Triticum aestivum L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities

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The *Triticum aestivum* L. endoxylanase inhibitor (TAXI) discovered by Debyser and Delcour [(1997) Eur. Pat. filed April 1997, published as WO 98/49278] and Debyser, Derdelinckx and Delcour [(1997) J. Am. Soc. Brew. Chem. **55**, 153–156] seems to be a mixture of two different endoxylanase inhibitors, called TAXI I and TAXI II. By using *Aspergillus niger* as well as *Bacillus subtilis* endoxylanases for assaying inhibition activity, both inhibitors could be purified to homogeneity from wheat (*Triticum aestivum* L., var. Soissons). TAXI I and TAXI II have similar molecular structures. They both have a molecular mass of approx. 40.0 kDa, are not glycosylated and occur in two molecular forms, i.e. a non-proteolytically processed one and a proteolytically processed one. However, the pI of TAXI II (at least 9.3) is higher than that of TAXI I (8.8). TAXI I and TAXI II clearly show different inhibition activities towards different endoxylanases. The N-terminal amino acid sequences of both inhibitors show a high degree of identity, which might indicate that there is an evolutionary relationship between them.

Key words: enzyme inhibitor characterization, proteolytic modifications, wheat proteins.

INTRODUCTION

Cereal grains contain three groups of important biopolymers: starch, proteins and non-starch polysaccharides. Starch and a large part of the protein fraction are located in the endosperm and serve as reserve material for the plant during germination and the initial stages of growth. They are degraded by amylases and proteases respectively [1]. The non-starch polysaccharides include mainly arabinoxylan (AX) and β-glucan, which are part of the cell walls and are hydrolysed by xylanolytic and β-glucanolytic enzymes respectively [1,2]. The degradation of these cell wall polysaccharides in the endosperm and aleurone layer during germination improves the accessibility of starch and protein for amylases and proteases [3,4]. Proteins that inhibit amylases [5–9] and proteases [10–13] have already been purified from cereals and have been characterized extensively. They possibly regulate the plant starch and nitrogen metabolism and/or have an important role in plant defence by inhibiting enzymic hydrolysis by micro-organisms and predators.

A new class of enzyme inhibitors, i.e. proteinaceous inhibitors of endo-β-1,4-xylanases (endoxylanases, EC 3.2.1.8), has been discovered in cereals by Debyser and Delcour [14] and Debyser et al. [15]. Inhibition activity against such xylanolytic enzymes was found in cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) [14,16].

An endoxylanase inhibitor, named TAXI (*T. aestivum* L. endoxylanase inhibitor), was purified from wheat flour and partly characterized by Debyser and Delcour [14] and Debyser et al. [17]. TAXI has a molecular mass of approx. 40.0 kDa and occurs in two molecular forms, A and B, the latter resulting from the proteolytic modification of the former [14,16,17]. After reduction with 2-mercaptoethanol, the modified molecular form (B) dissociates into two fragments with molecular masses of approx. 10.0 and approx. 30.0 kDa respectively, whereas the molecular mass of the unmodified form (A) does not change on reduction. The inhibitor is heat-sensitive and has a pI of 8.8 [14,16,17]. Rouau and Surget [18] also found evidence for the presence of endoxylanase inhibitors in normal and durum wheats. These authors detected high inhibition activity against microbial endoxylanases in both wheat flour and bran. McLauchlan et al. [19] purified a wheat endoxylanase inhibitor structurally quite different from TAXI. It is a monomeric, glycosylated and heat-sensitive protein with a pI of 8.7–8.9 and a molecular mass of 29.0 kDa. It was found to be a competitive inhibitor. Its N-terminal amino acid sequence is 87% identical with a sequence close to the N-terminus of the rice chitinase III polypeptide chain and shows no similarity with the amino acid sequences of TAXI.

