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Purification and characterization of a XIP-type endoxylanase inhibitor from Rice (*Oryza sativa*)

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Abstract

A rice XIP-type inhibitor was purified by affinity chromatography with an immobilized *Aspergillus aculeatus* family 10 endoxylanase. Rice XIP is a monomeric protein, with a molecular mass of *ca.* 32 kDa and a pI of *ca.* 5.6. Its N-terminal amino acid sequence was identical to that of a rice chitinase homologue, demonstrating the difficulty when using sequence information to differentiate between endoxylanase inhibitors and (putative) chitinases in rice. Rice XIP inhibited different endoxylanases to a varying degree. In particular, it most strongly inhibited family 10 endoxylanases from *A. niger* and *A. oryzae*, while several family 11 enzymes from *Bacillus subtilis*, *A. niger* and *Trichoderma* sp. were not sensitive to inhibition. The above mentioned *A. aculeatus* endoxylanase was not inhibited either, although gel permeation chromatography revealed that it complexed rice XIP in a 1:1 molar stoichiometric ratio.

Keywords: *Endoxylanase inhibitor, XIP, affinity chromatography, rice*

Introduction

Cereals contain specific proteins with inhibition activity against a number of polysaccharide hydrolysing enzymes, including α-amylases [1,2], limit dextrinases [3] and endoxylanases [4–8]. The majority of the latter enzymes belong either to glycoside hydrolase (GH) family 10 or to the structurally unrelated GH family 11 [9,10]. Presumably, endoxylanase inhibitors contribute to plant defence mechanisms [11]. Moreover, since their target enzymes are often applied in food and feed technologies to degrade or modify the arabinoxylan population in order to improve processing and/or end use quality of cereal products [12–14], these inhibitors could influence the biotechnological functionality of the enzymes [15].

Endoxylanase inhibitors occur in several cereals and different endoxylanases are affected to varying degrees. [6] A particular case is that of a GH family 10 endoxylanase from *Aspergillus aculeatus* (further referred to as XAA, NCBI accession number AAE69552 [16]) which apparently is not inhibited by cereal extracts from wheat, barley, rye, oats, maize and rice. [6] Two structurally different cereal endoxylanase inhibitors have fairly recently been documented in the literature, namely the TAXI-type (*Triticum aestivum x*ylanase *i*nhibitor) and the XIP-type (*X*ylanase *i*nhibiting *p*rotein) endoxylanase inhibitors. Their occurrence, properties and structures have recently been reviewed [6–8].

TAXI-type inhibitors are basic, monomeric (*ca.* 40 kDa) and dimeric (30 + 10 kDa) proteins. They have been purified from wheat [17,18], barley [19,21], rye [20,21] and durum wheat [21]. In general, the TAXI-type inhibitors are active against endoxylanases of GH family 11 with reported K_i values ranging from 5 to 30 nM. [7] They show no activity towards GH family 10 endoxylanases [7,18,21].

XIP-type inhibitors are basic, monomeric, glycosylated proteins of *ca.* 30 kDa [5,8]. They have been

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purified from wheat [5,22], rye [23,24], barley [24], durum wheat [24] and maize [24]. Wheat XIP-I specifically and competitively inhibits fungal endoxylanases (apart from the above cited XAA) with the formation of a stoichiometric (1:1) complex [25]. Reported K_i values ranged from 3.6 to 610 nM. Wheat XIP-I is inactive against bacterial enzymes [25]. Based on amino acid sequence homology and structural similarities, wheat XIP-I has been classified into the GH family 18, which mainly comprises chitinases. [10,26] However, the XIP-type inhibitors do not hydrolyse chitin [5,24].

