Antioxidant and proteinase inhibition activity of main oat avenanthramides

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Summary
Information on the antioxidant activity of oat avenanthramides is available but not in relation to other prospective biological activities. Data on the anti-inflammatory, antioxidant, anti-itch, anti-irritant and antiatherogenic activities of avenanthramides have been previously published. In the present study, surprisingly, mild inhibitory activity of avenanthramide A on thrombin, trypsin, plasmin and papain was detected. Avenanthramide B was shown to inhibit chymotrypsin and pepsin activities, while avenanthramide C was found to strongly inhibit chymotrypsin activity and moderately trypsin activity. The results obtained indicate prospective use of avenanthramides as building blocks for selective proteinase inhibitors and suggest the necessity of systematic research on other enzymes to complete a profile of their biological activity.

Keywords
avenanthramides; oat; antioxidants; proteinase inhibition

Natural phytochemicals are considered a prospective source of novel biologically active agents and interesting compounds for development of both natural and semi-synthetic drugs. Many natural compounds, including polyphenols such as polyphenolic acids [1, 2], catechins [3, 4], flavonoids [5], anthocyanins [6], coumarines [7], stilbenes [8] or terpenes [9], possess interesting enzyme inhibition activities on both in vitro and in vivo levels. The most prevalent and best described is their antioxidant activity [10–13].

Oat, a cereal of *Poaceae* family, is a biologically active ingredient of many foods. It is known for its antioxidant and anti-inflammatory activities [14]. Oat is rich in polyphenols and avenanthramides [15]. Avenanthramides (anthranilic acid amides) comprise a group of phenolic alkaloids found mainly in oats (*Avena sativa* L.) but also in white cabbage butterflies eggs (*Pieris brassicae* and *Pieris rapae*) and in fungus-infected carnation (*Dianthus caryophyllus*). The general formula of avenanthramide A, B and C, including categorization by Collins and Dimberg's modified system, is presented in Fig. 1.

A number of studies demonstrated antioxidant, anti-inflammatory, anti-itch, anti-irritant and antiatherogenic activities of avenanthramides [16–18]. The main goal of this study was to explore other possible biological activities, which are associated with antioxidant potency, mainly inhibition of pathological proteinases.

Trypsin (EC 3.4.21.4) is the serine protease able to cleave peptide chain mainly at the carboxyl side. It can act as pathophysiological agent in pancreatitis, as a clotting factor (prothrombin to thrombin), or activate other enzymes (phospholipase A2, elastase), tissue hormones (bradykinin, kallidin) and cytotoxic proteins (complement system) [19].

Thrombin (EC 3.4.21.5) is the principal enzyme of hemostasis catalysing conversion of fibrinogen to fibrin and activating pro-coagulant factors,

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platelets, regulation of endothelial cell function, and directly acting on other cells. Thrombin is a pathophysiological agent of coagulation disorder disease [20].

Urokinase (EC 3.4.21.75) is an important component of the extracellular protease system specifically converting plasminogen to plasmin. This enzyme is participating in a number of pathophysiological processes that require membrane and/or extracellular matrix remodelling, including tumour progression and metastasis [21].

Chymotrypsin (EC 3.4.21.1) is a digestive enzyme present in pancreatic juice, acting in the duodenum where it performs proteolysis, the breakdown of proteins and polypeptides [22]. Under certain circumstances it may act as a promoter of equine chronic obstructive pulmonary disease [23].

Plasmin is an important enzyme (EC 3.4.21.7) present in blood that degrades many blood plasma proteins, including fibrin clots by fibrinolysis. It could act as a promoter of cancer and rheumatoid arthritis [24].

Papain (E.C.3.4.22.2) is a cysteine proteinase belonging to a family of related proteins with a wide variety of activities, including endopeptidases, aminopeptidases, dipeptidyl peptidases, and enzymes with both exo- and endo-peptidase activities, including for example cathepsin B (E.C.3.4.22.1). Papain serves as their cheaper alternative. Inhibitors of papain-like family are potential drugs for treatment of diseases associated with inflammation [25].

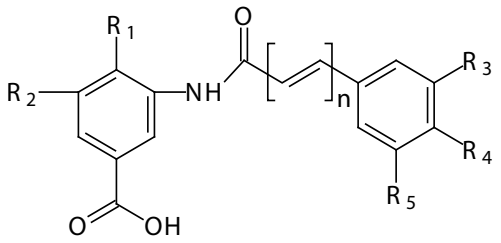
Similarly, pepsin (E.C. 3.4.23.1), an aspartic proteinase, can be used as an alternative for other aspartic proteinases, in particular for viral aspartic proteinases such as HIV proteinase (E.C. 3.4.23.16) or other viral aspartyl proteinases [26]. HIV proteinase belongs to retroviral aspartyl proteases and is essential for the life cycle of HIV, the retrovirus that causes AIDS [27].