The present paper demonstrates that TAXI, as described by Debyser and Delcour [14] and Debyser et al. [17], is in fact a mixture of two endoxylanase inhibitors, namely TAXI I and TAXI II, which differ from one another in pI and endoxylanase specificity. However, the inhibitors are shown to have comparable molecular masses and structures.

EXPERIMENTAL

Materials

All reagents were purchased from Sigma–Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise.

Endoxylanase M4 and α-L-arabinofuranosidase (arabinofuranosidase, EC 3.2.1.55) from *Aspergillus niger* and azurine-cross-linked wheat AX tablets were from Megazyme (Bray, Ireland). Endoxylanases from *Bacillus subtilis* and *Aspergillus aculeatus* were made available by F. Arnaut, NV Puratos (Groot-Bijgaarden, Belgium). BSA and a β-D-xylosidase (xylosidase,

Abbreviations used: AX, arabinoxylan; CEC, cation-exchange chromatography; GPC, gel-permeation chromatography; $IA^{B.s.}/IA^{A.n.}$, ratio of inhibition activity against *B. subtilis* endoxylanase to inhibition activity against *A. niger* endoxylanase; TAXI, *Triticum aestivum* L. endoxylanase inhibitor.

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EC 3.2.1.37) from *A. niger* were purchased from Sigma–Aldrich. The Digoxigenin Glycan Detection kit® and the Complete® protease inhibitor cocktail tablets from Boehringer (Mannheim, Germany) and pepstatin A from Sigma–Aldrich were used.

All electrophoresis media and markers, chromatographic media and nitrocellulose blot membranes were from Pharmacia Biotech (Uppsala, Sweden).

Wheat (*T. aestivum* L., var. Soissons), was from AVEVE (Landen, Belgium) and was milled with a Bühler MLU-202 mill (Uzwil, Switzerland).

Protein determination

Protein concentrations were determined in accordance with the Coomassie Brilliant Blue method of Bradford [20] with BSA as a standard.

Endoxylanase inhibition assay procedure

The inhibition activities of a set of samples were determined with the xylazyme-AX method as described by Debyser [16]. All endoxylanase solutions were prepared in sodium acetate buffer (25.0 mM, pH 5.0) with BSA (0.5 mg/ml) and contained 2.0 units per 1.0 ml. One enzyme unit corresponds to an increase in A_{590} of 1.0 with the xylazyme–AX method (see below).

Endoxylanase solution (0.5 ml) was preincubated for 30 min at room temperature with an equal amount of sample (same buffer as enzyme solution), possibly containing inhibition activity. The mixtures were kept at 30 °C and after 10 min an azurine-cross-linked wheat AX tablet was added. They were then incubated for 60 min at 30 °C. The reaction was terminated by the addition of 1.0 % (w/v) Tris solution (10.0 ml) and vigorous vortex-mixing. After 10 min at room temperature the tubes were shaken vigorously and the contents were filtered through a filter (diameter 90 mm) from Schleicher & Schuell (Dassel, Germany). The A_{590} values were measured against a control, prepared by incubating the sample with buffer instead of enzyme solution, with an Ultraspec III® UV/visible spectrophotometer (Pharmacia Biotech). The difference between the absorbance values of the samples and a control prepared by using buffer instead of sample was used as a measure of the inhibition activity, expressed as a percentage decrease in endoxylanase activity.

Protein electrophoresis

SDS/PAGE under non-reducing and reducing conditions was performed on 20 % (w/v) polyacrylamide gels with a PhastSystem® unit (Pharmacia Biotech), by the method of Laemmli [21]. 2-Mercaptoethanol [5 % (v/v)] was used as reducing agent. The low-molecular-mass markers used were α-lactalbumin (14.0 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), albumin (67.0 kDa) and phosphorylase *b* (94.0 kDa). The pI of the inhibitor was determined by using the same instrument with polyacrylamide gels containing ampholytes (pH 3–9) and with appropriate standards (Pharmacia Biotech calibration kit, pI 3.5–9.3). All gels were stained with silver in accordance with the instructions of the manufacturer (Development Technique file no. 210; Pharmacia Biotech).

