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A kinetic study of almond-β-glucosidase catalysed synthesis of hexyl-glycosides in low aqueous media Influence of glycosyl donor and water activity

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Abstract

A variety of alkyl and aryl glycosides were investigated as substrates for almond β-glucosidase catalysed synthesis of hexyl-β-D-glycosides in low aqueous hexanol media. The rate-limiting step in the organic media was determined to be the glycosylation of the enzyme. The kinetic constants V_{max}, K_m (glycosyl donor) and V_{max}/K_m were all influenced by the water activity and they all increased in value with increasing water activity. The increase in V_{max}/K_m was mainly determined by the increase in V_{max} and a plot of $\log(V_{max}/K_m)$ versus water activity resulted in a straight line with similar slopes for all glycosides but with different absolute values and thus the most reactive substrate *p*-nitrophenyl glucoside was the best one in the entire water activity range studied (0.53–0.96). The preference for the two competing acceptors, hexanol and water, was not affected by the aglycon part of the glucoside. Surprisingly, the ratio between *trans* glycosylation and hydrolysis increased with increasing water activity. A decrease in water activity caused an increase in equilibrium yield of hexyl glycoside, as expected, but was not beneficial for the kinetically controlled yield. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Almond-β-glucosidase; Water activity; Organic solvent; Kinetics; *Trans*-glycosylation

1. Introduction

Many enzymes express good catalytic activity in predominantly organic media provided that the thermodynamic water activity is high (>0.7) [1]. However, to be able to obtain high yields in synthetic reactions catalysed by hydrolytic enzymes much lower water activities are usually needed. Rantwijk et al. [2] conclude in an excellent survey of the latest literature in the field of glycosidase catalysed alkyl-glycoside synthesis, that in order to be able to fully explore the glycosidases for synthetic applications high operational stability and activity at low water activity are prerequisites. In contrast to the lipases, which work well at low water activities, the glucosidases do not [2,3]. One difference between these two enzyme groups is the polarity of the preferred substrates. In general the substrates for the glucosidases are more polar and only slightly soluble in non-aqueous solvents.

Almond-β-glucosidase hydrolyses the glycosidic bond with a net retention of the anomeric configuration through a double displacement mechanism [4]. In the first step, the glycosylation, the substrate binds to a pair of carboxylic acids and forms a covalent glycosyl–enzyme intermediate [5]. In the second step

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the anomeric centre is attached by a nucleophile (water or an alcohol) to yield a hydrolysis or a *trans* glucosylation product. The maximum product yield is determined by either the equilibrium constant (thermodynamically controlled yield) or the reactivity of the glycosyl donor (kinetically controlled yield) [5]. The almond-β-glucosidase is reported to express rather broad substrate specificity towards both the sugar moiety and to the aglycon part of the glycoside in a hydrolysis reaction [6]. Dale et al. [6] made a thoroughly structure activity study of the almond-β-glucosidase in aqueous media and they found a good correlation between k_{cat} and the pK_a of the leaving group of various aryl-glycosides. The broad substrate specificity of this enzyme makes it useful to study the influence of the structure of glycosides on the enzymatic activity at low water activities.

In this investigation we studied how less polar substrates than glucose (e.g. a more hydrophobic aglycon part) affected the activity and the kinetic constants at different water activities. This in order to elucidate, whether or not substrate engineering can be used to increase the performance of the glycosidase at low water activities. The reaction studied was the almond-β-glucosidase catalysed *trans* glycosylation of various alkyl and aryl-glycosides. All reactions were run in hexanol, which served both as substrate and solvent. The apparent K_m, V_{max} and V_{max}/K_m of the enzyme for various glycosides were determined.

2. Materials and methods

2.1. Chemicals

Almond β-glucosidase (E.C. 3.2.1.21) and *p*-nitrophenyl-β-D-glucopyranoside were bought from ICN (Costa Mesa, USA). Methyl, amyl, hexyl, octyl, phenyl, 2-hydroxy methyl phenyl (salicin) and naphtyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, triethylene glycol dimethyl ether (triglyme) and hexanol were bought from Sigma Chemicals (St. Louis, USA). Celite (30–80 mesh) was obtained from BDH Limited (Poole, England). Methyl-4,6-*O*-isopropylidene-β-D-glucopyranoside, *p*-nitrophenyl-4,6-*O*-isopropylidene-β-D-glucopyranoside and methyl-4,6-*O*-benzylidene-β-D-glucopyranoside were synthesised according to Wolfrom et al. [7].

2.2. Enzyme deposition

Almond-β-glucosidase was deposited on Celite [8]. An amount of 50 mg almond β-glucosidase in 1 ml sodium acetate buffer (50 mM, pH 5.0) was mixed with 1 g of washed Celite, dried by 2× 20 ml of cold acetone to remove the water and then finally under reduced pressure (10 mmHg, 2 h). The deposited enzyme was stored under argon at 5°C until use.

