounds together with pisatin in pea epicotyl assay (Kobayashi *et al.*, 1994a). The phenolics with weak or moderate antimicrobial activities against *Bacillus subtilis* and *Aspergillus candidus* were identified as (+)- $\alpha$ ,2',4,4'-tetrahydroxydihydrochalcone, (-)-4',7-dihydroxyflavanone, 4,4'-dihydroxy-2'-methoxychalcone. These compounds have also been reported as antimicrobial constituents in other plants (Ferrari *et al.*, 1983; Achenbach *et al.*, 1988).

Chitin and chitosan derivatives with a variety of molecular size and N-acetylation were examined for their antimicrobial flavonoid-inducing activity in pea epicotyl assay. The results revealed that the structural features required for the flavonoids induction were different from those required for pisatin induction. In the each class of the polysaccharides, intermediate-sized fragments, and oligosaccharides, DAC 32%,  $\text{NaNO}_2$ -degraded DAC 32%, and chitosan oligomer mixture containing monomer through nonamer possessed the most potent elicitor activity, respectively. We have reported that DAC 56%,  $\text{NaNO}_2$ -degraded chitosan, and the chitosan oligomer mixture exhibited the strongest activity in (+)-pisatin induction (Kobaya-

shi *et al.*, 1994a). This suggests that pea plants could distinguish challenging pathogens by the difference of structural features of fungal cell wall components, chitosaccharides. Thereby, the plant could produce antimicrobial compounds responsible for the chemical defense against invading pathogens. In our preliminary experiments using cotyledons of *Phaseolus vulgaris* as a model plant, we found that elicitor-active chitosan and DAC fragments caused severe browning on the cut surface of cotyledons and induced several phenolic compounds. The plant is known to produce prenylated phenolics, suggesting the possibility that the chitosaccharides could activate the biosynthesis of a different type of phenolics. The examination of the chitosan derivatives for species-specific elicitation in *Phaseolus vulgaris* and *Medicago sativa* is being undertaken in the assay systems (Kobayashi *et al.*, 1993b, 1993c).

(+)-2-Hydroxypisatin, a possible intermediate in (+)-pisatin degradation pathway in *Pisum sativum*, was not induced by the treatment with elicitor-active chitosan derivatives in the pea epicotyl assay (Kobayashi *et al.*, 1993a, Fig. 7). 2-Hydroxypisatin has been obtained from pea seedlings treated with an abiotic elicitor  $\text{CuCl}_2$  and also found in the pea seedlings obtained without seed sterilization process. This suggests that the compound might be not

only an intermediate in pisatin degradation pathway but also might play an important role in the chemical defense of pea plants. Most of pisatin induced by the elicitors was released from the epicotyl sections into the aqueous media in pea epicotyl assay. It is notable that 2-hydroxypisatin had much stronger phytotoxicity than pisatin. This compound might be rapidly converted into non-phytotoxic conjugates such as glycosides or malonylglycosides. Further research on 2-hydroxypisatin conversion should be conducted.

The three flavonoids as well as pisatin are thought to be biosynthesized *via* 2',4,4'-trihydroxychalcone (6) as a common intermediate (Fig. 7). The biosynthetic pathway of  $\alpha$ -hydroxydihydrochalcones including (+)- $\alpha$ ,2',4,4'-tetrahydroxydihydrochalcone (1), a new metabolite in pea plants, is not thoroughly understood because the natural occurrence of  $\alpha$ -hydroxydihydrochalcones are restricted to a few species (Malan and Swinny, 1990). There is no evidence to support that the  $\alpha$ -hydroxydihydrochalcone (1) and 2',4,4'-trihydroxychalcone (6) are biosynthetically related. However, the structural similarity of the two compounds suggests that the chalcone (6) is a precursor of the  $\alpha$ -hydroxydihydrochalcone (1). (–)-4',7-Dihydroxyflavanone (2) found in many spp. of Leguminosae, is a biosynthetic intermedi-

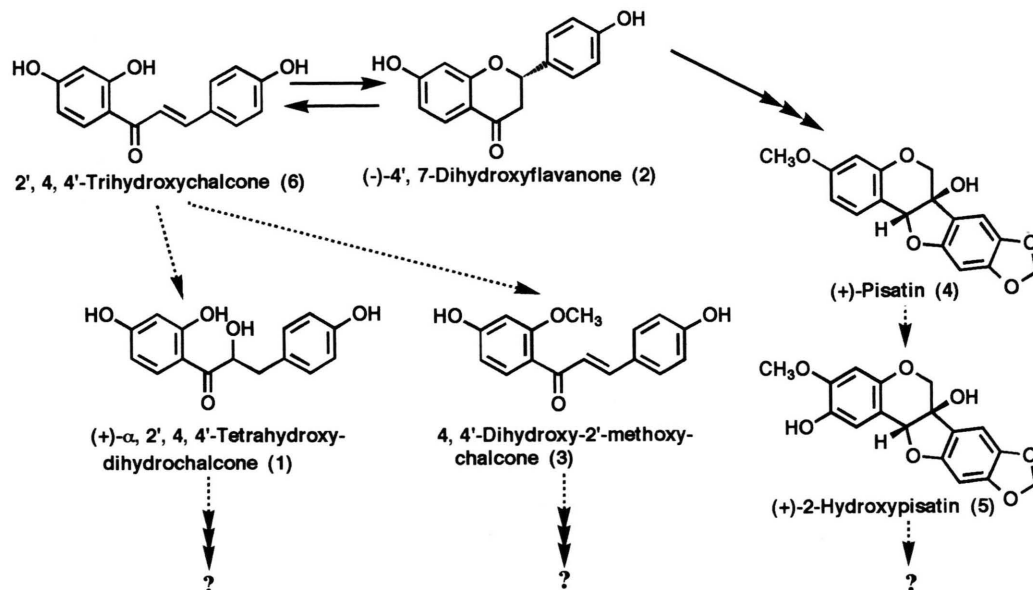


Fig. 7. The possible biosynthetic pathway of flavonoids induced by elicitor treatment of *Pisum sativum*.

ate of pterocarpanoid phytoalexins and reported to be induced as a stress metabolite in *Medicago sativa* following treatment with a pyridylaminated oligo- $\beta$ -glucoside elicitor (LN-3), prepared from an enzymatic hydrolysate of laminaran (Kobayashi *et al.*, 1993b). It is interesting that the time of accumulation maximum of **2** preceded that of (+)-pisatin (**4**) by 24 h. 4,4'-Dihydroxy-2'-methoxychalcone (**3**) is thought to be synthesized by 2'-O-methylation of 2',4,4'-trihydroxychalcone (**6**). The 2'-methoxychalcone (**3**) does not appear to be an intermediate in isoflavonoid synthesis since the formation of the isoflavone pyrane ring requires a free 2'-hydroxyl on the chalcone as mentioned previously (Carlson and Dolphin, 1982). Several derivatives of the  $\alpha$ -hydroxydihydrochalcone (**1**) and the 2'-methoxychalcone (**3**) have been found in other plants (Malan and

Swinny, 1990; Bezuidenhoudt *et al.*, 1981; Mabry *et al.*, 1970), suggesting that new metabolites originated from the two compounds may occur in pea plants. To clarify the networks of flavonoid biosynthesis responsible for the chemical defenses in pea plants, the chitosaccharide elicitor could be a useful tool for further investigation.

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