Inhibitor purification

TAXI I and TAXI II were purified on the basis of the method of Debyser and Delcour [14] and Debyser et al. [17] (see Figure 1). After each purification step, the resulting fractions were assayed for endoxylanase inhibition activity with *A. niger* and *B. subtilis* endoxylanases and the purity was checked by SDS/PAGE.

Step 1: preparation of wheat flour extract

Flour (10.0 kg) was suspended in 0.1 % ascorbic acid (50.0 litres), extracted overnight at 7 °C and centrifuged (10000 ***g***, 30 min, 7 °C). To the supernatant, 2.0 g/l $CaCl_2$ was added and the pH was raised to 8.5 with 2.0 M NaOH to precipitate the pectins. The extract was left overnight (7 °C) and centrifuged (10000 ***g***, 30 min, 7 °C). The pH was adjusted to 5.0 with 2.0 M HCl.

Step 2: concentration and partial purification by cation-exchange chromatography (CEC)

At pH 5.0, proteins with endoxylanase inhibition activity from the wheat extract were retained by CEC on an SP Sepharose® Fast Flow column (90 mm × 90 mm). The column was equilibrated with sodium acetate buffer (25 mM, pH 5.0; 500.0 ml) and a protein fraction was eluted with 0.5 M NaCl (1.0 litre). The eluate was dialysed against deionized water (7 °C, 48 h) and freeze-dried as the CEC material (17.0 g).

Step 3: purification by CEC

Batches of CEC material (4.0 g) in sodium acetate buffer (25 mM, pH 5.0; 400.0 ml) were applied to an SP Sepharose® Fast Flow column (26 mm × 100 mm) previously equilibrated with sodium acetate buffer (25 mM, pH 5.0; 200.0 ml). The proteins were eluted with a linear gradient of 0.0–0.5 M NaCl in 800.0 ml at a flow rate of 5.0 ml/min. Two separate fractions, one with high inhibition activity against *B. subtilis* and *A. niger* endoxylanases and one with high activity against *B. subtilis* endoxylanase but low activity against *A. niger* endoxylanase, were dialysed against deionized water (7 °C, 48 h) and freeze-dried as CEC I (4.7 g) and CEC II (2.9 g) respectively.

Step 4: purification by gel-permeation chromatography (GPC)

Batches of CEC I (20 mg) and CEC II (20 mg) in sodium acetate buffer (250 mM, pH 5.0; 1.0 ml) were fractionated by GPC on a Hiprep® Sephacryl® S-100 column (26 mm × 670 mm) with the same buffer at a flow rate of 0.7 ml/min. The active fractions were pooled as GPC I (590 mg in 2500 ml) and GPC II (320 mg in 1630 ml) respectively.

Step 5: purification by CEC

GPC I and GPC II were diluted 1:2. Batches of the diluted GPC I (100.0 ml) and diluted GPC II (100.0 ml) were fractionated by CEC on a MonoS® HR 5/5 column (5 mm × 50 mm) previously equilibrated with sodium acetate buffer (25 mM, pH 4.0; 5.0 ml) and sodium phosphate buffer (20 mM, pH 6.5; 5.0 ml) respectively. The bound proteins were eluted with a linear gradient of 0.0–0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min. The fraction thus derived from GPC I, containing inhibition activity against *B. subtilis* and *A. niger* endoxylanases, and the fraction derived from GPC II, containing activity against *B. subtilis* endoxylanase but not against *A. niger* endoxylanase, were used for further purification of TAXI I and TAXI II respectively. They were diluted 1:2, acidified to pH 4.0 with 1.0 M acetic acid and chromatographed again on the same MonoS® column equilibrated with sodium acetate buffer (25 mM, pH 4.0). The same flow rate and salt gradient were used. We finally obtained 12.0 mg of TAXI I and 9.5 mg of TAXI II.

