A novel class of protein from wheat which inhibits xylanases[1]

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We have purified a novel class of protein that can inhibit the activity of *endo*-β-1,4-xylanases. The inhibitor from wheat (*Triticum aestivum*, var. Soisson) is a glycosylated, monomeric, basic protein with a pI of 8.7–8.9, a molecular mass of 29 kDa and a unique N-terminal sequence of AGGKTGQVTVFWGRN. We have shown that the protein can inhibit the activity of two family-11 *endo*-β-1,4-xylanases, a recombinant enzyme from *Aspergillus niger* and an enzyme from *Trichoderma viride*. The inhibitory activity is heat and protease sensitive. The kinetics of the inhibition have been characterized with the *A. niger* enzyme using soluble wheat arabinoxylan as a substrate. The K_m for soluble arabinoxylan in the absence of inhibitor is 20 ± 2 mg/ml with a k_{cat} of 103 ± 6 s^{-1}. The kinetics of the inhibition of this reaction are competitive, with a K_i value of 0.35 μM, showing that the inhibitor binds at or close to the active site of free xylanase. This report describes the first isolation of a xylanase inhibitor from any organism.

Key words: arabinoxylan, glycosyl hydrolase, protein–protein interaction, *Triticum aestivum*, xylanase inhibitor.

INTRODUCTION

Plants contain a wide range of enzymes responsible for the hydrolysis of complex carbohydrate polymers. These enzymes facilitate the mobilization of reserves from storage organs and seeds during germination, and also direct the restructuring of the plant cell wall during growth and in response to microbial attack. Unregulated hydrolysis of carbohydrate polymers would be undesirable, so plants have evolved systems to ensure that such hydrolysis is tightly regulated in a tissue-specific and/or a temporal manner. This is generally carried out by groups of small inhibitor proteins that can specifically modulate the activity of carbohydrate hydrolyases. The presence of an inhibitor of α-amylase from cereals has been known for some time [1]. An inhibitor of polygalacturonase [2], of pectin methyl esterase [3] and of invertase [4] have all been reported from plants. However, there is no data on any inhibitors of xylanases, except for a report which stated the possible existence of a xylanase inhibitor of unknown type [5]. Xylanases (or, specifically, *endo*-β-1,4-xylanases) are produced by certain bacteria, fungi and plants, and hydrolyse the β-1,4-xylan linkages in the xylan component of plant cell walls. They have been grouped into two classes, family 10 (also called F) and family 11 (also called G) [6]. Family-11 xylanases are smaller (typically 20 kDa) and have a very characteristic and unique fold consisting of one α-helix and 13 β-strands in a single domain arrangement, which has been compared to the shape of a right hand [7]. In this report, we isolate and characterize a protein from wheat that binds to and inhibits two family-11 xylanases.

MATERIALS AND METHODS

Extraction of xylanase inhibitors from wheat flour

Wheat flour (1 kg; *Triticum aestivum*, var. Soisson) was suspended in 2 l of distilled water and stirred for 15 min at 4 °C. The slurry was centrifuged at 10000 ***g*** for 15 min at 4 °C and the supernatant was collected. The pH of the solution was adjusted to 7.0 and 10 g of carboxymethyl (CM)-Sephadex (Pharmacia Biotech, Uppsala, Sweden) was added as dry powder. The solution was stirred for 30 min, after which the ion-exchange resin was collected by sieving. A second 10-g batch of CM-Sephadex was added to the solution and treated as above. The combined resin was washed with distilled water, poured into a column and batch eluted with 200 ml of 0.5 M NaCl. The eluant was stirred at 4 °C and made to 80 % saturation by gradual addition of ammonium sulphate. This solution was then centrifuged at 10000 ***g*** for 30 min at 4 °C. The pellet was dissolved in 50 ml of distilled water and dialysed against 50 mM imidazole/HCl, pH 7.0, overnight.

