

# Levels of Avenanthramides and Activity of Hydroxycinnamoyl-CoA:Hydroxyanthranilate *N*-Hydroxycinnamoyl Transferase (HHT) in Steeped or Germinated Oat Samples

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## ABSTRACT

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Concentrations of avenanthramides and activity of the biosynthetic enzyme hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT) were analyzed in dry or, steeped nonmilled or milled, non-heat-treated (raw) or heat-treated oat samples (*Avena sativa* L.). Increased avenanthramide concentrations were found when intact raw groats were steeped. The increase was time- and temperature-dependent and maximal after 10 hr of steeping at 20°C. Continuous germination in air, after steeping, only contributed to a further increase in avenanthramides when steeping times were shorter than 10 hr.

Concentrations of avenanthramides and HHT activity were positively correlated during steeping of intact groats at 8 and 20°C. The increase in avenanthramides was suggested to be due to de novo synthesis and a whole grain structure seemed to be required as no increase was found when groats were milled before steeping. Avenanthramide levels also increased when heat-treated samples, lacking HHT activity, were steeped. This increase may be due to release of bound forms, possibly formed during the preceding heat treatment.

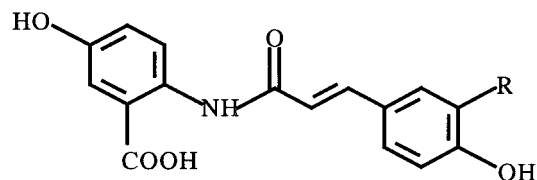
A correlation between whole grain products of cereals and protection against heart disease and certain kinds of cancer has been suggested from epidemiological studies (McCullough et al 2001; Renaud and Lanzmann-Petithory 2001; Roth and Mobarhan 2001; Truswell 2002; Chen et al 2002). This protection effect has partly been ascribed to dietary fibers and especially to soluble  $\beta$ -glucans. However, dietary antioxidants, which are mostly enriched in the outer parts of the grains, might also be possible health-promoting candidates (Miquel 2001; Visioli and Galli 2001). Antioxidants also stabilize colors and flavors and prolong the shelf life of food items.

Avenanthramides, a group of substituted *N*-cinnamoylanthranilic acids, are constitutive components of oat seeds and are found in both oat groats and hulls (Collins and Mullin 1988; Collins 1989; Dimberg et al 1993, 1996; Emmons and Peterson 1999; Bryngelsson et al 2002a; Matsukawa et al 2000). However, the concentration varies substantially with cultivar and cultivation conditions (Emmons and Peterson 2001). Avenanthramides have been characterized as antioxidants (Dimberg et al 1993; Peterson et al 2002; Bratt et al 2003) and the three major avenanthramides, derived from 5-hydroxy-anthranilic acid (**2**) and *p*-coumaric (**p**), caffeic (**c**) or ferulic (**f**) acid, namely **2p**, **2c** and **2f** (Fig. 1), are reported to be related to the fresh taste of oat products (Molteberg et al 1996). Avenanthramides are also well characterized as oat phytoalexins (Mayama et al 1981, 1982). Hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT) catalyses the condensation between anthranilic acids and hydroxycinnamoyl-CoA esters to form avenanthramides (Ishihara et al 1999). In oat leaves, the HHT is induced by the presence of elicitors (e.g., fungal toxins) (Ishihara et al 1997, 1998). In oat seeds, HHT activity has been detected in the endosperm as well as in the embryo (Matsukawa et al 2000).

For food purposes, oats are commonly subjected to heat processes such as steaming and autoclaving to inactivate lipolytic enzymes and thereby prevent rancidity during storage of the products. These processes also facilitate flaking of the groats and

develop the characteristic “oat taste”. Milled oat products may also be precooked by drum drying to provide products with a shorter cooking time. Previous studies showed that levels of avenanthramides were rather well preserved during steaming but decreased during autoclaving and drum drying (Dimberg et al 2001; Bryngelsson et al 2002b).

