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Section: Microbial and Enzyme Technology

Endogeneous β -D-xylosidase and α -L-arabinofuranosidase activity in flax seed mucilage

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Keywords: arabinofuranosidase, arabinoxylan, β -xylosidase, flax seed, mucilage

Purpose of work

Endogenous β -D-xylosidase (EC 3.2.1.37) and α -L-arabinofuranosidase (EC 3.2.1.55) activities have been detected and studied in flax seed mucilage (FM).

Abstract

Flax seed mucilage (FM) contains a mixture of highly doubly substituted arabinoxylan as well as rhamnogalacturonan I with unusual side group substitutions. Treatment of FM with a GH11 *Bacillus subtilis* XynA endo 1,4- β -xylanase (BsX) gave limited formation of reducing ends but when BsX and FM

were incubated together on different wheat arabinoxylan substrates and birchwood xylan, significant amounts of xylose were released. Moreover, arabinose was released from both water-extractable and water-unextractable wheat arabinoxylan. Since no xylose or arabinose was released by BsX addition alone on these substrates, nor without FM or BsX addition, the results indicate the presence of endogenous β -D-xylosidase and α -L-arabinofuranosidase activities in FM. FM also exhibited activity on both *p*-nitrophenyl α -L-arabinofuranoside (*p*NPA) and *p*-nitrophenyl β -D-xylopyranoside (*p*NPX). Based on K_M values, the FM enzyme activities had a higher affinity for *p*NPX (K_M 2 mM) than for *p*NPA (K_M 20 mM).

Introduction

Seeds from flax (*Linum usitatissimum*) are increasingly used in foods and are recognized to exert potentially positive effects on health due to their high contents of ω -3 lipids, antioxidants, and dietary fiber (Weill and Mairesse 2010). When flax seeds are wetted, the epidermal cells of the flax seed coats release large quantities of mucilage that form a gel-like capsule surrounding the seeds (Naran et al. 2008). This flax seed mucilage (FM) contains a mixture of arabinoxylan (AX) and rhamnogalacturonan 1 polysaccharides having rare side chain substitutions such as single terminal D- and L-galactose and L-fucose residues (Warrand et al. 2005a; 2005b; Naran et al. 2008). In the AX isolated from FM the ratio of branched to unbranched xylose residues is ~1.1:1 and the doubly substituted β -1,4-linked D-xylopyranosyl units, that have single, L-arabinosyl units attached via (α 1 \rightarrow 2) and (α 1 \rightarrow 3) bonds, constitute almost 80 mol% of the arabinofuranosyl substituted xylose residues in the AX backbone (Naran et al. 2008).

Enzyme-catalyzed degradation of cereal-derived AX is currently receiving significant attention both in relation to biofuel processing and prebiotic food ingredients production (Sørensen et al. 2006; 2007; Pastell et al. 2009). Due to its heterogenous structure, the enzymatic degradation of AX requires

coordinated action of several different enzymes: Endo-1,4- β -xylanases (xylanases, EC 3.2.1.8) catalyze the hydrolysis of the β -1,4 linkages in the backbone of AX (and other xylans), generating a mixture of xylo-oligosaccharides (Biely, 1985). β -Xylosidases (EC 3.2.1.37) catalyze the cleavage of xylobiose and the hydrolytic liberation of the terminal xylose unit from the non-reducing end of the xylo-oligosaccharides arising from endo-1,4- β -xylanase activity (Rasmussen et al. 2006). Enzymes exhibiting β -xylosidase activity are categorized into seven different GH families: 3, 30, 39, 43, 51, 52 and 54 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). Certain bifunctional β -xylosidases may also exert α -L-arabinofuranosidase activity (and vice versa) – those are mainly categorized in GH family 3 (<http://www.cazy.org/CAZY>; Cantarel et al. 2009).

α -L-Arabinofuranosidases (EC 3.2.1.55), categorized in GH families 3, 10, 43, 51, 54, and 62, catalyze the hydrolysis of terminal non-reducing α -L-arabino-furanosyl residues from the xylopyranosyl residues in AX and may also catalyze the hydrolytic release of terminal α -L-arabinofuranosyl residues from other structures (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). A particular issue in relation to arabinoxylans is the presence of similarly linked α -L-arabinofuranosyls that require different α -L-arabinofuranosidase activities depending on whether the α -L-arabinofuranosyls are linked to singly or doubly substituted xylose residues or are linked via (α 1 \rightarrow 2) or (α 1 \rightarrow 3)-links to xylose (Sørensen et al. 2007).

We here report on the finding of endogenous β -xylosidase and α -L-arabinofuranosidase activities in flax seed mucilage. To our knowledge, this is the first time that the presence these endogenous activities in FM have been reported.

Materials and methods

Chemicals

Xylo-oligosaccharide standards (X2, X3, X4, X5 and X6) from birchwood xylan were purchased from Megazyme (Bray, Ireland). Pullulan with average molecular weights of 0.13×10^4 , 0.6×10^4 , 1×10^4 and $40 \times 10^4 \text{ g mol}^{-1}$ were from Fluka Chemie AG (Buchs, Switzerland).

