Pergamon

Tetrahedron Letters 43 (2002) 2005–2008

TETRAHEDRON LETTERS

Synthesis of octyl glucopyranoside by almond β-glucosidase adsorbed onto Celite R-640®

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Received 18 December 2001; revised 23 January 2002; accepted 25 January 2002

Abstract—The synthesis of octyl glucoside from *p*-nitrophenyl glucopyranoside (*p*-NPG) and 1-octanol was carried out with almond β-glucosidase adsorbed onto Celite R-640®. The influence of the amount of water added to the system as well as the addition of co-solvents have been studied. The presence of small percentages of DMF has a strong denaturing effect on the enzyme adsorbed onto Celite R-640®. Denaturation is less pronounced with other co-solvents such as acetonitrile, 2-methyl-2-butanol or ethyl acetate. Crown Copyright © 2002 Published by Elsevier Science Ltd. All rights reserved.

The use of glycosidases to catalyze the synthesis of alkyl-glycosides (by reversed hydrolysis or transglycosylation) has attracted considerable interest recently due to the numerous advantages offered by such an enzymatic process (mild reaction conditions, high specificity of enzymes, no protection/deprotection steps required for the sugar hydroxyl groups, production of anomerically pure products, no need for expensive cofactors, etc).[1] However, the obtained yields remain limited as glycosidases lose activity at low water activities (a_W).[2,3] The presence of water is necessary to maintain the enzymatic activity but on the other hand causes substrate and product hydrolysis. Therefore, the hydration level of the media has to be carefully controlled.

It has been reported that Celite R-640® can efficiently control the hydration level of the media during peptide and amide synthesis catalyzed by thermolysin[4] and penicillin G amidase, respectively.[5] Celite is a chemically inert silica-based matrix which consists of diatomaceous earth broken up and subsequently recalcined to create porous particles with controlled pore sizes.[6] Celite R-640® has been characterized by its capacity to adsorb great quantities of water, and in toluene Celite R-640® adsorbs and releases water so that a_W is maintained constant in a reaction system within wide ranges of water concentrations.[7] Thus, the enzyme activity as well as the reaction equilibrium are effectively controlled leading to complete conversions and very high isolated yields.[4] The aim of this work is to expand the applicability of Celite R-640® as a support for the immobilization of almond β-glucosidase for the synthesis of octyl-β-D-glucopyranoside by reaction between 1-octanol and *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) as glucosyl donor.

The water adsorption capacity of Celite R-640® in 1-octanol was first verified at 50°C by adding an aqueous solution (sodium acetate buffer 0.05 M, pH 5.0) to dry 1-octanol containing Celite R-640®. Equilibration of the system was monitored by following the quantity of water dissolved in the organic phase (determined by coulometric Karl Fischer titration) as well as the water activity of the system (measured with a

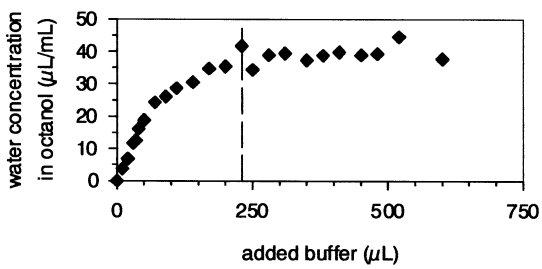

Figure 1. Water distribution in 1-octanol with Celite R-640® at equilibrium (2 mL 1-octanol; 200 mg Celite R-640® (Fluka); 0–700 μL of sodium acetate buffer 0.05 M, pH 5.0; temperature: 50°C; equilibration time: 48 h, orbital agitation: 280 rpm). Dashed line represents the amount of water needed to saturate the octanol.

Keywords: β-glucosidase; Celite R-640®; immobilization; water activity; octyl glucoside; organic solvent.

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PII: S0040-4039(02)00197-1

DARAI hygrometer at 50°C). In the absence of Celite, octanol saturated with buffer contains 40 μL of water per mL at 50°C. As can be seen in Fig. 1, 240 μL of water can be added to the mixture 1-octanol/Celite R-640® before the octanol phase becomes saturated. Above this value, an aqueous phase appears. This means that Celite R-640® can adsorb up to 80% of its weight in water under our conditions.

