

Purification of an Arabinofuranosidase and Two β -Xylopyranosidases from Germinated Wheat

Melissa M. Grant^{1,3}, Dennis E. Briggs¹, Colin S. Fitchett²,
Elaine Stimson² and Michael J. Deery²

ABSTRACT

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Arabinosidase and β -xylosidase activities were detected in germinated wheat grain, and both increased over seven days of germination, under malting conditions. Arabinosidase was partially purified by anion exchange chromatography, chromatofocusing and gel filtration chromatography. The pH optimum of the partially purified enzyme was 4.2 and the K_M was 1.90 mM p-NP-Ara. Edman degradation, MALDI-TOF mass spectrometry and nano-ESI mass spectrometry were used to identify the two major proteins in the partially purified arabinosidase mixture. The two proteins were a β -amylase with an amino acid sequence partially homologous to a barley β -amylase, and three wheat serine protease inhibitors. Further purification, by affinity chromatography and hydrophobic interaction chromatography, removed the identified contaminating proteins. At this point an 80 kDa protein was detected by SDS-PAGE. No identity could be assigned to this protein by MALDI-TOF mass spectrometry by reference to electronic protein databases. Similarly, the β -xylosidase was partially purified by anion exchange chromatography followed by chromatofocusing and gel filtration chromatography. The first step separated the mixture into two distinct fractions with K_M values of 3.35 and 4.01 mM p-NP-Xyl and pH optima of 4.5. The latter fraction also displayed xylanase activity against RBB-xylan.

Key words: Arabinofuranosidase, germinated, wheat, purification, xylopyranosidase.

¹ Malting and Brewing Group, Department of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, England,

² Cereals Innovation Centre, DuPont (UK) Ltd., Station Road, Cambridge, CB1 2UJ, England.

³ Corresponding author. Email: m.m.grant@aston.ac.uk

Current addresses of authors:

Melissa M. Grant, LHS, Aston University, Aston Triangle, Birmingham, B4 7ET.

Dennis E. Briggs, 66 Sandhills Lane, Barnt Green, Birmingham, B45 8NX, UK.

Colin S. Fitchett, Cambridge Biopolymers Ltd, 13 Sedgwick Street, Cambridge, CB1 3AJ.

Elaine Stimson, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW.

Michael J. Deery, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW.

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Abbreviations

DHB, 2,3-Dihydroxybenzoic acid

p-NP-Ara, *p*-nitrophenyl α -L-arabinofuranoside

p-NP Xyl, *p*-nitrophenyl β -D-xylopyranoside

PVPP, polyvinyl polypyrrolidone

SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

MALDI-TOF-MS, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

nano-ESI-MS, Nano-electrospray mass spectrometry

PVDF, polyvinylidene fluoride

RBB-xylan, 4-O-methyl-D-glucurono-D-xylan Remazol Brilliant Blue

Trizma, Tris (hydroxymethyl) aminomethane

INTRODUCTION

Cereal grain arabinoxylan is composed of chains of xylopyranose residues linked by β -1,4 glycosidic bonds. This xylan backbone is most commonly substituted with α -L-arabinofuranose residues at positions C2 and/or C3. The frequency of this substitution occurs between 1 arabinose every 2 to 5 xylose residues¹¹, isolated and adjacent substitutions are both common. Substitution occurs less often on three or more consecutive xylose residues¹⁰. Depending on the origin of the arabinoxylan it may or may not be substituted with acetyl-, feruloyl- and glucuronyl-residues^{1,5,18}. In wheat grain, arabinoxylan is found in the cell walls. The most highly substituted versions are found in the outer, 'bran' layers, which may resist the invasion of the grain by micro-organisms. During germination the arabinoxylan in the cell walls of the starch-containing endosperm is broken down to allow access of hydrolytic enzymes to the starch granules and protein stored within the cells. Acetyl- and feruloyl-substituents are removed by esterases^{2,14,15,19}. The core arabinoxylan polysaccharide is broken down by a number of glycoside hydrolases which include α -L-arabinofuranosidase (arabinosidase), endo-xylanase and β -D-xylopyranosidase (β -xylosidase)²⁰. The arabinosidase removes arabinose residues and the latter remove single xylose units from the non-reducing termini of either whole chains or from smaller xylo-oligosaccharides. Tenkanen *et al.*²² reported that an arabinofuranosyl group, linked α -1,3 to the xylopyranosyl ring, protected the β -1,4-xylosidic linkage before the substituted xylose unit from being cleaved by a fungal β -xylosidase. The enzyme is essential for complete xylan hydrolysis²³. Endo-xylanases cleave the xylan backbone in unsubstituted regions.