Although analysis of sequence data indicated the presence of both TAXI-like [6,27] and XIP-like [6] proteins in rice and revealed the high homology of wheat XIP-I with several rice chitinase homologues, neither endoxylanase inhibitors nor endoxylanase inhibition activity could be found in rice. [6] In addition, western blotting with anti-XIP-I antibodies could not detect XIP-I homologues in rice [28]. In the past, we developed and successfully applied an affinity chromatography (AC) based purification procedure with different immobilised endoxylanases for the purification and characterisation of cereal TAXI-type [21,29] and XIP-type endoxylanase inhibitors. [22,24] We here describe the affinity based purification, using the above cited XAA, and characterization of a XIP-type endoxylanase inhibitor from rice. This is the first detailed report on an endoxylanase inhibitor in rice.

Materials and methods

Materials

Brown rice (*Oryza sativa* L. 'Puntal') was from Masterfoods (Olen, Belgium) and was ground into whole meal using a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden).

The endoxylanases used, their source and some of their properties are summarised in Table I. All reagents were from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless specified otherwise. Chitine azure and the *Streptomyces griseus* chitinase was also from Sigma-Aldrich. Azurine-cross-linked wheat arabinoxylan tablets (Xylazyme AX) were purchased from Megazyme (Bray, Ireland). All chromatographic and electrophoresis media and markers were from Amersham Biosciences (Uppsala, Sweden).

Preparation of the affinity chromatography (AC) column

XAA was purified from Shearzyme 500L (Novozymes, Bagsvaerd, Denmark) following a single anion exchange chromatography purification step. The enzyme preparation (25 mL) was first dialysed against a 25 mM Tris/HCl buffer and centrifuged

Table I. Overview of endoxylanases used.

GH	Organism	Accession no.	MM	pI	pH_{opt}	Concentration used	Source
11	*Aspergillus niger*	P55329[a]	20 kDa	3.5	3.5	ca. 230 ng/mL	M4 (Megazyme, Bray, Ireland)
11	*Bacillus subtilis*	P18429[a]	20 kDa	9.3	6.0–7.0	ca. 270 ng/mL	Grindamyl H640 (Danisco, Brabrand, Denmark)
11	*Penicillium funiculosum*	CAC15487[b]	23.6 kDa	3.7	5.0	ca. 100 ng/mL	Institute of Food Research (Norwich, UK)[37]
11	*Trichoderma viride*	n.i.	20 kDa	8.4	4.5–5.0	ca. 82 ng/mL	M1 (Megazyme)
11	*T. longibrachiatum*	P36218[a]	19 kDa	5.5	4.5	ca. 132 ng/mL	M2 (Megazyme)
11	*T. longibrachiatum*	P36217[a]	21 kDa	9.0	6.0	ca. 100 ng/mL	M3 (Megazyme)
10	*A. aculeatus*	AAE69552[b]	56 kDa	4.5	4.0	ca. 5 μg/mL	Shearzyme 500 L (Novozymes, Bagsvaerd, Denmark)
10	*A. nidulans*	Q00177[a]	34 kDa	3.4	6.0	ca. 900 ng/mL	Institute of Food Research (Norwich, UK)
10	*A. niger*	n.i.	36 kDa	n.i.	n.i.	ca. 220 ng/mL	*A. niger* culture filtrate[c]
10	*A. oryzae*	n.i	36 kDa	4.7	n.i.	ca. 200 ng/mL	VTT (Espoo, Finland)

GH: Glycoside hydrolase family; MM: molecular mass; n.i.: no information; [a]SWISS-PROT accession number; [b]NCBI accession number; [c]purified from a culture filtrate according to Vynck *et al.* (2003)[36]

(10,000 × *g*; 20 min; 6°C). Next, the supernatant was loaded on a Q Sepharose Fast Flow column (26 × 100 mm), equilibrated with the same buffer. Bound enzyme was eluted with a linear gradient from 0–1.0 M NaCl in 300 mL at a flow rate of 5.0 mL/min. The isolated XAA was dialysed against deionised water and lyophilised.