Purification of xylanase inhibitors

The dialysed solution was then applied to an 80 ml CM-Sepharose column equilibrated with 50 mM imadazole/HCl, pH 7.0. The column was run at 2 ml/min and eluted with a linear gradient of 0–0.3 M NaCl in equilibration buffer. Fractions (2 ml) were collected and assayed for their ability to inhibit xylanase activity. Column fractions containing inhibitory activity were dialysed overnight at 4 °C against 50 mM Tris/HCl, pH 10.0, and then loaded on to a DEAE-Sepharose column equilibrated with the same buffer. The column was run at 1 ml/min and eluted with a linear gradient of 0–0.3 M NaCl in equilibration buffer. Fractions (1 ml) were collected and assayed for their ability to inhibit xylanase activity. Column fractions containing inhibitory activity were dialysed overnight at 4 °C against 50 mM sodium phosphate buffer, pH 7.0, and made to 1 M with respect to ammonium sulphate before being loaded on to a Hi-Load Phenyl-Superose column equilibrated with the same buffer containing 1 M ammonium sulphate. The column was run at 5 ml/min and eluted by reducing the ammonium sulphate concentration in a linear gradient from 1.0 to 0.0 M. Thirty fractions (5 ml) were collected and assayed for their ability to inhibit xylanase activity.

Abbreviation used: CM, carboxymethyl.

[1] This paper is dedicated to the memory of Martinus Roza.

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Column fractions containing inhibitory activity were dialysed overnight at 4 °C against 20 mM Tris/HCl buffer, pH 7.0, and then loaded on to a Mono S (1 ml) cation-exchange column (Pharmacia Biotech) equilibrated with the same buffer. The column was run at 1 ml/min and eluted with a gradient of 0–0.3 M NaCl in equilibration buffer. Then, 55 fractions (1 ml) were collected and assayed for their ability to inhibit xylanase activity.

Preparation of soluble wheat-flour arabinoxylan

Wheat flour (100 g; var. Soisson) was mixed with 200 ml of water and stirred at 4 °C for 1 h. The solution was then centrifuged at 15000 ***g*** for 30 min at 4 °C and the soluble arabinoxylan fraction obtained by sequential enzyme treatment [8]. At the end of the purification, the arabinoxylan was freeze-dried, which was followed by freeze-milling to a fine powder.

Screening column fractions for inhibition of xylanase activity

Routine assays during purification were performed using a microtitre-plate-based colorimetric assay to screen column fractions for their ability to inhibit a partially pure xylanase activity. The xylanase was partially purified from a commercially available *Aspergillus* hemicellulase preparation by elution from an anion-chromatography column (Q-Sepharose, Pharmacia Biotech) using a linear salt gradient (0–1.0 M NaCl). Column fractions (30 μl) were mixed with xylanase (30 μl) and the reaction initiated by the addition of 30 μl of a 1% solution of soluble wheat arabinoxylan dye linked with Remazol Brilliant Blue (Megazyme International Ireland Ltd, Co. Wicklow, Ireland). The microtitre plate was incubated for 10 min at 37 °C and the reaction terminated by the addition of 200 μl of absolute ethanol. The plate was then incubated at room temperature for a further 10 min, centrifuged at 1000 ***g*** for 20 min at 10 °C, and 150 μl of the supernatant was carefully transferred to a fresh microtitre plate. The xylanase activity and any inhibitory activity was related directly to dye release as measured by the absorbance at 610 nm. The procedure was as reported in the product information from Megazyme International.

Electrophoresis

The molecular masses of purified proteins under reducing and non-reducing conditions were estimated on 12.5% discontinuous SDS/PAGE [9] by comparison with a set of molecular-mass markers (Low-molecular-mass markers, Sigma, Poole, Dorset, U.K.), which ranged from 66 to 14.1 kDa. Sample buffer under reducing conditions contained dithiothreitol at a final concentration of 40 mM. The iso-electric points of purified proteins were estimated on a pH gradient generated in pre-cast 5% polyacrylamide gels (Clear-Gel IEF, Pharmacia Biotech) rehydrated in 10.5 ml of 10% (w/v) sorbitol containing 0.7 ml Pharmalyte (pH 8.5–10.0) (Pharmacia Biotech) and run on a Multiphor apparatus (Pharmacia Biotech). The gel was prefocused for 20 min at 700 V, the samples were entered into the gel at 500 V for 20 min, followed by focusing at 2000 V for 4 h. The run was finished by sharpening the bands for 10 min at 2500 V.