Arabinosidase and β -xylosidase activities were found in wheat flour by Lee and Ronalds¹⁷. The activity of arabinosidase increased during the first five days of germination⁹. Cleemput *et al.*⁸ partially purified an arabinosidase from ungerminated wheat and SDS-PAGE analysis of the partially purified material showed that there were at least four proteins present. The molecular masses of these were 6, 8, 40 and 64 kDa. Isoelectric focusing revealed that at least 13 proteins with pI values ranging from 5.0 to 7.0 were present. Cleemput *et al.*⁸ isolated a β -xylosidase from ungerminated wheat flour and characterized the enzyme, which had a molecular mass of 64 kDa and a pI of 5.5. It was capable of attacking xylobiose, xylotriose, xylo-tetraose and xylopentaose and could release xylose from xylan, but its activity was hindered by arabinose substituents on arabinoxylan chains.

This paper reports the partial purification of an arabinosidase and two β -xylosidases from extracts of germinated wheat grain. Arabinosidase activity was measured using the artificial substrate *p*-nitrophenyl α -L-arabinofuranoside (p-NP-Ara), and β -xylosidase activity was measured using the artificial substrate *p*-nitrophenyl β -D-xylopyranoside (p-NP-Xyl).

MATERIALS AND METHODS

Micromalting

Wheat samples (Variety Abbott, 75 g) were steeped in distilled water (500 mL, 23 h, 15°C). The samples were drained and surface sterilized by immersion in a filtered solution of calcium hypochlorite (2%, 1 h) before washing twice with distilled water (500 mL) and steeping again in distilled water (500 mL, 23 h, 15°C). After steeping the samples were drained and placed in a malting germination chamber^{4,16}. The samples were kept in the chamber, in a water saturated atmosphere at 15°C, and were rotated for 5 min in every 6 h, for between 1 and 7 days. At the end of each germination period, the samples of malted grain were removed and frozen (-20°C) prior to freeze drying.

At the same time a control experiment was conducted to check that no micro-organisms were growing on the surfaces of the grains. WL medium (38.5 g WL nutrient agar and 10 g agar technical No. 3 dissolved in 500 mL distilled water) was autoclaved (20 min, 120°C), poured into Petri dishes (9 cm diameter) and allowed to set. Grains (50) were placed on the surface and left at room temperature for the duration of the experiment. Any microbial growth was detected as colonies on or around the grains. The dishes were left at room temperature so that no one organism was favoured as the temperature would fluctuate over the course of the experiment. Grains from this experiment were discarded.

Enzyme extraction

Wheat grain (malted, de-rooted and freeze dried, or unmalted, 50 g) was ground for 30 s, to a flour, in a Moulinex coffee grinder. The flour was mixed with PVPP (3.33 g) and succinate buffer (150 mL, pH 4.2, 75 mM) and was dispersed and ground for 30 s with a Polytron high speed homogenizer (Polytron, Switzerland; at power setting 10,

using a 12 mm saw tooth head). The resulting slurry was shaken for 15 min at room temperature and then centrifuged (15 min, 12000 g, room temperature, MSE super minor centrifuge). The supernatant was retained and filtered through a 0.45 μ m syringe top filter (Gellman), before use¹².

Assay of arabinosidase and β -xylosidase activity¹²

Enzyme extract (50 μ L) was added to p-NP-Ara (Sigma-Aldrich, 100 μ L, 10 mM, in succinate, 75 mM, pH 4.2) to detect arabinosidase activity or to p-NP-Xyl (Sigma-Aldrich, 100 μ L, 10 mM, in succinate, 75 mM, pH 4.2) to detect β -xylosidase activity. The mixture was incubated at 30°C and at the end of the chosen incubation period the reaction was stopped by the addition of ice-cold Trizma (Sigma-Aldrich, 1 mL, 1%). The absorbance of the *p*-nitrophenate ions in solution was measured at the peak absorbance wavelength, 399 nm. Arabinosidase and β -xylosidase activities are recorded as μ U, where a unit represents μ mol *p*-nitrophenol released *per* min under the conditions described.

Assay of endo-xylanase activity

The method of Biely *et al.*³ was used for the detection of endo-xylanase activity with RBB-xylan (Sigma-Aldrich).