Consumer concern regarding synthetic food additives has prompted research to focus on antioxidants from natural sources and a long-term goal for the food industry is to produce food products with maximum conservation of endogenous antioxidants, both for food stabilization and for nutritional purposes. It is, therefore, of interest to find mild processes and technologies that may be used for future food production. Different types of bioprocesses, (e.g., germination [commonly initiated by steeping of grains], malting, and fermentation) are less commonly used for oats today but may be of future interest if they are found to be examples of such mild processes. Very few studies have been performed to investigate the effects of bio-processing on levels of antioxidants in cereals (e.g., Peterson 1994; Yang et al 2001; Subba Rao and Muralikrishna 2002). Levels of avenanthramides increased during germination of oat seeds (Matsukawa et al 2000). As HHT activity was also elevated in germinated seeds, the increase in avenanthramides was suggested to originate from de novo synthesis. Concentrations of free avenanthramides have increased during preparation of various oat-based products such as muffins containing 9% commercial oat bran (particle size  $\geq 0.5$  mm) and the increase was suggested to result from either a de novo synthesis, a release of bound forms or increased extractability after processing (Dimberg et al 2001). However, no increased



### Avenanthramide

**2p**  
**2c**  
**2f**

### R

H  
OH  
OCH<sub>3</sub>

Fig. 1. Chemical structure of three major avenanthramides in oats.

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avenanthramide concentrations were found in muffins containing oat groats milled to a particle size <0.5 mm (*unpublished data*). Those results led to the hypothesis that fewer disrupted cell compartments of the oat tissue than that present in finely milled material are needed to obtain an increased concentration of avenanthramides in the presence of water. The present study aimed to investigate whether this assumption is valid and whether the increase is due to *de novo* synthesis. Furthermore, the effect of continuous germination in air following steeping on levels of avenanthramides in oat grains was investigated.

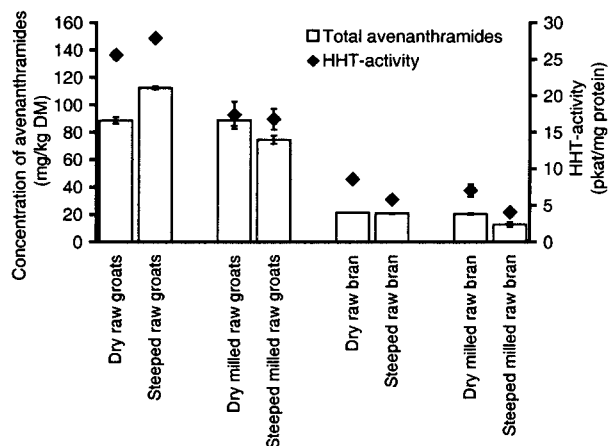
## MATERIALS AND METHODS

### Oat Materials

Raw grains of covered oats (*Avena sativa* L., cv Sang) were supplied by Kungsörnen AB, Järna, Sweden, and raw groats of naked oats (*A. sativa* L. *nuda*, cv Bullion) were provided by Svalöf-Weibull (Svalöv, Sweden). Steamed groats, autoclaved grains, and raw bran (0.5–1.1 mm) of Sang were supplied by Kungsörnen AB; raw bran was made from non-heat-treated groats by using a pilot-plant mill (Falling Number Laboratory Mill 3303) and sieve (Bühler Miag rotating unit). Commercially available rolled oats and oat bran, manufactured from steamed groats, were purchased in a grocery store. Grain is defined as the whole seed including the hull, while groat is defined to naked or dehulled seed. Raw-unprocessed samples are defined as non-heat-treated samples not further processed in our laboratory before steeping. Dry samples are not steeped. Milled samples are milled in our laboratory using a ultra centrifugal mill type ZM 1 (Retsch, Haan, Germany) to a particle size  $\leq 0.5$  mm.

