

Tentative Avenanthramide-Modifying Enzyme in Oats

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ABSTRACT

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A decrease of the concentration of the synthetic avenanthramide N-(4'-hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid in a buffered slurry of milled oat groats (*Avena sativa* L.) was temperature and pH-dependent, with a maximum rate at 30°C and pH 9. The reaction was inhibited in the presence of 2-mercaptoethanol, acetic acid and at high temperature; suggestive of enzymatically catalyzed nature. Among eight

different synthetic avenanthramides tested, the tentative enzyme had highest affinity for avenanthramides comprising caffeic and *p*-coumaric acids and lowest for those comprising sinapic and ferulic acids. The activity was found in samples from several oat cultivars and was equally pronounced in both bran and endosperm flour of oats. Steeping of oat grains did not influence the reaction.

Dietary antioxidants, which are often enriched in the outer part of cereal grains, might be important factors for the health-promoting effect reported for whole-grain cereal products (Miquel 2001; Visioli and Galli 2001). Antioxidants also stabilize colors and flavors and prolong the shelf life of food items. 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.

Avenanthramides, a group of substituted *N*-cinnamoylanthranilic acids, are constitutive components in oat seeds (Collins and Mullin 1988; Collins 1989; Dimberg et al 1993, 1996; Emmons and Peterson 1999; Matsukawa et al 2000; Bryngelsson et al 2002a). Some avenanthramides have been characterized as antioxidants in vitro (Dimberg et al 1993; Peterson et al 2002; Bratt et al 2003), and the three major avenanthramides, **2p**, **2c** and **2f** (Fig. 1), are reported to be related to fresh taste of oat products (Molteberg et al 1996). The enzyme hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT) catalyses the condensation between anthranilic acids and hydroxycinnamoyl-CoA esters to form avenanthramides (Ishihara et al 1997, 1998, 1999), and HHT activity has been detected in the endosperm as well as in the embryo of oat seeds (Matsukawa et al 2000).

Enhanced levels of avenanthramides were found in groats after germination (Matsukawa et al 2000; Bryngelsson et al 2003), as well as after processing of certain products (e.g., muffins, tea cakes, pasta) containing different oat fractions (e.g., oat bran) (Dimberg et al 2001). Further, avenanthramides are well retained during commercial steaming, commonly used to inactivate lipolytic enzymes in oats used for food production (Dimberg et al 1996; Bryngelsson et al 2002b), and they are rather stable toward different temperatures, pH levels, and during UV-light exposure (Dimberg et al 2001). However, when homogenized raw oat groats were mixed with water there was a decrease of avenanthramide concentrations (Bryngelsson et al 2003).

The aim of the present study was to investigate whether or not the loss of avenanthramides in homogenized oat samples steeped in water, is caused by an enzymatically catalyzed reaction.

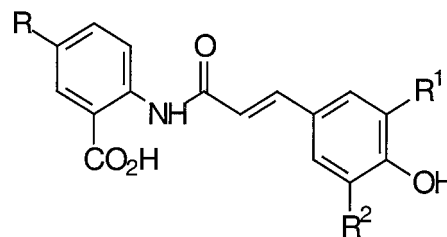
MATERIALS AND METHODS

Dry raw groats of naked oats (*Avena sativa* L. nuda cv Bullion, Svalöf Weibull, Sweden) were used in all experiments. In addition,

samples of Bullion were steeped (10 hr, 20°C, 5 mL of tap water/g of oat sample), either as intact or as milled groats and after freeze-drying the samples were used in Experiment E. Milling of groats (particle size ≤ 0.5 mm), was performed at our laboratory using an ultra centrifugal mill (ZM 1, Retsch, Haan, Germany). Experiment E also included dry samples of raw bran (particle size 0.5–1.1 mm), raw endosperm flour (particle size < 0.5 mm), steamed groats (100°C, 1 hr) and autoclaved grains (2.4 bar, 100–120°C, 16 min) from covered oats (cv Sang, Cerealia AB, Sweden). The raw bran and endosperm were made from non-heat-treated groats by using a pilot-plant mill (Falling Number Laboratory Mill 3303) and sieve (Bühler Miag rotating unit). In the present study, grain equals whole seed including the hull, and groat equals naked or dehulled seed.

Synthetic avenanthramides derived from anthranilic acid (**1**) or 5-hydroxyanthranilic acid (**2**) and *p*-coumaric (**p**), caffeic (**c**), ferulic (**f**), or sinapic (**s**) acids, respectively (**1p**, **1c**, **1f**, **1s**, **2p**, **2c**, **2f**, and **2s**) (Fig. 1) were synthesized according to Bratt et al (2003) or to Collins (1989). All other chemicals used were purchased from Merck (Germany) and were of analytical grade.

