Occurrence of Avenanthramides and Hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase Activity in Oat Seeds

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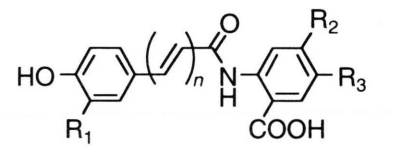
Z. Naturforsch. **55c,** 30–36 (2000); received October 26/November 5, 1999

Avena sativa, Oats, Biosynthesis, Phytoalexin, Avenanthramide

Oat phytoalexins, avenanthramides, occur as constitutive components in seeds. The amounts of each avenanthramide were analyzed. The composition of avenanthramides in dry seeds was different from that in elicitor-treated leaves. In seeds, avenanthramide C was most abundant with an amount two times larger than that of avenanthramide A or B. On the other hand, avenanthramide A was the major component in elicitor-treated leaves. The total amount of avenanthramides in seeds increased 2.5 times during imbibition for 48 h although the composition did not change. The hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT, EC 2.3.1.-) activity, which is responsible for the final condensation step in the avenanthramide biosynthesis, was detected in dry seeds. The activity was localized in endosperm and scutellum, and slightly increased during 48-h imbibition. The enzyme was partially purified by anion exchange chromatography from both dry seeds and elicitor-treated leaves. The activity was separated into two peaks by chromatography, indicating that HHT consists of at least two isoforms. The substrate specificities of HHT isoforms from seeds were different from each other.

Introduction

Avenanthramides (**1–6**), a series of substituted *N*-cinnamoylanthranilic acids, have been well characterized as oat phytoalexins (Mayama *et al.*, 1982; Mayama *et al.*, 1981; Miyagawa *et al.*, 1995). They structurally belong to a group of hydroxycinnamic acid amides. Hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT, EC 2.3.1.-) which catalyses the condensation between anthranilic acids and hydroxycinnamoyl-CoA esters to form avenanthramides is induced in elicitor-treated oat leaves (Ishihara *et al.*, 1997; Ishihara *et al.*, 1998) as well as other upstream enzymes in the biosynthetic pathway (Ishihara *et al.*, 1999). An involvement of hydroxycinnamic acid amides in stress responses has also been found in barley. The production of *p*-coumaroyl- and feruloylagmatines, which are precursors to the antifungal compounds known as hordatines (Negrel and Smith, 1984), are reportedly induced by infection with an arbuscular mycorrhizal fungus (Peipp *et al.*, 1997) and a powdery mildew fungus (von Röpenack *et al.*, 1998). This induction has been reproduced by treatment with methyl jasmonate (Lee *et al.*, 1997).



		R_1	R_2	R_3	*n*
1:	Avenanthramide A	H	H	OH	1
2:	B	OMe	H	OH	1
3:	C	OH	H	OH	1
4:	D	H	H	H	1
5:	G	H	OH	H	1
6:	L	H	H	OH	2

Structures of avenanthramides.

On the other hand, the hydroxycinnamic acid amides have also been known to occur at specific stages of growth in the Gramineae family. Avenanthramides occur in oat groats and hulls as constitutive components (Collins, 1986; Collins, 1989; Col-

Abbreviations: HHT, hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase; THT, tyramine *N*-hydroxycinnamoyltransferase; $(GlcNAc)_5$, penta-*N*-acetylchitopentaose.

0939–5075/2000/0100–0030 $ 06.00

lins *et al.*, 1991). *p*-Coumaroylagmatine accumulates in barley shoots and roots immediately after germination together with the coupled dimers, hordatines A and B (Smith and Best, 1978). In maize, *N*-hydroxycinnamoyltyramines (Martin-Tanguy *et al.*, 1982) and *N*-hydroxycinnamoyltryptamines (Ehmann, 1974) were identified in reproductive organs. These findings indicated that the amide synthesis is activated at specific developmental stages although the biological functions are unclear.