We have studied the application of Celite R-640® as an immobilization support for almond glucosidase. The enzyme adsorption on Celite is simple and does not require any stressful treatment.[4] An enzyme solution in aqueous buffer is added to octanol containing a few rods of Celite and the system is kept closed at 50°C under agitation until equilibrium is reached (48 h). The enzymatic reaction is then started by adding *p*-NPG which was chosen as glycosyl donor in order to provide information on hydrolysis, synthesis and transglycosylation capacities of the biocatalyst.[8] When the same immobilization technique was used in toluene, the equilibrium was reached after 24 h. This different behavior can be attributed to the higher hydrophilicity of octanol.

In the absence of added water, the a_W of the system Celite/1-octanol at 50°C is 0.22. The water activity of the system progressively increases when water is added. However, as can be seen in Table 1, within a small range of water concentrations (30–60 μL of added water), Celite R-640® is able to maintain a_W constant at 0.40 in the system. This lower buffering capacity, as compared to toluene, may come from the higher polarity of 1-octanol and some interactions with the Celite, but we have no evidence for it. The capacity of Celite R-640® to control the water activity has already been used to foster the reaction during peptide synthesis in toluene.[7] Nevertheless, almond β-glucosidase is known to be very sensitive to the hydration level of the media and it is reported that a water activity of at least 0.7 is needed to carry out the synthesis of octyl glucoside.[1,2] Therefore, at a water activity as low as 0.40, the enzyme is not sufficiently hydrated to show good catalytic activity. Moreover, even if Celite R-640® is able to control the water activity at 0.40 during the reaction, the obtained hydrolytic and synthetic rates are very much influenced by the amount of added water, which is quite surprising. Below 50 μL of added water, there was no detectable hydrolysis or synthesis. Above 50 μL, the more water is added, the faster both reaction rates get. As expected, the equilibrium yield decreases when the water activity increases. It should be noted that above 280 μL of added water, hydrolytic as well as synthetic rates decrease (2.5- and 3-fold, respectively) to be the same whatever the quantity of water added, this latest observation being due to the low partition coefficient of octyl glucoside at 50°C between water and octanol (<1.6% in the aqueous phase). In fact, as mentioned above, a separate water phase appears in the system above 240 μL of water added, and the lower rates obtained can be explained by the rapid denaturation of the enzyme at high water content in the medium during the 48 h of equilibration.[9] Moreover, initial reaction rates do not decrease when reactions are performed after 24 h of equilibration.

It is obvious in Table 1 that hydrolysis of *p*-NPG occurs much faster than the synthesis of octyl glucoside, implying that a high quantity of glucose is released before it is converted into octyl glucoside. However, when performing the same reaction directly with free glucose instead of *p*-NPG, the obtained syn-

Table 1. Effect of water adsorbed by Celite R-640 on the synthesis of octyl-glucoside by almond β-glucosidase[a]

Water added (μL)	a_W (−)	*p*-NPG hydrolysis initial rate (μmol h^{-1}·mg $prot^{-1}$)	Oc-Glc synthesis initial rate (μmol h^{-1}·mg $prot^{-1}$)	Equilibrium yield in Oc-Glc[b] (%)	α Hydrolytic rate/synthetic rate (−)
480	0.77	1.2	6.8×10^{-2}	10.6	18
280	0.74	1.0	6.8×10^{-2}	10.1	15
140	0.70	2.7	2.0×10^{-1}	10.9	14
110	0.68	2.8	1.7×10^{-1}	21.9	16
100	0.63	2.6	1.7×10^{-1}	24.4	15
90	0.60	1.9	1.4×10^{-1}	29.1	14
80	0.58	8.0×10^{-1}	5.6×10^{-2}	33.1	14
70	0.52	5.8×10^{-1}	3.4×10^{-2}	37.2	17
60	0.40	1.4×10^{-1}	9.1×10^{-3}	34[c]	15
50	0.40	3.6×10^{-2}	2.2×10^{-3}	8.5[c]	16
40	0.41	$<2\times10^{-4}$[d]	$<1\times10^{-4}$[d]	–	–
35	0.40	$<2\times10^{-4}$[d]	$<1\times10^{-4}$[d]	–	–
30	0.40	nd	nd	–	–
20	0.29	nd	nd	–	–
10	0.26	nd	nd	–	–
0	0.21	nd	nd	–	–