Inhibitor purification in the presence of protease inhibitors

The purification method described above was repeated on a small scale (100 g of wheat flour) with a protease inhibitor cocktail dissolved in all solutions used. For every 50 ml of solution one Complete® protease inhibitor tablet and 1.0 mg of pepstatin A were used. Because the tablets contained EDTA to inhibit metalloproteases, no $CaCl_2$ was added in step 1.

Protein sequencing

TAXI I (25 μg) and TAXI II (25 μg) were subjected to SDS/PAGE under reducing conditions [21] in a SE 600 Series gel electrophoresis unit (Hoefer Pharmacia Biotech, San Francisco, CA, U.S.A.). The slab gel (140.0 mm × 160.0 mm × 1.5 mm) consisted of a stacking gel [total acrylamide, 3.88 % (w/v); cross-linker, 1.33 % (w/v) of total acrylamide] and a running gel [total acrylamide, 17.57 % (w/v); cross-linker, 0.46 % (w/v) of total acrylamide]. Separation was achieved with a current of 30 mA for 4 h at room temperature. The proteins were electroblotted to a nitrocellulose membrane with the Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Nazareth, Belgium), with an electric potential difference of 10 V for 1 h at room temperature, and were then subjected to Edman degradation. The N-terminal amino acid sequences were determined with an Applied Biosystems 477 A Protein Sequencer, connected on-line with a 120 A phenylthiohydantoin-amino-acid analyser (Perkin Elmer, Lennik, Belgium). All amino acid sequence determinations were performed in duplicate.

Glycan detection

For glycan detection, the digoxigenin glycan assay was performed as described by Roels and Delcour [22]. TAXI I (1.0 mg/ml), TAXI II (1.0 mg/ml), the positive control protein transferrin (1.0 mg/ml) and the negative control protein creatinase (1.0 mg/ml) were separated by SDS/PAGE under reducing conditions as described above, but with the sample buffer advised by the manufacturer of the Digoxigenin Glycan Detection Kit®. The proteins were electroblotted to a nitrocellulose membrane with a semi-dry PhastTransfer® unit (Pharmacia Biotech), with an electric potential difference of 20 V for 30 min at 15 °C. On the blot the vicinal diols of the glycans were converted to aldehydes with metaperiodate and labelled with the steroid hapten digoxigenin via hydrazide. The labelled glycoconjugates were detected with a digoxigenin-specific antibody conjugated with alkaline phosphatase. In the presence of the appropriate substrate, blue–purple bands appeared where the phosphatase was present. Oxidation, labelling and detection were performed in accordance with the kit instructions (method B).

Arabinofuranosidase and xylosidase inhibition assay procedure

The method used was based on that by Cleemput et al. [23]. *p*-Nitrophenyl arabinose and *p*-nitrophenyl xylose were used as substrates for measuring the arabinofuranosidase and xylosidase activities respectively, in the presence and the absence of inhibitor. Substrate (0.05 mmol), arabinofuranosidase (50 μl), xylosidase (1.0 ml), TAXI I (234 μg) and TAXI II (580 μg) were dissolved separately in Mes buffer (50 mM, pH 5.5; 5 ml). Enzyme (25 μl) and endoxylanase inhibitor (25 μl) solutions were preincubated for 30 min at room temperature. Substrate (100 μl) was added and after 30 min at 30 °C the reaction was terminated by the addition of 1.0 % (w/v) Tris solution (1.5 ml). Finally, A_{410} was measured against a control.