In the present study, we investigated the accumulation of avenanthramides and the occurrence of HHT activity in oat seeds to elucidate the biochemical basis of dual expression, i.e., stress-dependent and development-dependent expressions, of amide compounds. In addition, HHT from both oat seeds and elicitor-treated leaves was partially purified and characterized.

Materials and Methods

Plant materials

Oat (*Avena sativa* L. cv. Shokan 1) seeds were used in the experiments after sterilization by 0.5% hypochlorous acid. The seeds were placed on two wet paper layers in a Petri dish, and incubated for 48 h at 20 °C in growth chambers under continuous fluorescent lights (40 W m^{-2}). The germinated seeds were divided with a razor blade, and enzyme activity was extracted. For the preparation of the enzyme solution from elicitor-treated leaves, the seeds were sown in wet vermiculite after an imbibition for 12 h. Thereafter, oat plants were grown for 7 days at 20 °C in growth chambers under continuous fluorescent lights (40 W m^{-2}). The elicitor treatment of oat leaves has been previously described (Ishihara *et al.*, 1998). Penta-*N*-acetylchitopentaose [$(GlcNAc)_5$, Seikagaku Kogyo, Tokyo, Japan] was used as a elicitor at 1 mM.

Chemicals

Avenanthramides A (**1**), B (**2**) and D (**4**) were synthesized according to the method of Collins (1989). The synthesis of avenanthramides C (**3**), G (**5**), L (**6**) and other related amides has been previously reported (Ishihara *et al.*, 1998; Miyagawa *et al.*, 1996; Miyagawa *et al.*, 1995). Hydroxycinnamoyl-CoA thioesters were prepared by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters (Stöckigt and Zenk, 1975).

Analyses of avenanthramides in seeds

Twenty oat seeds were homogenized with sea sand and extracted with 10 ml/g of methanol. After centrifugation (12,000×*g*, 4 °C, 15 min), the pellet was reextracted twice with 10 ml/g of methanol, and then the pooled supernatant was evaporated *in vacuo*. The residue was dissolved in 800 µl of methanol, and a 20 µl aliquot was analyzed by HPLC (column: Wakosil II 5C 18 HG, 4.6 mm i.d. × 150 mm long, Wako, Osaka, Japan). The following linear gradient elution system was applied: 0–60 min, 15–30% solvent A (water containing 0.5% trifluoroacetic acid) in acetonitrile; 60–75 min, 30% solvent A. Avenanthramides were detected at 340 nm.

Enzyme extraction and partial purification of HHT

Plant materials frozen in liquid nitrogen were ground with sea sand (40–80 mesh) and homogenized in 10 ml/g of 100 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.5) containing 14.4 mM mercaptoethanol. The homogenate was centrifuged (12,000×*g*, 4 °C, 10 min), and the supernatant was used as a crude enzyme solution. All operations were carried out at 4 °C. The crude enzyme solution was fractionated by $(NH_4)_2SO_4$ precipitation. The proteins that precipitated between 30 and 45% saturation were dissolved in 20 mM *tris-HCl* (tris[hydroxymethyl]aminomethane) buffer (pH 8.0) containing 14.4 mM mercaptoethanol (buffer A), and dialyzed against the same buffer. The solution was loaded on a DEAE Sepharose column (16 mm i.d. × 20 mm long, Pharmacia), and the proteins were eluted by a NaCl gradient (0–40 ml, 0 mM NaCl; 40–400 ml, 0–300 mM NaCl) in buffer A at a flow rate of 0.8 ml/min. Two peaks of activity were observed in the chromatogram. The active fractions corresponding to the two peaks were separately applied to a Mono-Q HR 5/5 column (Pharmacia). The bound proteins were eluted with a 0 to 250 mM NaCl linear gradient (200 ml) in buffer A at a flow rate of 0.5 ml/min. The active fractions were concentrated by ultrafiltration (Centriplus-10, Amicon). After adding 1 volume of glycerol, the enzyme solution was stored at −20 °C prior to characterization. For

estimation of the apparent molecular weight, parts of the active fractions obtained by anion exchange chromatography were separately applied onto a Superdex 75 HR 10/30 column (Pharmacia). Proteins were eluted with buffer A at a flow rate of 0.5 ml/min. The following proteins were used as molecular weight markers: transferrin (81 kDa), ovalbumin (43 kDa), myoglobin (17.6 kDa), ribonuclease A (13.7 kDa), and aprotinin (0.65 kDa).