[a] Same conditions as Fig. 1, except that the buffer contains 20 mg of lyophilized almond β-glucosidase (Sigma 2.85 U/mg); *p*-NPG: 140 μmol.
[b] Equilibrium verified by addition of fresh lyophilized enzyme preequilibrated at the proper water activity with saturated salt solutions. (NaI, $a_W=0.39$; $Mg(NO_3)_2$, $a_W=0.53$; NaBr, $a_W=0.58$; $NaNO_2$, $a_W=0.64$; NaCl, a_W 0.75).
[c] Equilibrium not reached yet, value obtained after 722 h.
[d] Less than detection limit.
nd: not determined.

thetic rate are decreased by a factor of 2 showing that some transglucosylation may occur (results not shown). Table 1 reports also the values of the ratio between the initial rates of hydrolysis and synthesis (α). As can be seen, this value is reasonably constant for all the reactions. This suggests that once the hydrolysis of *p*-NPG starts, the availability of free glucose for the enzyme is the same independently of the water amount present in the system. The concentration of glucose in octanol, measured by HPLC, is around 3 mM in all cases. This means that once *p*-NPG is hydrolyzed into *p*-nitrophenol and glucose, most of the latter is adsorbed onto the surface of Celite rods in the vicinity of the enzyme and this increases the synthetic reaction rate.

A positive effect of the presence of solubility-enhancing co-solvents such as DMF to the reaction system has been reported.[10] Table 2 reports results obtained with the enzymatic preparation β-glucosidase/Celite R-640® assayed in the presence of different percentages of DMF as a co-solvent. The addition of a co-solvent results in a decrease of the water activity of the system and, as a result, in a decrease of the initial rates. However, the observed reduction of the initial rates as well as the lower yield obtained can also be ascribed to an additional denaturing effect of DMF. Indeed, for the same water amount (70 μL), in the presence of 10% DMF, the reaction stops rapidly and only 53% of *p*-NPG is hydrolyzed with synthesis of traces of octyl glucoside, while with 20% of DMF, only 12% of *p*-NPG is hydrolyzed without any octyl glucoside synthesis. The addition of fresh enzyme, pre-equilibrated at the proper water activity, starts again hydrolytic and synthetic reactions in both cases, confirming the denaturation of the enzyme. Moreover, at a fixed DMF concentration, the inactivation occurred faster when more water was present in the media and the enzyme is completely denatured after 48 h of equilibration. Previous results have shown that the presence of DMF up to 20% greatly improves hydrolysis of *p*-NPG, synthesis of octyl glucoside, as well as the obtained yields when using almond β-glucosidase either lyophilized[8] or adsorbed onto XAD-4 resin (unpublished results). The presence of Celite alters the reaction conditions as some DMF is adsorbed with the enzyme and water onto the solid (verified by measuring a small decrease of the DMF/octanol ratio by HPLC after the addition of Celite in the reaction media). As a result, the local DMF concentration in the vicinity of the enzyme is enhanced. In addition, when a large water amount is added (lines 5, 6 and 9 in Table 2), a small separate aqueous phase is present and DMF partitions between the octanol and aqueous phases, leading to a still greater DMF concentration around the enzyme.

We have studied the influence of other co-solvents, such as acetonitrile, 2-methyl-2-butanol and ethyl acetate, which are not adsorbed onto Celite, with the aim of increasing the polarity of the reaction media with a subsequent enhancement of the solubility of the glucose formed during hydrolysis of *p*-NPG. As can be seen in Table 3, the obtained water activities in the three reaction media are comparable. The enzyme seems to tolerate these co-solvents better than DMF. Indeed, 10% of each co-solvent leads to complete hydrolysis of *p*-NPG in just a few hours in all cases and high equilibrium yields are obtained (about 30%). However, although yields remain similar, the presence of the co-solvents leads to lower rates at a same a_w of the system (compared with Table 1). When present at 50% in the media, the three co-solvents have a different effect. Almond β-glucosidase is strongly inhibited by acetonitrile and 2-methyl-2-butanol and the measured reaction rates decrease many fold. On the opposite, the presence of either 10 or 50% ethyl acetate has a less pronounced effect and does not influence the initial rate of hydrolysis of *p*-NPG. However, the α ratio between hydrolytic and synthetic initial rates slightly increases when going from 10 to 50% of ethyl acetate in the medium. We hypothesize that the observed decrease in the synthetic initial rate can be attributed to an enhanced glucose solubility in octanol, and thus, to a lower availability of the glucose (less adsorbed on Celite) to the enzyme. This can be one of the reasons for the impressive increase of α observed in Table 2 when increasing the DMF amount.