Chromatography

Enzyme extract (50 mL) was dialysed against imidazole buffer (50 mM, pH 7.4, overnight at 10°C). The dialysed extract was pumped (1 mL/min) onto a DEAE-cellulose (Sigma-Aldrich) column (2.6 \times 40 cm, volume 145 mL) that had been equilibrated in imidazole buffer (50 mM, pH 7.4). The column was washed with the same buffer (2 column volumes) before the linear elution gradient was applied (imidazole buffer (50 mM, pH 7.4) to imidazole buffer (50 mM, pH 7.4) + NaCl (0.75 M)). Fractions containing arabinosidase activity were pooled, dialysed against distilled water, then freeze dried. Similarly, fractions containing β -xylosidase activity were pooled and dialysed against distilled water prior to freeze drying. Dried samples were reconstituted in imidazole buffer (25 mM, pH 7.4, 3 mL) and then pumped (1 mL/min) on to a chromatofocusing column of Polybuffer Exchange 92 (Pharmacia, 0.9 \times 13 cm, volume 27 mL) equilibrated in imidazole buffer (25 mM, pH 7.4). Proteins were eluted with Polybuffer 74 (Pharmacia, pH 4, diluted 1:8 with distilled water). Fractions containing arabinosidase activity were pooled and dialysed against distilled water prior to freeze drying. Similarly, fractions containing β -xylosidase activity were pooled and dialysed against distilled water before freeze drying. Dried samples were reconstituted in succinate buffer (75 mM, pH 4.2) prior to gel filtration on a Bio-Gel P-100 column (BioRad, 20 \times 1.6 cm, volume 56 mL) equilibrated with succinate buffer (75 mM, pH 4.2). Samples were applied to the column at 1 mL/min, which was washed with succinate buffer (75 mM, pH 4.2). Fractions containing arabinosidase activity were pooled and, similarly, fractions containing β -xylosidase activity were pooled. The pool of active arabinosidase fractions is referred to as 'partially purified arabinosidase'.

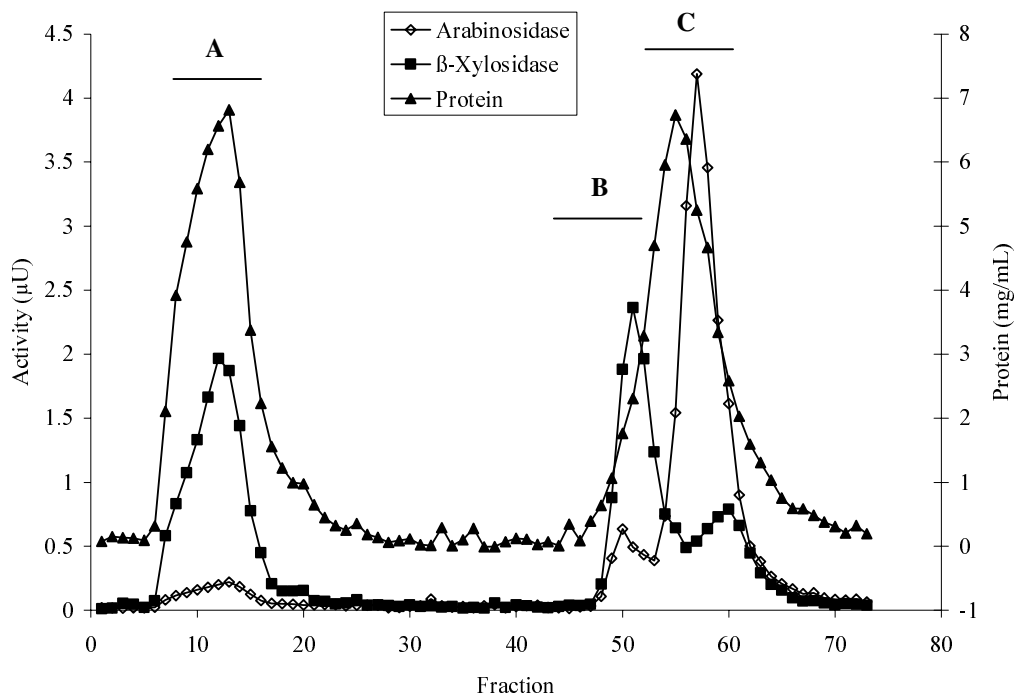


Fig. 1. Anion exchange chromatography, on DEAE-cellulose of extract from germinated wheat. A, Non-DEAE-cellulose-binding β -xylosidase. B, DEAE-cellulose-binding β -xylosidase. C, Arabinosidase.

Further purification of arabinosidase activity

Partially purified arabinosidase was further purified using Sepharose 6B-cyclohexaamylose affinity medium to remove contaminating β -amylase and by hydrophobic interaction chromatography on phenyl agarose. Sepharose 6B-cyclohexaamylose was made by the method of Buttmer and Briggs⁷. Samples were dialysed overnight against sodium acetate buffer (50 mM, pH 5.7) containing ammonium sulphate (1 M) before being applied (1 mL/min) to the column (0.9 \times 13 cm), which had been equilibrated in sodium acetate buffer (50 mM, pH 5.7) containing ammonium sulphate (1 M). The column was washed with sodium acetate buffer (50 mM, pH 5.7) containing ammonium sulphate (1 M), then bound proteins were eluted with sodium acetate buffer (50 mM, pH 5.7). Fractions containing arabinosidase activity were pooled and dialysed against distilled water prior to freeze drying. Dried samples were reconstituted in succinate buffer (pH 5.2, 75 mM) containing ammonium sulphate (25% w/v). Samples were then pumped (1 mL/min) on to a phenyl agarose column (Sigma, 0.9 \times 10 cm, volume 21 mL), which had been equilibrated in succinate buffer (pH 5.2, 75 mM) containing ammonium sulphate (25% w/v). The column was washed with succinate buffer (pH 5.2, 75 mM) containing ammonium sulphate (25% w/v) to remove unbound proteins. Bound proteins were eluted with a decreasing linear gradient of succinate buffer (pH 5.2, 75 mM) containing ammonium sulphate (25% w/v) to succinate buffer (pH 5.2, 75 mM).