A methanolic solution of a synthetic avenanthramide (50 μ L) was carefully mixed with a slurry made of 25 mg of milled oat sample and 450 μ L of 0.1M phosphate buffer and thereafter incubated in a water bath. After incubation, 100 μ L of acetic acid was added to stop the reaction and the volume was brought to 2,500 μ L by addition of methanol. Aliquot of the sample was centrifuged (18,000 $\times g$, 5 min) and the supernatant was analyzed by HPLC. In the reference sample, acetic acid was added to the oat slurry before the synthetic avenanthramide was added. All



Avenanthramide	R	R ¹	R ²
1p	H	H	H
1c	H	OH	H
1f	H	OCH ₃	H
1s	H	OCH ₃	OCH ₃
2p	OH	H	H
2c	OH	OH	H
2f	OH	OCH ₃	H
2s	OH	OCH ₃	OCH ₃

Fig. 1. Chemical structures of avenanthramides used in the present study.

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TABLE I
Added Avenanthramides and Incubation Conditions for Experiments

Experiment ^a	Avenanthramide ^b	pH ^c	Temperature (°C)	Time (hr)
A	2p	7.5 ^d	30	1, 2, 3, 4, 5, 6, 24
B	2p	5.7, 6.4, 7.0, 7.8, 8.4, 9, 10, 11, 12	30	1
C	2p	9	20, 30, 40, 60, 90	1
D	1p, 1c, 1f, 1s, 2p, 2c, 2f, 2s	7	30	2
E ^e	2p	7.5	30	1

^a Oat sample (25 mg): dry intact raw groats.

^b Added volume: 50 μ L. Concentrations of methanolic solutions of the avenanthramide (mM): **1p** (0.56), **1c** (0.63), **1f** (0.72), **1s** (1.05), **2p** (0.50), **2c** (0.77), **2f** (0.65), and **2s** (0.66).

^c 450 μ L of 0.1M phosphate buffer.

^d Buffer with (14 mM) or without 2-mercaptoethanol.

^e Oat samples (25 mg): steeped intact raw groats, dry milled raw groats, steeped milled raw groats, dry raw bran, dry raw endosperm flour, dry steamed groats, dry autoclaved grains.

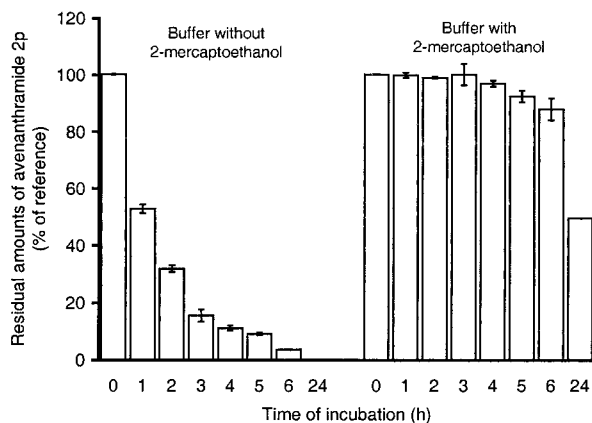


Fig. 2. Concentrations of **2p** in buffered oat slurries after different times of incubation (30°C, pH 7.5) with or without 2-mercaptoethanol (Experiment A). Amount added synthetic **2p**: 1.1 μ mol/g of oat sample. Bars and dots show mean of duplicates; error bars \pm SD.

samples were analyzed as duplicates; results are presented as percentage of residual amount of avenanthramide compared with the reference sample. Further details about added avenanthramides and incubation conditions are given in Table I. For each batch of samples, a control containing the oat slurry, but not the synthetic avenanthramide, was included. The concentrations of the endogenous avenanthramides present in these samples were too low to interfere with the analysis. Two other controls contained only the synthetic avenanthramide and methanol or buffer, respectively, but no oat slurry. Those controls were included to confirm that the avenanthramides were stable during the incubation conditions used.

HPLC Analysis

HPLC analysis was performed according to Bryngelsson et al (2002b) using a reversed-phase column (HP; ODS Hypersil, 5 μ m, 125 \times 4 mm). An isocratic mobile phase system consisting of 19% acetonitrile (A) in 0.01M phosphate buffer (pH 2.8) (B), was used for **2p**, **2c**, **2f**, and **2s** and 24% A in B for **1p**, **1c**, **1f**, and **1s**. Avenanthramides were detected at 340 nm and quantified using external standards.