The objective of this paper was to prove a hypothesis about inhibition activity of avenanthramides A, B and C on selected proteinases, and to start a systematic research focused on beneficial effects of oat in prevention of diseases related with proteinase hyperactivity.

MATERIAL AND METHODS

Chemicals

Common laboratory chemicals, including dimethyl sulfoxide (DMSO), methanol p.a., ethanol p.a., 2-propanol, chloroform p.a., Tween 40, β-carotene type IV, sodium carbonate, sodium acetate and acetic acid were supplied by Mikrochem (Pezinok, Slovakia). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox), 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB), phosphate buffer tablets, chromogenic substrate N-α-Cbz-L-lysine thiobenzyl ester hydrochloride (Z-L-Lys-SBzl hydrochloride), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), linoleic acid, ammonium persulfate, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and sodium dodecyl sulphate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All the enzymes used in the experiments were also supplied by Sigma-Aldrich. Avenanthramides A, B and C were purchased from ReseaChem (Burgdorf, Switzerland).



Collin's system	Dimberg's modified system	n	R_1	R_2	R_3	R_4	R_5
A	2p	1	H	OH	H	OH	H
B	2f	1	H	OH	OCH_3	OH	H
C	2c	1	H	OH	OH	OH	H
O	$2p_d$	2	H	OH	H	OH	H
P	$2f_d$	2	H	OH	OCH_3	OH	H

Fig. 1. General formula of avenathramides and categorization of derivatives.

Microplate assays

Assays were carried out in 96-well microplates using spectrophotometric methods. All the assays were performed in triplicates.

Antioxidant activity determined by DPPH method

The DPPH radical (•DPPH) scavenging activity of avenanthramides was measured using the method of Brand-Williams et al. [28], modified for a microplate screening system. Decrease in absorbance indicated higher free-radical-scavenging potency. Briefly, to 25 μl of the extract/standard solution/ethanol, 100 μl of 0.3 mol·l^{-1} methanolic solution of •DPPH was added. After 10 min of incubation in the dark, the optical density (*OD*) was measured at 490 nm in a microplate reader

OPSYS (Dynex, Chantilly, Virginia, USA). Antioxidant activity was expressed in micrograms of tested avenanthramide per microgram of Trolox.

Antioxidant activity determined by ABTS method

ABTS radical ($ABTS^{\bullet+}$) scavenging activity of avenanthramides was measured by the method of FERRI et al. [29], modified for a microplate screening system. $ABTS^{\bullet+}$ reagent was prepared by reaction of 7 μmol·l^{-1} ABTS and 2.45 μmol·l^{-1} ammonium persulfate in the final volume of 10 ml. The mixture was incubated for 16 h in the dark to produce $ABTS^{\bullet+}$ radical. *OD* of the mixture was measured at 630 nm after adjustment to a value of 0.7 by dilution with deionized water. Decrease in absorbance indicated higher free-radical-scavenging activity. Briefly, 100 μl of $ABTS^{\bullet+}$ reagent was added to 25 μl of extract/standard solution/ethanol. After 10 min of incubation in the dark, the optical density was measured at 630 nm in microplate reader OPSYS. Antioxidant activity was expressed in micrograms of tested avenanthramide per microgram of Trolox.

Antioxidant activity determined by FRAP method

Ferric reducing antioxidant power (FRAP) as the ability to change the oxidation status of transition metals of avenanthramides was measured by reduction of Fe(III)-TPTZ complex. The method by STRATIL et al. [30], modified for microplate screening system, was used. Fe(III)-TPTZ complex was prepared by mixing 10 ml TPTZ solution with 1 ml of sodium acetate buffer (pH 3.6) and 1 ml of 20 mmol·l^{-1} $FeCl_3$. Increase of absorbance indicated a higher reducing ability. Briefly, 35 μl of extract/standard solution/ethanol was mixed with 165 μl of Fe(III)-TPTZ reagent. After 5 min of incubation in the dark at 37 °C, the optical density was measured at 630 nm in microplate reader OPSYS. Antioxidant activity was expressed in micrograms of tested avenanthramide per microgram of Trolox.