Detection of β -amylase activity was performed according to Buttmer and Briggs⁶. All protein measurements were made using the bicinchoninic acid assay²¹.

Edman sequencing

Protein samples were prepared by electroblotting onto PVDF membranes²⁴. Proteins on the PVDF membrane were stained with Coomassie blue. Excised protein bands were given to AltaBioscience (University of Birmingham) for gas phase Edman sequencing. All sequences were compared to the NCBI database.

Tryptic digestion

Proteins were digested by the method of Hellman¹³.

MALDI-TOF-MS

Peptide solutions (1 μ L) were mixed with DHB (1 μ L, 10 mg/mL in acetonitrile (20% v/v); trifluoroacetic acid (0.1% v/v)) and 1 μ L of this mixture was spotted onto a 100 well stainless steel MALDI plate and allowed to air dry before being placed in the mass spectrometer (Tof Spec 2E from Micromass, Manchester, UK) fitted with a nitrogen laser (337 nm). Reflectron mass spectra were recorded by accumulating data from 20 laser shots. A two point calibration was carried out using trypsin autolysis products at m/z 842.3 and m/z 2212.0.

Nano-ESI-MS

Peptide solutions (3 μ L) were pipetted with gel loading pipette tips (Eppendorf) into a gold tipped capillary and loaded into a nanospray source (Protana, Denmark). Nano-ESI MS/MS was performed using an ion trap mass spectrometer (LCQ Deca, Thermoquest, San Jose, CA, USA) operated in positive ion mode. During the MS/MS ex-

periment, doubly charged peptide ions were isolated in the trap and subjected to collision induced dissociation.

RESULTS

Arabinosidase and β -xylosidase activities extracted from germinated wheat grains were found to increase 7 and 5 fold respectively over 7 days germination¹². To validate that there was no microbial contamination during germination, grains that had been surface sterilized and grains that had not been surface sterilized were placed on WL medium and allowed to germinate for five days. Microbial colonies were counted. All non-surface sterilized grains (100%) sustained microbial growth in comparison to surface sterilized grains, only 2% of which sustained microbial growth. Thus it can be assumed that the enzyme activities studied are from wheat and not from microbial contamination. Extraction of these activities was more efficient when combined with the use of a Polytron homogenizer. Yields (mean \pm standard deviation) were: arabinosidase without homogenisation 2.80 ± 0.08 (n = 3), with homogenisation 3.83 ± 0.14 (n = 3); β -xylosidase without homogenisation 2.48 ± 0.11 (n = 3), with homogenisation 2.60 ± 0.10 (n = 3)¹². pH optima for both enzymes were also measured: the arabinosidase optimum was pH 4.2 with a symmetrical curve finishing at pH 3.0 and 6.0; the β -xylosidase optimum was pH 4.5 with a symmetrical curve finishing at pH 3.0 and 7.0¹².

Arabinosidase and β -xylosidase activities were separated by anion exchange chromatography on DEAE-cellulose. The β -xylosidase activity was split – one fraction binding to the column and thus named DEAE-cellulose binding β -xylosidase and the other, non-DEAE-cellulose binding β -xylosidase, not being retained by the column (Fig. 1). Each of the three separate enzyme activities; arabinosidase, DEAE-cellulose binding β -xylosidase and non-DEAE-cellulose binding β -xylosidase were separately subjected to chromatofocussing and gel filtration chromatography. The pH values at which the enzymes were eluted from the chromatofocussing column were: arabinosidase, 5.2; DEAE-cellulose binding β -xylosidase, 6.0; non-DEAE-cellulose binding β -xylosidase, >7.4. Following these three purification steps the enzymes had been partially purified: arabinosidase 26.3 fold, DEAE-cellulose binding β -xylosidase 12.8 fold and non-DEAE-cellulose-binding β -xylosidase 22.9 fold. All the partially purified enzymes were tested for endo-xylanase activity with RBB-xylan. Only non-DEAE-cellulose β -xylosidase had any associated endo-xylanase activity ($0.066 \pm 0.009 \Delta A_{492}$ nm/min) The preparations were subjected to SDS-PAGE and IEF (Fig. 2). The arabinosidase preparation contained at least 2 proteins with molecular masses of approximately 40 and 60 kDa. The β -xylosidase preparations contained at least 3 proteins each. IEF showed the arabinosidase preparation to contain 3 proteins around pI 5, and the DEAE-cellulose binding β -xylosidase preparation 3 proteins around pI 6. Another property of the enzymes that was assessed after partial purification were the K_M values: arabinosidase 1.90 mM p-NP-Ara, DEAE-cellulose binding β -xylosidase 4.01 mM p-NP-Xyl and non-DEAE-cellulose binding β -xylosidase 3.35 mM p-NP-Xyl. The pH

optima agreed with those determined on the unpurified material.