Substrates

Water-unextractable arabinoxylan (WUAX) (arabinose:xylose (A/X): 0.61) and water-extractable arabinoxylan (WEAX) medium viscosity (A/X: 0.61), both from wheat, were obtained from Megazyme (Bray, Ireland). Birchwood xylan was purchased from Sigma-Aldrich and used as is. *p*-nitrophenyl β -D-xylopyranoside (*p*NPX) and *p*-nitrophenyl α -L-arabinofuranoside (*p*NPA) were obtained from Sigma-Aldrich (Bornem, Belgium).

Extraction of flax seed mucilage

Brown species of flax seed were bought from Sun Island (JAN Import A/S, Hadsted, Denmark). The mucilage was extracted from the flax seeds with water for 24 h at ambient temperature according to Naran et al. (2008).

Enzymes

Viscozyme L9 was obtained from Novozymes (Bagsværd, Denmark). This enzyme preparation contained different enzymes including arabanase, cellulase, β -glucanase, hemicellulase, and xylanase (Sørensen et al. 2003). The *Bacillus subtilis* xylanase (BsX) was obtained from Danisco A/S (Brabrand, Denmark). BsX carries a Swiss Prot entry P18429, XynA_BACSU and has been classified as a glycosyl hydrolase family 11

xylanase according to sequence homology (<http://www.cazy.org/CAZY>; Cantarel et al. 2009). The xylanase was purified from a Grindamyl H 640 enzyme preparation according to Sørensen and Sibbesen (2006). BsX had a molecular weight of 20 kDa, pH optimum at 6, and temperature optimum at 50 °C.

SDS-PAGE

Protein content was analyzed by SDS–PAGE using a Criterion XT Precast Gel, 12% Bis-Tris (Bio-Rad, Hercules CA, US). SDS-PAGE was run at 200 V for 60 min. The running buffer was XT MOPS (Bio-Rad) and the gel was stained with Bio-Safe Coomassie (Bio-Rad) according to the manufacturer’s procedure.

Enzymatic hydrolysis of FM and other xylan substrates

Enzyme-catalyzed hydrolysis, by BsX, of the four substrates (FM, WEAX, WUAX, and birchwood xylan) was done as described in Rasmussen et al. (2010): Substrate (1% dry wt), 0.1 M sodium acetate pH 6 with 0.02 % sodium azide. BsX was added to the final reaction at 0.12 $\mu\text{mole} \cdot \text{enzyme g}^{-1} \text{DM substrate}$. Samples were withdrawn at set times during the incubation and the enzymatic hydrolysis was stopped immediately by heating (100°C for 10 min). After centrifugation, the supernatant was analyzed further. For studies of the endogenous activities in FM, the FM was preincubated for 10 min at 40°C with the substrates (WEAX, WUAX, and birchwood xylan) prior to addition of BsX. The final concentration of FM was 0.5 mg DM ml⁻¹. All procedures were performed in triplicate against a reference (no enzyme).

Enzyme assay and kinetics

Enzyme activity in the FM was assayed by the hydrolysis of 44 mM *p*NPX and *p*NPA, respectively in 0.1 M sodium acetate, pH 6 at 40°C for 30 min. FM was added in amounts sufficient to promote an increase in

absorbance between 0.1 and 1 at 410 nm. The reaction was terminated by the addition of 1 M Na₂CO₃. One unit (U) of β-xylosidase or α-L-arabinofuranosidase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of pNPX or pNPA min⁻¹, respectively. Specific activities are given as units (U) per mg total protein in the FM. For determination of the *K_M* values for the β-xylosidase and arabinofuranosidase activities in FM, activity assays were performed on pNPX and pNPA at concentrations between 3.7 and 44 mM at pH 6 and 40°C. *K_M* and *V_{max}* values for both substrates were calculated from Hanes plots, i.e. [S]/v_i vs. [S].

High performance size exclusion chromatography (HPSEC)

HPSEC was carried out as described in Rasmussen and Meyer (2010).

High performance anion exchange chromatography (HPAEC)

Separation and quantification of xylo-oligosaccharides in the hydrolysates were performed as described in Rasmussen et al. (2010).

Results

Composition of flax seed mucilage (FM)

Determination of the monosaccharide content of FM by HPAEC (Table 1) revealed that xylose was the most abundant monosaccharide of the FM constituting 43% (equivalent to 47 mol %) of the carbohydrate fraction. Galactose was the second most abundant monosaccharide making up about 20% by weight (17 mol %) of the carbohydrate fraction, with arabinose and galacturonic acid at relatively

lower levels, and fucose, rhamnose and glucose present in lower amounts (Table 1). By summing up xylose, galactose, arabinose, fucose, and glucose the neutral polysaccharides of flax seed mucilage (FM) were estimated to make up about 83 % by weight (equivalent to 85 mol %) of the carbohydrates. Acidic polysaccharides calculated as rhamnogalacturonan 1 were estimated to constitute about 17 % by weight with rhamnose and galacturonic acid as the main constituents. The proportion of acidic to neutral polysaccharides was similar to that previously reported by Warrand et al. (2003) and Guilloux et al. (2009), but the A/X ratio of 0.31 was slightly lower than that reported by Guilloux et al. (2009) (they reported A/X 0.36).

The FM also contained about 9.5 % by (w/w) protein as determined by a BCA Protein Assay Kit (Table 1). The protein was also seen when a sample of FM was analyzed by SDS-PAGE, where a smear of protein bands occurred, but with a band between 20 and 25 kDa being predominant (data not shown).