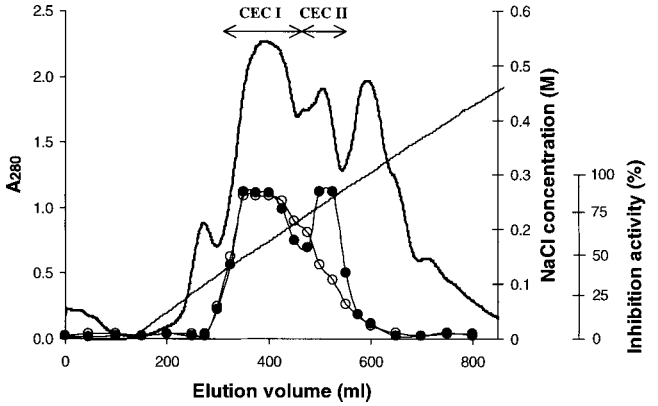

Figure 1 SP Sepharose® Fast Flow CEC chromatogram of CEC material

CEC material resulting from the fractionation of wheat flour extract by CEC on SP Sepharose® Fast Flow (90 mm × 90 mm) was separated again on SP Sepharose® Fast Flow (26 mm × 100 mm) (thick line, A_{280}) with increasing NaCl concentration (thin straight line), as described in the Experimental section. This resulted in CEC I and CEC II. The inhibition activities were measured against *A. niger* (○) and *B. subtilis* (●) endoxylanases.

RESULTS

Inhibitor purification

With the purification method described in the Experimental section, and *A. niger* and *B. subtilis* endoxylanases for assaying inhibition activity, TAXI I and TAXI II were purified to homogeneity from wheat flour. After initial fractionation by CEC on SP Sepharose® Fast Flow columns, two protein fractions, one with high inhibition activity against *B. subtilis* and *A. niger* endoxylanases (CEC I) and one with high activity against *B. subtilis* endoxylanase but much lower activity against *A. niger* endoxylanase (CEC II), were obtained (Figure 1). They were eluted at NaCl concentrations of 0.12–0.22 M and 0.23–0.27 M respectively. Neither CEC I nor CEC II contained significant inhibition activity against *A. aculeatus* endoxylanase. The ratio of inhibition activity against *B. subtilis* endoxylanase to inhibition activity against *A. niger* endoxylanase ($IA^{B.s.}/IA^{A.n.}$) for diluted (1:100) CEC I and CEC II was 1.11 and 3.21 respectively. This difference in $IA^{B.s.}/IA^{A.n.}$ indicated that a mixture of two endoxylanase inhibitors was present, subsequently referred to as TAXI I and TAXI II.

From CEC I, TAXI I was purified by GPC on a Hiprep® Sephacryl® S-100 column, from which it was eluted at a volume of 127.5–138.5 ml (GPC I), followed by CEC on a MonoS® column at pH 4.0, from which it was eluted at NaCl concentrations of 0.27–0.36 M. GPC I also contained TAXI II but in much lower levels than TAXI I. With CEC on MonoS®, TAXI II, characterized by a much higher $IA^{B.s.}/IA^{A.n.}$ than TAXI I (see below), resulted in an additional but smaller inhibition activity peak in the chromatogram. The final purification step was performed twice at the same pH to increase the purity of TAXI I. The chromatogram of the final separation on MonoS® is displayed in Figure 2(A).

TAXI II was isolated from CEC II in a similar way, but CEC on MonoS® was performed first at pH 6.5 and secondly at pH 4.0. CEC II contained, in contrast with CEC I, much more TAXI II than TAXI I. With GPC, TAXI II was eluted at the same volume as TAXI I and with CEC on MonoS® at pH 6.5 and 4.0, TAXI II eluted at NaCl concentrations of 0.08–0.11 M