The arabinosidase preparation was taken for the identification of the proteins present after these three purification steps. The 40 kDa protein was identified as three

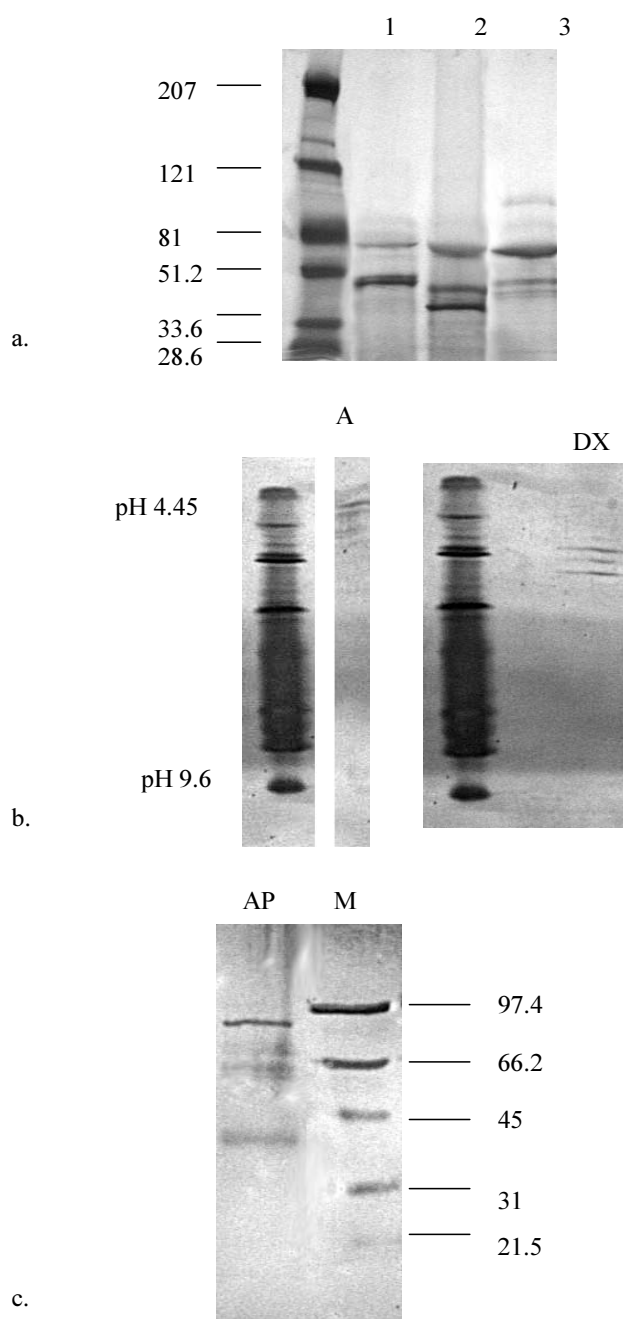


Fig. 2. Separation of purified enzyme-containing fractions by SDS-PAGE and IEF.

- SDS-PAGE of
 - Arabinosidase,
 - Non-DEAE-cellulose-binding β -xylosidase,
 - DEAE-cellulose-binding β -xylosidase.
- IEF of A, arabinosidase; DX, DEAE-cellulose binding β -xylosidase.
- SDS-PAGE of AP, arabinosidase after removal of β -amylase and serpins; M, markers.