Enzymatic hydrolysis of FM

During treatment with BsX the reducing ends assay showed a modest increase in xylose equivalents released from FM as compared to from WEAX as a result of the xylanase activity (Fig. 1). The low degree of hydrolysis of FM was also observed with HPSEC where only a slight change in the molecular weight took place during enzymatic hydrolysis as compared to the hydrolysis of WEAX (Fig. 2A and 3A). In order to analytically quantify the progress of the enzymatic hydrolysis, the HPSEC chromatograms were divided into fixed time intervals and the refractive index response (the area under the curve) was used for quantification (Rasmussen and Meyer, 2010)(Fig. 2B and 3B). The main change in area on both substrates, representing the effect of the BsX activity, took place during the first 4 hours. Thereafter, no significant change was observed (Fig. 2B and 3B), but the evolution of the area response levels within the time intervals differed for the two substrates (Fig. 2B and 3B). Analysis by HPAEC of the activity of

BsX on FM also revealed that the BsX was unable to catalyze the release of significant amounts of small xylooligosaccharides (DP 2-6) from FM during 24 h of incubation (data not shown).

Endogeneous activity

In order to examine if FM contained any endogenous activities or inhibitors for the BsX, FM was added together with BsX to different AX substrates which differed in structure: WEAX, WUAX and birchwood xylan. In all three cases significant amounts of small XOS (DP 2-6) were released from the substrates during 24 h of incubation (Fig. 4). The XOS were mainly contributed by xylobiose (X2), xylotriose (X3) and to some extent xylo-tetraose (X4), but xylose was released from all substrates and arabinose from WEAX and WUAX (Fig. 4). Previous results have shown that this BsX xylanase catalyzes the liberation of significant amounts of small XOS (DP 2-6) from these substrates, but no xylose or arabinose (Rasmussen et al. 2010). Therefore, the observed release of xylose and arabinose suggested the presence of endogenous β -xylosidase and α -L-arabinofuranosidase activities in the FM. Further analysis revealed that initially the arabinose was released faster than the xylose from both substrates. Nonetheless, after 24 h more xylose than arabinose had been released (Fig 4.). This pattern was confirmed when the initial rates of arabinose, xylose and XOS release in the reactions with BsX and FM added together were compared for all three substrates (Table 2).

The finding that more xylo-oligosaccharides, xylose, and arabinose were released from WUAX than from WEAX during the combined BsX and FM catalyzed hydrolysis (Figures 4B and C) is in accordance with previous data signifying a higher propensity of BsX to attack WUAX than WEAX (Rasmussen et al. 2010). The highest amounts of XOS and xylose were released from birchwood xylan (Fig. 4A). This can be related to the structure of this substrate since it has a much lower substitution degree than WEAX and WUAX hence presumably providing better access for the BsX enzyme to the xylan backbone. Moreover, the xylose content in birchwood xylan was higher than 90% (by weight) where WEAX and WUAX, both

had an A/X ratio of 0.61. No arabinose was released from birchwood xylan which is accordance with the low (negligible) arabinose levels in birchwood xylan and confirming that while the BsX action on this substrate produced attack points for the β -xylosidase activity in the FM there were hardly any bonds to attack for the putative α -L-arabinofuranosidase activity.

The effect of the addition of FM to the BsX endo-1,4- β -xylanase reaction on WEAX was also evident on the HPSEC chromatogram when compared to that without addition of FM (Fig. 3A and 5A). According to Fig. 2A, the FM contained polymers with a molecular weight larger than $400 \cdot 10^3$ Da and these polymers were also seen in Fig. 5A (appear less pronounced because of the scale difference on the vertical axes). During the incubation period, the molecular weight of WEAX changed as result of the endo-1,4- β -xylanase treatment, and an increase in low molecular weight molecules was also observed. These smaller molecules were not released when the WEAX was treated with the BsX alone. The areas in the 23-26 min and 26-29 min intervals changed most significantly during the incubation (Fig. 3B and 5B). The decrease in the 23-26 min interval after 4 h incubation indicated that the molecules eluting within this time interval were substrates for the enzyme activities in FM since this decrease was not observed when WEAX was hydrolyzed by BsX alone. Furthermore, the increase in the area between 26 and 29 min, representing molecules smaller than 1.3×10^3 g mol⁻¹, was 46% higher than the area in the same time interval when hydrolysis of WEAX was catalyzed by BsX without addition of FM.

Kinetic parameters

The putative endogenous β -xylosidase and α -L-arabinofuranosidase activities in FM were assayed on *p*NPX and *p*NPA substrates in order to estimate the specific activity and kinetic parameters (Table 3). The specific activities were calculated based on the total protein concentration in FM. Both the specific activity and the substrate affinity (as judged from the K_M values) were higher on *p*NPX than on *p*NPA

(Table 3). These findings contrast the HPAEC results that indicated that the putative α -L-arabinofuranosidase activity in FM catalyzed a faster release of arabinose than xylose on the genuine polymeric substrates (Fig. 4). The discrepancy may be due to the generally faster rate of β -xylosidase on short oligomers, xylobiose and *p*NPX than on longer AX chains. Nonetheless, since the β -xylosidase activity had a higher specific activity and also higher maximal velocity, more xylose than arabinose was released after 24 h incubation (Fig. 4).