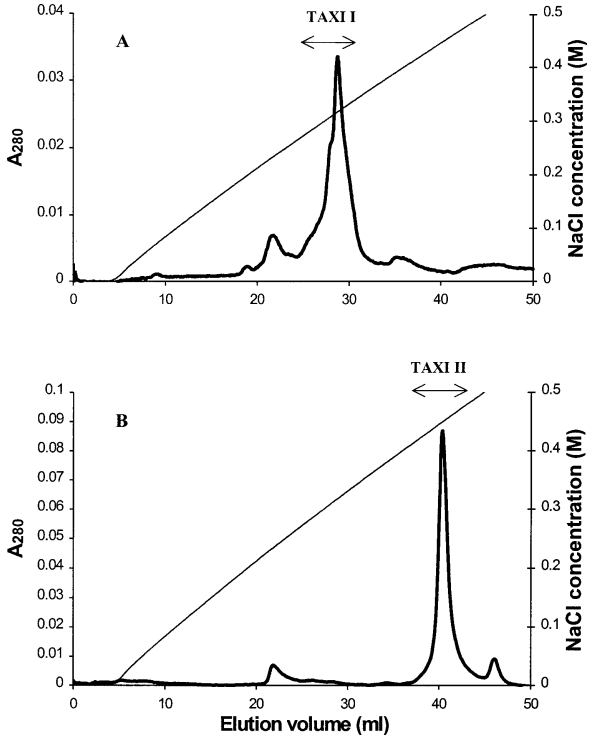

Figure 2 MonoS® CEC chromatogram of purified TAXI I (A) and TAXI II (B)

With the use of CEC on MonoS® with increasing NaCl concentration (thin straight line), as described in the Experimental section, TAXI I and TAXI II were purified to homogeneity from GPC I and GPC II respectively. Only the last separations on MonoS®, performed at pH 4.0, resulting in pure TAXI I and TAXI II are shown (thick line, A_{280}).

and 0.42–0.49 M respectively. By analogy with the above, a small additional activity peak, caused by the presence of TAXI I, was observed with CEC on MonoS® at pH 6.5. Figure 2(B) displays the MonoS® CEC chromatogram of almost pure TAXI II, separated at pH 4.0.

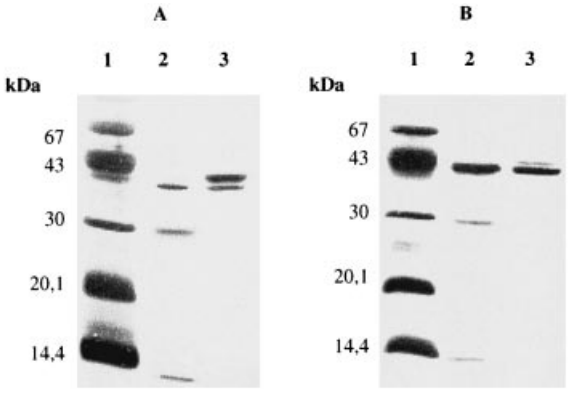

Figure 3 SDS/PAGE profiles of TAXI I (A) and TAXI II (B)

The purity and the molecular mass of TAXI I and TAXI II were assessed by SDS/PAGE, as described in the Experimental section. The gels were stained with silver. The sizes of the molecular markers are indicated at the left. Lanes 1, low-molecular-mass markers under reducing conditions; lanes 2, TAXI I (**A**) and TAXI II (**B**) under reducing conditions; lanes 3, TAXI I (**A**) and TAXI II (**B**) under non-reducing conditions. The gels were scanned with a Vuego Scanner (Brisa 610 S) and iPhoto Plus (version 4.0) software.

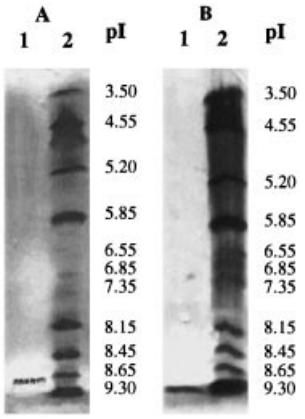

Figure 4 Isoelectric focusing gels of TAXI I (A) and TAXI II (B)

The pI of TAXI I and TAXI II was determined by isoelectric focusing, as described in the Experimental section. The gels were stained with silver. The pI values of the markers (lanes 2) are indicated at the right. Lanes 1, TAXI I (**A**) and TAXI II (**B**). The gels were scanned with a Vuego Scanner (Brisa 610 S) and iPhoto Plus (version 4.0) software.