The purified endoxylanase was linked to N-hydroxysuccinimide activitated Sepharose 4 Fast Flow matrix, according to the manufacturer's instructions [29], using 10–15 mg enzyme for 1.0 mL matrix. The coupling reaction was performed in a 200 mM $NaHCO_3$ buffer (pH 8.3; 7.0 mL) containing 0.5 M NaCl and proceeded for 2.5 h at room temperature while the mixture was shaken continuously. After removal of excess endoxylanase by rinsing with 0.5 M ethanolamine (pH 8.3) containing 0.5 M NaCl and incubation with the same ethanolamine solution for 4 h at room temperature, the matrix was washed with a 0.1 M glycine solution (pH 3.0) containing 0.5 M NaCl. Finally, the matrix was equilibrated with 25 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl (buffer A). This way, an affinity column (10 × 70 mm) with XAA as ligand was obtained.

Protein purification

Step 1. Preparation of the Rice whole meal extract. Rice whole meal (500 g) was suspended in 25 mM sodium acetate buffer (pH 5.0) (1:5 w/v) and was extracted for 30 min at 6°C by mechanical shaking. After centrifugation (5,000 × *g*, 30 min, 6°C), the supernatant was filtered to clarify the extract and the pH of the extract was adjusted to 5.0 with 1.0 N HCl.

Step 2. Concentration by cation exchange chromatography (CEC). The crude extract was applied to a SP-Sepharose Fast Flow column (26 × 300 mm), equilibrated with 25 mM sodium acetate buffer (pH 5.0). The bound protein fraction was eluted in one step with 1.0 M NaCl. The eluate was dialysed against deionised water (48 h, 6°C) and lyophilised, yielding the CEC-fraction.

Step 3: Inhibitor purification by AC. The CEC-fraction was first dissolved in buffer A. The resulting solutions (10–20 mg CEC-fraction/mL) were loaded (flow rate of 0.33 mL/min) on the AC column with immobilised XAA, previously equilibrated with buffer A. Elution of proteins interacting with the XAA affinity column was first with 7.0 mL deionised water and, following reequilibration with buffer A, with 7.0 mL of a 200 mM sodium phosphate buffer (pH 12.0). Elution was at a flow rate of 1.0 mL/min and fractions (1.0 mL) were collected. Peak fractions were pooled and subjected to a buffer exchange (25 mM sodium acetate buffer, pH 5.0), using a PD-10 column, yielding fractions further referred to as AC-XAA and AC-XAA12.

Protein determination

Protein concentrations were estimated according to the Coomassie Brilliant Blue method of Bradford [30] using bovine serum albumin as a standard.

Endoxylanase inhibition assay procedure

The endoxylanase inhibition activity was determined colorimetrically with the Xylazyme AX method (Megazyme product sheet Xyl 7/01) based on the method of Gebruers *et al.* (2001) [18]. Thus, (diluted) inhibitor (0.250 mL) and enzyme (0.250 mL) solutions, prepared in 25 mM sodium acetate buffer (pH 5.0), were incubated for 30 min at room temperature and an additional 10 min at 30°C prior to addition of the Xylazyme AX substrate tablets. The reaction proceeded for 60 min at 30°C and was then stopped by addition of 5.0 mL 2% (w/v) Tris solution with vigorous vortex stirring. After 10 min at room temperature, the suspension was shaken, and the absorbance at 590 nm (A_{590}) of the filtrate was measured. Inhibitory activity was expressed as the reduction (in %) of the endoxylanase activity determined in the absence of inhibitor. Before use, all endoxylanases were appropriately diluted in a sodium acetate buffer (25 mM, pH 5.0) containing 0.05% (w/v) bovine serum albumin to stabilise the enzymes. Under the conditions of the assay, the enzyme concentrations, presented in Table I, correspond to an increase in A_{590} of *ca.* 1.0. All measurements were made in duplicate.