Discussion

Previous analysis of the BsX catalyzed hydrolysis of WEAX, WUAX, wheat bran, and birchwood xylan have confirmed that this enzyme is a pure endo-1,4- β -xylanase that catalyzes the generation of significant amounts of small XOS (DP 2-6) without any liberation of xylose (or arabinose) from these substrates (Rasmussen et al. 2010). The finding that BsX treatment on FM only produced a limited degradation - as judged from the evolution of reducing ends - was presumably a consequence of the unique substitutions of the AX in the FM. The wheat AX employed for comparison had an A/X ratio of 0.61 with a distribution of doubly and singly substituted xylopyranosyls of approximately 50:50 and no other main chain substitutions (Sørensen et al. 2007). The compositional analysis of FM showed an A/X ratio of only \sim 0.31. However, relatively high levels of galactose to xylose were found (Table 1), and previous data have shown that the flax seed FM contains D-galactose (Naran et al. 2008). Guilloux et al. (2009) also suggested that the high ramification of flax seed AX polysaccharides could decrease the efficiency of enzyme catalyzed hydrolysis of flax seed AX. Co-incubation of FM and BsX produced xylose from birchwood xylan and both xylose and arabinose from the wheat arabinoxylan substrates (Fig. 4). This indicated that FM contained β -xylosidase and α -L-arabinofuranosidase activities that catalyzed the release of xylose and arabinose, respectively, from the xylan substrates. The presence in FM of these endogenous enzyme activities were confirmed by the activity measurements on *p*NPX and *p*NPA, respectively. The presence of active enzymes in the FM is most likely related to the extraction method

where in this study, the mucilage was extracted at room temperature. In other studies of FM extraction was done at significantly higher temperatures, i.e. 80°C (Guilloux et al. 2009) and 100°C (Diederichsen et al. 2006). The concentrations (and/or activities) of these endogenous enzymes were apparently relatively low and only a smear of bands and only a relatively low molecular weight band (25-30 kDa) appeared on the SDS PAGE. The approximate molecular weights of the fungally derived enzymes are generally much higher, e.g. approximately 100 kDa for β -xylosidase from *T. reesei* and 60-92 kDa for different fungal α -L-arabinofuranosidases (Sørensen et al., 2007). Also, relatively high concentrations of *p*NPX and *p*NPA and a long incubation period (30 min) were necessary to measure the kinetic parameters. Whether FM contained two different enzymes or rather a bi-functional β -xylosidase/ α -L-arabinofuranosidase requires further study. Previous results have suggested the existence of bi-functional β -xylosidase/ α -L-arabinofuranosidase enzymes, e.g. in roots of alfalfa (Xiong et al. 2009).

Conclusion

FM mucilage contains endogenous β -xylosidase and α -L-arabinofuranosidase activities being active on synthetic substrates like *p*NPX and *p*NPA and natural substrates like birchwood xylan, WEAX and WUAX. Limited degradation of FM by a pure endo-1,4- β -xylanase, BsX, was observed, which may be ascribable to the high proportion of substitution of the FM xylan, notably a possible higher galactose substitution degree compared to e.g. wheat arabinoxylan. The finding of these endogenous activities was probably due to the relatively mild extraction method.

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Figure legends

Fig. 1 Progress curves of formation of reducing ends assessed as xylose equivalents (xylose moles/mucilage mass (mmol/g) during the enzyme catalyzed hydrolysis by BsX of FM (Δ) and WEAX (\square). The reducing ends were measured by the 4-hydroxybenzoic acid hydrazide (PAHBAH) assay with absorbance measurements at 410 nm (Lever, 1977). Values represent the mean of three independent measurements and are shown \pm 1 standard deviation.

Fig. 2 BsX catalyzed hydrolysis of FM. **(A)** HPSEC chromatographic profile after 0 min (—), 1 h (- - - -), 2 h (- · -), 4 h (·····) and 24 h (- - -). Molecular weight markers (+) from left to right are: $400 \cdot 10^3$, $110 \cdot 10^3$, $10 \cdot 10^3$, and $1.3 \cdot 10^3 \text{ g mol}^{-1}$. Vertical lines in the chromatogram represent the time intervals used in figure B. **(B)** Progression curves of the area in the following time intervals: 15.5-18.1 min (\blacktriangle), 18.1-20.8 min (\blacksquare), 20.8-23.4 min (Δ), 23.4-26.1 min (\bullet) (no peaks were seen beyond 26 min).

Fig. 3 BsX catalyzed hydrolysis of WEAX. **(A)** HPSEC chromatographic profile after 0 min (—), 1 h (- - - -), 2 h (- · -), 4 h (·····) and 24 h (- - -). Molecular weight markers (+) from left to right are: $400 \cdot 10^3$, $110 \cdot 10^3$, $10 \cdot 10^3$, $1.3 \cdot 10^3 \text{ g mol}^{-1}$, and xylose (150 g mol^{-1}). Vertical lines in the chromatogram represent the time intervals used in figure B. **(B)** Progression curves of the area in the following time intervals: 15.5-18.1 min (\blacktriangle), 18.1-20.8 min (\blacksquare), 20.8-23.4 min (Δ), 23.4-26.1 min (\bullet), 26.1-28.7 (\square). Values represent the mean of three independent measurements and are shown \pm 1 standard deviation.