### Chemicals

Synthetic avenanthramides, N-(4'-hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (**2p**), N-(3',4'-dihydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (**2c**), and N-(4'-hydroxy-3'-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (**2f**) were provided by Sunnerheim, Department of Chemistry, Uppsala University, Sweden. *p*-Coumaroyl-CoA thioester was synthesized by transesterification of *p*-coumaroyl-*N*-succinimide ester according to Stöckigt and Zenk (1975). 5-Hydroxyanthranilic acid was supplied by Aldrich Chemie (Steinheim, Germany). NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, 2-mercaptoethanol, methanol, and acetonitrile were supplied by Merck (Darmstadt, Germany). All solvents were of analytical grade and used without further purification. Commercial sodium hypochloride (Klorin brand), manufactured by Colgate Palmolive, was purchased in a grocery store.



**Fig. 2.** Concentrations of avenanthramides and activity of HHT in dry or steeped intact or milled raw naked groats or raw oat bran. Bars and dots show mean of duplicates; error bars  $\pm$ SD.

### Sample Preparation

**Experiment A.** Steeping of nonmilled and milled raw groats and raw bran of naked oats. Samples (10 g) of nonmilled or milled raw groats or raw bran of naked oats were steeped in 50 mL of tap water or stored dry for 10 hr at 20°C and thereafter freeze-dried and stored at -20°C until time of extraction. Milling was performed either before steeping or before extraction.

**Experiment B.** Steeping of raw groats of naked oats. Surface sterilized (1% sodium hypochloride, 30 sec) samples (30 groats) were steeped in 5 mL of tap water for 6, 8, 10, 24, or 48 hr at temperatures of 8, 20, or 40°C, or for 15, 30, or 60 min at 80°C. The samples were thereafter freeze-dried and stored at -20°C until time of extraction.

**Experiment C.** Steeping and continuous germination in air of raw grains of covered oats. Surface-sterilized raw grains of covered oats (5 g) were steeped in 15 mL of tap water for 2, 6, 10, 24, or 30 hr at room temperature. After steeping, the grains were either used directly for avenanthramide extraction or placed on wet filter papers in petri dishes for a continuous germination in air (24 hr in darkness).

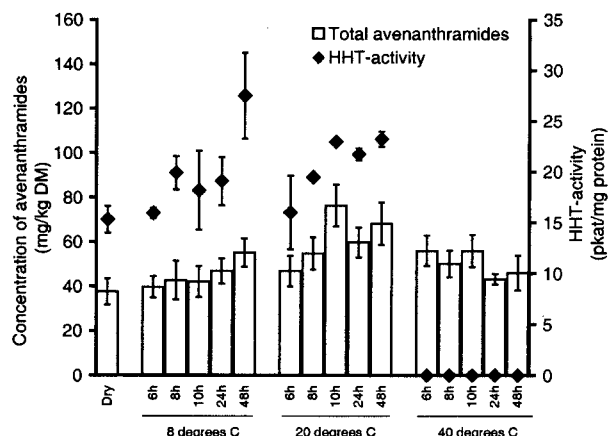
**Experiment D.** Steeping of heat-treated samples. Samples of steamed groats, autoclaved grains, or commercial rolled oats or bran were prepared as described in Experiment A.

### Extraction of Avenanthramides

Avenanthramides were extracted by 2  $\times$  16 mL (Experiment C) or 3  $\times$  5 mL (Experiment A, B, D) of methanol/g of sample either from premilled samples (Experiments A and D) or by homogenization of samples directly in methanol using an Ultra Turrax homogenizer (Experiments B and C). After intermittent centrifugation, the supernatants were pooled and the solvent was evaporated under vacuum (40°C). The residues were dissolved in 1 mL of methanol, centrifuged, and the supernatants were analyzed by HPLC as described below. The concentrations of avenanthramides are based on dry matter (dm) in Experiment A, B and D and on fresh weight (fw) in Experiment C. All extractions were performed in duplicate, except Experiment B which includes triplicate extractions.

