

Purification and Partial Characterization of an Endoxylanase Inhibitor from Barley

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ABSTRACT

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Hordeum vulgare L. xylanase inhibitor (HVXI), an endoxylanase inhibitor with a protein structure, was purified to homogeneity from barley (*Hordeum vulgare* L.). HVXI is a nonglycosylated monomeric protein, with a molecular weight of $\approx 40,000$ and a $pI \geq 9.3$. Although it inhibits different endoxylanases to a varying degree, the activities of an α -L-ara-

binofuranosidase and a β -D-xylosidase were not inhibited. Apparently, HVXI occurs in two molecular forms. These characteristics and the N-terminal sequences of the composing polypeptides show that HVXI is homologous with *Triticum aestivum* L. xylanase inhibitor I, an endoxylanase inhibitor from wheat flour.

Arabinoxylan (AX) is one of the major nonstarch polysaccharides present in cereal grain cell wall material. The total AX content is $\approx 6.6\%$ (w/w) for barley (*Hordeum vulgare* L.) (Henry 1988) and varies between 6.4 and 6.93% (w/w) for barley malt (Debyser et al 1997a). The water-extractable fractions of barley and its malt AX vary between 0.2 and 0.8% (w/w) (Woolard et al 1977) and 0.49 and 0.69% (w/w) (Debyser et al 1997a) of the kernel weights, respectively. AX hydrolysis requires several types of enzymes, including endo-(1-4)- β -xylanases (EC 3.2.1.8), hereafter referred to as endoxylanases, β -D-xylosidases (EC 3.2.1.37), and α -L-arabinofuranosidases (EC 3.2.1.55) (Biely 1985). Based on amino acid sequence similarities, the (microbial) endoxylanases have been grouped into two classes: family 10 (F) and family 11 (G) (Henrissat 1991). The two families have different molecular structures, molecular weights, and catalytic properties (Jeffries 1996; Biely et al 1997).

Although AX is a minor barley constituent, it is important for the functionality of the cereal. During malting, AX is degraded to a lesser extent than β -glucan (Viëtor et al 1991). Such degradation and a limited solubilization of water-unextractable AX during mashing (Debyser et al 1997b) lead to a certain AX profile in wort and beer. Undegraded AX, extracted or solubilized from malt, contributes to wort viscosity and lowered filtration rates and is involved in the formation of certain types of beer hazes (Steiner 1968; Coote and Kirsop 1976; Viëtor et al 1993; Leclercq et al 1999).

Debyser et al (1997b) reported the presence of endoxylanase inhibitors in cereals. They found evidence for the inhibition of the barley malt xylanolytic system by wheat water extractables during the production of Belgian white beer. In the same year, Debyser and Delcour (1997) disclosed more data on the structure of *Triticum aestivum* L. xylanase inhibitor (TAXI) in a patent application. The inhibitor is characterized by two molecular forms. A possible model was proposed in which one of the forms is proteolytically derived from the other one (Debyser and Delcour 1997; Debyser 1999; Debyser et al 1999). Recently, it was shown that TAXI consisted of a mixture of two structurally similar inhibitors (i.e., TAXI I and TAXI II), with different specificities (Gebruers et al 2001). Furthermore, endoxylanase inhibiting activities have been found in rye (*Secale cereale* L.) and barley (Debyser and Delcour 1997; Debyser 1999; Debyser et al 1999).

Other groups are also active in this new domain. Rouau and Surget (1998) confirmed the existence of an endoxylanase inhibit-

ing compound in wheat flour and McLauchlan et al (1999) purified and characterized a wheat flour endoxylanase inhibitor which was structurally distinctively different from the TAXI type inhibitors (TAXI I and II).

As the addition of endoxylanases leads to a reduced wort viscosity and a small increase in extract yield (Ducroo and Frelon 1989; Viëtor et al 1993; Cach and Annemuller 1995), an efficient enzymic degradation of (barley) AX in beer production is highly desirable. Our objective was to study one of the factors which can affect such hydrolysis. We here describe the purification and characterization of *Hordeum vulgare* L. xylanase inhibitor (HVXI), an endoxylanase inhibitor from barley, which seems to be the barley homologue of TAXI I from wheat. This is the first detailed report on endoxylanase inhibitors in barley.