Fig. 4 Progress curves of the products generated from the catalyzed hydrolysis of different xylan substrates by BsX ($0.12 \mu\text{mole g}^{-1} \text{ DM substrate}$) in the presence of FM ($0.5 \text{ mg DM} \cdot \text{ml}^{-1}$). **(A)** WEAX, **(B)** WUAX, **(C)** Birchwood xylan. Reaction: 1% weight/volume substrate in sodium acetate buffer of pH 6 at 40°C . \blacktriangle : arabinose, \blacksquare : xylose, Δ : xylobiose, x: xylo-oligosaccharides (DP 2-6). Values represent the mean of three independent experiments and are shown \pm 1 standard deviation.

Fig. 5 Enzyme catalyzed hydrolysis of WEAX by BsX and FM together. **(A)** HPSEC chromatographic profile after 0 min (————), 1 h (- - - - -), 2 h (- · -), 4 h (········) and 24 h (- - -). Molecular weight markers (+) from left to right are $400 \cdot 10^3$, $110 \cdot 10^3$, $10 \cdot 10^3$, $1.3 \cdot 10^3$, and xylose. The vertical lines in the chromatogram represent the time intervals used in figure B. **(B)** Progression curve of the area in the following time intervals: 15.5-18.1 min (\blacktriangle), 18.1-20.8 min (\blacksquare), 20.8-23.4 min (\triangle), 23.4-26.1 min (\bullet), 26.1-28.7 (\square). Values represent the mean of three independent measurements and are shown ± 1 standard deviation.

Tables

Table 1 Monosaccharide content of FM as determined by HPAEC-PAD analysis (Arnous and Meyer, 2008)

after acid and enzymatic hydrolysis (Garna et al., 2004). The data are expressed as mg dehydrated monosaccharide g⁻¹ dry matter (DM), mmole dehydrated monosaccharide g⁻¹ DM, and as relative percentage weight and molar distributions, respectively, of the carbohydrate fraction. Arabinose/xylose ratio (A/X) as calculated from HPAEC data and protein content as determined by the BCA Protein Assay Kit (Thermo Scientific, Rockford, US).

	mg g ⁻¹ DM	mmol g ⁻¹ DM	Distribution (wt%) within the carbohydrate fraction	Distribution (mol %) within the carbohydrate fraction
Xylose	281±9	2.13±0.07	43.1±0.8	46.8±0.7
Galactose	127±3	0.78±0.13	19.5±0.3	17.2±2.1
Arabinose	87±0.9	0.66±0.01	13.3±0.5	14.5±0.5
Fucose	32±0.5	0.22±0.00	4.9±0.2	4.8±0.2
Rhamnose	33±1.3	0.23±0.01	5.1±0.1	5.0±0.1
Galacturonic acid	78±2.9	0.45±0.02	12.1±0.8	9.8±0.6
Glucose	14±0.6	0.09±0.00	2.1±0.1	1.9±0.1
Protein	95			
A/X ratio	0.308			

Table 2 Initial rates (v_i) [$\mu\text{M min}^{-1}$] of arabinose, xylose and XOS release by the hydrolysis of individual polymeric substrates by co-inclubation with BsX and FM.

	v_i		
	Arabinose	Xylose	XOS
WEAX	1.8±0.13	0.3±0.01	3.1±0.02
WUAX	1.5±0.02	0.5±0.01	20.3±0.28
Birchwood xylan	*	1.1±0.03	34.1±0.72

* No arabinose was released from this substrate

Table 3 Kinetic parameters for FM on *p*NPX and *p*NPA substrates \pm standard deviation (n=3).

	Specific activity [U mg ⁻¹]	K _m [mM]	V _{max} [U min ⁻¹]
<i>p</i> NPA	0.053 \pm 0.014	20 \pm 2.3	1.1 \times 10 ⁻³ \pm 0.1 \times 10 ⁻³
<i>p</i> NPX	0.075 \pm 0.005	2 \pm 1.2	0.9 \times 10 ⁻³ \pm 0.2 \times 10 ⁻³