Glycosylation and protein assays

The presence of glycosylation on purified proteins was determined using the Glyco-Track® kit (Oxford GlycoSciences, Abingdon, Oxon, U.K.). In this procedure the proteins of interest were run on 12.5% SDS/PAGE as described above. They were then electroblotted to a 0.2 μm nitrocellulose membrane (Trans-Blot®, Bio-Rad, Richmond, CA, U.S.A.) using the buffer system of Kyhse-Anderson [10] at 2.5 mA/cm^2 for 30 min on a semi-dry blotter (Transblot SD®, Bio-Rad). The membrane was then treated according to the manufacturer's instructions, which leads to the sugar residues of glycoproteins becoming biotinylated. They were then tagged with a streptavidin/alkaline phosphatase conjugate and detected colorimetrically.

Protein concentrations of purified inhibitor were estimated using a commercial protein-assay kit (Coomassie Plus, Pierce, Rockford, IL, U.S.A.) based on the method of Bradford [11] and with BSA as the standard. The method was validated by correlating the response of the assay to the actual protein concentration of purified inhibitor, as determined by quantitative amino acid analysis.

N-terminal amino acid sequencing and amino acid analysis

N-terminal amino acid sequence was determined by automated Edman degradation performed by Alta Bioscience, Birmingham, U.K. Amino acid composition was determined in the Department of Molecular and Cell Biology, University of Aberdeen, Aberdeen, U.K. Samples were hydrolysed with 6 M HCl in the vapour phase under argon at 160 °C for 40 min. Derivatization was carried out on an Applied Biosystems 420A Amino Acid Analyser. The phenylthiocarbamoyl amino acids generated were identified online employing a C_{18} reverse-phase narrow-bore cartridge.

Protease digestion of inhibitor

The purified inhibitor (19.2 μg) was incubated with either 20 m-units of Pronase or 20 m-units of Pronase and 20 m-units of subtilisin (Roche Diagnostics, Welwyn Garden City, Herts, U.K.) for 3 h at 37 °C in a total volume of 100 μl. For both proteases, 1 unit is the enzyme activity that liberates Folin-positive amino acids and peptides corresponding to 1 μmol of tyrosine in 1 min. The proteolytic digestion was stopped by the addition of 5 μl of protease inhibitor cocktail (Complete®, Roche Diagnostics), prepared by dissolving one tablet in 0.5 ml of water. The efficiency of the digest was monitored by SDS/PAGE.

Chitinase assay

The purified xylanase inhibitor was assayed for chitinase activity by measuring the release of Remazol Brilliant Violet 5R from dyed chitin [12]. Three different concentrations (3.8, 19.2 and 38.4 μg) of purified inhibitor were incubated with 5 mg of chitin azure (Sigma) suspended in McIlvane's buffer, pH 5.5, in a total volume of 1 ml. Chitinase (0.05, 0.25 and 0.5 units) from *Streptomyces griseus* (Sigma) was used as a positive control. All samples were incubated at 35 °C on a rotating incubator for either 2 or 4 h. Samples were then centrifuged at 16000 ***g*** and the absorbance of the supernatant at 550 nm was measured on a Beckman DU 70 spectrophotometer.

Kinetic analysis

The effect of the purified inhibitor on xylanase activity was characterized by the dinitrosalicylic acid assay [13] using a family-11 xylanase from *Aspergillus awamori* var. Awamori, which was expressed in *A. niger*, purified to homogeneity [14], and soluble wheat-flour arabinoxylan as substrate. Preliminary studies were also carried out on a family-11 xylanase from *Trichoderma viride* [15]. The sequence of the *A. niger* protein used in this study is very similar to one for which a three-dimensional structure is available [7]. The two xylanases only differ by three

amino acids at the sequence level. Protein concentration of the *A. niger* xylanase was calculated using an absorption coefficient of 0.39 mg/ml for $A_{280} = 1$ based on the amino acid composition. Substrate (180 μl) and inhibitor were mixed and the reaction initiated by adding enzyme to give a final volume of 200 μl. The mixture was incubated for 5 min at 30 °C, terminated by the addition of 300 μl of dinitrosalicylic acid reagent and boiled for 5 min. The solution was then cooled and centrifuged for 5 min at 13000 ***g***, and 200 μl transferred to a microtitre plate. The absorption at 550 nm was measured relative to a xylose standard curve (0–180 μg/ml). Units were μmol of substrate/min. The kinetic constants from xylanase activity on different concentrations of substrate and in the presence of various amounts of inhibitor were estimated using the Enzfitter programme (Biosoft, Cambridge, U.K.).

