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In situ enzymatic aided formation of xylan hydrogels and encapsulation of horse radish peroxidase for slow release

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ABSTRACT

Xylans have special gelling and film forming properties for production of hydrogels that can be used as encapsulation matrices for slow release of bioactive agents. Hydrogels with particle sizes ranging from 18 nm to >10 μm and zeta potential of up to −19 mV were formed by selective removal of arabinose side chains from oat spelt xylan by recombinant α-L-arabinofuranosidase (AbfB). The xylan concentration significantly ($P < 0.05$) influenced both the particle size and zeta potential of the oat spelt xylan hydrogels, and the later was also influenced significantly ($P < 0.05$) by the hydrolysis time. The horse radish peroxidase was encapsulated *in situ*, both during and after formation of the xylan hydrogels and was released in an active form for a period of 180 min. Thus, the recombinant AbfB presents a biological tool for modification of xylan into biodegradable encapsulation matrices for slow or targeted release of bioactive agents.

1. Introduction

Biodegradable polymers are on high demand for production of hydrogels for applications in industries including pharmaceutical, food and medical industries. Among other uses, hydrogels produced from polysaccharides are used for micro/nano encapsulation and controlled, slow and targeted release of bioactive agents (Reis, Neufeld, Ribeiro, & Veiga, 2006). The functional properties of hydrogels for use as encapsulation matrices are influenced by the properties of the raw materials and preparation methods. Hydrogels prepared as slow release delivery systems of biological materials are required to protect the functional properties of the encapsulated bioactive agents (Coviello, Matricardi, Marianecci, & Alhaique, 2007) that have to be delivered active, pure and untransformed. The major challenges in developing such systems have been in finding suitable biomaterials and preparation methods that would ensure non-toxicity and biocompatibility (Reis et al., 2006).

Among the polysaccharides, hydrogels are often prepared from (1-4)-glycosidic-linked polysaccharides such as cellulose and starch that exhibit gelling properties when in solution (Dumitriu, 2002; Ebringerová & Heinze, 2000). Xylans have special gelling and film forming properties suitable for production of the biodegradable hydrogels (Ebringerová & Heinze, 2000). Although xylans are also (1-4)-glycosidic-linked polysaccharides; cellulose and starch are most preferred for preparation of biodegradable hydrogels because cellulose and starch have limited solubility in water. Cellulose and starch are high molecular weight biopolymers with relatively low degree or no side chain substitution (Ebringerová & Heinze, 2000), a characteristic that leads to reduced water solubility. In contrast, xylans are relatively high in water solubility due to low molecular weight and high degree of side chain substitution, which is a limiting factor for their industrial application as hydrogels. However, utilisation of xylans for production of hydrogels has become necessary because starch and cellulose have other existing competing uses such as additives in food, fibre for paper and source of fermentable sugars for biofuels production. Additionally, xylans are available in abundance, accounting for 25–35% biomass dry weight in higher plants (Timell, 1967).

The transformation of the water soluble xylan into micro/nanohydrogels would allow these xylans to be used in many industrial applications. Insoluble xylan forms chemically and physically crosslinked hydrophilic polymer networks (hydrogels) capable of imbibing large amounts of water or biological fluids (Coviello et al., 2007). Therefore, the xylan hydrogels have potential to perform in a similar manner as hydrogels prepared from cellulose and starch.

The biodegradable encapsulation and delivery systems so far developed for slow or targeted release of oral drugs in the human gut system have a high risk of being degraded prematurely before being delivered to the target place and in the right dosage. Xylan

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nanohydrogels are known to be suitable for sustained targeted release of encapsulated products in the human digestive system because of being chemostable and resistant to digestion in the human stomach and intestines (Ebringerová, Kardošová, Hromádková, Malovíková, & Hříbalová, 2002), but hydrolysable only by enzymes present in the human colon (Nagashima et al., 2008). A study by Silva et al. (2007) showed that xylan coated magnetite microparticles were protected from gastric dissolution when ingested orally, which improved the performance of the magnetic resonance imaging (MRI) that required sustained presence of the magnetite in the body. The xylan-derived micro or nanohydrogels have added advantages because they posses antiviral, anti-ulcer and antitumour activities, which are not found in other natural polymers such as cellulose and starch (Cipriani, Mellinger, De Souze, Baggio, & Freitas, 2008; Ebringerová & Heinze, 2000; Ebringerová et al., 2002; Saitô, 2005). Therefore, the xylan hydrogels have potential wide application in the biomedical, pharmaceutical, agriculture, water treatment, nutriceutical, bioremediation, production of functional packaging, and sanitary and textiles products.

The important features for efficient functioning of the polysaccharide hydrogels as encapsulation matrices of bioactive agents include swelling ability, gas or liquid permeability, stable morphology (particle size, surface charge, and assembling behaviour) and chemostability. The importance of these hydrogel functional properties have been described for specific industrial use in detail by a number of workers for example, the use of hydrogel foam for medical applications (Cole, Grab & Woodson, 1992), controlled drug delivery and pharmaceutical formulations (Peppas, 1997; Peppas, Bures, Leobandung, & Ichikawa, 2000), production of engineered poly multilayer capsules (Caruso, Trau, Möhwald, & Renneberg, 2000), biomedical devices (Domb et al., 2002; Hans & Lowman, 2002; Kishida & Ikada, 2002; Lindblad, Ranucci & Albertsson, 2001) and microspheres for encapsulation and immobilisation of bioactive agents (Hornig, Bunjes & Heinze, 2009; Vaghefi & Savitzky, 2005; Wang et al., 2006; Yi, Neufeld & Poncelet, 2005). Therefore, finding a preparation method for xylan hydrogels would be particularly relevant for the development of biocompatible encapsulation and slow release systems for delivering bioactive agents.

Depending on the source, the xylan backbone chains of 1,4-linked β-D-xylose residues have L-arabinose, D-MeGlcA, short oligosaccharide side chains, *O*-acetyl ferulic and ρ-coumaric groups attached (Timell, 1967; Wilkie, 1979), which are responsible for the water solubility. Therefore, the formation of xylan micro/nanohydrogels from water soluble xylans can be achieved by reducing the degree of side chain substitution and altering the side chain distribution pattern (Ebringerová & Heinze, 2000; Kabel, Van Den Borne, Vincken, Voragen, & Schols, 2007; Linder, Bergamon, Bodin, & Gatenholm, 2003a; Linder, Roubrocks, & Gatenholm, 2003; Sternemalm, Höije & Gatenholm, 2008).