### Extraction of Crude Enzyme

For extraction of proteins, the samples described above were homogenized in 0.1M phosphate buffer (pH 7.5, containing 14.4 mM 2-mercaptoethanol) (5 mL/g of sample) for 30 sec using an Ultra Turrax homogenizer and were thereafter centrifuged (12,000  $\times$  g, 10 min, 4°C). The supernatants were used as the crude enzyme extracts. All steps in the preparation of the crude enzyme extract were performed on ice.



**Fig. 3.** Concentrations of avenanthramides and activity of HHT in dry or steeped raw naked groats steeped for different times and at different temperatures. Bars and dots show mean of duplicates; error bars  $\pm$ SD.

## HHT Assay

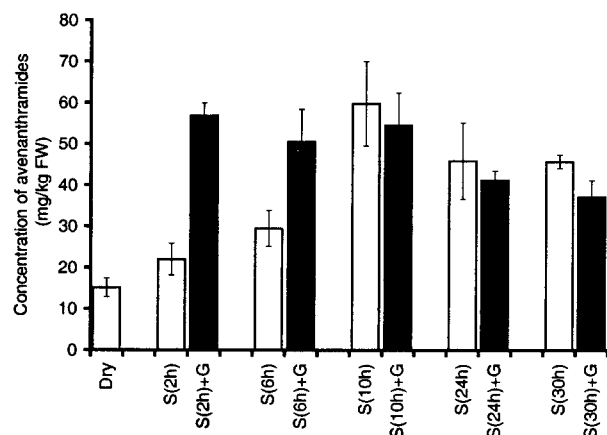
The HHT assay was modified from the method described by Matsukawa et al (2000). The crude enzyme solution (10  $\mu$ L), 2 mM *p*-coumaroyl-CoA (10  $\mu$ L), 10 mM 5-hydroxyanthranilic acid (10  $\mu$ L), and 100 mM phosphate buffer (pH 7.5) (70  $\mu$ L) were mixed in Eppendorf tubes and incubated (30 min, 30°C). Addition of 20  $\mu$ L of acetic acid stopped the reaction and the volume was set to 500  $\mu$ L by adding MeOH. After centrifugation (18,000  $\times$  g, 10 min) the enzymatically formed **2p** was analyzed in the supernatant by HPLC. The protein content of the crude enzyme solutions was determined according to the method of Bradford (1976). The HHT activity is reported as pkat/mg of protein (kat = mol **2p** formed per sec).

In the present study, *p*-coumaroyl-CoA and 5-hydroxyanthranilic acid were chosen as substrates. However, the HHT also has an affinity for other hydroxycinnamic acid CoA-esters and anthranilic acids (Matsukawa et al 2000).

All HPLC analyses were performed on a reverse-phase column (HP ODS Hypersil column, 5  $\mu$ m, 125  $\times$  4 mm) using a combination of 0.01M phosphate buffer (pH 2.8, 5% acetonitrile) and acetonitrile as a mobile phase and a flow rate of 1 mL/min. Samples from the HHT assay were analyzed using 15% acetonitrile in buffer, while methanol extracts of oat samples were analyzed using a gradient of 0–40% acetonitrile in buffer (60 min) (Dimberg et al 2001). The avenanthramides were detected at 340 nm. Identification of the analyzed compounds was made by comparison of retention times and UV-spectra with those of synthetic avenanthramides. Quantification was performed using external standards and the results are presented as the sum of the three analyzed avenanthramides (**2c**, **2f**, and **2p**).

## RESULTS

*Experiment A.* During steeping of intact raw groats, both avenanthramide concentrations and HHT activity increased (27 and 9%, respectively) (Fig. 2). No change in avenanthramide concentration was found in the nonmilled raw bran after steeping but the HHT activity was reduced by 33%. Steeped milled raw groats as well as steeped milled raw bran had lower concentrations of avenanthramides and lower HHT activity compared with the dry intact groats and bran, respectively. However, the HHT activity, but not the concentrations of avenanthramides, was also equally decreased in dry-milled groats stored for 10 hr at 20°C (Fig. 2), indicating that HHT was rather unstable during dry storage of homogenized samples at room temperature. This should be taken into consideration when analyzing the activity of this enzyme in various samples.