2.3. Pre equilibration of water activity

The hexanol and the substrates were equilibrated with saturated salt solutions at 40°C [9]. The salts used were NaBr (water activity, $a_w = 0.53$), NaCl ($a_w = 0.75$), KCl ($a_w = 0.82$) and K_2SO_4 ($a_w = 0.96$). Equilibration was performed for 3 days. The enzyme preparation was only equilibrated against NaCl ($a_w = 0.75$) at 25°C for 20 h, since the amount of water associated with the enzyme preparation compared to the amount of water dissolved in the hexanol was very low.

2.4. Enzymatic reaction

The reaction mixture consisted of one single phase that contained glycoside, hexanol and water in a total volume of 2 ml. The reaction was carried out in 4 ml vials equipped with teflon/rubber septa and placed on a rotational shaker (200 rpm) at 40°C. Each vial contained 20 mg of the enzyme preparation. The reaction was followed by withdrawing samples which were analysed by quantitative thin-layer chromatography (TLC).

2.5. Determination of kinetic constants

The glycoside concentration was usually varied between 1.5 and 60 mM (depending on the solubility of the glycoside in hexanol). The obtained initial activities (usually based on the total activity, but in some cases only the *trans* glycosylation activity was used) for the different substrate concentrations were fitted to the Michaelis-Menten equation by non-linear

regression by using a PC software, Enzfitter, to elucidate V_{max}, K_m and V_{max}/K_m. When V_{max}/K_m was determined graphically (i.e. the initial part of a plot of v versus $[s]$) the specificity constant is denoted V/K.

Based on a molecular weight of 135 000 [10] and assuming all enzyme molecules to be active with accessible active sites, the k_{cat}/K_m can be calculated from the following equation: $k_{cat}/K_m = (V/K)/[E]$ ($M^{-1}\,min^{-1}$).

2.6. Analyses

The water concentration in the reaction media was determined by water analysis using a Karl Fischer titrator. The *trans* glucosidase and the hydrolytic activity were determined by analysing the produced hexyl-β-glycoside and glucose (fucose and xylose was not quantified) using a quantitative TLC equipment from CAMAG. The samples were applied on Silica gel 60 or RP-18 TLC plates (20× 10 cm) from Merck, Darmstadt, Germany. The samples were eluted using a solvent system of chloroform–methanol–water (65:15:2) (Otto et al., 1998) for silica coated plates and acetonitrile–water (2:5) for RP-18 coated plates. The products and substrate were developed and visualised by dipping the plate in a vanillin solution (1 g vanillin, 25 ml ethanol, 25 ml water and 35 ml 85% phosphoric acid) [11] and heating to 190°C for 6 min. The spots were quantified densitometrically by a Camag TLC scanner using an absorbance/reflection mode. The slit dimension was set at 4 mm × 0.3 mm and the wavelength was set to 500 nm. Concentrations were calculated from calibration curves.

3. Results and discussion

3.1. Screening of suitable substrates

The activity of the almond-β-glucosidase catalysed *trans* glycosylation of various alkyl and aryl-glycosides in hexanol was measured at a fixed initial water activity of 0.82 and a fixed substrate concentration of 10 mM. The obtained initial activities are shown in Table 1. Almost all derivatives at the aglycon part of the glucosides were converted except for the very hydrophobic undecyl and naphtyl glucosides.

Table 1
Initial activities for almond-β-glucosidase catalysed hexyl-glycoside synthesis using various alkyl and aryl glycosides (10 mM) in hexanol at a water activity of 0.82

Substituent	Initial activity (mU/mg)
Aglycon derivatives	
Hydrogen	2
Methyl	9.1
Amyl	8.5
Octyl	0.38
Undecyl	0
Phenyl	0.52
Naphtyl	0
p-Nitrophenyl	36
2-Hydroxy methyl phenyl	11
Sugar derivatives	
1-Methyl-4,6-isopropylidene	0
1-Methyl-4,6-benzylidene	0
1-*p*-Nitrophenyl-4,6-isopropylidene	0
Xylose (1-*p*-nitrophenyl)	1.7
Fucose (1-*p*-nitrophenyl)	24

Highest activity was obtained for *p*-nitrophenyl glucoside (36 mU/mg). The enzyme was more sensitive to modification of the sugar part of the glycoside, see Table 1, and no activity was observed for the isopropylidene or benzylidene protected methyl glycosides. Modification at position 5 of the sugar part was more successful and *p*-nitrophenyl glycosides of xylose and fucose were both accepted as substrates. With the *p*-nitrophenyl-fucoside the activity was almost as high as with the corresponding glucoside.

The difference in activity upon changing the aglycon part, Table 1, can either be due to differences in leaving group efficiency (i.e. *p*-nitrophenyl is a very good leaving group) or to that the aglycon part is actually involved in the binding of the substrate.