MATERIALS AND METHODS

Materials

Barley (*Hordeum vulgare* L. 'Hiro') was obtained from AVEVE (Landen, Belgium) and was ground into whole meal using a Cyclo-tec 1093 sample mill (Tecator, Hogänäs, Sweden).

All reagents were from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless specified otherwise. Azurine-cross-linked wheat arabinoxylan (Xylazyme AX tablets), an endoxylanase (M4) and an α -L-arabinofuranosidase from *Aspergillus niger*, an endoxylanase (M1) from *Trichoderma viride* and an endoxylanase (M6) from a rumen microorganism culture filtrate were obtained from Megazyme (Bray, Ireland). The endoxylanases from *Bacillus subtilis* and *A. aculeatus* were from Puratos (Groot-Bijgaarden, Belgium). A β -D-xylosidase from *A. niger* was from Sigma-Aldrich. All chromatographic and electrophoresis media and markers were from Pharmacia Biotech (Uppsala, Sweden).

Endoxylanase Inhibition Assay Procedure

Before use, all endoxylanases were appropriately diluted in buffer containing bovine serum albumin (0.5 mg/mL) to stabilize the enzymes. Depending on the pH optimum of the enzyme, the buffer used was a sodium acetate buffer (25 mM, pH 5.0) or a sodium phosphate buffer (25 mM, pH 6.0).

In a standard assay, modified from the method of Debyser et al (1999), the activity of an endoxylanase solution in the presence or absence of the inhibitor was measured colorimetrically using the Xylazyme AX tablets as substrate. The endoxylanase solution (0.5 mL) was mixed with the sample (0.5 mL, same buffer as the enzyme solution). Substrate was added after a preincubation of 30 min at room temperature. The mixture was incubated for 60 min at 30°C and the reaction was stopped by the addition of 10.0 mL of 1.0% (w/v) Tris solution and vigorous vortex stirring. After 10 min at room temperature, the suspension was shaken, filtered through a Whatman No. 1 filter, and the absorbance (590 nm) was measured. Inhibition of the enzyme activity was expressed as percent reduction of the

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control (in absence of inhibitor). One inhibitor unit (IU) was defined as the amount that inactivated 50% of a fixed activity (1.0 U) of the *A. niger* endoxylanase M4. Under the conditions of the assay (incubation for 1 hr, 30°C), one enzyme unit corresponds to an increase in absorbance at 590 nm of 1.0. All measurements were made in duplicate.

Inhibitory activity determinations during purification were as described by Debyser et al (1999) using the *A. niger* endoxylanase M4.

Inhibitor Purification

Step 1: Preparing crude extract. Barley whole meal (2,500 g) was suspended in 12.5 L of 0.1% (w/v) ascorbic acid in water and was extracted overnight at 6°C. After centrifugation (10,000 × g, 30 min, 6°C), CaCl₂ at 2.0 g/L was added to the supernatant, pH was adjusted to 8.5 with 2.0N NaOH, and the extract was kept overnight (6°C). The precipitated material (pectins) was removed by centrifugation (10,000 × g, 30 min, 6°C) and pH was adjusted to 5.0 with 2.0N HCl.

Step 2: Concentration by cation exchange chromatography (CEC). The crude extract was applied to a SP-Sepharose Fast Flow column (90 × 90 mm) equilibrated with a 25 mM sodium acetate buffer (pH 5.0). A protein fraction containing inhibitory activity against endoxylanase M4 was eluted with 800 mL of 0.5M NaCl. The eluate was dialyzed against deionized water (48 hr, 6°C) and lyophilized. This material (4.95 g) is hereafter referred to as the CEC-I fraction.

Step 3: Purification by CEC. The CEC-I material was dissolved in 495 mL of a 25 mM sodium acetate buffer (pH 5.0) and fractionated by CEC on a SP-Sepharose Fast Flow column (26 × 300 mm) equilibrated with the same buffer. The column was eluted with a linear gradient of 0–0.5M NaCl in 800 mL at a flow rate of 5.0 mL/min. Fractions (10 mL) were collected; those containing endoxylanase inhibiting activity were pooled, dialyzed against deionized water (6°C overnight), and lyophilized, yielding fraction CEC-II (1.71 g).