**Fig. 4.** Concentrations of avenanthramides in dry, steeped (S) or steeped and germinated (G) raw oat grains. Bars and dots show mean of duplicates; error bars  $\pm$ SD.

*Experiment B.* Steeping of raw naked groats for different times at different temperatures showed a 50% increase in avenanthramides obtained by 48, 8, or 6 hr of steeping at 8, 20, or 40°C, respectively, and already after 15 min at 80°C (Fig. 3 and Table I). A maximal concentration was obtained after 10 hr of steeping at 20°C. After steeping at 80°C, small amounts of **2f** and **2p**, but not **2c**, were detected in the steeping water. This was probably due to the alkaline pH (8.5) of the water after steeping. The **2c** is completely broken down in alkaline conditions even at moderate temperatures, while **2f** is unstable in alkaline conditions only at elevated temperatures (Dimberg et al 2001). The concentrations of avenanthramides in the water were taken into account when calculating the final concentrations in the steeped samples. Dry naked groats had an HHT activity of 15.3 pkat/mg of protein and steeping significantly increased this activity (Fig. 3). The highest activity (27.5 pkat/mg of protein) of HHT was found in the groats steeped for 48 hr at 8°C. However, due to a large variation in HHT activity among samples steeped under these conditions, this activity was not significantly different from the activity found after 10 hr of steeping at 20°C, which were the conditions resulting in the highest avenanthramide concentration. Steeping for more than 10 hr at 20°C did not result in any further changes in HHT activity. A good correlation between the avenanthramide concentrations and HHT activity was found during steeping of naked groats at 8 and 20°C ( $R^2 = 0.92$  and  $0.90$ , respectively). No HHT activity was detected in samples steeped at 40 or 80°C.

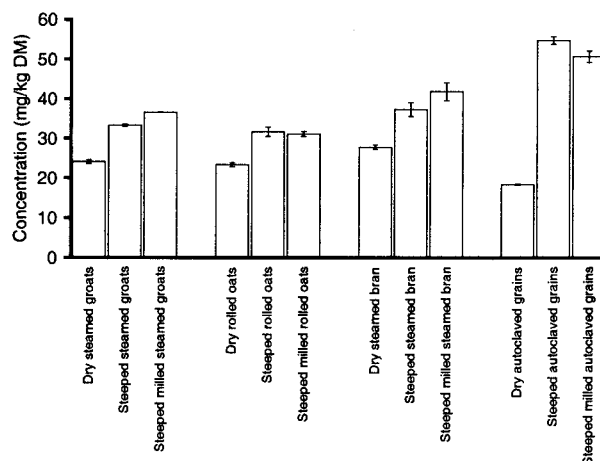
*Experiment C.* As in experiment B, a maximal concentration of avenanthramides was obtained after 10 hr of steeping (20°C) (Fig. 4). The continuous germination in air contributed to a further increase in avenanthramides after steeping only when the steeping time was shorter than 10 hr. However, the maximal level of avenanthramides was obtained by 10 hr of steeping only.

*Experiment D.* The avenanthramide concentrations were increased during steeping of all the heat-treated samples, and steeping of autoclaved grains gave a much higher increase compared with the other samples (Fig. 5). A similarly large increase

**TABLE I**  
Concentrations of Avenanthramides  
in Dry or Steeped (80°C) Raw Naked Groats<sup>a</sup>

Steeping Time	Total Avenanthramides (mg/kg of dry matter)
Dry	37.6 $\pm$ 5.9
15 min	56.9 $\pm$ 12.7
30 min	54.0 $\pm$ 5.7
60 min	61.4 $\pm$ 8.2

<sup>a</sup> Means of duplicates  $\pm$  SD.