Antioxidant activity determined by BCLM method

β-Carotene linoleate model (BCLM) reflects the ability of a compound to prevent/repair products of lipid peroxidation process in linoleate suspension with β-carotene supplementation. The method published by WETTASINGHE and SHAHIDI [31] was modified for microplate screening system. β-carotene linoleate (BCL) suspension reagent was prepared by mixing 800 mg linoleic acid, 4 g Tween 40 and 16 mg β-carotene in chloroform (20 ml), which was subsequently removed by evaporation. Reaction mixture (300 µl) was dissolved in 10 ml of air-saturated water. The emulsion formed (90 µl) was mixed with 10 µl of extract/standard solution/methanol in appropriate wells on microplate, incubated for 2 h at 60 °C and cooled to room temperature. Optical density was measured at 490 nm in microplate reader OPSYS. Antioxidant activity was expressed in micrograms of tested avenanthramide per microgram of Trolox.

Enzyme inhibition assay

To determine protease inhibiton activities of avenanthramides, a simple spectrophotometric method was used. For this purpose, chromogenic substrate Z-L-Lys-SBzl.2HCl in equimolar combination with DTNB was applied. The substrate was cleaved by the corresponding enzymes [32] releasing DTNB-S-Bzl detectable at 405 nm. Each well contained substrate (0.06 mmol·l^{-1}), tested compounds (10–200 µmol·l^{-1}), and 1% (v/v) DMSO in phosphate buffer, pH 7.0. Reaction was started by the addition of enzyme solution and incubated at 37 °C for 1 h. The enzyme solution activities were as follows: trypsin from bovine pancreas, 1 U·ml^{-1}; thrombin from bovine plasma, 0.6 U·ml^{-1}; urokinase from human urine, 2.5 U·ml^{-1}; chymotrypsin from bovine pancreas, 0.4 U·ml^{-1}; plasmin from bovine plasma, 0.1 U·ml^{-1}; papain from *Carica papaya*, 0.21 U·ml^{-1}; pepsin from porcine gastric mucosa, 2.5 U·ml^{-1}.

Differences in optical density (ΔOD) were measured by microplate reader OPSYS at 405 nm and were calculated according to the equation:

$$\Delta OD = OD_{61} - OD_1 \quad (1)$$

where OD_1 and OD_{61} is an optical density in 1st and 61st minute, respectively.

The percentage of inhibition of activity (*IA*) was calculated according to the equation:

$$IA = \left(1 - \frac{\Delta OD_S}{\Delta OD_C}\right) \times 100 \quad (2)$$

where ΔOD_S is optical density of sample and ΔOD_C is optical density of control (blank).

Data processing

Main parameters were calculated for each sample, i.e. Trolox equivalent antioxidant capacity (*TEAC*), parameter for radical-scavenging activity by DPPH method, and percentage of inhibition of activity (*IA*) for all enzyme inhibition assays. Inhibition concentration responsible for 50% inhibition of an enzyme (IC_{50}) was calculated from the relation:

$$IA = f(c) \quad (3)$$

where c is the concentration of avenanthramide.

At least five relevant concentrations versus *IA* values were taken into account.

Statistical analysis

Statistical analysis was performed on the basic data set composed from experimental variables (inhibition of activity) and individual measurements. The main goal of this analysis was to find similarities or differences in studied avenanthramides (types A, B and C). Statistical methods such as correlation analysis using Spearman correlation coefficients, analysis of variance (ANOVA) with post hoc least significant difference (LSD) test, principal component analysis (PCA) and cluster analysis (CA) were applied. This statistical approach was carried out by JMP 9.0 software (SAS, Cary, North Carolina, USA). Due to several repeating values of some inhibitory activities, not all variables could be included in statistical analysis. Statistical analysis made use of screening data obtained for standard chemicals.

RESULTS AND DISCUSSION

Antioxidant activity is the ability of a compound to provide free electron (single electron transfer – SET mechanism) [33] or to provide electron with hydrogen proton (hydrogen atom transfer – HAT mechanism) [34], and thus balance the molecule changes by electron shifting, e.g. by a system of conjugated bonds. This ability is expressed as one of the antioxidant mechanisms, such as termination, scavenging, quenching of radicals, transition metals oxidation status changing or preventing the lipoperoxidation processes. Tab. 1 shows data on antioxidant activities of three main avenanthramides (A, B and C), namely the ability to terminate $^{\bullet}$DPPH, ABTS$^{\bullet+}$ radicals, the ability to change Fe^{3+} to Fe^{2+} in complex with TPTZ, as well as the ability to prevent the lipid peroxidation process. Based on this data, avenanthramide C is the most effective compound. From the general point of view, the antioxidant activities of individual avenanthramides are different. This could be related to higher hydrophilicity and the presence of three OH groups as donors of electron (SET mechanism), or partially to acidic hydrogen atom (HAT mechanism). However, avenanthramides seem to be effective in lipophilic models, with the logarithm of the partition coefficient log$P \sim 2$ for *n*-octanol–water system. Interestingly, different activities in DPPH opposite to ABTS test were found in spite of the common mechanism, i.e. the ability to scavenge a model radical. Although antioxidant activity of selected avenanthramides is well known, it is difficult to compare our data with literature due to different experimental conditions and different interpretation of results. However, our results correspond to the literature in avenanthramide C being found the most effective antioxidant [35, 36].