Protein electrophoresis

SDS-PAGE under non-reducing and reducing conditions was performed on 20% (w/v) polyacrylamide gels with a PhastSystem unit (Amersham Biosciences) and low molecular weight markers (LMW marker kit; molecular mass 14.4 kDa–97.0 kDa) [31]. 2-Mercaptoethanol [5% (v/v)] was used as the reducing agent. The pI was determined by isoelectric focusing with the same instrument using polyacrylamide gels containing ampholytes (pH 3.0–9.0) and appropriate standards (Broad pI kit, pI 3.5–9.3, Amersham Biosciences). All gels were silver stained according to the manufacturer's instructions (Amersham Biosciences Development Technique file no. 210).

Protein sequencing

For the determination of the N-terminal amino acid sequence, proteins were separated by SDS-PAGE in an SE 600 Series Gel Electrophoresis Unit (Hoefer

Pharmacia Biotech, San Francisco, CA, USA) [17]. Next, they were electroblotted onto a PVDF membrane with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad Laboratories, Nazareth, Belgium) by applying an electric potential difference of 10 V for 1 h at room temperature, and subjected to Edman degradation. The sequence analysis was performed on a capillary Procise 491 cLC Protein Sequencer (Applied Biosystems, Foster City, CA, USA).

Chitinase assay procedure

Potential chitinase activity of the isolated AC-XAA and AC-XAA12 fractions was evaluated colorimetrically by measuring the solubilisation of fragments from dyed chitin. [5] Chitine azure (5 mg), suspended in McIlvaine's buffer (0.1 M citrate/0.2 M Na_2HPO_4; pH 5.5) was added to the different inhibitor fractions, as well as to the positive control samples, i.e. 0.05, 0.25 and 0.5 units of a chitinase from *Streptomyces griseus*, in a total volume of 1.0 mL. All mixtures were incubated for 4 h at 35°C and shaken at regular time intervals. Following centrifugation (13,000 × *g*; 10 min), the extinction (550 nm) of the supernatant was measured.

Gel permeation chromatography (GPC)

A mixture of purified XAA and rice XIP (ratio *ca.* 10:1), dissolved in a 10 mM sodium acetate buffer (pH 5.0) containing 0.2 M sodium chloride, was analysed by gel permeation chromatography (GPC) on a Superdex 75 HR 10/30 column (10 × 300 mm). Separations were at a flow rate of 1.0 mL/min.

Column calibration was with the Gel Filtration LMW Calibration Kit (Amersham Biosciences), containing Blue Dextran (2,000 kDa; for the determination of the void volume), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa) and ribonuclease A (13.7 kDa). In addition, XAA and rice XIP were each individually separated under identical conditions, which allowed peak identification in the chromatograms.

Results

Inhibitor purification and identification

Following extraction and concentration by CEC, a rice extract was applied to an affinity column with immobilised XAA. Although no inhibitory activity against XAA could be detected in crude extracts from rice [6], specific rice proteins were nevertheless capable of interacting with the immobilised XAA. These proteins eluted with deionised water (0.93 mg) and with the alkaline buffer (pH 12) (0.67 mg). Under the conditions of the assay, the isolated proteins, much like the crude rice extract, did not inhibit XAA. However, other endoxylanases were inhibited to a variable extent (*cf. infra*) demonstrating that a rice endoxylanase inhibitor was specifically retained on the XAA-AC column. In addition, significant inhibitory activity against a GH family 11 *Penicillium funiculosum* endoxylanase (XPF) and GH family 10 *Aspergillus niger* (XAN) and *A. oryzae* (XOA) endoxylanases still remained in the run-through of the XAA affinity column.

SDS-PAGE of the AC-XAA- and AC-XAA12-fractions revealed that, both under reducing and non-reducing conditions, the isolated rice inhibitors migrated as a distinct protein band of *ca.* 32 kDa (Figure 1), which had a pI value of 5.5–5.6, as estimated by IEF (result not shown). Following SDS-PAGE and blotting, the N-terminal amino acid sequence DDPGLAVYWGRHKEEGSLREAXDT-GRYTTV (with X representing an unidentified amino acid) was determined. A standard BLASTP (version 2.2.8) [32] revealed this sequence to be highly identical to those of several chitinase and chitinase-like proteins from various plant sources, especially from rice (Table II). In particular, when replacing 'X' by a cysteine, the obtained amino acid sequence was identical to the N-terminal region of a (possibly inactive) rice chitinase III homologue (OsChib3H-h; NCBI accession number BAA77780) [32] and, except for one amino acid residue, identical to the sequence of a putative rice chitinase III (NCBI accession numbers NP_911042 and BAC79552). However, none of the AC-XAA- and the AC-XAA12-fractions had detectable chitinase activity. In addition, the N-terminal sequence of the isolated rice inhibitor shows high homology to that of wheat XIP-I [26]. When the above described purification procedure was