Figures

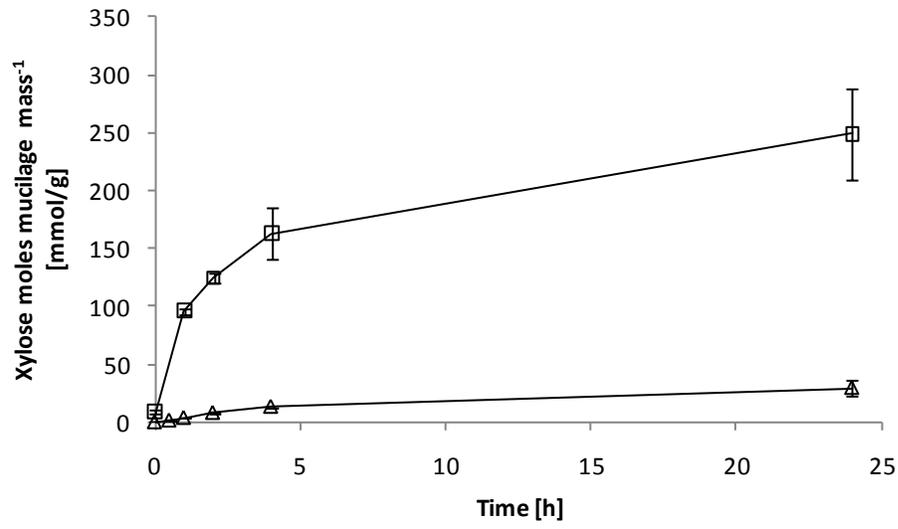


Figure 1

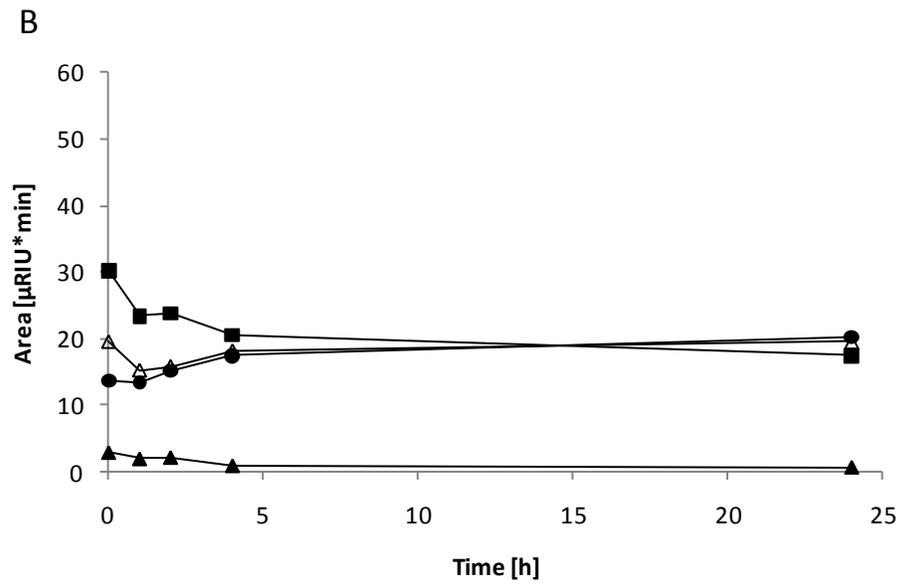
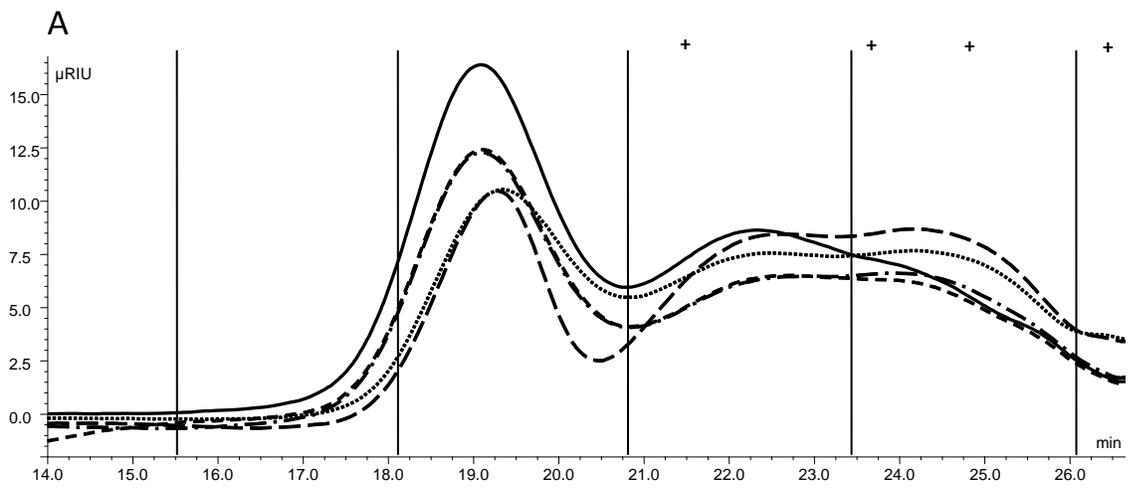