The removal of these side chains from water soluble xylans is often achieved through hydrothermal treatment, supercritical antisolvent precipitation, and manipulation of pH and ionic charges in coacervation process (Coviello et al., 2007; Gabrielii, Gatenholm, Glasser, Jain, & Kenne, 2000; Garcia et al., 2001; Haimer, Wendland, Potthast, Rosenau, & Liebner, 2008; Hans & Lowman, 2002; Linder, Bergamon, et al., 2003; Linder, Roubrocks, et al., 2003; Sternemalm et al., 2008). Other workers have shown that xylan hydrogels could alternatively be produced by chemical crosslinking of water soluble polymers with other functional groups or by polymerisation of the water soluble monomers (Hans & Lowman, 2002; Lindblad, Ranucci & Albertsson, 2001; Nagashima et al., 2008). However, the chemical methods and application of supercritical conditions (150 bar) (Haimer et al., 2008) and hydrothermal treatments (>100 °C) (Henriksson & Gatenholm, 2001; Linder, Bergamon, et al., 2003; Linder, Roubrocks, et al., 2003) may not allow the coupled *in situ* formation of xylan hydrogels and encapsulation of sensitive bioactive agents because of being destructive and non-selective to the bioactive agents. Although the morphology of the xylan hydrogels is often stabilised, thus preventing self assembling tendency of the nanoparticles, by complexation procedures (Akiyoshi et al., 1998) and addition of plasticisers such as polyethylene glycol 1000 (PEG 1000) (Garcia et al., 2001; Hans & Lowman, 2002), the hydrogels produced by crosslinking methods might be incompatible for direct use in pharmaceutical applications (Van Tomme, Storm & Hennik, 2008) because of traces of residual chemicals. Therefore, non-toxic and biocompatible methods for production of xylan hydrogels are needed to allow wider industrial applications of the xylan hydrogels.

Enzymes provide non-invasive methods that would enable preparation of biocompatible and less toxic xylan hydrogels compared to those prepared by chemical methods. Berlanga-Reyes et al. (2009) reported preparation of hydrogels through oxidative cross linking of feurolylated arabinoxylans by laccasse. However, the use of enzymes for formation of hydrogels has not been studied comprehensively. In this study, hydrogels were prepared from oat spelt xylan by selective removal of arabinose side chains by recombinant α-L-arabinofuranosidase (AbfB) (EC3.2.1.55) with polymeric xylan substrate specificity. In particular, the potential of using the recombinant AbfB for combined *in situ* formation of micro/nanohydrogels from water soluble oat spelt xylan and encapsulation of horse radish peroxidase (HRP) as a model bioactive agent for slow release, was evaluated. The degree of arabinose release, stability of the morphology in particular the particle size and surface charge (zeta potential) of the oat spelt xylan hydrogels were evaluated at different oat spelt and polyethylene glycol 1000 (PEG 1000) concentrations and hydrolysis time. In addition, the release characteristics of the HRP from xylan hydrogels were assessed for HRP added to the oat spelt xylan during and after xylan hydrogel formation.

2. Materials and methods

2.1. Materials

The oat spelt xylan (X-0627) containing arabinose, glucose and xylose in a 10:15:75 ratio, was purchased from Sigma. The recombinant AbfB with volumetric activity of 1.1 U mL^{-1} against *p*-nitrophenyl arabinofuranoside (*p*NPA) was produced in house. The horse radish peroxidase (HRP) (EC 1.11.1.7) (Type VI-A P-6782) with specific activity of 1000 U mg^{-1} against 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), # A-1888 was purchased from Sigma. The H_2O_2 for use in the HRP activity assay was prepared from a 30% (w/w) stock solution purchased from Merck. The polyethylene glycol (PEG) 1000 (product # 81190) plasticiser was purchased from Merck. Citrate and potassium phosphate buffers were prepared according to Gomori (1955). Unless specified otherwise, the water used in the study was de-ionised (dH_2O).

2.2. Production of oat spelt xylan hydrogels by selective enzyme hydrolysis

Oat spelt xylan solutions were prepared according to De Wet, Matthew, Storbeck, Van Zyl, and Prior (2008). About 2.5 mL oat spelt xylan solutions (1%, w/v) was placed in test tubes into which 1 mL of the recombinant AbfB was added. The volume of the reaction mixture was adjusted to 5 mL by addition of 0.05 M citrate buffer pH 5.0. The reaction was performed at 40 °C in a shaking water bath (20 revolutions per minute) for 16 h. Precipitation of the oat spelt xylan was assessed visually and pictures were taken. The liberated arabinose was determined using a high pH anion exchange

Table 1
Experimental design evaluating the effect of oat spelt xylan concentration, enzyme hydrolysis time and polyethylene glycol 1000 (PEG 1000) on arabinose release, particle size and zeta potential of oat spelt xylan hydrogels in a central composite design (CCD).