On the other hand, the same aromatic hydroxyl groups located on the partial double bond are able to offer/provide H bond donors/acceptors for a non-covalent complex with various enzymes, in particular proteinases [37]. Since molecular weight of avenanthramides is about 300 g·mol^{-1}, each possesses one carboxy group, one pseudopeptidic bond consisting of NH-CO moiety, at least two hydroxyl groups and one methoxy group (6–7 H bond acceptors and 4 H bond donors), and the log*P* value is approx. 2, these compounds fulfill the requirements defined as LIPINSKI rule of 5 [38]. Avenanthramide molecules seem to be ideal building blocks for proteinase inhibitors, e.g. trypsin and chymotrypsin [39]. Despite this fact, there is no record about avenanthramides as inhibitors of proteinases in literature. Our results show that avenanthramide A, B and C exhibit inhibition activities towards trypsin, chymotrypsin, thrombin, urokinase, plasmin, papain and pepsin in the micromolar and milimolar concentration range. Inhibition activities on individual enzymes are presented in Tab. 2. As can be seen, all the three avenanthramides tested expressed significant specificity in proteinase inhibition activity. In fact, the testing model was improved to supress two important chromogenic interferences. The first one was the interaction of avenanthramides with one component of the substrate tandem (DTNB). However, the more important contribution was the breakdown of avenanthramide by proteolytic enzymes, which produce anthranilic acid and phenolic acid. Anthranilic acid expresses false chro-

Tab. 1. Antioxidant activity of avenanthramides A, B and C in four different tests.

Avenanthramide	Antioxidant activity [$\mu g \cdot \mu g^{-1}$]			
	DPPH	ABTS	FRAP	BCLM
A	100.02	1.75	16.61	0.88
B	25.06	2.54	5.22	2.08
C	8.33	1.96	3.13	0.86

Antioxidant activity is expressed in micrograms of tested avenanthramide per microgram of Trolox.
DPPH – 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical scavenging activity, FRAP – Fe(III)-2,4,6-tris(2-pyridyl)-*s*-triazine complex reducing antioxidant power, BCLM – β-carotene linoleate model, the ability to prevent/repair products of lipo-peroxidation.

Tab. 2. Inhibition activities of avenanthramides A, B and C as proteinase inhibitors.

Avenanthramide	IC_{50} [mmol·l^{-1}]						
	Trypsin	Thrombin	Urokinase	Plasmin	Chymotrypsin	Papain	Pepsin
A	0.62	0.16	> 10	0.67	> 10	0.67	> 10
B	> 10	> 10	> 10	> 10	0.77	> 10	0.28
C	0.46	> 10	> 10	> 10	0.03	> 10	> 10

Tests were carried out in triplicate, standard deviation was less than 5%. IC_{50} – concentration responsible for 50% of enzyme inhibition.

mogenic contribution under the used wavelength, and the released phenolic acids express inhibition activity, too.

Avenanthramide A appears to be the most prospective compound from those tested. Its inhibition activity towards proteinases decreased in the order: thrombin > trypsin > plasmin = papain. It seems to be a prospective inhibitor of three members of basic serine proteinases of trypsin-like family in the hundred-micromolar range. Moreover, avenanthramide A is a moderate inhibitor of papain, whereas it has no inhibitory effect on urokinase, chymotrypsin and pepsin. Surprisingly, avenanthramide B seems to be a weak inhibitor of chymotrypsin. However, it was shown to be an even more effective inhibitor of pepsin than a representative of the whole family of aspartic enzymes. Similarly, avenanthramide C shows weak inhibition activity on trypsin and mild inhibition activity on chymotrypsin.