Step 4: Purification by gel-permeation chromatography (GPC). Batches of the CEC-II material (12 mg) were dissolved in 1.0 mL of 250 mM sodium acetate (pH 5.0) and were further purified by GPC on a Sephacryl S100 column (26 × 670 mm). The column was eluted with the same buffer at a flow rate of 0.7 mL/min and fractions (2.1 mL) were collected. The endoxylanase inhibiting fractions of four runs were pooled, yielding the GPC solution.

Step 5: Purification by CEC. The GPC solution was diluted four times and was subsequently fractionated by CEC on a MonoS HR 5/5 column (5 × 50 mm). After sample application, the column was washed with a 20 mM sodium phosphate buffer (pH 6.5), removing noninhibiting proteins. The column was then eluted with a linear gradient from 0 to 0.6M NaCl in 40 mL at a flow rate of 0.75 mL/min. Fractions (0.5 mL) were collected and assayed for their ability to inhibit endoxylanase activity. Inhibiting fractions were pooled and pH was adjusted to 4.0 with 1.0N acetic acid. This solution was chromatographed a second time on the same MonoS column, equilibrated with a 25 mM sodium acetate buffer (pH 4.0) and using the same gradient as above.

Protein Determination

Protein concentrations were estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

Protein Electrophoresis

SDS-PAGE under nonreducing and reducing conditions was performed on 20% polyacrylamide gels with a PhastSystem unit (Pharmacia Biotech) (Laemmli 1970) and low molecular weight markers (LMW; Pharmacia Biotech LMW marker kit; *M_r* 14,400–97,000). 2-Mercaptoethanol (5% v/v) was used as the reducing agent. The pI was determined with the same instrument using polyacrylamide gels containing ampholytes (pH 3.0–9.0) and appropriate standards (Pharmacia Biotech broad pI kit, pI 3.5–9.3). All gels were silver stained using a technique adapted for the PhastSystem mini gels (Heukeshoven and Dernick 1985; Blum et al 1987).

Protein Sequencing

For determination of the N-terminal amino acid sequence, proteins were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane with a semi-dry electrophoretic transfer cell (Biorad Laboratories, Nazareth, Belgium), and subjected to Edman degradation. Sequence analysis was performed on an Applied Biosystems 477A Protein Sequencer (Perkin Elmer, Lennik, Belgium).

Glycosylation

Possible glycosylation of the purified inhibitor was studied using the digoxigenin-glycan assay (Boehringer Mannheim, Brussels, Belgium) as described by Roels and Delcour (1996). The inhibitor and the control proteins were separated by SDS-PAGE as described above and transferred to a nitrocellulose membrane with a semidry transfer unit (Pharmacia Biotech). Oxidation, labeling, and detection of the sugar residues of glycoproteins were performed directly on the membrane according to the kit instructions (Method B).

α-L-Arabinofuranosidase and β-D-Xylosidase Inhibition Assay Procedure

p-Nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-β-xylopyranoside solutions (10 mM in 50 mM 2(N-morpholino)-ethanesulfonic acid [MES] buffer, pH 5.5) were used as substrates for the α-L-arabinofuranosidase and β-D-xylosidase activities, respectively, in the presence or absence of inhibitor. The method was modified from that by Cleemput et al (1997). Enzyme solution (25 μL in 50 mM MES buffer pH 5.5) and the sample (25 μL, same buffer) were preincubated (30 min, room temperature). Substrate (100 μL) was added and, after 30 min at 30°C, the reaction was terminated by the addition of 1.5 mL of 1.0% (w/v) Tris solution. *p*-Nitrophenol released by the enzymes was determined colorimetrically (410 nm).

RESULTS AND DISCUSSION

Inhibitor Purification

By modifying the purification procedure for TAXI (Debyser and Delcour 1997; Debyser et al 1999), HVXI was purified from barley whole meal. Following a selective extraction and concentration by CEC on a SP-Sepharose Fast Flow column, the procedure sequentially included CEC on SP-Sepharose Fast Flow, GPC on Sephacryl S100 and, finally, CEC on MonoS. The final purification step was performed twice under different pH conditions in order to remove the last impurities. The endoxylanase inhibiting activity was enriched ≈900-fold, with an overall yield of ≈18% (Table I).