RESULTS

Experiment A. Decrease of the concentration of **2p** over time and dependence of the presence of 2-mercaptoethanol. In reactions without 2-mercaptoethanol (containing 1.1 μ mol **2p**/g oat sample), the amount of **2p** was decreased 50% within 1hr of incubation and after 6 hr, the added **2p** was almost completely vanished (Fig. 2). In a buffer containing 2-mercaptoethanol, however, only a slight decrease was detected after 6 hr of incubation and a 50% loss was found first after 24 hr of incubation.

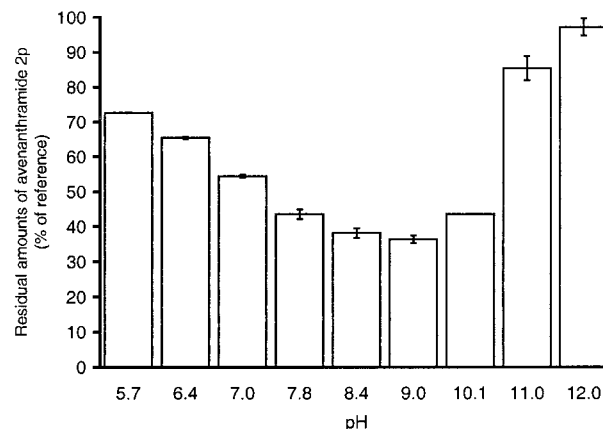


Fig. 3. Concentrations of **2p** in buffered oat slurries after incubation (30°C) in buffers with different pH levels (Experiment B). Amount added synthetic **2p**: 1.1 μ mol/g of oat sample. Bars and dots show mean of duplicates; error bars \pm SD.

Experiment B. Decrease of the concentration of **2p** at different pH. The decrease of the amount of **2p** was pH dependent. The lowest concentration was obtained when the reaction was run at pH 9 (Fig. 3). At pH 12 almost all **2p** was retained.

Experiment C. Decrease of the concentration of **2p** at different temperatures. Based on the result found in Experiment B, pH 9 was chosen in this experiment. The decrease of the amount of **2p** was temperature-dependent. The lowest concentration was obtained when the reaction was run at 30°C (Fig. 4). The concentration was diminished to some extent at 60°C, but at 90°C no reaction took place.

Experiment D. Decrease of the concentration of different avenanthramides. In this experiment, pH 7 was chosen because **2c** (and **1c**) is destroyed at alkaline conditions (Dimberg et al 2001). To compensate for the less optimal pH 7, the incubation time was extended to 2 hr. The decrease of the concentrations of the different avenanthramides were of the order: **2c** \geq **2p** \geq **1c** $>$ **1p** $>$ **2s** \geq **1s** $>$ **2f** \geq **1f** (Table II). The declining was, thus, most pronounced for the avenanthramides containing caffeic (c) and *p*-coumaric (p) acids and least for those containing sinapic (s) and ferulic acids (f). This was also confirmed by a higher retention of **2f**, compared with **2p** and **2c**, when all three compounds were incubated in the same sample (data not shown). The sensitivity for the avenanthramides containing 5-hydroxyanthranilic acid (**2**) seemed to be higher compared with those containing anthranilic acid (**1**) for the pair **2p**/**1p**, while the other pairs showed about equal sensitivity toward the reaction.

Experiment E. Decrease of the concentration of **2p** by dry and steeped samples of different oat fractions. Incubation of **2p** in slurries made of dry or steeped intact groats resulted in equal diminishing of **2p** concentrations (Table III). Nor was there any

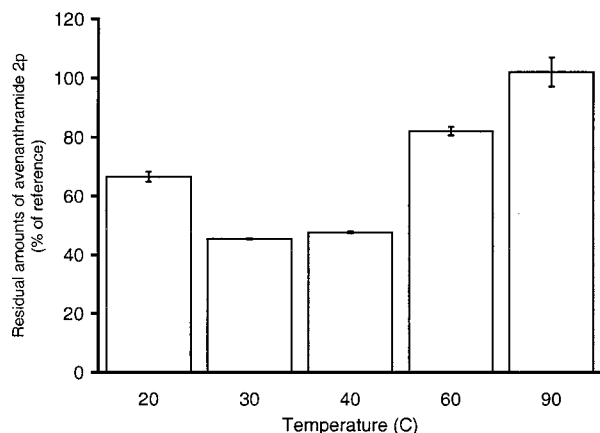


Fig. 4. Concentrations of **2p** in buffered oat slurries after incubation (pH 9) at different temperatures (Experiment C). Amount added synthetic **2p**: 0.9 $\mu\text{mol/g}$ of oat sample. Bars and dots show mean of duplicates; error bars $\pm\text{SD}$.

difference in the result between those two slurries and the slurries made from groats that had been milled before steeping. This indicates that the reaction was not affected by 10 hr of steeping of milled groats. Furthermore, the raw bran slurry was not different when compared with the raw endosperm flour slurry (Table III). However, the slurries made from steamed groats or autoclaved grains did not exert any activity toward the avenanthramide.