RESULTS

Purification of the xylanase inhibitor

Following an aqueous extraction from flour, a batch elution from CM-Sephadex and concentration by ammonium sulphate, the xylanase inhibitor was purified to homogeneity by sequential chromatography on a cation-exchange column, an anion-exchange column, a hydrophobic-interaction column and, finally, a second cation-exchange column. The final analytical Mono S cation-exchange column was performed in five runs to avoid over-saturation of the column. All five runs produced similar elution profiles with two distinct peaks at 280 nm, both of which contained approximately equal amounts of inhibitory activity against xylanase. The specific inhibitory activities of each peak were almost identical and they are referred to below as inhibitors 1 and 2. Subsequent characterization of the inhibition with purified xylanase was carried out using inhibitor 1 only.

Characterization of xylanase inhibitors

SDS/PAGE analysis (Figure 1) demonstrated after staining with Coomassie Brilliant Blue that the two fractions from the Mono S column containing the inhibitory activity against the partially purified xylanase were homogeneous proteins, each with an apparent molecular mass of 29 kDa, which did not change under non-reducing conditions. Isoelectric focusing showed that inhibitors 1 and 2 had apparent pI values of 8.7 and 8.9 respectively. The latter contained a small amount of a protein which ran with the same pI as the former. Glycosylation analysis of the purified inhibitors (Figure 2) showed that both were glycosylated, and that the level of glycosylation was comparable with the positive control ovalbumin, which contains 2% (w/w) bound sugars. Analysis of both inhibitors revealed they shared the same N-

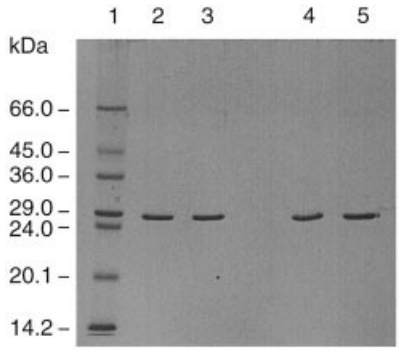

Figure 1 SDS/PAGE of the purified xylanase inhibitors from wheat

The purity of the two inhibitors was assessed by SDS/PAGE as described in the Materials and methods section. Gels were stained with Coomassie Brilliant Blue. The sizes of the molecular-mass markers are indicated to the left. Lane 1, low-molecular-mass markers (Sigma); lanes 2 and 3, 1 μl of the fractions corresponding to the first and second peaks eluted from the Mono S column run under reducing conditions. Lanes 4 and 5, as lanes 2 and 3 respectively, but run under non-reducing conditions. The gel was photographed and then digitally scanned for annotation using a Microtek Scanmaker III and Microscan (version 1.0.5) software.

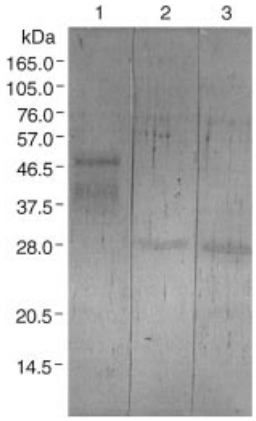

Figure 2 Glycoprotein analysis of the purified xylanase inhibitors from wheat

The presence of glycosylation on the purified inhibitors was determined using the Glyco-Track® kit (Oxford GlycoSciences) as described in the Material and methods section. The sizes of the molecular-mass markers are indicated on the left. Lane 1, ovalbumin, glycosylation positive control (1 μg). Lanes 2 and 3, 1 μl of the fractions corresponding to the first and second peaks eluted from the Mono S column. The membrane was photographed and then digitally scanned for annotation using a Microtek Scanmaker III and Microscan version 1.0.5 software.