TABLE I
Purification of *Hordeum vulgare* L. Xylanase Inhibitor (HVXI) from Barley Whole Meal

Fraction	Total Protein (mg)	Total Activity (IU) ^a	Specific Activity (IU/mg) ^a	Purification (fold)	Recovery (%)
Crude extract	12,000	95,000	7.9	1	100
CEC-I	2,700	88,000	32.9	4.2	93
CEC-II	825	69,800	84.6	10.7	73
GPC	137	38,200	279	35.3	40
MonoS (pH 6.5)	4.1	27,600	6,700	848	29
MonoS (pH 4.0)	2.4	17,100	7,100	899	18

^a One inhibitor unit (IU) inactivates 50% of a fixed activity of *Aspergillus niger* endoxylanase M4.

Inhibitor Characterization

The purified inhibitor migrated as a double protein band with a molecular weight of $\approx 40,000$ (SDS-PAGE under nonreducing conditions). In the presence of 2-mercaptoethanol, the SDS-PAGE gel showed three protein bands with M_r of $\approx 40,000$; 29,000 and 12,000 (Fig. 1). The pI of the inhibitor, estimated by isoelectric focusing, was at least 9.3.

The M_r 40,000 and M_r 29,000 polypeptides both share the same N-terminal amino acid sequence KALPVLAPVTKDAATSLYTI, indicating that the M_r 29,000 polypeptide is proteolytically derived from the M_r 40,000 protein. The N-terminal amino acid sequence obtained for the M_r 12,000 polypeptide is GALAAXGVNPVAPFG. These sequences are compared with their TAXI I and II equivalents in Fig. 2 (Gebruers et al 2001). The HVXI polypeptides of M_r 40,000 and M_r 29,000 have a high sequence similarity with TAXI I and II (94% identity in an 18-amino acid overlap and 90% identity in a 20-amino acid overlap, respectively). The sequence of the smaller polypeptide is less similar to TAXI I and II (60 and 67% identity, respectively). We believe that, much as TAXI I and II, HVXI occurs as two molecular forms, both with a molecular weight of $\approx 40,000$ (Debyser 1999). The first form exists as a single polypeptide chain. After proteolytic modification, it is transformed

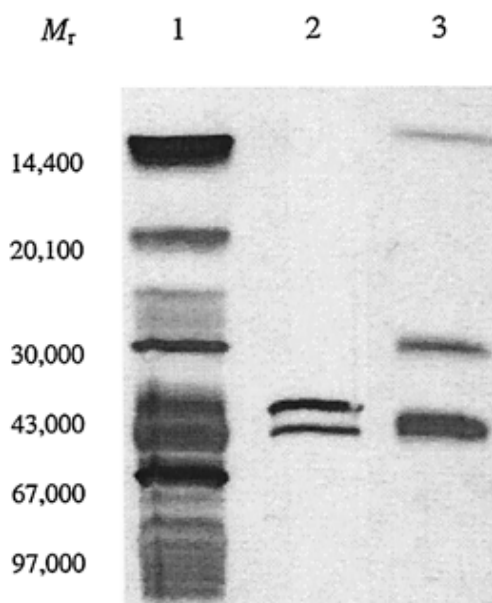


Fig. 1. SDS-PAGE (20% w/v) of purified inhibitor with silver-stained proteins. Lane 1, low molecular weight markers; lane 2, pure inhibitor under nonreducing conditions; lane 3, pure inhibitor under reducing conditions (5% v/v, 2-mercaptoethanol).

A			
HVXI	K A L P V L A P V T K D A A T S L Y T I		
TAXI I	* * * * * p * * * * *	94%	
TAXI II	* G * * * * * T * * * * *	90%	

B			
HVXI	G A L A A X G V N P V A P F G		
TAXI I	* * P V * R A * E A * * * * *	60%	
TAXI II	* * P V * R A * I * * * * *	67%	

Fig. 2. Comparison of N-terminal amino acid sequences of polypeptides of *Hordeum vulgare* L. xylanase inhibitor (HVXI) with molecular weights of 40,000 and 29,000 (A) and 12,000 (B) with *Triticum aestivum* L. xylanase inhibitor (TAXI) I and II equivalents (Gebruers et al 2001). X = nondefined amino acid, * = residues identical with those of HVXI, % = identity with HVXI sequence.

into the second form, which is composed of two disulfide-linked subunits with molecular weights of $\approx 29,000$ and $\approx 12,000$ (Fig. 3). This second form is not the result of proteolytic activity during extraction because, as evidenced by TAXI I and II, the purification of these inhibitors in the presence of protease inhibitors gave similar results (Gebruers et al 2001). We have strong indications that the second form is active, but to what extent the former is active is still unclear. It is reasonable to assume that the first form is the precursor of the second and that, following proteolytic modification, the inhibitor becomes (more) active. After all, proteins are often synthesized as proproteins which require proteolytic processing in order to be converted into their active mature forms. In this