DISCUSSION

It is well known that denaturation of proteins (e.g., by extreme pH, high temperatures, or 2-mercaptoethanol [breaking S-S bridges]) often cause enzymes to lose their activities. It is also well known that most enzyme reactions are active only in narrow pH and temperature intervals. The studied reactions were inhibited by addition of 2-mercaptoethanol or acetic acid, and showed clear pH and temperature optima. Furthermore, no reactions took place when the oat samples were heat-treated (steamed or autoclaved) before incubation with the avenanthramides. Therefore, we assume that the decrease of the avenanthramide concentrations was enzyme-catalyzed. To further elucidate whether or not the reaction was due to any component attached to the solid matrix, a buffer extract of milled oat samples was also analyzed. The added **2p** was affected by this extract as well, although to a lesser extent (23% decrease for the extract compared with 75% decrease for the oat slurry), indicating that the tentative avenanthramide modifying enzyme (TAME) might be only partly water extractable.

The substrate specificity shown in this study is in good agreement with other experiments (*unpublished data*), where we found endogenous **2f** to be more retained compared with **2p** and **2c** during steeping of homogenized oat samples. In addition to the two cultivars (Bullion and Sang) used in the present study, activity was also found in two North American cultivars (Jim and Vista) (data not shown). TAME, therefore, seemed to be generally present in oats and to be evenly distributed throughout the groat.

While the activity of the avenanthramide-synthesizing enzyme HHT is enhanced in intact groats during steeping and suggested to be, at least partly, responsible for the reported increase of avenanthramides in germinated grains (Matsukawa et al 2000; Bryngelsson et al 2003), it is obvious that TAME is equally present in intact dry or steeped groats. However, it is not known whether TAME exerts any activity simultaneously to HHT during steeping (Bryngelsson et al 2003). Possibly, TAME is compartmentalized and thereby separated from its substrates, the avenanthramides, in intact oat groats and this might explain the net increase of avenanthramide concentrations found after steeping of intact groats. However, in homogenized samples, the enzyme may

TABLE II
Avenanthramide Concentrations^a Left After Incubation (30°C) in Buffered (pH 7) Slurries of Oat Groats (Experiment D)^{a,b}

Avenanthramide	Residual Amounts of Avenanthramides (% of references)
1p	58 \pm 0
2p	39 \pm 0
1c	40 \pm 0
2c	37 \pm 2
1f	100 \pm 1
2f	99 \pm 0
1s	76 \pm 2
2s	73 \pm 1

^a Amount of synthetic avenanthramides added ($\mu\text{mol/g}$ of oat sample): **1p** (1.1), **2p** (0.9), **1c** (1.3), **2c** (1.5), **1f** (1.4), **2f** (1.3), **1s** (2.1), and **2s** (1.3).

^b Means of duplicates $\pm\text{SD}$.

TABLE III
Concentration of **2p** Left After Incubation (30°C) in Buffered (pH 9) Oat Slurries (Experiment E)^{a,b}

Oat Sample	Residual Amounts of Avenanthramide 2p (% of reference)
Bullion cultivar	
Dry intact raw groats	48 \pm 0
Steeped intact raw groats	47 \pm 1
Dry milled raw groats	49 \pm 3
Steeped milled raw groats	49 \pm 0
Sang cultivar	
Dry raw bran	62 \pm 1
Dry raw endosperm flour	66 \pm 1
Dry steamed groats	96 \pm 7
Dry autoclaved grains	92 \pm 5

^a Amount of synthetic **2p** added: 0.9 $\mu\text{mol/g}$ of oat sample.

^b Means of duplicates $\pm\text{SD}$.

be released from its compartment and may thereby be able to decrease the present avenanthramides when milled oat samples are soaked in water. Furthermore, in contrast to HHT (Bryngelsson et al 2003), TAME is not deactivated during longer steeping of milled samples at room temperature; this higher stability of TAME compared with HHT might explain the net decrease of avenanthramides found after steeping of milled samples.