Table 1 The amino acid composition of xylanase inhibitor proteins 1 and 2

The amino acid composition of the purified xylanase inhibitors from the final purification step (Mono S column) from wheat flour was determined as described in the Materials and methods section. The values shown for aspartic acid and glutamic acid are sums with their respective amides asparagine and glutamine. ND, not determined.

	Composition (%)	
Amino Acid	Peak 1	Peak 2
Aspartic acid	11.7	10.0
Glutamic acid	5.9	8.3
Serine	7.6	9.6
Glycine	14.5	16.5
Histidine	4.2	3.9
Arginine	4.2	4.1
Threonine	5.2	5.0
Alanine	8.8	7.3
Proline	5.5	4.9
Tyrosine	5.4	5.1
Valine	6.1	5.2
Methionine	1.8	1.6
Cysteine	ND	ND
Isoleucine	2.0	2.2
Leucine	8.3	7.6
Phenylalanine	3.6	3.9
Tryptophan	ND	ND
Lysine	5.2	4.8

Table 2 Chitinase activity of purified xylanase inhibitor 1

The chitinase activity of the purified inhibitor was measured by the release of Remazol Brilliant Violet 5R from chitin and compared with the activity of *Streptomyces griseus* chitinase, which was used as a positive control.

Sample	Incubation ...	Absorbance of digest supernatant at 550 nm	
		2 h	4 h
Buffer blank		0.046	0.046
Inhibitor (3.8 μg)		0.052	0.052
Inhibitor (19.2 μg)		0.056	0.054
Inhibitor (38.4 μg)		0.054	0.054
S. griseus chitinase (0.05 units)		0.180	0.287
S. griseus chitinase (0.25 units)		0.353	0.524
S. griseus chitinase (0.50 units)		0.477	0.700

terminal amino acid sequence, AGGKTGQVTVFWGRN. We also determined the amino acid sequence of three internal peptides of inhibitor 1, representing a total of 44 residues, generated by digestion with trypsin (results not shown). Allowing for the obvious limitations of amino acid analysis, such as the destruction of certain residues and conversion of asparagine and glutamine residues to their respective acids, both inhibitors also had a very similar amino acid composition (Table 1). We predict that inhibitor 2 is a post-translationally modified product of inhibitor 1 that has arisen either *in vivo* or as an artefact of the purification process. It cannot be ruled out, however, that the proteins are closely related to each other but the products of different genes. The yield of inhibitor 1 was 6 mg from 1 kg of flour, although this may be an underestimate if indeed the second protein is a modified product of the first or the initial aqueous extraction conditions used were not optimal. Using a BLASTP (Version 2.0.6) search [16], the N-terminal sequence of the inhibitors showed 86% identity with the sequence AAGKTGQ-MTVFWGRN, of chitinase III from rice, *Oryza sativa* [17]. This sequence is probably at or close to the N-terminus of the mature chitinase, as there is a consensus sequence [18] for a cleavable signal sequence downstream from it. A search using an earlier version of BLAST [19] revealed a 53% identity with the N-terminal sequence GATGQANQFYGQ of an unidentified protein from *Arabidopsis thaliana*, and a 40% identity with the sequence AGTRAGDIAIYWGQN from another rice chitinase, IIIa. When the sequences of three internal peptides of inhibitor 1 were submitted to a BLASTP search however, they showed no similarity with any of the above proteins or any significant similarity with any other sequence in the databases. It can be seen from Table 2 that purified inhibitor 1 had no detectable chitinase activity. In contrast, the chitinase positive control from *S. griseus* showed activity in this assay.

Table 3 shows that inhibitor 1 inhibited family-11 xylanases from two filamentous fungi. It inhibited the enzyme from *A. niger* by 83% and the *T. viride* enzyme by 54% and the inhibition was heat sensitive, since boiled inhibitor showed no inhibitory activity. The xylanase inhibitory activity was also protease sensitive (Table 4). Pronase treatment reduced the inhibition of the *A. niger* enzyme from 79 to 14% of the control level, whereas combined Pronase/subtilisin treatment reduced the inhibition to zero. Both proteases together with the protease inhibitor cocktail had no effect on control xylanase activity in the absence of the inhibitor (results not shown).