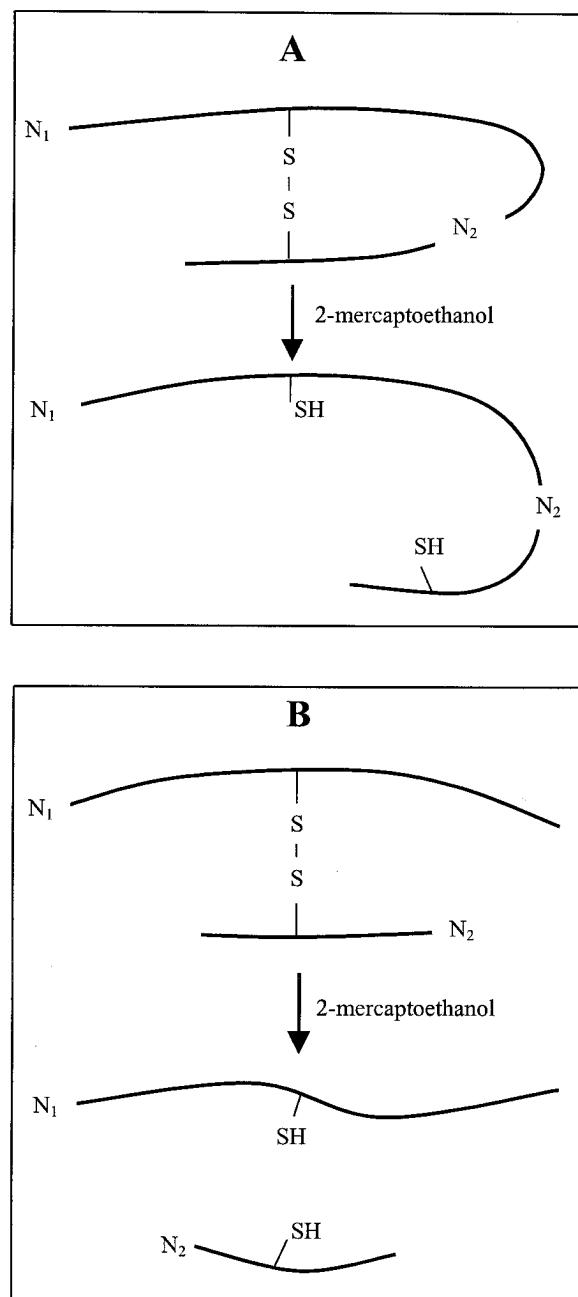


Fig. 3. Hypothetical model for two molecular forms of endoxylanase inhibitor (Debyser and Delcour 1997; Debyser 1999). **A**, Nonproteolytically modified form before and after reduction: a polypeptide with M_r $\approx 40,000$ and N-terminal amino acid sequence N_1 . **B**, Proteolytically modified form before and after reduction: two polypeptides with M_r $\approx 29,000$ and $\approx 12,000$ and N-terminal amino acid sequences N_1 and N_2 , respectively. For *Hordeum vulgare* L. xylanase inhibitor (HVXI), N_1 and N_2 are KALPVLAPVTKDAATSLYTI and GALAAXGVNPVAPFG, respectively.

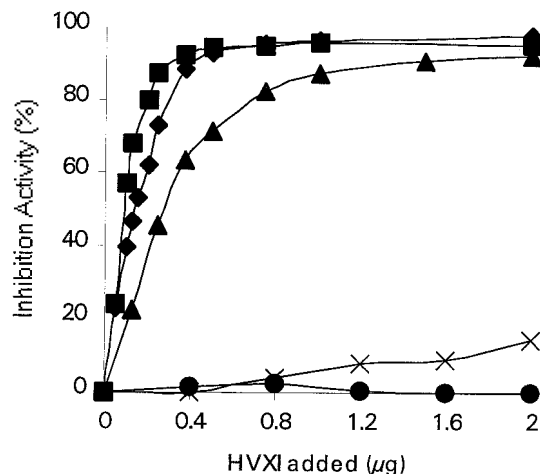


Fig. 4. Inhibition activities of *Hordeum vulgare* L. xylanase inhibitor (HVXI) against endoxylanases from *Asperillus aculeatus* (●), *A. niger* (◆), *Bacillus subtilis* (▲), *Trichoderma viride* (■), and a rumen microorganism culture filtrate (×).

context, there is a possible resemblance with an α -amylase inhibitor from bean (*Phaseolus vulgaris* L.) seeds. Endoproteolytic cleavage of the precursor of this inhibitor results in an active form, composed of two subunits (Santino et al 1992; Pueyo et al 1993).