Inhibitor characterization

The SDS/PAGE profiles (non-reducing conditions) of purified TAXI I and TAXI II show two polypeptides of approx. 40.0 kDa (Figure 3, lanes 3). Under reducing conditions, additional polypeptides of approx. 30.0 and approx. 10.0 kDa can be seen (Figure 3, lanes 2). These findings are in agreement with those of Debyser and Delcour [14] and Debyser et al. [17]. The pI of TAXI II is at least 9.3 and is therefore higher than that of TAXI I, which has a pI of 8.8 (Figure 4).

The N-terminal amino acid sequences of the TAXI I and TAXI II 10.0 kDa polypeptides are GAPVARAVEAVAPFG-VXYDT and GAPVARAVIPVAPFELXYXTKSLGN (single-letter amino acid codes) respectively. The 30.0 and 40.0 kDa polypeptides have the same N-terminal amino acid sequences, which for TAXI I and TAXI II are LPVLAPVTKDPATSLYT-IPFXDXA and KGLPVLAPVTKDTATSLYTIPF respectively. These results confirm the molecular structure model of TAXI by Debyser and Delcour [14] and Debyser et al. [17]. Because the N-terminal sequences of the 30.0 and 40.0 kDa polypeptides are identical, the 10.0 and 30.0 kDa polypeptides, held together by one or more disulphide bonds, are probably derived from the 40.0 kDa polypeptide by proteolytic modification. Elimination of endogenous wheat proteases during inhibitor purification by using Complete® protease inhibitor cocktail tablets together with pepstatin A had no observable effect on the ratio of proteolytically to non-proteolytically modified TAXI I and TAXI II. The SDS/PAGE profiles under reducing and non-reducing conditions were identical with those depicted in Figure 3. In addition, no difference in N-terminal amino acid sequences of the polypeptides of TAXI I and TAXI II from those described above could be observed.

TAXI I and TAXI II are not glycosylated, as evidenced from the Digoxigenin Glycan Detection Kit® results. Even after 15 h of colour development, no bands appeared on the blot for both inhibitors. The positive and negative control proteins, transferrin and creatinase respectively, gave the expected results.

Inhibition activities against xylanolytic enzymes

The activities of TAXI I and TAXI II were determined on three different endoxylanases, namely those of *A. aculeatus*, *A. niger*

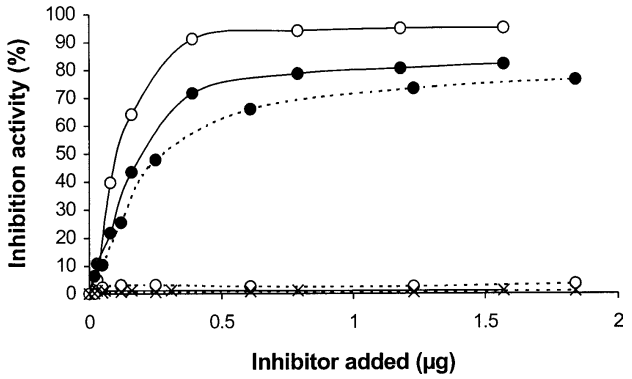

Figure 5 Inhibition activities of different quantities of TAXI I and TAXI II against *A. niger*, *A. aculeatus* and *B. subtilis* endoxylanases

For different quantities of TAXI I (solid lines) and TAXI II (dotted lines), the inhibition activities were determined against 1 unit of *A. niger* (○), *A. aculeatus* (×) and *B. subtilis* (●) endoxylanases. For this purpose the xylazyme-AX method, as described in the Experimental section, was used.

and *B. subtilis* (Figure 5). Under the test conditions, TAXI I inhibited only the *A. niger* and *B. subtilis* endoxylanases, the former more than the latter. Different quantities of TAXI I (0.10 and 0.20 μg respectively) decreased 1 unit of these endoxylanases by 50%. TAXI II inhibited *B. subtilis* endoxylanase but had no significant activity on both *A. aculeatus* and *A. niger* endoxylanases; 0.28 μg was needed for 50% inhibition of 1 unit of *B. subtilis* endoxylanase. At 50% inhibition of *B. subtilis* endoxylanase, TAXI I and TAXI II were characterized by $IA^{B.s.}/IA^{A.n.}$ values of 50:70 and 50:3 respectively. Both inhibitors were totally inactivated by 15 min of boiling.