Kinetics of xylanase inhibition

Figure 3(a) demonstrates that *Aspergillus* xylanase exhibits a K_m of 20 ± 2 mg/ml and a k_{cat} of 103 ± 6 s^{-1} on soluble wheat arabinoxylan. In the presence of inhibitor 1 up to 1.02 μM, the

Table 3 The inhibition of fungal xylanases by xylanase inhibitor 1

Xylanases from *Aspergillus niger* (0.39 μg) and *Trichoderma viride* (0.23 μg) were incubated with purified inhibitor 1 (5.4 μg) from wheat flour as described in the Materials and methods section. The *A. niger* enzyme was also treated with an equal amount of inhibitor that had been boiled for 15 min. Xylanase activity and percentage inhibition are expressed as means $\pm$ S. D., $n = 3$.

Treatment	Xylanase activity (μmol of xylose/min)	Inhibition (%)
A. niger xylanase	0.086 ± 0.004	Not applicable
A. niger xylanase + inhibitor	0.015 ± 0.003	83 ± 4
A. niger xylanase + boiled inhibitor	0.092 ± 0.010	None
T. viride xylanase	0.068 ± 0.005	Not applicable
T. viride xylanase + inhibitor	0.031 ± 0.005	54 ± 7

Table 4 The effect of proteolytic digestion on the inhibition of a fungal xylanase by xylanase inhibitor 1

Aspergillus niger xylanase (0.39 μg) was incubated with purified xylanase inhibitor 1 (5.4 μg) and an equal amount of inhibitor that had been treated with either Pronase (20 m-units) or a mixture of Pronase and subtilisin (20 m-units each) as described in the Materials and methods section. Xylanase activity is expressed as the mean $\pm$ S.D., $n = 3$. All treatments produced results that were statistically different from the control xylanase activity (Student's *t*-test, $P < 0.05$), except Pronase/subtilisin.

Treatment	Xylanase activity (μmol of xylose/min)	Inhibition (%)
A. niger xylanase	0.080 ± 0.002	Not applicable
A. niger xylanase + inhibitor	0.017 ± 0.002	79 ± 3
A. niger xylanase + Pronase-digested inhibitor	0.069 ± 0.005	14 ± 6
A. niger xylanase + Pronase/subtilisin-digested inhibitor	0.076 ± 0.008	None

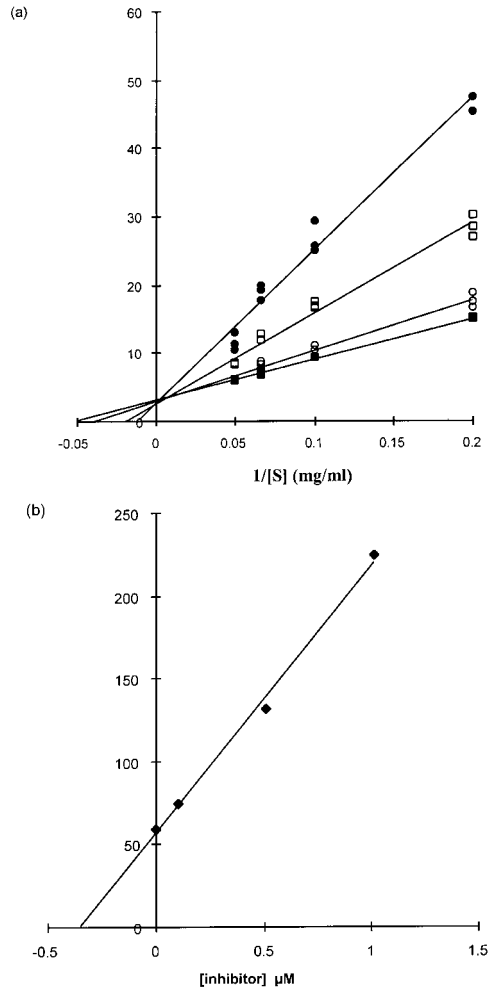

Figure 3 Inhibition kinetics

(**a**) Inhibition of *A. niger* family-11 *endo*-β-1,4-xylanase by purified xylanase inhibitor 1 with soluble wheat arabinoxylan as substrate. Xylanase activity is expressed as a double reciprocal plot with no inhibitor (■), or in the presence of 0.10 (○), 0.51 (□) or 1.02 μM (●) inhibitor. (**b**) Secondary plot of slopes from the double reciprocal plot against inhibitor concentration to give K_i.