	<u>TDVR</u>	<u>LSIA</u>		<u>SAASNAAF</u>
Serpin Y11485	MATTLATDVR	LSIAHQTRFA	LRLASTISSN	PKSAASNAAF
Serpin Y11486	MATTLATDVR	LSIAHQTRFA	FRLASAISSN	PESTVNNAAF
Serpin Z49890	MATTLATDVR	LSIAHQTRFA	LRLASTISSN	PKSAASNAAF
	<i>SPVSLHSALS</i>	<i>LLAAGAGSAT</i>	<i>R</i>	
Serpin Y11485	SPVSLHSALS	LLAAGAGSAT	RDQLVATLGT	GEVEGG
Serpin Y11486	SPVSLHVALS	LITAGAGGAT	RNQLAATLGE	GEVEGL
Serpin Z49890	SPVSLYSALS	LLAAGAGSAT	RDQLVATLGT	GKVEGL
Serpin Y11485	EQVVQFVLAD	ASSAGGPRVA	FANGVFVDAS	LLLKPSFQEL
Serpin Y11486	EQVVQFVLAD	ASNIGGPRVA	FANGVFVDAS	LQLKPSFQEL
Serpin Z49890	EQVVQFVLAD	ASSTGGSACR	FANGVFVDAS	LLLKPSFQEI
		<i>AAE</i>	<i>VTTQVNSWVE</i>	<i>K</i>
Serpin Y11485	AVCKYKAETQ	SVDFQTKAAE	VTTQVNSWVE	KVTSGR
Serpin Y11486	AVCKYKAEAQ	SVDFQTKAAE	VTAQVNSWVE	KVTTGL
Serpin Z49890	AVCKYKAETQ	SVDFQTKAAE	VTTQVNSWVE	KVTSGR
		<i>LVLANALYF</i>	<i>KGAWTDQFDS</i>	<i>YGTK</i>
		<i>LVLGNALYF</i>	<i>KGAWTDQFDP</i>	<i>R</i>
Serpin Y11485	LPSGSVDNTT	KLVLANALYF	KGAWTDQFDS	YGTKNDYFYL
Serpin Y11486	LPAGSIDNTT	RLVLGNALYF	KGAWTDQFDP	RATQSDDFYL
Serpin Z49890	LPPGSIDNTT	KLVLANALYF	KGAWTEQFDS	YGTKNDYFYL
Serpin Y11485	LDGSSVQTPF	MSSMDDQYI	SSSDGLKVLK	LPYKQG
Serpin Y11486	LDGSSIQTPF	MYSSEEQYIS	SSDGLKVLKL	PYKQGG
Serpin Z49890	LDGSSVQTPF	MSSMDDQYLL	SSDGLKVLKL	PYKQGG
			<i>LSAEPDFLE</i>	<i>R</i>
			LSAEPDFLEQ	HIPR
Serpin Y11485	QFSMYILLPE	APGGLSSLAE	KLSAEPDFLE	RHIPRQRVAI
Serpin Y11486	FSMYILLPEA	LSGLWSLAEK	LSAEPDFLEQ	HIPRQKVALR
Serpin Z49890	FFMYILLPEA	PGLSSLAEK	LSAEPDFLER	HIPRQRVALR
	<i>IS</i>	<i>LGIEASDLLK</i>		
Serpin Y11485	RQFKLPKFKI	SFGMEASDLL	KCLGLQLPFS	DEADFS
Serpin Y11486	QFKLPKFKIS	LGIEASDLLK	GLGLLLPFGA	EADLSE
Serpin Z49890	QFKLPKFKIS	FGIEASDLLK	CLGLQLPFGD	EADFSE
	<i>VS</i>	<i>SVFQAFVEV</i>	<i>NEQGTEAAAS</i>	<i>TAIK</i>
		AFVEVN	ETGTEAAATT	IAK
Serpin Y11485	SPMPQGLRVS	SVFQAFVEV	NEQGTEAAAS	TAIKMVPQQA
Serpin Y11486	PMAQONLYISS	IFHKAFVEVN	ETGTEAAATT	IAKVVLQRAP
Serpin Z49890	LMPQGLRVSS	VFHQAFVEVN	EQGTEAAAST	AIKMVLQQAR
Serpin Y11485	RPPSVMDFIAD	HPFLFLLRE	DISGVVLFMG	HVVNPL
Serpin Y11486	PPSVLDFIVD	HPFLFLIRED	TSGVVLFIGH	VVPLL
Serpin Z49890	PPSVMDFIAD	HPFLFLVRED	ISGVVLFMGH	VVNPLL

Fig. 3. Comparison of sequences for three wheat serine protease inhibitors (serpins), from the NCBI database, and peptide data from Edman sequencing (underlined), MALDI-TOF MS (italics) and tandem nano-ESI MS (bold), obtained from the 40 kDa protein in partially purified arabinosidase.

serine protease inhibitors or serpins by MALDI-TOF MS and Edman sequencing (Fig. 3). The 60 kDa protein was shown to be similar to a wheat β -amylase but homologous to a barley β -amylase by MALDI-TOF MS and tandem nano-ESI MS (Fig. 4). The β -amylase activity was detected by assessing the breakdown of starch using the method of Buttimer and Briggs⁶. The β -amylase was removed by (i) cyclohexaamylose-sepharose chromatography and (ii) the serpins were removed by hydrophobic interaction chromatography. The proteins removed from the partially purified arabinosidase fraction by the cyclohexaamylose-sepharose column exhibited β -amylase activity. SDS-PAGE of the further purified arabinosidase showed that the most abundant protein present was 80

kDa in mass. MALDI-TOF MS analysis of this protein produced a mass fingerprint (Fig. 5) but no protein was identified by comparison to the NCBI or SWISS-PROT electronic databases. Edman sequencing and tandem nano-ESI MS were not utilized.