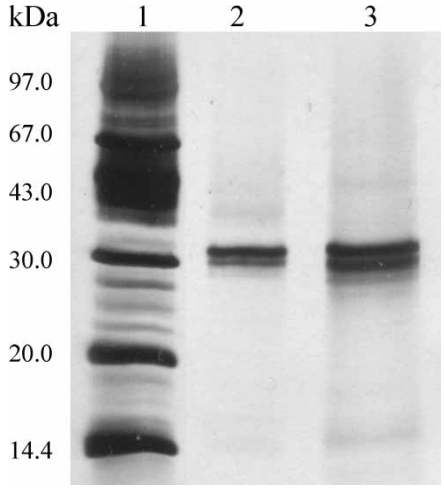

Figure 1. SDS-PAGE gel (20% w/v) of the rice AC-XAA and AC-XAA12 fractions with silver stained proteins. The sizes of the markers are indicated at the side of the gel. Lane 1, low molecular mass markers; Lane 2, AC-XAA fraction; Lane 3, AC-XAA12 fraction.

Table II. N-terminal amino acid sequence of rice XIP and comparison with other chitinase and chitinase-like proteins from rice, and with wheat XIP-I.

Protein	NCBI accession no.	Amino Acid Sequence																													
Rice XIP	–	NH_2-	D	D	P	G	L	A	V	Y	W	G	R	H	K	E	E	G	S	L	R	E	A	X[a]	D	T	G	R	Y	T	T	V
OsChib3 H-h[b,c]	BAA77780		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*
Putative chitinase III[c]	NP_911042; BAC79552		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	N	*	*
OsChib3H-g[b,c]	BAA77779		W	Y	G	P	*	*	*	*	*	*	*	*	*	DY	*	*	*	*	*	*	*	C	*	*	*	*	*	N	*	*
Chitinase IIIC00481	JC5841		N	T	G	E	T	V	*	F	*	*	*	N	*	D	*	*	*	*	*	*	*	C	*	*	*	L	*	*	S	*
Chitinase III C10701[d]	JC5845	A	A	G	K	T	G	Q	M	T	*	F	*	*	*	N	*	*	T	*	K	*	T	C	*	*	*	L	*	*	*	*
wheat XIP-I[e]	CAD19479	NH_2-	A	G	G	K	T	G	Q	V	T	*	F	*	*	N	*	A	*	*	*	*	*	C	*	S	*	M	*	*	M	*

*amino acid residue, identical to that of rice XIP; [a]non-defined amino acid; [b]rice chitinase class III homologue; [c]chitinase activity not known; [d]identical to OsChiB3H-a (NCBI accession number BAA77769) with N-terminal region highly homologous to wheat XIP-I; [e]Elliott *et al.* (2002)[26]

repeated with extracts of other cereals (*i.e.* wheat, durum wheat, rye, barley, maize, oats and buckwheat), which likewise did not inhibit XAA [6], only proteins from wheat could be isolated and were identified as XIP-type inhibitors (results not shown). These findings clearly demonstrate that the isolated rice chitinase homologue is an endoxylanase inhibitor belonging to the XIP-type inhibitor family and we hence refer to this inhibitor as rice XIP.