Sample[c] identification	Coded variables[a]			Natural values			Composition of reaction mixture				Output		
	X_1	X_2	X_3	Xylan (%, w/v)	Time (h)	PEG 1000 (%, xylan wt)	Xylan (μL)	Citrate buffer (μL)	PEG 1000 plasticiser (μL)	AbfB[b] (μL)	Arabinose release (% available)	Mean particle size (nm)	Zeta potential (mV)
XEP_1	−1	−1	−1	1.00	7.00	15.00	2500	1475	25	1000	18	81	−13.0
XEP_2	−1	−1	1	1.00	7.00	40.00	2500	1433	67	1000	16	21	−13.2
XEP_3	−1	1	−1	1.00	24.00	15.00	2500	1475	25	1000	10	103	−10.4
XEP_4	−1	1	1	1.00	24.00	40.00	2500	1433	67	1000	61	235	−19.1
XEP_5	1	−1	−1	3.00	7.00	15.00	2500	1425	75	1000	31	10,000	−1.03
XEP_6	1	−1	1	3.00	7.00	40.00	2500	1300	200	1000	24	10,000	−2.35
XEP_7	1	1	−1	3.00	24.00	15.00	2500	1425	75	1000	46	10,000	−2.37
XEP_8	1	1	1	3.00	24.00	40.00	2500	1300	200	1000	54	10,000	−9.16
XEP_9	−1.68	0	0	0.32	15.50	27.50	2500	1485	15	1000	0	292	−15.5
XEP_10	1.68	0	0	3.68	15.50	27.50	2500	1332	168	1000	22	10,000	−9.5
XEP_11	0	−1.68	0	2.00	1.20	27.50	2500	1408	92	1000	19	4378	−5.18
XEP_12	0	1.68	0	2.00	29.80	27.50	2500	1408	92	1000	41	74	−8.41
XEP_13	0	0	−1.68	2.00	15.50	6.48	2500	1478	22	1000	48	144	−17.1
XEP_14	0	0	1.68	2.00	15.50	48.52	2500	1339	161	1000	40	126	−10.8
XEP_15	0	0	0	2.00	15.50	27.50	2500	1408	92	1000	100	114	−12
XEP_16	0	0	0	2.00	15.50	27.50	2500	1408	92	1000	nd	114	−12

[a] Three factor standard two cubic plus star surface central composite design consisting of 16 runs, with the number of cube points (n_c) = 8, number of star points (n_s) = 6 and combined number of centre points in the cube portion of the design and the number of centre points in the star portion of the design (n_0) = 2.
[b] α-L-Arabinofuranosidase (AbfB) with volumetric activity of about 18.00 nkat mL^{-1}.
[c] XEP denotes mixture of xylan, enzyme and plasticiser.

chromatography coupled with pulsed electrochemical detection (HPAEC-PAD) (Dionex) equipped with a gradient pump GP 50, a Carbopac™ PA 10 (4 mm × 250 mm) column and electrochemical detector (ED40) for pulsed amperometric detection (PAD). Stability of the hydrogels was assessed by measuring the change in mean particle sizes of the hydrogels at 30 min, 3 h and 7 days (stored at 4 °C) after termination of the AbfB hydrolysis. The measurement was done using a Zetasizer (Nano ZS90) that determines particle size by measuring Brownian motion of the particles in a sample using Dynamic Light Scattering (DLS) Principle (Malvern Instruments Ltd., 2004).

2.3. Effect of process parameters on arabinose release, particle size and surface charge (zeta potential) of oat spelt xylan hydrogels

The effects of PEG 1000 plasticiser, oat spelt xylan concentrations and AbfB hydrolysis time on arabinose release, particle size and surface charge (zeta potential) of the xylan hydrogels were evaluated in a standard three factor-two level cubic plus star surface central composite design (CCD). The design consisted of 16 runs of which 8 were cube points (n_c), 6 were star points (n_s) and 2 were a combination of centre points in the cube portion of the design and centre points in the star portion of the design (n_0) is presented in Table 1. The experimental region for xylan concentrations (X_1) was $1\% \le X_1 \le 3\%$, oat spelt xylan hydrolysis time (X_2) was $7\,h \le X_2 \le 24\,h$ and PEG plasticiser concentration (X_3) was $15\% \le X_3 \le 40\%$ (Table 1). The central points for xylan concentration (X_1), oat spelt xylan hydrolysis time (X_2) and PEG 1000 plasticiser concentration (X_3) were set at 2%, 15.5 h and 27.5%, respectively. The independent variables in the study were coded using Eqs. (1)–(4).

$$X_1 = \frac{C1-2}{1} \quad (1)$$

$$X_2 = \frac{C3-15.5}{8.5} \quad (2)$$

$$X_3 = \frac{C2-27.5}{12.5} \quad (3)$$

where X and C = coded and natural independent values, respectively, 1, 2 and 3 = subscripts for xylan concentration hydrolysis time and plasticiser. α = rotatability was calculated from Eq. (4):

$$\alpha = (n_c)^{1/4} = 1.6818 \quad (4)$$

where n_c = number of cube points in the design (*i.e.*, points in the factorial portion of the design) (Statsoft Inc., 2011). The $\pm\alpha$ values for oat spelt xylan concentration in natural form were 0.32 and 3.68%, for plasticiser 6.48 and 48.52% and for time 1.2 and 29.8 h. The experimental data was fitted with a second order polynomial (Eq. (5)).

$$Z = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \varepsilon \quad (5)$$

where Z = particle size (nm) or zeta potential (mV). $\beta_0 + \beta_1, \ldots, \beta_n$ = linear regression coefficients. $\beta_{11}, \ldots, \beta_{nn}$ = quadratic regression coefficients. ε = error, X_1, X_2, X_3 = xylan concentration, hydrolysis time (h) and PEG 1000 concentration, respectively.

The sizes of effects for xylan concentration, hydrolysis time and PEG 1000 concentration and their interactions on arabinose release, particle size and zeta potential were determined by regression analysis. In addition, a desirability plot was used to determine the optimal set point for the parameters that would give the highest arabinose release, smallest particle size and the lowest zeta potential.

A mixture of oat spelt xylan solution and PEG 1000 plasticiser of specified volume and concentration according to the experimental design (Table 1) were treated with recombinant AbfB as described in Section 2.2. The oat spelt xylan hydrogels were analysed for mean particle size and zeta potential. The determination of the mean particle size followed the same protocol as described in Section 2.2. The zeta potential of the xylan hydrogels was measured by a Zetasizer (Nano ZS90), which operated based on a combination of electrophoresis and laser Doppler velocimetry techniques (sometimes called laser Doppler electrophoresis). The results were analysed using an inbuilt Malvern software programme.

2.4. Encapsulation and release of horse radish peroxidase in AbfB formed oat spelt xylan hydrogels

The oat spelt xylan and recombinant AbfB reaction mixtures were prepared as described in Section 2.2 in two sets of test tubes. In one set, 50 and 100 μL of HRP supernatant (HRP Type VI-A P6782, EC 1.11.1.7) with a protein concentration of 1 mg mL^{-1} and specific activity of 1000 U mg^{-1} against 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), was added. The volumes of the reaction mixtures were adjusted to 5 mL by adding 0.05 M citrate buffer (pH 5.0). The test tubes were incubated at 40 °C for 24 h. The reactions were stopped by placing the test tubes in water containing ice. After 1 h of terminating the reactions, 50 and 100 μL of HRP supernatants were added to the second set of the test tubes that were initially incubated without the HRP. Subsequently, both sets were stored at 4 °C for at least 24 h prior to testing for HRP positive encapsulation and release. Two control samples, one containing oat spelt xylan solution and the recombinant AbfB in the absence of HRP and another containing the HRP and oat spelt xylan but without the recombinant AbfB, were incubation under similar conditions.