**Fig. 5.** Concentrations of avenanthramides in dry or steeped steamed groats, rolled oats, commercial oat bran, or autoclaved grains. Bars and dots show mean of duplicates; error bars  $\pm$ SD.

was found when autoclaved grains were dehulled before steeping (data not shown) showing that the hulls did not contribute much to the increase. As expected, no HHT activity was detected in those samples, either before or after steeping.

## DISCUSSION

The hypothesis that a less disrupted oat structure would be necessary to obtain increased avenanthramide concentrations during steeping was partly confirmed in this study. An increase was found after steeping of intact raw groats while no increase, but actually a decrease, was found after steeping of both milled raw groats and milled raw bran. However, no increase was found during steeping of nonmilled raw bran, which was contradictory to the hypothesis. The increase during steeping of intact groats might, at least partly, be due to a de novo synthesis, as a good correlation between avenanthramide concentrations and HHT activity was found during steeping at <40°C, which was the limit for the enzyme activity. A de novo synthesis of avenanthramides was previously suggested to take place during germination of seeds and the HHT in oat grains was reported to have higher affinity for the *p*-coumaroyl-CoA-ester compared with the caffeoyl- and feruloyl-CoA-esters (Matsukawa et al 2000). However, in the present study the relative percentage of the three analyzed avenanthramides was more or less similar before and after the increase that took place during steeping, both in raw and heat-treated samples. As HHT also had some activity in steeped raw bran, the lack of increase in avenanthramides might be due to a shortage of endogenous enzyme substrates in the bran structure. In intact grains, the substrates might be released by regulated reactions during the initiated germination process and such reactions might not take place in the separated bran fraction. A decrease after steeping of milled samples might possibly be due to a combination of decreased HHT activity and release of enzymes able to either degrade the avenanthramides or incorporate them into insoluble components. Such enzymes have not previously been described but unregulated enzyme-mediated reactions might take place when cell tissue compartments are broken (e.g., by milling) and enzymes come into contact with new substrates. As food products are commonly manufactured by mixing homogenized ingredients with water, the mechanism responsible for the decrease in avenanthramides during steeping of milled samples might be of importance. This is under further investigation in our laboratory.

Because avenanthramide concentrations were also increased during steeping of samples at 40°C, which resulted in a loss of HHT activity, as well as during steeping of heat-treated samples, in which HHT was already inactivated, the question was raised whether mechanisms other than biosynthesis by HHT were also involved. In samples losing HHT activity during steeping, biosynthesis by HHT during the initial steeping phase (i.e., before the temperature reached the inactivation temperature of HHT) might possibly contribute to an increase in avenanthramide concentrations. However, when heat-treated (steamed or autoclaved) samples, lacking HHT activity, are steeped this could not be the case. In a previous study, decreases in avenanthramides were found during certain heat treatments, especially autoclaving, of oat samples (Bryngelsson et al 2002b). Those decreases may possibly be due to heat-induced formation of complexes between avenanthramides and other molecules. The apparently large increase in avenanthramides found during steeping of the heat-treated samples in the present study may therefore be an effect of hydrolysis and release of bound forms. However, in the present study we have no data on avenanthramide concentrations in the raw materials used for steaming or autoclaving or used for preparation of commercial rolled oats and bran. Therefore, a comparison of the assumed decrease during the heat treatments and the apparent increase in avenanthramide concentrations found during steeping cannot be made.

In conclusion, whole groat oat products with elevated avenanthramide contents due to de novo synthesis can be manufactured by soaking intact raw grains or groats in water at moderate temperatures. The results also stress that if oat products with highly retained avenanthramides are desired, finely ground non-heated oat material should be avoided when the production process includes water addition. Furthermore, oat-based foods with increased levels of free avenanthramides, possibly due to release of bound forms, may also be obtained by soaking heat-treated oat fractions.

## ACKNOWLEDGMENTS

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