Inhibition activity against endoxylanases

Different amounts of rice XIP were assayed for their ability to inhibit a range of GH family 10 and 11 microbial endoxylanases. Under the conditions of the assay, the activity of the different enzymes was affected to varying extents (Figure 2). The GH family 10 endoxylanases XAN and XAO were the most strongly inhibited. For these enzymes, inhibitor level and inhibitory activity were linearly correlated up to *ca.* 45% inhibition. This inhibition activity corresponded for both enzymes to a molar inhibitor/-endoxylanase ratio of about 2.2–2.4. The GH family 11 endoxylanase XPF was less sensitive to inhibition by rice XIP. The linear correlation between inhibitor level and inhibitory activity extended only to *ca.* 20% at an inhibitor/endoxylanase ratio of *ca.* 4. Even with a molar inhibitor/enzyme ratio of *ca.* 25, XPF was only inhibited to about 40%. Based on these data and assuming a 1:1 enzyme/inhibitor complex (*cf. infra*), we estimated the association constant of the rice XIP/XPF complex to be 6 to 7 fold smaller than that of the rice XIP/XAN_{10} and rice XIP/XAO complexes. Furthermore, under the conditions of the assay and using molar inhibitor/endoxylanase ratios of at least 40:1, rice XIP had little if any effect on the activity of several family 11 endoxylanases from *Bacillus subtilis*, *A. niger*, *Trichoderma longibrachiatum*

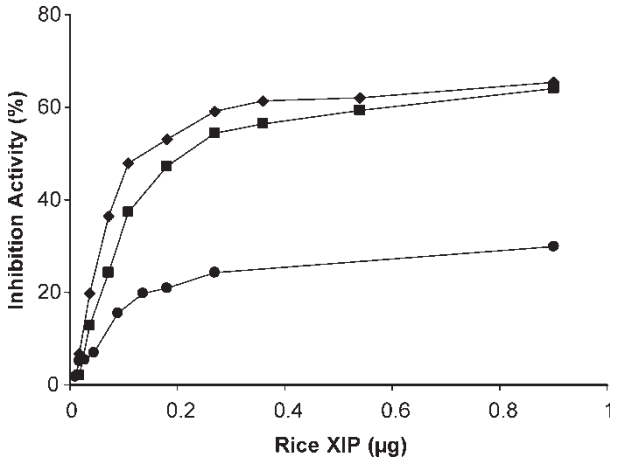

Figure 2. Inhibition activities of rice XIP against GH family 10 endoxylanases from *A. niger* (■) and *A. oryzae* (◆) and a GH family 11 endoxylanase from *P. funiculosum* (●).

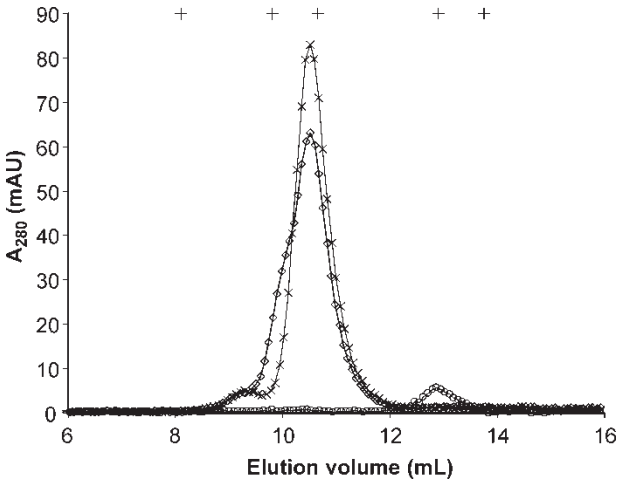

Figure 3. Gel permeation chromatography of the purified rice XIP (o), the *A. aculeatus* family 10 endoxylanase XAA (×) and the rice XIP/XAA mixture (◇). The volumes of the markers of the Gel Filtration LMW Calibration Kit are indicated (+).

and *T. viride*. The family 10 endoxylanases from *A. aculeatus* (XAA) and *A. nidulans* were not inhibited either.