Assay for HHT activity

The reaction mixture consisted of 10 µl of enzyme solsution, 10µl of 1 mM *p*-coumaroyl-CoA, 10 µl of 10 mM 5-hydroxyanthranilic acid and 70 µl of 100 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0). After 15 min incubation at 30 °C, the reaction was stopped by adding 20 µl of acetic acid. The mixture was filled up to 500 µl with methanol, and a 10 µl aliquot was analyzed by HPLC as described previously (Ishihara *et al.*, 1998). Apparent K_m and relative V_{max} values were obtained from the [s]/v versus [s] plots. The protein content in the enzyme solution was determined according to the method of Bradford (1976).

Results

Analyses of avenanthramide composition

Avenanthramide contents in dry seeds were analyzed by HPLC (Table I), and compared with those in leaves treated with $(GlcNAc)_5$ at 1 mM which was reported previously (Ishihara *et al.*, 1998). The total amount of avenanthramides (**1–6**) in seeds was one-fifth of the amount in leaves treated with $(GlcNAc)_5$ on a fresh weight basis. The composition of the avenanthramides in seeds was different from that in elicitor-treated leaves. In dry seeds **3** was most abundant, followed by **2** and **1**, while **1** was the major component in elicitor-induced avenanthramides, and **2–6** represented only a minor portion. Under our experimental conditions, oat seeds germinated 18–36 h after imbibition. The total amount of avenanthramides in an oat seed increased approximately 2.5 times during the 48 h-imbibition, although the composition did not change.

Table I. Occurrence of avenanthramides in oat seeds.

Avenanthramide	Dry seeds [nmol/g fr.wt (%)]	Germinated seeds[a] [nmol/g fr.wt (%)]
A (**1**)	76.6 (100[b])	97.5 (100[b])
B (**2**)	80.7 (105)	101 (104)
C (**3**)	140 (183)	198 (203)
D (**4**)	2.73 (3.6)	2.98 (3.1)
G (**5**)	52.7 (69)	73.9 (76)
L (**6**)	17.4 (23)	35.8 (37)

[a] Avenanthramides extracted from seeds after 48-h imbibition during which average weight of a seed increased from 21.6 to 43.0 mg.
[b] Amount of avenanthramide A set to 100%.

Occurrence of HHT activity in seeds

The activity of HHT which catalyses the condensation reaction of hydroxyanthranilic acids and hydroxycinnamoyl-CoA esters was investigated in seeds either before or after imbibition (Table II). The HHT activity occurred in dry seeds, and was detected in both embryo (0.62 pkat/seed) and endosperm (1.7 pkat/seed). The total activity in the germinated seeds (2.75 pkat/seed) after 48 h imbibition was slightly larger than that in a dry seed. A germinated seed was divided into endosperm, scutellum, shoot, and root. Eighty-five percent of the total HHT activity was localized in the endosperm (2.33 pkat/seed, 5.5 pkat/mg protein) though the highest activity per mg protein was recorded in scutellum (7.5 pkat/mg protein). Little activity was detected in the shoots (0.03 pkat/seed), and no activity was noted in the roots.

Table II. HHT activity in dry seeds and germinated seeds.

		HHT activity	
		[pkat/mg protein]	[pkat/seed]
Dry seed	Embryo	3.8	0.62
	Endosperm	5.4	1.7
Germinated seed[a]	Shoot	0.48	0.03
	Root	n.d.[b]	n.d.
	Scutellum	7.5	0.39
	Endosperm	5.5	2.33

[a] Oat seeds imbibed on wet filter paper for 48 h before measuring enzyme activity.
[b] n.d., not detectable.