Statistical processing revealed strong correlations between inhibition activity on chymotrypsin and antioxidant activities determined by DPPH and BCLM (for both, $R = 0.915$). Another strong correlation was observed between inhibitory activity on trypsin and antioxidant activity determined by BCLM ($R = 0.911$). Further strong correlation was observed between antioxidant activities determined by different mechanisms, DPPH and FRAP ($R = 0.917$). This correlation was expected, due to polar environment of both methods, which was also published [40]. This can be easily explained by relations between antioxidant activities, by SET or HAT mechanisms, and proteinase inhibition activities mediated by aromatic hydroxyl groups [37].

The similarities between inhibition potencies of particular avenanthramides (type A, B and C) were studied using cluster analysis (CA). In the dendrogram, similarities between avenanthramide B and C were found, while avenanthramide A formed its own cluster. Only one particular structural feature could be responsible for this fact, i.e. hydroxyl or methoxy group as R_3 substituent. Common oxygen atom indicates that the hydrogen bond acceptor atom in this position distinguishes avenanthramides B and C from avenanthramide A.

In Fig. 2, PCA results are shown with all the measured data and variables. Here, significant differences are evident between activities in vitro. The conclusions are supported by analysis of variance (ANOVA) and by post hoc test (LSD). The main output from PCA, the biplot, showed natural grouping of objects for each studied avenanthramide type (A, B and C), explaining 99.8% of data variance. Position of vectors (studied variables) indicated strong correlations between DPPH, FRAP and inhibition activity on chymotrypsin (*IA*_chymotrypsin). Another strong correlation was observed between inhibition activity against trypsin (*IA*_trypsin) and antioxidant activity by ABTS. Orthogonal position (right angles) of these vector groups and inhibition activities of pepsin and thrombin indicated very weak or no correlations between those variables. However, the discussed correlations should be studied on more samples.

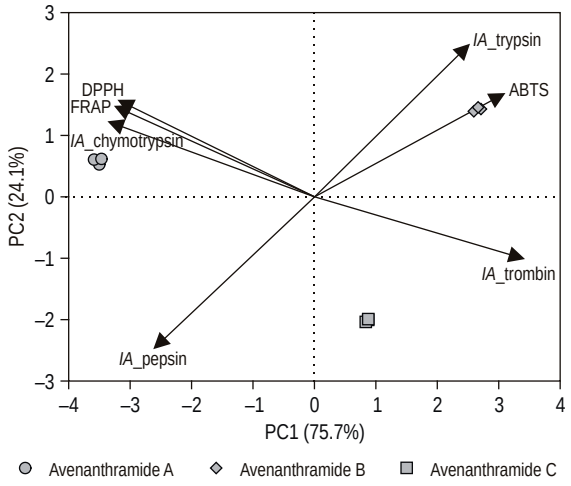


Fig. 2. PCA of oat avenanthramides.

All antioxidant activity variables (DPPH, ABTS, FRAP, BCLM) and proteinase inhibition activity variables were used.

Since this is the first report on proteinase inhibition activity of avenanthramides, the interpretation of the results should be cautious. Avenanthramides seem to be very interesting compounds expressing a wide spectrum of proteinase inhibition activities. Similar findings are very rarely observed in case of natural compounds in the role of enzyme inhibitors. The avenanthramide skeleton could serve as a base for selective proteinase inhibitors, which is the subject of intensive scientific efforts [41–43]. The molar weight of avenanthramide molecules (about 300 $g \cdot mol^{-1}$) allows the application of these compounds as building blocks, and also there is a significant space for enhancement of their inhibition potency by derivatization.

Avenanthramides may be useful to prevent diseases by oats consumption or if inhibition activities of avenanthramides would increase. The diseases are those in which proteinases play a significant role in disease progression. For example, strong inhibition activity of avenanthramide C on chymotrypsin (elastase family) may prevent joint tissue destruction, like rheumatoid arthritis. Another example is mild trypsin inhibition, which may prevent the chronic form of pancreatitis [37]. The prospects of avenanthramides are connected with preparation of synthetic avenanthramides with improved biological properties [44].

CONCLUSION

This study revealed new interesting properties of avenanthramide A, B and C, namely their inhibition potency to seven proteinases: trypsin, thrombin, plasmin, chymotrypsin, urokinase, papain and pepsin. These results may form the basis for further research and utilization of these compounds. Results suggest that consumption of oat products may be beneficial from dietetic and nutraceutical point of view in diseases accompanied by hyperactivity of proteinases. Further systematic research is necessary regarding inhibition activity of avenanthramide on other enzymes in vitro and also studies in vivo. Derivatization of the avenanthramide skeleton seems an attractive way to prepare new selective proteinase inhibitors as prospective drugs.

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