Other xylanolytic enzymes, an arabinofuranosidase and a xylosidase from *A. niger*, were not inhibited by TAXI I or TAXI II.

DISCUSSION

Two endoxylanase inhibitors (TAXI I and TAXI II) were purified to homogeneity from wheat, and partly characterized. Both are non-glycosylated and have similar N-terminal amino acid sequences and SDS/PAGE profiles, indicating that there might be an evolutionary relationship between them. Their pI values are respectively approx. 8.8 and approx. 9.3 or higher. Whereas TAXI I inhibits *A. niger* endoxylanase to a greater extent than the *B. subtilis* enzyme, of these two enzymes TAXI II inhibits only *B. subtilis* endoxylanase. The activity of *A. aculeatus* endoxylanase was not affected by either TAXI I or TAXI II.

With a BLAST (version 2.0.10) search [24], the N-terminal amino acid sequences of the 40.0, 30.0 and 10.0 kDa polypeptides of TAXI I and TAXI II were found to have no significant similarities to amino acid sequences of other documented proteins.

The present results show that Debyser and Delcour [14] and Debyser et al. [17], in their reports on TAXI, probably studied a mixture of TAXI I and TAXI II. Indeed, mixtures of these two proteins with the above-mentioned inhibition specificities might very well have resulted in the observation that TAXI inhibited *B. subtilis* endoxylanase more effectively than the corresponding *A. niger* enzyme. Because the authors only screened with *A. niger* endoxylanase to purify TAXI, they probably picked up CEC I together with some material of CEC II with CEC on SP Sepharose® Fast Flow (Figure 1). Nevertheless, our observations are in line with the published [17] model for the molecular structure of the TAXI-type endoxylanase inhibitors, maintaining that these proteinaceous inhibitors occur in two molecular forms, A and B, with a molecular mass of approx. 40.0 kDa. According to the model, after reduction with 2-mercaptoethanol, form B dissociates into two fragments of approx. 10.0 and approx. 30.0 kDa, whereas the molecular mass of form A is not affected by the treatment. Because the N-terminal sequences of the 30.0 and 40.0 kDa polypeptides were identical, the 10.0 and 30.0 kDa polypeptides of form B, held together by one or more disulphide bonds, are probably derived from the 40.0 kDa polypeptide (form A) by proteolytic modification. We have strong indications that form A is active as endoxylanase inhibitor but it is not clear at present to what extent form B is active. It seems reasonable to assume that the first form (A) is a precursor of the second form (B) and that the inhibitor needs to be proteolytically modified to become (more) active. Such a mechanism has indeed been observed for an α-amylase inhibitor from bean (*Phaseolus vulgaris* L.) seeds [25,26]. However, the described results suggest that no proteolytic modification by endogenous wheat proteases occurs during purification and that forms A and B are already present as such in wheat flour.

In contrast with TAXI-type endoxylanase inhibitors, the more recent inhibitor described by McLauchlan et al. [19] is monomeric and glycosylated and has a molecular mass of 29.0 kDa. Its N-terminal amino acid sequence is 87% identical with a sequence of rice chitinase III in a 15-residue overlap and shows no similarity with the amino acid sequences of either TAXI I or TAXI II.

In wheat, endoxylanase inhibitors might have a dual function. They are possibly important in the regulation of plant metabolism by inhibiting endogenous endoxylanases and/or in plant defence by inhibiting exogenous endoxylanases produced by micro-organisms and predators. In contrast with exogenous endoxylanases, endogenous endoxylanases of wheat are less well documented, although Cleemput [27] and Cleemput et al. [23,28] have purified two endoxylanases with different substrate specificities.

Further work will be needed to study the interaction of the novel proteins described here with the latter enzymes.

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