DISCUSSION

As with the study of Lee and Ronalds¹⁷, but in contrast to that of Cleemput *et al.*⁸, the grain utilized in this work was germinated, as germination yielded higher activities of both arabinosidase and β -xylosidase. Homogenization with a Polytron was used during the preparation of the en-

Barley β -amylase	MEVNVKGNVY	QVYVMLPLDA	VSVNNRFEKG	DELRAQRKL
Wheat β -amylase	MAGNMLANVY	QVYVMLPLDV	VSVDKFEKG	DEIRAQLKKL
Barley β -amylase	VEAGVDVMV	DVWWGLVEGK	GPKAYDWSAY	KQLFELVQKA
Wheat β -amylase	TEAGVDGVMV	DVWWGLVEGK	GPKAYDWSAY	KQVFDLVHEA
Barley β -amylase	GLKLQAIMSF	HQCGGNVGDV	VNIPIQWVR	DVGTRDPIF
Wheat β -amylase	GLKLQAIMSF	HQCGGNVGDV	VNIPIQWVR	DVGATDPDIF
Barley β -amylase	YTDGHGTRNI	<i>NIEYLTLGVNDQ</i>	<i>PLFHGRSAVQ</i>	<i>MYADYMTSFR</i>
Wheat β -amylase	YTNRGGTRNI	EYLTLGVNDQ	PLFHGRSAVQ	MYADYMTSFR
Barley β -amylase	<i>ENMK</i>		<i>YPSY</i>	<i>PQSHGWSFPG</i>
Wheat β -amylase	ENMKDFLDAG	VIVDIEVGLG	PAGEMRPSY	PQSHGWSFPG
Barley β -amylase	ENMKKFLDAG	TIVDIEVGLG	PAGEMRPSY	
Wheat β -amylase	PQPSQGWVFP			
Barley β -amylase	<i>IGEFICYDK</i>			
Wheat β -amylase	IGEFICYDKY	LQADFKA AAA	AVGHPEWEFP	NDAGQYNDTP
Barley β -amylase	IGEFICYDKY	LEADFKA AAA	KAGHPEWELP	DDAGEYNDTP
Barley β -amylase	ERTQFFRDNG	<i>FF</i>	<i>LAWYSNNLIK</i>	HGDRILDEAN
Wheat β -amylase	EKTQFFKDNG	TYLSEKGRFF	LAWYSNNLIK	HGDKILDEAN
Barley β -amylase	<i>KVQL</i>	<i>AIKISGIHWW</i>	<i>YK</i>	
Wheat β -amylase	KVFLGYKVQL	AIKISGIHWW	YKVP SHAAEL	TAGYYNLHDR
Barley β -amylase	KVFLGCRVQL	AIKISGIHWW	YRVPNHAAEL	TAGYYNLDDR
Barley β -amylase	DGYRTIARML	KRHRASINFT	CAEMRDLEQS	SQAMSAPEEL
Wheat β -amylase	DGYRTIARML	TRHHASMNFT	CAEMRDSEQS	EEAKSAPEEL
Barley β -amylase	VQQVLSAGWR		YDTAYN	TILR
Wheat β -amylase	VQQVLSAGWR	EGLNVACENA	LPRYDTAYN	TILRNARPHG
Barley β -amylase	INQSGPPEHK	LFGFTYLRLS	NQLVEGQNYV	NFK
Wheat β -amylase	INKNGPPEHK	LFGFTYLRLS	NQLVEGQNYV	NFKTFVDRMH
Barley β -amylase	ANLPRDPYVD	SGP	EISIEMILQA	AQPK
Wheat β -amylase	ANLGHDPYVD	PMAPLPRSGP	EISIEMILQA	AQPKLQPPFF
Barley β -amylase	QEHTDLPVGP	PVAPLERSKP	EMPIEMILKA	AQPKLEPPFF
Wheat β -amylase	DKNTDLPVKD	TGGMGGQAEQ	PTCMGGQVK	GPTGGMGGQA
Barley β -amylase	EDPTSGIGGE	HTDVGDEVLV	APV	
Wheat β -amylase		LPATM		

Fig. 4. Comparison of sequences for barley β -amylase and wheat β -amylase, both from the NCBI database, and peptide data from MALDI-TOF MS (italics) and tandem nano-ESI MS (bold), obtained from the 60 kDa protein in partially purified arabinosidase.

zymes and was found to increase the efficiency of enzyme extraction. Buttimer and Briggs⁶ compared, by transmission electron microscopy, grists of barley, prepared using a coffee mill, that had been homogenized with a Polytron homogenizer, with grists that had not been so treated. The unhomogenized samples contained whole, undisrupted cells. However, in the homogenized grists the cells were completely disrupted. Polytron homogenisation increased the extraction of β -amylase from ungerminated barley grists. Thus total cellular destruction is needed to achieve maximal enzyme extraction.

The pH optima of the enzymes studied were pH 4.2 for the arabinosidase and pH 4.5 for both β -xylosidase activities. These values are slightly different to those found by Lee & Ronalds¹⁷ (arabinosidase pH 4.6–4.7 and β -xylosidase pH 4.8–4.9, using p-nitrophenyl derivatives). No pH

optima data were published by Cleemput *et al.*⁸ who measured the arabinosidase and β -xylosidase activities, when using p-nitrophenyl derivatives, at pH 6.0, a value that is at the very edge of the pH activity curves.