Samples (2 mL) of the reaction mixtures were centrifuged at 23,600 × *g* using a Sorvall Super T21 centrifuge (Kendall) for 5 min at 4 °C. The liquid was decanted and stored to recover the pellet (hydrogels). The pellets were washed by re-suspending them in one volume Milli-Q H_2O and centrifugation was repeated under similar conditions. The process was repeated until there were no traces of HRP activity in the waste wash water.

2.5. Horse radish peroxidase assay

HRP activity was tested in the initial decanted solutions and hydrogels (pellet). In both cases, the liquid and the hydrogel, about 20 μL samples, were placed in Eppendorfs in which 100 μL of 9.1 mM ABTS (prepared in 100 mM potassium phosphate buffer pH 5.0) and 100 mM potassium phosphate buffer pH in de-ionised water to make total volume of 265 μL, was added. About 5 μL H_2O_2 (0.3%) prepared from 30% (w/w) stock solution (Merck) were added to the mixture. Thereafter, the reaction mixtures were incubated in the dark for 10 min at room temperature (25 °C). The HRP activity was measured from the optical density of the reaction mixture measured at 405 nm (OD_{405nm}) in a microplate reader (xMark™ Bio-rad) against a reagent blank, which contained ABTS, buffer and H_2O_2.

The degree of encapsulation of the HRP in the oat spelt xylan hydrogels for which HRP was added before and after AbfB xylan hydrogel formation, was determined from the residual activity of the HRP in the initial decanted solution whereas the rate of release of the HRP encapsulated from the hydrogel was measured from the change in the OD_{405nm} of the HRP assay of the hydrogel at 10, 30, 60, 120 and 180 min. The samples were stored in a dark place during the OD_{405nm} measurement intervals. In the assay, ATBS oxidation by non-encapsulated HRP was used as a positive control whereas a mixture of ATBS and the oat spelt xylan hydrogels without encapsulated HRP was used as a negative control. The rate of release of the HRP out of the oat spelt xylan hydrogel was defined as the rate of change of the OD_{405nm} per unit time.

3. Results

3.1. Physical characteristics of recombinant AbfB formed oat spelt xylan hydrogels

Oat spelt xylan hydrogel precipitates were formed after selective removal of the arabinose side chains by the recombinant AbfB (Fig. 1a). The oat spelt xylan hydrogel precipitates aggregated and settled as evident from the visible two phase solid–liquid separation in the test tubes (Fig. 1a). The release of the arabinose was the maximum when the oat spelt xylan was treated with the recombinant AbfB at xylan and PEG 1000 concentrations of 2% (w/v) and 27.5% (v/v) for 15.5 h (Fig. 1b i–iii). The oat spelt concentration, hydrolysis time and PEG 1000 concentration significantly influenced ($P < 0.05$) the degree of arabinose release (Fig. 1b iv). The release of the arabinose fitted the polynomial quadratic equation with a regression coefficient (R^2) of 0.94 (adjusted $R^2 = 0.86$).

The mean particle size distribution of the hydrogels was after 30 min of terminating the hydrolysis, estimated to be between 150 and 200 nm for the control sample (unhydrolysed oat spelt xylan) (Fig. 2a i) and 18 and 700 nm for the hydrolysed sample (Fig. 2a ii). About 3.0 h later, the mean particle size distribution of the hydrogels in the hydrolysed sample ranged between 28 nm and 1 280 nm (Fig. 2b ii) and 7 days later (stored at 4 °C), the mean particle size range for the control sample after storage at 4 °C was from 100 nm to >1 μm (1000 nm) (Fig. 2c i) whereas the mean particle size range for the hydrolysed sample was from 78 nm to 1.48 μm (Fig. 2c ii).

3.2. Dependency particle size and surface charge (zeta potential) properties of oat spelt hydrogels on xylan concentration, enzyme hydrolysis time and PEG 1000 plasticiser concentration.

The effects of xylan concentration, enzyme hydrolysis time and PEG 1000 concentration on arabinose release are presented in particle size and zeta potential of the oats spelt xylan hydrogels were reflected in contour desirability plots shown in Fig. 3a and b, respectively. The response contour plots showed that the minimum mean particle size of the hydrogels was obtained at hydrolysis time of 15.5 h and xylan and PEG 1000 concentrations of about 1.0% and 27.7%, respectively (Fig. 3a i and ii). The mean particle size experimental data fitted the polynomial quadratic equation with a regression coefficient (R^2) of 0.88 (adjusted $R^2 = 0.81$). The Pareto chart plot showed that oat spelt xylan had a significant effect on the mean particle size of the hydrogels at $P < 0.05$ (Fig. 3a iv).

The oat spelt xylan hydrogels had a negative surface charge, which was reflected by the negative zeta potential. The values for the zeta potential ranged from −19 to −1.03 mV (Table 1). The lowest zeta potential for the oat spelt xylan hydrogels was obtained at the concentrations of oat spelt xylan and PEG 1000 of 1 and 40%, respectively, for AbfB hydrolysis time of 24 h (Table 1). However, the desirability contour plot (Fig. 3b i) showed the lowest optimal zeta potential of −14.3 mV could be estimated at xylan and PEG 1000 concentrations of 1 and 27.5%, respectively, for the AbfB hydrolysis time between 15 and 20 h. The zeta potential experimental data fitted the polynomial quadratic equation with R^2 of 0.74 and adjusted $R^2 = 0.56$ at $P < 0.05$. The Xylan concentration and AbfB hydrolysis time were the significant parameters at $P < 0.05$ that influenced the zeta potential for the xylan hydrogels (Fig. 3b iv).