Partial purification of HHT from seeds and elicitor-treated leaves

For characterization of HHT, the crude extract from dry seeds was fractionated by salting-out

Table III. Purification of HHT isoforms in dry seeds and elicitor-treated leaves by ion exchange chromatography on DEAE Sepharose and Mono Q.

Purification step	Total activity [pkat]		Protein [mg]		Specific activity [pkat/mg protein]	
	Seed	Leaf[a]	Seed	Leaf[a]	Seed	Leaf[a]
Crude extract	645	2230	228	81	2.83	27.5
$(NH_4)_2SO_2$ precipitation						
30–45%	201	736	39.6	14.9	5.09	49.5
DEAE Sepharose						
S1/E1	110	464	1.48	0.533	74.7	871
S2/E2	21.6	331	1.48	0.648	14.6	511
Mono Q						
S1/E1	68.1	172	0.139	0.060	489	2880
S2/E2	8.80	111	0.099	0.0427	88.9	2600

[a] Primary leaves of oats were treated with 1 mM penta-*N*-acetylchitopentaose for 12 h.

(Table III). The active fraction (precipitate between 30 and 45% $(NH_4)_2SO_4$ saturation) was subjected to anion exchange chromatography using DEAE Sepharose. The activity obtained from seeds was separated into two peaks (Fig. 1a). These peaks were referred to as S1 and S2 in order of elution. In the elution profiles, S1 was six times larger than S2. For further purification, the active fractions were separately subjected to strong anion exchange chromatography using a Mono Q HR 5/5 column (Fig. 2a). On this column, S1 and S2 were eluted as a single peak. The molecular weight of HHT was estimated at 40 kDa by gel filtration of each active fraction using a Superdex 75 HR 10/30 column, and no detectable difference in molecular weight was observed among the fractions. To compare the HHT composition between dry seeds and elicitor-treated leaves, extracts from elicitor-treated leaves were also fractionated in a similar way (Table III). On anion exchange chromatography with DEAE Sepharose (Fig. 1b) and Mono Q (Fig. 2b), the activity from elicitor-treated leaves were also separated into two peaks, E1 and E2, the activity of E1 being two times larger than that of E2 on the elution profile on DEAE Sepharose. Fractions S1 and E1 eluted at a similar NaCl concentration on anion exchange chromatography as well as S2 and E2. These results indicated that HHT from both origins consist of at least two isoforms.

Substrate specificity of HHT from seeds

The substrate specificity of the two active fractions obtained by anion exchange chromatography

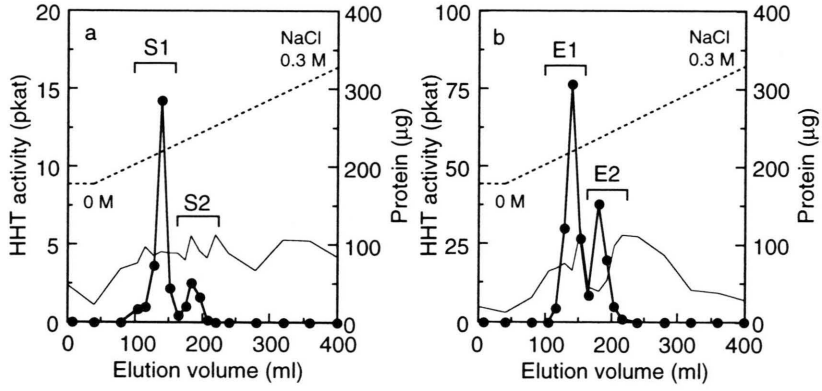


Fig. 1. Elution profiles of HHT activity obtained from dry seeds (a) and elicitor-treated leaves (b) on DEAE Sepharose. Solid lines represent protein contents. Bars on peaks of activity indicate pooled fractions.