Figure 2

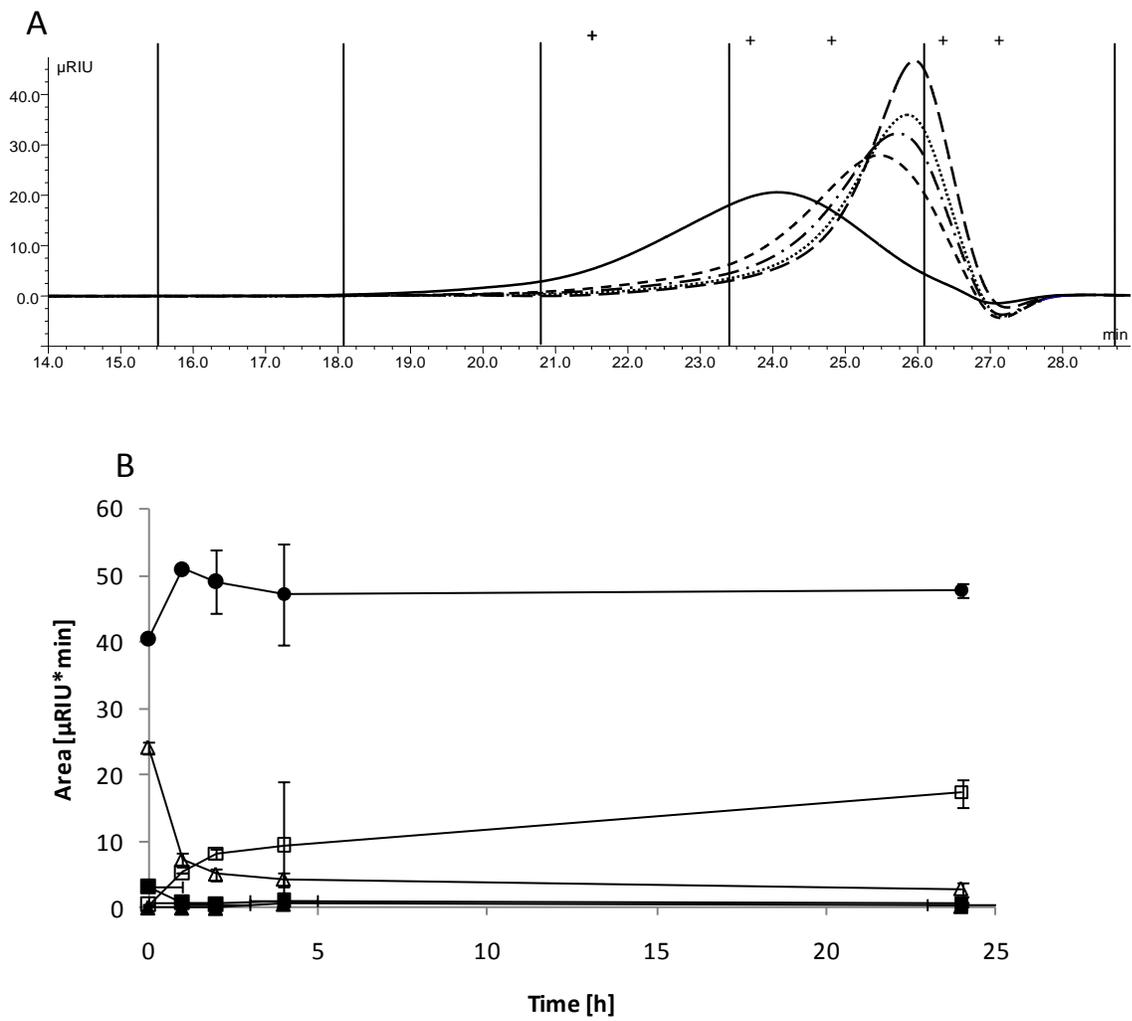


Figure 3

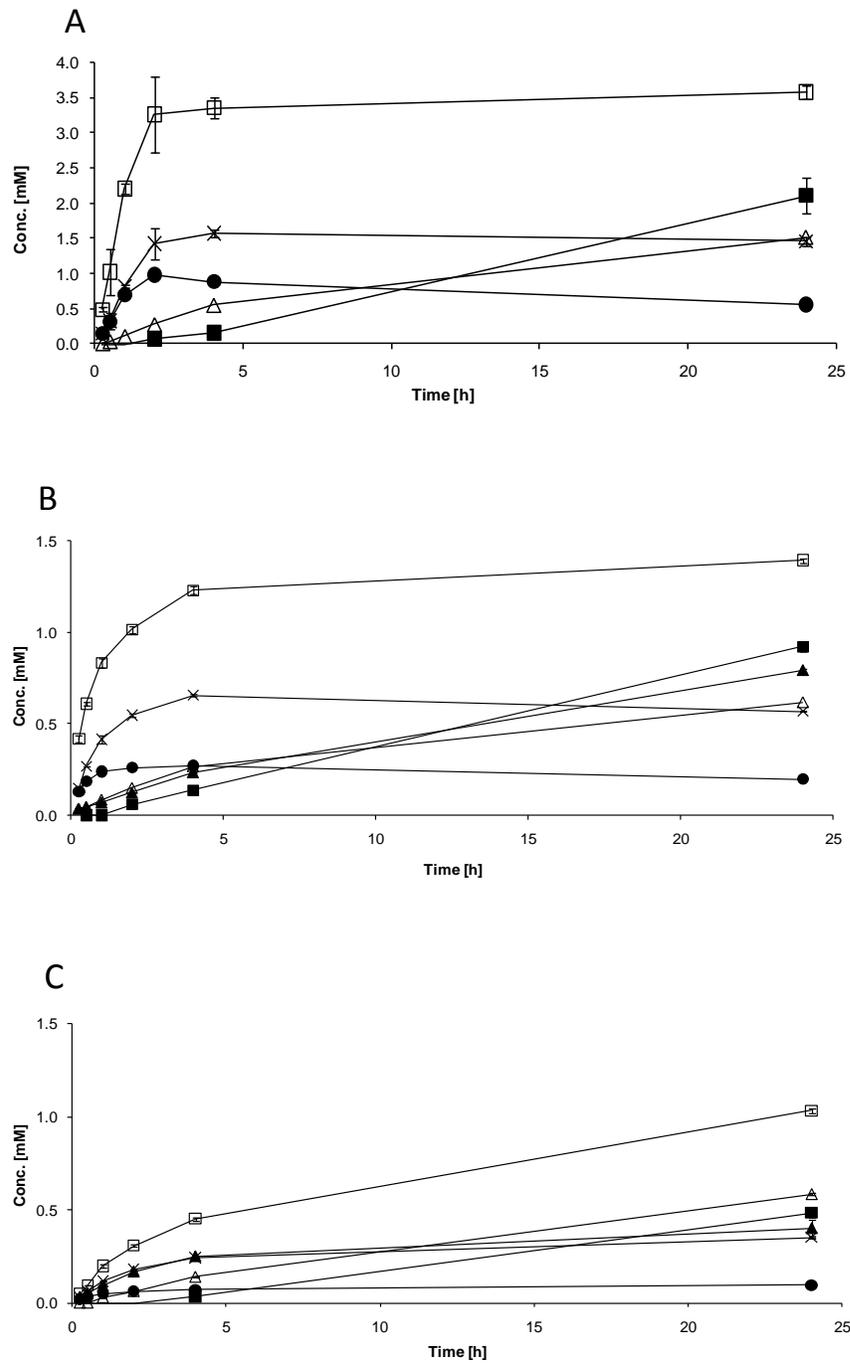