3.3. Encapsulation and release of HRP in oat spelt xylan hydrogels

The HRP activity was detectable in the oat spelt xylan hydrogels after multiple rinsing with water. The measured HRP activity (OD_{405nm}) in the initial decanted solutions was in the ratio of 1.0:11.0:5.1 for reaction mixtures where the HRP was added before formation of oat spelt xylan hydrogels, after formation of hydrogels and in unhydrolysed oats spelt xylan, respectively (Fig. 4a). A similar trend was observed at HRP dosage level of 50 μL whereby the ratio of the HRP activity (OD_{405nm}) was 1:6.5:1.7, respectively (Fig. 4a).

The release of the HRP from oat spelt xylan hydrogels, monitored by the change of the OD_{405nm} for a period of 180 min, showed

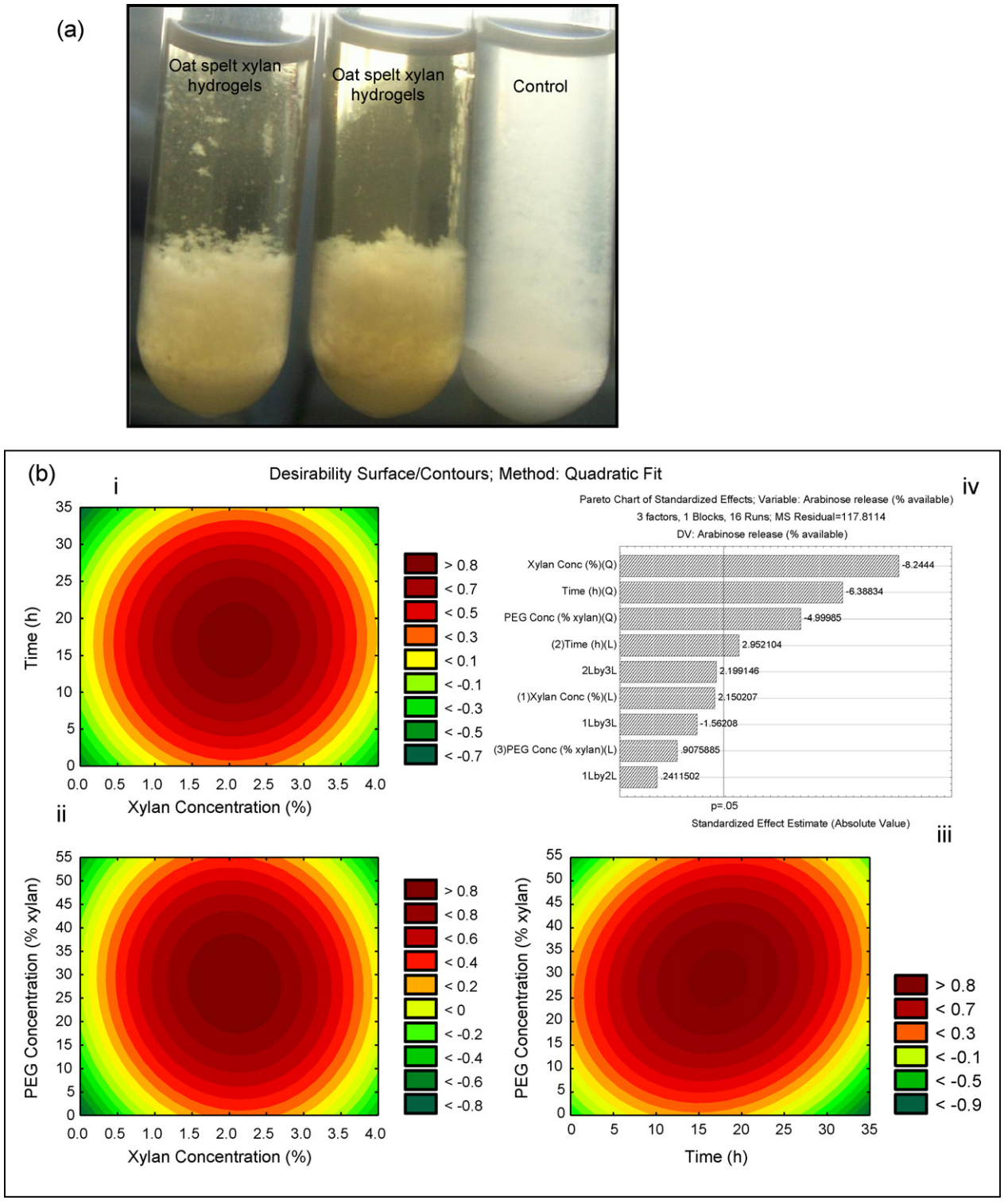

Fig. 1. Hydrolysis of water soluble oat spelt xylan by recombinant α-L-arabinofuranosidase (AbfB): (a) oat spelt xylan hydrogels formed. (b) Desirability contour plot showing the effects of xylan concentration, enzyme hydrolysis time and Poly Ethyl Glycol (PGE) 1000 concentration on the degree of arabinose release from the oat spelt xylan. Plot (i) shows interaction between time and xylan concentration; (ii) PEG 1000 concentration and xylan concentration, (iii) PEG 1000 and time and (iv) Pareto chart showing the size and significance of the main effects and interactions of the independent variables on arabinose release. In the Pareto Charts (iv) the vertical dotted line denotes significant level at $P < 0.05$. The model fitted the arabinose release data with $R^2 = 0.94$; Adjusted $R^2 = 0.86$.

that after 10 min of terminating the encapsulation process, the OD_{405nm} of the assay mixture for HRP that was added before and after formation of the oat spelt xylan hydrogels were 1.276 and 0.882, respectively (Fig. 4b). The OD_{405nm} of the assay mixture for HRP added before and after formation of the oat spelt xylan nanohydrogels further increased to 3.74 and 5.0, respectively, after the 180 min (Fig. 4b). During the same time period, the OD_{405nm} for the positive control assay mixture thus the non-entrapped (encapsulated) HRP incubated under similar conditions remained constant at approximately OD_{405nm} of 11 (Fig. 4b).