Analysis by gel permeation chromatography

Although rice XIP is able to interact with an AC column with immobilized XAA, it does not inhibit this endoxylanase. In order to visualize the rice XIP/XAA interaction, a mixture of rice XIP and XAA was analyzed by GPC (Figure 3). The molecular mass of the eluting proteins was estimated by calibrating the GPC column. When separately analyzed by GPC, XAA and rice XIP are proteins of *ca.* 50 kDa and *ca.* 25 kDa, respectively. The GPC separation of the XAA/rice XIP mixture (with an excess of XAA) showed the disappearance of the inhibitor peak and the appearance of a distinct shoulder of the XAA peak. SDS-PAGE analysis showed that the shoulder contained both the endoxylanase and the rice inhibitor, demonstrating their interaction (result not shown). The position of the new shoulder indicates a molecular mass of *ca.* 64 kDa. A comparison with the molecular masses of XAA and rice XIP suggests that the XAA/rice XIP complex comprises one enzyme and one inhibitor molecule.

Discussion

In contrast to earlier reports describing the absence of endoxylanase inhibitors in rice [6,22,24,28], a XIP-type endoxylanase inhibitor was purified from this cereal using an AC-based purification procedure. Surprisingly, it interacted with the immobilized XAA, but did not inhibit the enzyme. Database screening revealed that its N-terminal amino acid sequence matched that of a putative rice chitinase homologue. However, this protein can now be classified as a XIP-type endoxylanase inhibitor.

Substantial inhibitory activity against several endoxylanases still remained in the run-through of the XAA affinity column, indicating that presumably other endoxylanase inhibitors occur in rice. In this respect, it remains to be seen whether the chitinase-like rice proteins found in the databases have chitinase and/or endoxylanase inhibition activity. Nagasaki *et al.* [33] suggested that these chitinases and chitinase homologues comprise a super family, with some members lacking chitinase activity. Indeed, XIP-type inhibitors and chitinases belonging to GH family 18 are closely related proteins: the XIP-I crystal structure revealed that XIP-I has structural features typical of GH family 18 [34]. However, there are significant differences in the region homologous with the active site of the chitinases [34]. A thorough study of the rice chitinase homologues and XIP-type endoxylanase inhibitors is therefore necessary to gain more insight into the structure-function relationship of these proteins. Alternatively, part of the inhibitory activity remaining in the run through might be due to the presence of TAXI-type inhibitors. After all, database screening and a genomic PCR approach yielded rice sequences with significant homology to wheat TAXI I [6,27].

Only a limited number of the enzymes tested in this paper were inhibited by rice XIP. These results explain to a large extent why, in the past, no inhibition activity has been detected in rice extracts. [6] Indeed, in these studies, measurement of the inhibitory activity was against the family 11 endoxylanases from *Bacillus*, *Aspergillus* and *Trichoderma* sp., which are not inhibited by the purified inhibitor. The above findings demonstrate the necessity to choose an arsenal of enzymes when studying enzyme inhibition, in particular by cereal extracts.

In addition, the observed specificity differs from wheat XIP-I and its rye, barley and maize homologues, which specifically inhibited all fungal endoxylanases tested, except for XAA [25]. In line with the above, no XIP-I homologues could be detected in rice by immunodetection and analysis of EST data [6,28]. However, the latter technique indicated that different XIP-type sequences occur in a variety of cereals and a classification into three subfamilies, *i.e.* XIP-I, XIP-II and XIP-III sequences, was suggested. [6] In this respect, the presence of XIP-II or XIP-III homologues was predicted in rice. Therefore, rice XIP possibly belongs to one of these subfamilies of the XIP-type inhibitor family.

Although the purified inhibitor did not inhibit XAA, GPC analysis revealed that they form a 1:1 molar complex. Because wheat XIP-I also interacted with endoxylanases in a 1:1 molar ratio [25], it is tempting to assume the rice XIP/XAA interaction to be similar

to the XIP/endoxylanase interaction. However, the molecular basis for interaction without inhibition is at present still unclear.

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