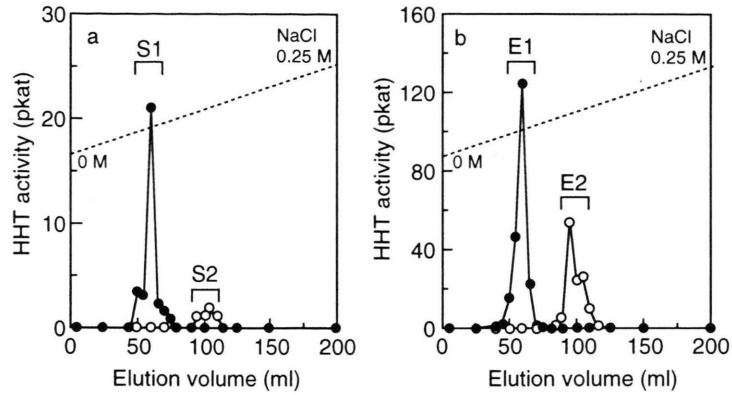


Fig. 2. Elution profiles of HHT activity obtained from dry seeds (a) and elicitor-treated leaves (b) on a Mono Q HR 5/5 column. Pooled fractions obtained by chromatography on DEAE Sepharose, S1 (●) and S2 (○) from seeds, and E1 (●) and E2 (○) from elicitor-treated leaves, were separately applied onto column. Bars on peaks of activity indicate pooled fractions.

from seeds was investigated. The apparent K_m and relative V_{max} values for anthranilic acid derivatives were determined with *p*-coumaroyl-CoA as a common acyl donor (Table IV). They accepted only anthranilate derivatives which constitute naturally occurring avenanthramides. The most prominent difference between S1 and S2 was the kinetic values for anthranilate. The K_m value of S1 was five times larger than that of S2, and the relative V_{max} value of S1 was three times smaller than that of S2. The kinetic constants for hydroxycinnamoyl-CoA esters determined with 5-hydroxyanthranilic acid as a common acyl acceptor showed that both enzyme preparations had similar broad specificities for hydroxycinnamoyl-CoA esters; however, the K_m and V_{max} values for each substrate somewhat varied between S1 and S2 (Table V).

Table IV. Kinetic constants (K_m and relative V_{max}) of HHT isoforms from seeds for hydroxyanthnilates. *p*-Coumaroyl-CoA (0.4 mM) was used as common acyl donor.

Substrate	S1		S2	
	K_m [μM]	Relative V_{max} (%)	K_m [μM]	Relative V_{max} (%)
4-Hydroxyanthranilate	3500	100[a]	5200	100[a]
5-Hydroxyanthranilate	640	68	720	58
Anthranilate	2800	35	14000	100
3-Hydroxyanthranilate	n.d.[b]	–	n.d.[b]	–
Tyramine	n.d.[b]	–	n.d.[b]	–

[a] Relative V_{max} values expressed as percentages based on V_{max} value obtained with 4-hydroxyanthranilate.
[b] n.d., not detectable.

Table V. Kinetic constants (K_m and relative V_{max}) of HHT isoforms from seeds for hydroxycinnamoyl-CoA esters. 5-Hydroxyanthranilic acid (1 mM) was used as common acyl acceptor.

Substrate	S1		S2	
	K_m [μM]	Relative V_{max} (%)	K_m [μM]	Relative V_{max} (%)
Feruloyl-CoA	210	100[a]	180	100[a]
Cinnamoyl-CoA	170	74	130	45
p-Coumaroyl-CoA	160	54	99	24
Caffeoyl-CoA	140	16	87	9.5
Avenalumoyl-CoA	21	8.9	56	23
4-Hydroxybenzoyl-CoA	72	3.1	270	1.6

[a] Relative V_{max} values expressed as percentage based on V_{max} value obtained with feruloyl-CoA.