The mechanisms behind the reactions modifying the avenanthramides are not known but it does not seem to be a break of the amide bonds of the avenanthramides, as no cinnamic acids were detected in the incubated samples. Preliminary results from ongoing studies indicate that, at least initially, an oxidative process seems to be involved. Purification and further characterization of the tentative enzyme will be undertaken in our laboratory.

ACKNOWLEDGMENTS

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LITERATURE CITED

- Bratt, K., Sunnerheim, K., Bryngelsson, S., Fagerlund A., Engman, L., Andersson, R. E., and Dimberg, L. H. 2003. Avenanthramides in oats (*Avena sativa* L.) and structure-antioxidant activity relationships. *J. Agric. Food Chem.* 51:594-600.
- Bryngelsson, S., Fogelfors, B., Kamal-Eldin, A., Andersson, R., and Dimberg, L. H. 2002a. Lipids and antioxidants in groats and hulls of Swedish oats (*Avena sativa* L.). *J. Sci. Food Agric.* 82:606-614.
- Bryngelsson, S., Dimberg, L. H., and Kamal-Eldin, A. 2002b. Effects of

- commercial processing on levels of antioxidants in oats (*Avena sativa* L.). *J. Agric. Food Chem.* 50:1890-1896.
- Bryngelsson, S., Ishihara, A., and Dimberg, L. H. 2003. Levels of avenanthramides and activity of hydroxycinnamoyl-CoA: hydroxyanthranilate N-hydroxycinnamoyltransferase (HHT) in steeped or germinated oat samples. *Cereal Chem.* 80:357-361.
- Collins, F. W. 1989. Oat phenolics: Avenanthramides, novel substituted N-cinnamoylanthranilate alkaloids from oat groats and hulls. *J. Agric. Food Chem.* 37:60-66.
- Collins, F. W., and Mullin, W. J. 1988. High-performance liquid chromatographic determination of avenanthramides, N-aroylantranilic acid alkaloids from oats. *J. Chromatogr.* 445:363-370.
- Dimberg, L. H., Theander, O., and Lingnert, H. 1993. Avenanthramides—A group of phenolic antioxidants in oats. *Cereal Chem.* 70:673-641.
- Dimberg, L. H., Molteberg, E. L., Solheim, R., and Frølich, W. 1996. Variation in oat groats due to variety, storage and heat treatment. I. Phenolic compounds. *J. Cereal Sci.* 24:263-272.
- Dimberg, L. H., Sunnerheim, K., Sundberg, B., and Walsh, K. 2001. Stability of oat avenanthramides. *Cereal Chem.* 78:278-281.
- Emmons, C. L., and Peterson, D. M. 1999. Antioxidant activity and phenolic contents of oat groats and hull. *Cereal Chem.* 76:902-906.
- Ishihara, A., Matsukawa, T., Miyagawa, H., Ueno, T., Mayama, S., and Iwamura, H. 1997. Induction of hydroxycinnamoyl-CoA: hydroxyanthranilate N-hydroxycinnamoyltransferase (HHT) activity in oat leaves by victorin. *Z. Naturforsch.* 52c:756-760.
- Ishihara, A., Miyagawa, H., Matsukawa, T., Ueno, T., Mayama, S., and Iwamura, H. 1998. Induction of hydroxyanthranilate hydroxycinnamoyl transferase activity by oligo-N-acetylchitoooligosaccharides in oats. *Phytochemistry* 47:969-974.
- Ishihara, A., Ohtsu, Y., and Iwamura, H. 1999. Biosynthesis of oat avenanthramide phytoalexins. *Phytochemistry* 50:237-242.
- Matsukawa, T., Isobe, T., Ishihara, A., and Iwamura, H. 2000. Occurrence of avenanthramides and hydroxycinnamoyl-CoA:hydroxyanthranilate N-hydroxycinnamoyltransferase activity in oat seeds. *Z. Naturforsch. C-A J. Biosci.* 55:30-36.
- Miquel, J. 2001. Nutrition and ageing. *Public Health Nutrition.* 4:1385-1388.
- Molteberg, E. L., Solheim, R., Dimberg, L. H., and Frølich, W. 1996. Variation in oat groats due to variety, storage and heat treatment. II. Sensory quality. *J. Cereal Sci.* 24:273-282.
- Peterson, D. M., Hahn, M., and Emmons, C. L. 2002. Oat avenanthramides exhibit antioxidant activities in vitro. *Food Chem.* 79:473-478.
- Visioli, F., and Galli, C. 2001. The role of antioxidants in the Mediterranean diet. *Lipids.* 36:S49-52.

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