HVXI was not glycosylated as was revealed by the digoxigenin-glycan assay (results not shown). Although the control proteins gave the expected results, no band appeared on the blot even after developing overnight. After boiling of the inhibitor solution (15 min, pH 5.0), no inhibitory activity could be found, indicating that the inhibitor is heat sensitive.

Inhibition of Arabinoxylan Hydrolyzing Enzymes

The inhibition of several AX degrading enzymes by HVXI was investigated. Using the standard assay, the activity of different levels of the inhibitor against five different microbial endoxylanases (*A. aculeatus*, *A. niger*, *B. subtilis*, *T. viride*, and rumen microorganism culture filtrate endoxylanases), depended on the enzyme used (Fig. 4). HVXI strongly inhibited the *T. viride*, *A. niger*, and *B. subtilis* endoxylanases (all family 11), with the first being the strongest inhibited and the latter being the least inhibited. Different levels of HVXI (≈ 0.08 , 0.14, and 0.27 μg , respectively) lowered the activity of one unit of these enzymes by 50%. In our assay, the inhibitor showed little if any activity towards the *A. aculeatus* endoxylanase (family 10) or the enzyme from a rumen microorganism culture filtrate (family unknown). Other AX hydrolyzing enzymes, like an α -arabinofuranosidase and a β -xylosidase from *A. niger*, were not inhibited either. Part of this selectivity toward endoxylanases may be due to the fact that the enzymes tested belong to different families. However, other factors should not be ignored because, in the presence of the inhibitor, not all family 11 enzymes tested reacted in the same way.

These biological characteristics, N-terminal amino acid sequences, and SDS-PAGE profiles under reducing and nonreducing conditions are similar for the TAXI type endoxylanase inhibitors of wheat (Debyser and Delcour 1997; Debyser 1999; Debyser et al 1999; Gebruers et al 2001). Therefore, HVXI is their barley homologue, in particular of TAXI I, which showed a similar effect on the activities of various endoxylanases (Debyser et al 1999; Gebruers et al 2001). In contrast, the inhibitor described by McLaughlan et al (1999) is a glycosylated, monomeric (single-chained), basic protein with a M_r of 29,000. In addition, its N-terminal amino acid sequence showed 86% identity with the sequence of chitinase III from rice (*Oryza sativa* L.), whereas the N-termini of HVXI did not reveal such high identity with known proteins. Using an advanced BLAST (version 2.0.10) search (Altschul et al 1997),

the N-terminal sequence of the M_r 40,000 and M_r 29,000 polypeptides (amino acids 8–18) revealed a 73% identity in an 11-amino acid overlap with an internal sequence PITKDAHTSIY of a hypothetical protein from *Arabidopsis thaliana* (amino acids 344–354). The sequence of the M_r 12,000 polypeptide showed only 60% identity with the sequence GALATPGYPAAPYG of “osr40g3”, a rice protein.

In general, proteinaceous enzyme inhibitors may be involved in plant defense mechanisms or in regulating certain metabolic activities in the plant (Garcia-Olmedo et al 1987; Täufel et al 1991). The endoxylanase inhibitor in barley may have both functions. On the one hand, it can prevent the degradation of AX by phytopathogenic microorganisms; on the other hand, it can regulate the AX degradation during germination. In extracts of germinating barley, xylan hydrolase activity appears several days later than (1-3),(1-4)- β -glucanases (Slade et al 1989). Although the endoxylanase genes are transcribed ≈ 24 hr after those of the (1-3),(1-4)- β -glucanases (Banik et al 1997), it has been suggested that the late appearance of the endoxylanase activity is due to a strong binding of these enzymes with the (aleurone) cell walls (Benjavongkulchai and Spencer 1989; Slade et al 1989). However, the presence of endoxylanase inhibitors in barley may also explain these observations.

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