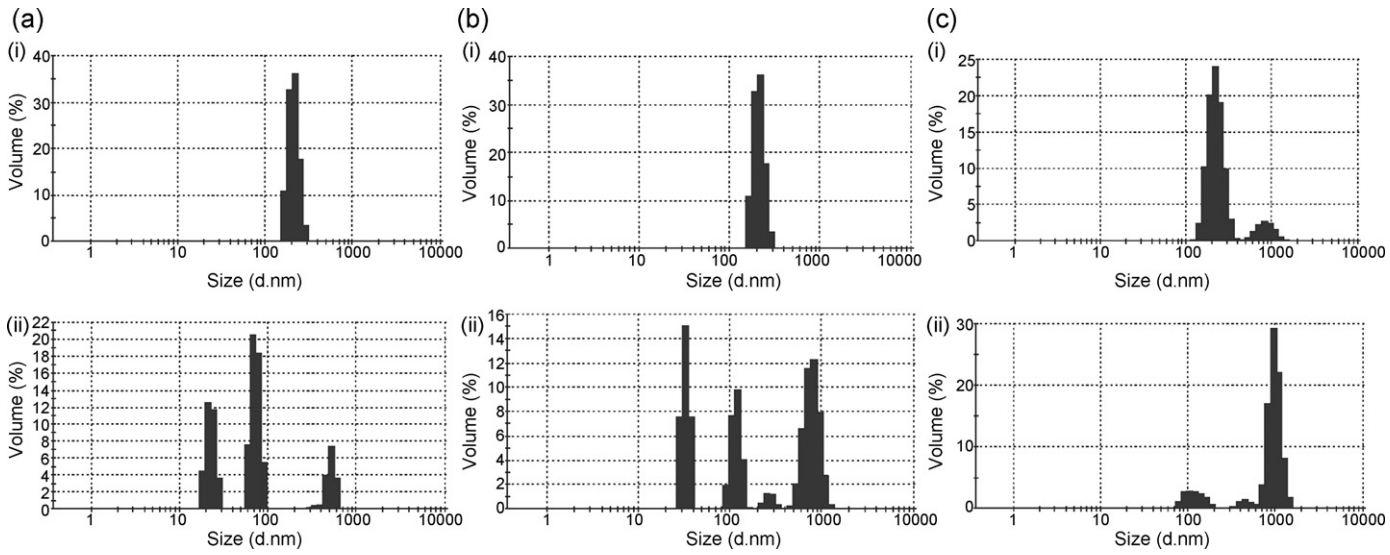

Fig. 2. Mean particle size distribution plotted by volume statistics, of oat spelt xylan hydrogel formed from selective hydrolysis of oat spelt xylan by recombinant α-L-arabinofuranosidase (AbfB): (a) measured after 30 min of terminating hydrolysis, (b) after 3 h of terminating hydrolysis and (c) after 7 days of terminating the hydrolysis. In the figures (i) and (ii) represent unhydrolysed oat spelt xylan and hydrolysed xylan, respectively, under the specified conditions. In the figure, the "peak splitting" is an indication of the diversity of the particles sizes for a given particle size range.

4. Discussion

4.1. Characteristics of hydrogels produced by hydrolysis of oat spelt xylan by α-L-arabinofuranosidase (AbfB)

The hydrolysis of oat spelt xylan by recombinant AbfB presented an alternative way of preparing micro- and nanohydrogels from water soluble xylan. The partial hydrolysis of the oat spelt xylan by the selective removal of arabinose side chains by the recombinant AbfB, resulted into formation of a mixture of micro and nano hydrogels. The formation of the hydrogels is as a result of the reduction in the steric hindrance caused by the arabinose between and within the xylan polymers (Ebringerová & Heinze, 2000). Therefore, the recombinant AbfB with polymeric xylan substrate specificity presents an alternative biological method of preparing xylan hydrogels to the chemical and physical methods. Furthermore, there is potential to use the recombinant AbfB in nano and microparticle engineering where bioactive substances are required to be either delivered or released in micro or nano sized doses. The results showed that smaller oat spelt xylan hydrogels with mean particle size of 18 nm were obtained from the AbfB hydrolysis (Table 1) whereas in esterification (Daus & Heinze, 2009) and coacervation (Garcia et al., 2001) of corn cob xylan, the smallest nanoparticles formed were 162 nm and 371 nm, respectively.

The xylan nanohydrogels aggregated at higher xylan concentration (Fig. 3a) and longer residence time (Fig. 2a–c) during enzyme hydrolysis and storage, respectively. The oat spelt xylan hydrogels mean particle sizes measured in the range from 18 to 700 nm after 30 min (Fig. 2a ii) and was After 7 days of storage at 4 °C, ranged from 78 nm to >1 μm (Fig. 2c ii). The increase in the mean particle size of