After the first three stages of purification similar protein patterns were observed by SDS-PAGE when compared to those found during the purification for arabinosidase by Cleemput *et al.*⁸. In both gels shown here, and in the paper by Cleemput *et al.*⁸, proteins were detected at approximately 60 and 40 kDa. When the proteins found in the partially purified arabinosidase preparation were sequenced, neither was identified as an arabinosidase, when compared to the NCBI database. Removal of the β -amylase and serpin proteins did not remove the arabinosidase activity. The most abundant protein, which remained after further purification, has a mass of 80 kDa, as seen by

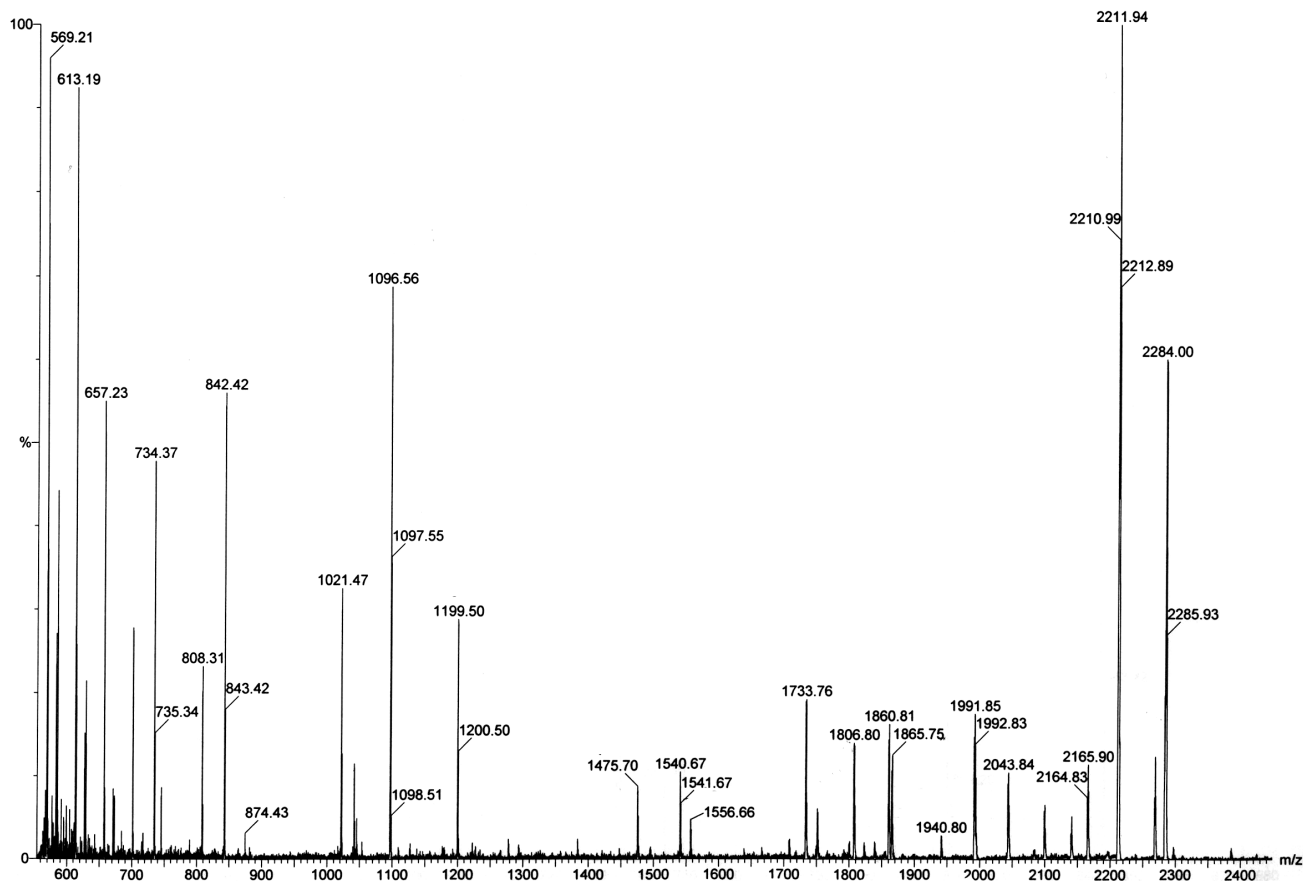


Fig. 5. MALDI-TOF MS mass fingerprint of unidentified 80 kDa protein found after further purification of arabinosidase.

SDS-PAGE. However, a MALDI-TOF MS mass fingerprint did not identify it within the NCBI database.

In the β -xylosidase SDS-PAGE separations the protein patterns are different to those reported by Cleemput *et al.*⁸. However, the purification of β -xylosidase activity was not complete.

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