V_{max} did not change but the K_m increased with increasing concentrations of inhibitor protein. Hence, the lines on the double reciprocal plot converge at the x axis. This shows that the inhibition is competitive, i.e. the inhibitor binds to the free enzyme in competition with the substrate. A secondary plot of slope against inhibitor concentration (Figure 3b) gives an inhibition constant, K_i, of 0.35 μM at pH 5.5 in sodium citrate/acetic acid buffer.

DISCUSSION

We have isolated a new class of protein from wheat flour that inhibits xylanases. The inhibitory activity is heat and protease sensitive, which excludes the possibility of a non-proteinaceous agent. Some enzymes, most notably glycosidases, are efficiently inhibited by Tris. Although we have used this buffer in our experiments, it is unlikely to be the inhibitory species in this case. During the purification procedures the inhibitory activity was fraction specific, despite Tris being present in all fractions. It is a relatively small, glycosylated protein with a basic isoelectric point, which is physically similar to the polygalacturonase inhibitor from tomato [2]. The inhibitor of pectin methylesterase from kiwi fruit [3] is also a glycoprotein, but has an acidic isoelectric point. In comparison, the inhibitor of invertase from tomato [20] is the least similar physically, being non-glycosylated and having a near-neutral iso-electric point. Although the N-terminal sequence of the inhibitor has 86 and 40 % identities with two rice chitinases and 53 % identity with an unidentified protein from *Ar. thaliana*, none of three regions of internal amino acid sequence from inhibitor 1 have any similarity to any chitinases or any sequence in the databases. In addition, the inhibitor itself has no intrinsic chitinase activity. We think that this is good evidence that the inhibitor is a new class of protein. The N-terminal similarity shared by the xylanase inhibitor, two rice chitinases and an unidentified protein from *Ar. thaliana* does not correspond to any known motif in the protein databases. If on further study the xylanase inhibitor and the unidentified *Arabidopsis* protein are shown to have cleavable signal sequences, then perhaps the motif represents a sequence required at the C-terminal side of the cleavage point. The inhibitor is active in the sub-micromolar concentration range. The inhibition is competitive, suggesting it binds at or close to the xylanase active site. The inhibition of polygalacturonase from the plant pathogen *Botrytis cinerea* by pear polygalacturonase inhibitor is also reported to be competitive [21], while in contrast, that of *Colletotrichum lindemuthianum* polygalacturonase by bean polygalacturonase inhibitor is non-competitive [22]. This ability of some proteinaceous plant inhibitors to modulate the activity of hydrolytic enzymes from plant pathogens has led to the proposal that they play a role in plant defence as well as the control of intrinsic enzyme activity. It is not unreasonable to speculate that the xylanase inhibitor may also have these functions in wheat. In barley grain, the α-amylase inhibitor has also been shown to inhibit the proteinase, subtilisin [23], and so it is possible that the xylanase inhibitor may also have other activities. Whereas the activity of the purified inhibitor was characterized using a pure xylanase, a partially purified xylanase extract was used to screen column fractions for inhibition during the purification from wheat flour. If the partially purified xylanase extract contained accessory enzyme activity, such as arabinofuranosidase, then it is possible that, using soluble wheat arabinoxylan as the substrate, the screening procedure may have isolated a protein that can inhibit both enzymes. Further work is in progress to determine if the purified xylanase inhibitor has any activity against other hydrolytic enzymes. We suggest that this class of protein is termed XI (for xylanase inhibitor), and if found in other species, then an abbreviated species name could be placed before XI.

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