Table 2. Effect DMF on the synthesis of octyl-glucoside by almond β-glucosidase[a]

DMF (% v/v)	Water added (μL)	a_w (−)	*p*-NPG hydrolysis initial rate (μmol h^{-1}·mg $prot^{-1}$)	Oc-Glc synthesis initial rate (μmol h^{-1}·mg $prot^{-1}$)	Equilibrium yield in Oc-Glc[b] (%)	α hydrolytic rate/synthetic rate (−)
0	70	0.52	5.8×10^{-1}	3.4×10^{-2}	37.2	17
1	70	0.55	3.7×10^{-1}	1.8×10^{-2}	38.2	21
5	70	0.45	6.8×10^{-2}	6.1×10^{-4}	5.5[c]	111
10	70	0.40	3.7×10^{-2}	1.5×10^{-4}	2.5[c]	247
10	140	0.65	$<2\times10^{-4}$[d]	$<1\times10^{-4}$[d]		–
10	280	0.70	$<2\times10^{-4}$[d]	$<1\times10^{-4}$[d]	–	–
20	70	0.40	2.8×10^{-3}	$<1\times10^{-4}$[d]	–	–
20	140	0.68	3.4×10^{-4}	$<1\times10^{-4}$[d]	–	–
20	280	0.72	$<2\times10^{-4}$[d]	$<1\times10^{-4}$[d]	–	–

[a] Same conditions as Table 1 except for organic phase = 2 mL of 1-octanol/DMF.
[b] True equilibrium (see Table 1, footnote b).
[c] Equilibrium not reached yet, value obtained after 650 h.
[d] Less than detection limit.

Table 3. Influence of co-solvents on the synthesis of octyl glucoside by almond β-glucosidase[a]

Co-solvent	% of co-solvent	a_w (−)	*p*-NPG hydrolysis initial rate (μmol h^{-1}·mg $prot^{-1}$)	Oc-Glc synthesis initial rate (μmol h^{-1}·mg $prot^{-1}$)	Equilibrium yield in Oc-Glc[b] (%)	α hydrolytic rate/synthetic rate (−)
Acetonitrile	10	0.60	2.5×10^{-1}	1.1×10^{-2}	33.2	23
2-Methyl-2-butanol	10	0.62	3.5×10^{-1}	1.1×10^{-2}	31.3	32
Ethyl acetate	10	0.59	3.8×10^{-1}	1.4×10^{-2}	33.9	27
Acetonitrile	50	0.53	1.5×10^{-2}	5.6×10^{-4}	5.5[c]	27
2-Methyl-2-butanol	50	0.53	5.5×10^{-2}	2.3×10^{-4}	2.4	239
Ethyl acetate	50	0.62	3.5×10^{-1}	9.0×10^{-3}	21.8	39

[a] Same conditions as Table 2 with organic phase = 2 mL of 1-octanol/co-solvent, volume of buffer containing enzyme fixed at 70 μL and equilibration time = 24 h.
[b] True equilibrium (see Table 1, footnote b).
[c] Yield obtained after 650 h (equilibrium not reached, the synthesis starts again when adding fresh lyophilized enzyme preequilibrated at the proper water activity).

In conclusion, it has been shown that it is possible to carry out the synthesis of octyl glucoside from *p*-NPG and 1-octanol with almond β-glucosidase adsorbed onto Celite R-640®. The influence of the amount of water added to the system as well as the presence of co-solvents have a great effect on the enzyme activity. Particularly, the addition of small percentages of DMF has a strong denaturing effect on the almond glucosidase adsorbed onto Celite R-640® compared to the biocatalyst lyophilized or adsorbed onto XAD-4,[8] probably due to the adsorption of DMF onto Celite in the vicinity of the enzyme.

Acknowledgements

Alessandra Basso is grateful to MURST (Rome) for financial support to Professor Paolo Linda.

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