Discussion

Avenanthramides are produced as phytoalexins in oat leaves infected with crown rust fungus (*Puccinia coronata* f. sp. *avenae*) in incompatible combinations (Mayama *et al.*, 1982; Mayama *et al.*, 1981). The induction of avenanthramides can be reproduced by treatment with elicitors such as *N*-acetylchitooligosaccharides (Bordin *et al.*, 1991), victorin C (Mayama *et al.*, 1986), heavy metal ions (Fink *et al.*, 1990), and the calcium ionophore A23187 (Ishihara *et al.*, 1996). In addition, the occurrence of avenanthramides in oat grains and hulls as constitutive components has been found (Collins, 1986; Collins, 1989; Collins *et al.*, 1991). In the present study, we quantitatively analyzed the avenanthramide composition in seeds and

compared the results with those in previous reports of elicitor-treated leaves (Ishihara *et al.*, 1998). In the seeds, compound **3** was most abundant, followed by **1** and **2**, whereas **1** was the major component; other avenanthramides accounted for only a minor portion in elicitor-treated leaves. The avenanthramide content increased 2.5 times during imbibition, though the composition was unchanged, indicating that avenanthramides are produced in the germination process in seeds.

In the elicitor-treated leaves, avenanthramides are synthesized from hydroxyanthranilates and hydroxycinnamoyl-CoA esters through condensation catalyzed by hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT) (Ishihara *et al.*, 1997; Ishihara *et al.*, 1998). The enzyme activity was also detected in the seed before imbibition, although the activity was much lower than that in the elicitor-treated leaves. The HHT activity in dry seeds is probably responsible for the avenanthramide production in the seed maturation process, and remained active even after the seeds were dried. Since avenanthramide production was observed in imbibed seeds, the enzyme activity is also considered to be involved in the avenanthramide production in imbibed seeds.

The presence of tyramine *N*-hydroxycinnamoyltransferase (THT), which catalyses the condensation between hydroxycinnamoyl-CoA esters and tyramine, has been reported in barley and wheat (Louis and Negrel, 1991). THT activity occurred in shoots and roots immediately after germination and thereafter gradually decreased. This activity has also been assayed in oats, but scarcely detected. On the basis of these previous findings, we hypothesized that HHT operates in oat seedlings in place of THT in wheat and barley. However, localization of HHT was different from THT; no HHT activity was detected in the roots and little activity in the shoots after 48-h imbibition. It is thus suggested that HHT has a different function than THT in barley and wheat although both enzymes catalyze similar reactions.

Purification of HHT by two anion exchange chromatography steps revealed that HHT consists of at least two isoforms in both dry seeds (S1 and S2) and elicitor-treated leaves (E1 and E2). The isoforms, S1 and S2, showed different specificities for anthranilate derivatives, while they had similar, broad substrate specificities for hydroxycinnamoyl-CoA esters. In carnation, it has been indicated that hydroxycinnamoyl/benzoyl-CoA:anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT) which catalyses almost identical reaction with HHT is encoded as a small family of genes, and that at least three of these genes are activated under the conditions of fungal elicitation (Yang *et al.*, 1997). The presence of isoforms of enzyme which catalyses the amide formation has also been demonstrated in tobacco by anion exchange chromatography (Fleurence and Negrel, 1989). However, the biological meanings of presence of the isoforms have been unclear. On anion-exchange chromatography, we observed the difference of relative activity of isoforms between dry seeds and elicitor-treated leaves; the relative activity of S1 to S2 was larger than that of E1 to E2, suggesting different regulation of HHT between seeds and elicitor-treated leaves, although the identity of each isoform remains to be elucidated. The most characteristic difference of avenanthramide composition between in seeds and elicitor-treated leaves was the higher percentages of **2** and **3** in seeds. Compounds **2** and **3** have feruloyl and caffeoyl moieties, respectively. However, the substrate specificity of HHT does not explain the difference in the avenanthramide composition because the kinetic constants of HHT for hydroxycinnamoyl-CoA esters in elicitor-treated laves (Ishihara *et al.*, 1998) were similar to those in dry seeds. Therefore, other factors should be taken into consideration. For instance, differences in the supply of substrates and the metabolism of avenanthramides in seeds and elicitor-treated leaves can affect the avenanthramide composition. This hypothesis is supported by the discrepancy between the substrate specificity of HHT *in vitro* and the composition of avenanthramide accumulated in seeds.

Acknowledgements

The authors grateful to Dr. Hisashi Miyagawa, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, for helpful discussion.

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