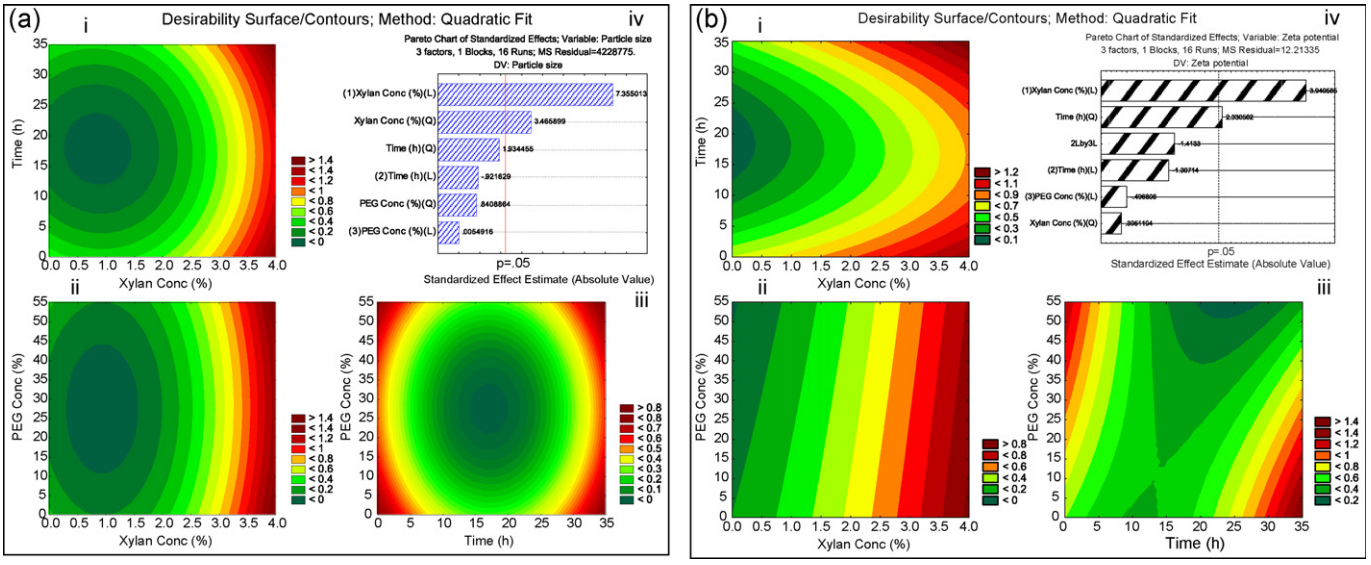

Fig. 3. Desirability contour plot showing the effects of xylan concentration, enzyme hydrolysis time and Poly Ethyl Glycol (PGE) 1000 concentration on (a) particle size and (b) zeta potential. Plot (i) shows interaction between time and xylan concentration; (ii) PEG 1000 concentration and xylan concentration, (iii) PEG 1000 and time and (iv) Pareto charts showing size and significance of the main effects and interactions of the independent variables. In the Pareto Charts (iv) the vertical dotted line denotes significant level at $P < 0.05$. The model fitted the particle size data with $R^2 = 0.88$; Adjusted $R^2 = 0.81$ and the zeta potential data with $R^2 = 0.74$; Adjusted $R^2 = 0.56$.

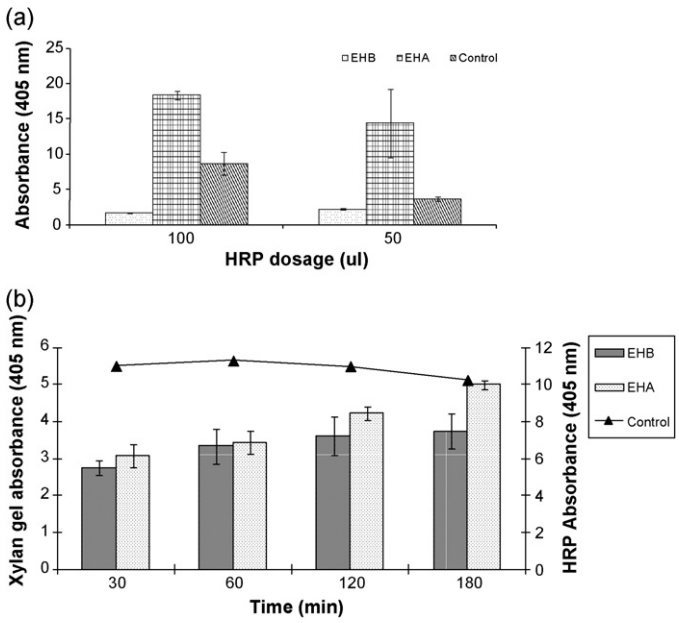

Fig. 4. Encapsulation of horse radish peroxidase (HRP) in oat spelt xylan hydrogel formed by selective hydrolysis of oat spelt xylan by recombinant α-L-arabinofuranosidase. In (a) residual activity in the solution decanted after centrifugation from oat spelt enzyme hydrolysate in which the HRP was added before (EHB) and after (EHA) the oat spelt xylan hydrogel formation. The HRP dosage levels were 50 and 100 μL. The control is the residual activity of HRP in unhydrolysed oat spelt xylan. (b) The left *y*-axis is the progression of diffusion of HRP out of oat spelt xylan hydrogel for HRP added before formation of oat spelt xylan hydrogel (EHB) and after formation of hydrogels (EHA). The line graph with the *y*-axis on the right represents the activity of the positive control (non encapsulated HRP) during the assessment period.

the xylan nanohydrogels was due to the tendency of self aggregation (Linder, Bergamon, et al., 2003). The self aggregation behaviour of the xylan hydrogels might not be desirable for encapsulation and delivery of bioactive agents that may be required to be released in specific constant dosages (Kumar, 2000). Therefore, for improved functional performance of the xylan hydrogels as delivery systems, the self aggregation behaviour may need to be controlled during enzymatic hydrolysis. The manipulation of process parameters such as xylan concentration and hydrolysis time, addition of anticoagulants/antiflocculant chemicals, and application of physical methods such as sonication, might reduce the degree of self aggregation by the xylan hydrogels.

4.2. Effect of oat spelt xylan and PEG 1000 concentrations and hydrolysis time on degree of arabinose release, particle size and zeta potential of xylan hydrogels

The results showed that the release of the mean particle sizes of the oat spelt xylan hydrogels were significantly affected ($P < 0.05$) by the oat spelt xylan concentration (Fig. 3a iv). Such effect was evident from the differences between the mean particle size range (21–292 nm) of the oat spelt xylan hydrogels formed at xylan concentration of ≤1% (w/v) and that of xylan hydrogels (74–4378 nm) formed at a xylan concentration of 2% (w/v) (Table 1). The higher xylan concentration favoured formation of microhydrogels rather than nanohydrogels. Therefore, controlling the oat spelt xylan concentration during the AbfB hydrolysis can tailor made the mean particle sizes of the xylan hydrogels for specific uses. In coacervation of corn cob, Garcia et al. (2001) found that at xylan concentration of higher than 100 mg mL^{-1} (10% w/v) hydrogels of micro sizes were formed. The larger mean particle size at higher xylan concentration can be attributed to the phenomenon of self aggregation discussed in Section 4.1, which is influenced partly by the reduction in repulsion electrostatic forces between the xylan nanohydrogels (Ebringerová, Hromádková & Heinze, 2005; Hornig & Heinze, 2008; Kabel et al., 2007; Linder, Bergamon, et al., 2003; Linder, Roubrocks, et al., 2003; Muller, Jacobs & Kayser, 2001). The electrostatic repulsion forces are responsible for keeping the particles in separation, which reduces the tendency of the hydrogels to self aggregate (Hornig & Heinze, 2008; Muller et al., 2001). However, at higher xylan concentration, the hydrogels are inherently forced into self assembling behaviour through inter and intra polymer bonding. Consequently, bigger and irregular shaped hydrogels are formed compared to those formed at lower xylan concentration. Additionally, the self aggregation behaviour is facilitated by presence of small amounts of aromatic substituents covalently bonded to lignin residues in the xylan (Linder, Bergamon, et al., 2003). According to Linder, Bergamon, et al. (2003) and Kabel et al. (2007) the varying degrees of aggregation behaviour of xylan may also be a consequence of changes in the chemical structures of the xylan. Such chemical changes in the xylan chain structure resulted in a loss of gel firmness of up to 43% therefore enhanced self aggregation in wheat arabinoxylan hydrogels formed by oxidation methods (Carvajal-Millan, Gugliarelli, Belle, Rouau, & Micard, 2005).