Figure 4

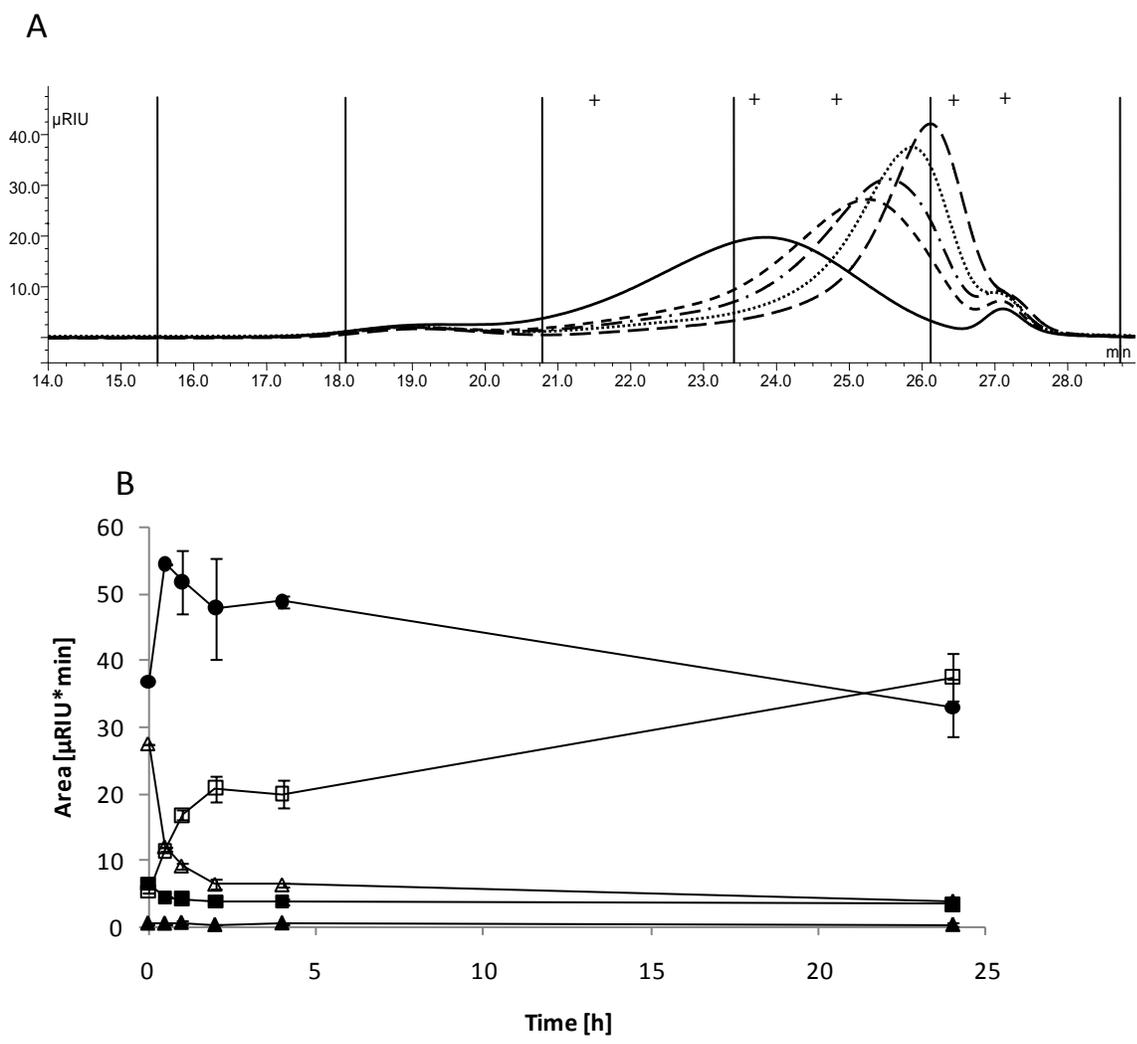


Figure 5