To obtain xylan hydrogels with stable morphology, either xylan concentration or the hydrolysis time has to be controlled because of their significant influence on the surface charge. The results in Table 1 showed that the xylan nanohydrogels formed at higher xylan concentrations resulted in high zeta potential values (thus, less negative zeta potential values) than at lower xylan concentration. For example, at xylan concentrations of 1% and 3%, the zeta potential values were up to −19 and −9.5 mV, respectively (Table 1). Furthermore, the Pareto Chart plot (Fig. 3b iv) showed that the zeta potential for the xylan hydrogels was significantly affected ($P < 0.05$) by both the xylan concentration and recombinant AbfB hydrolysis time. The higher zeta potential values are related to the reduced electrostatic repulsion forces on the surface of the xylan hydrogels. The zeta potential values of at least −30 to −20 mV lead to formation of hydrogels with stable physical structures (morphology) due to increased electrostatic repulsion (Hornig & Heinze, 2008; Muller et al., 2001). In this study, the lowest zeta potential for the xylan hydrogels of −19 mV (Table 1) was −1 mV outside the stable range, hence the observed self aggregation behaviour with time and increased xylan concentration (Fig. 3b iv).

Although, the self aggregation should be less prevalent in arabinoxylans such as oat spelt xylan compared to arabinoglucuronoxylan such as corn cob xylan because of their shear thinning and absence of thixotropic properties (Ebringerová & Heinze, 2000), the reduction in self aggregation of the xylan hydrogels in enzymatic methods can be addressed possibly by considering the saturation kinetics of the xylan hydrolysing enzymes.

The response contour plots (Fig. 3a i–iii) showed that oat spelt xylan hydrogels of nano sizes of less than 100 nm could be optimally obtained at oat spelt xylan concentrations ranging from 0.5 to 1.5% for hydrolysis time ranging from 15 to 20 h. There was no significant effect ($P < 0.05$) of PEG 1000 plasticiser on the stability of the particle size (Fig. 3a iv) and zeta potential (Fig. 3b iv) of the oat spelt xylan hydrogels. Therefore, the results of this study are in contradiction with those of Garcia et al. (2001) who showed stability of the morphology of the corn cob xylan hydrogels by addition the PEG 1000 plasticiser. According to Hornig and Heinze (2008), the hydrogel aggregation can be prevented by creating a balance between the hydrophilic and hydrophobic parts of the xylan polymer. Similarly, Garcia et al. (2001) showed a positive effect of PEG 1000 plasticiser on the stability of the morphology of the xylan hydrogels, which through covalent bonding or surface adsorption stabilised the morphology of corn cob xylan hydrogels. However, according to Garcia et al. (2001) such an effect was enhanced by

the variation of pH or ionic strength. Therefore, it is possible that the effect of the PEG 1000 plasticiser is specific to the method of preparing the xylan hydrogels and therefore was not applicable to formation of xylan hydrogels by the enzyme hydrolysis.

4.3. Horse radish peroxidase encapsulation and release

Horse radish peroxidase (HRP) was encapsulated and released in active form from the oat spelt xylan hydrogels formed by selective hydrolysis of the oat spelt xylan by the recombinant AbfB. The encapsulation of the HRP occurred simultaneously *in situ* both during and after formation of the oat spelt xylan hydrogels (Fig. 4a). Such flexible encapsulation suggests a technology for encapsulation of bioactive agents that are both chemical and heat sensitive. Therefore, the encapsulation procedure demonstrated by this study is considered advantageous over the chemical and hydrothermal methods of preparing hydrogels.

The xylan hydrogels released the encapsulated HRP in its active form over a period of 180 min (Fig. 4b). The sustained release of the HRP is an indication that the xylan hydrogels can potentially be used as encapsulation matrix for targeted delivery and slow release of bioactive substances. Although the initial rate of release from the xylan hydrogel of the HRP added after xylan hydrogel formation was 1.5 times higher than the rate of release of the HRP added before formation of the hydrogel (data not shown). The rate of release of the HRP added after formation of the xylan hydrogel stabilised within the first hour, in contrast to the rate of release of the HRP encapsulated before the formation of the hydrogel, which continued to decline with time (Fig. 4b). This phenomenon implies that the HRP encapsulated after the hydrogel formation was released with less restriction from the xylan hydrogel matrix than the one encapsulated during the formation of the hydrogel. Steady rate of release of the HRP demonstrated in the case where the HRP was encapsulated after hydrogel formation is critical for efficient and sustained release of constant dosage of the bioactive agent. Therefore, choice of the encapsulation method is critical for customised slow and targeted release of the bioactive agent from the xylan hydrogels.

5. Conclusion

Simultaneous *in situ* formation of xylan nanohydrogels and encapsulation of the HRP was achieved through selective hydrolysis of oat spelt xylan by recombinant AbfB with polymeric xylan substrate specificity. The oat spelt xylan hydrogels formed were a mixture of micro and nano particles with particle sizes as small as 18 nm. Stability and the morphological characteristics of the xylan nanohydrogels were significantly influenced by the oat spelt xylan concentration and hydrolysis time. In addition, sustained release of the encapsulated HRP occurred over a period of 180 min. The characteristic of the rate of release of the encapsulated HRP depended on whether the HRP was added before or after oat spelt xylan hydrogel formation. Such characteristic release shows that different encapsulation methods were discovered for customised slow release of the bioactive agent. Therefore the recombinant AbfB has presented a flexible novel method for simultaneous *in situ* formation of hydrogels and encapsulation of soluble bioactive substances for slow and targeted release.

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