3.2. Glycosylation and deglycosylation steps in the reaction

The *trans* glycosylation reactions reported in Table 1 were in all cases accompanied by hydrolysis yielding the monosaccharides. A typical time course of a reaction is shown in Fig. 1.

The initial ratio of *trans* glycosylation to hydrolysis was similar for glycosyl donors giving widely different total reaction rates. The almond-glucosidase catalysed reactions in organic media (i.e. hexanol containing low amount of water) thus seem to occur

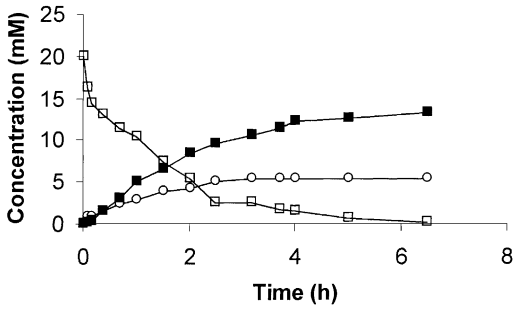

Fig. 1. Progress curve for almond-β-glucosidase catalysed transglucosylation (—■—) and hydrolysis (—○—) of 20 mM *p*-nitrophenyl glucoside (—□—) in hexanol with a water activity of 0.96 at 40°C and 200 rpm.

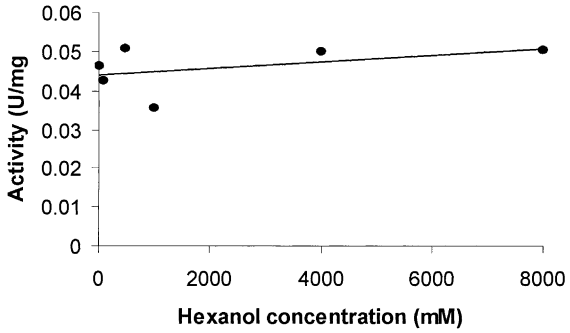

Fig. 2. The total activity (hydrolysis and transglucosylation) of almond β-glucosidase converting 10 mM *p*-nitrophenyl glucoside as a function of the hexanol concentration in triglyme with a water activity of 0.96 at 40°C and 200 rpm.

via a similar common glycosyl–enzyme intermediate as has been reported for almond-glucosidase in aqueous media [12]. The total activity (hydrolysis and *trans* glycosylation) using 10 mM *p*-nitrophenyl glucoside as glucosyl donor was also measured as a function of the hexanol concentration (20–8000 mM) in triglyme. As is evident in Fig. 2, varying the hexanol concentration did not influence the total activity significantly. This shows that the glycosylation was the rate-limiting step and it is appropriate to describe the kinetics using a one-substrate model.

3.3. Influence of water content on the trans-glucosylation and hydrolysis reactions

In Fig. 3 the initial activity for the transglucosylation and hydrolysis of 20 mM *p*-nitrophenyl glucoside as a function of the water activity is shown. Surprisingly, the hydrolysis was kinetically favoured over the transglucosylation at water activities below 0.96 and this is quite peculiar. The hydrolysis to transglucosylation ratio increased thus by decreasing water activity. At low water activities the enzyme is less flexible and maybe this restriction in flexibility was the reason for the enzyme to favour the smaller water molecule over the larger hexanol as glycosyl acceptor.

The synthesis of hexyl-glucoside was dependent on the water activity of the system. In Fig. 4 the progress curves obtained for the synthesis of hexyl-glucoside from *p*-nitrophenyl glucoside in three reactions that only differ in the amount of water (17% water and controlled water activity of 0.96 and 0.75, respectively) is shown. The highest initial activity for formation of

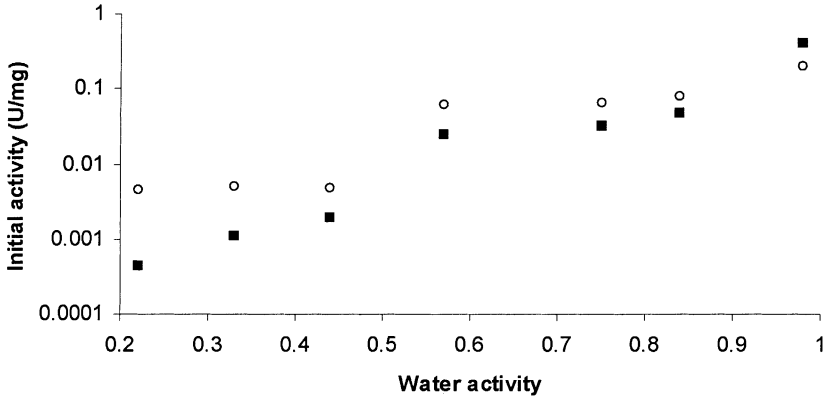

Fig. 3. Initial activity for the transglucosylation (—■—) and hydrolysis (—○—) of almond β-glucosidase converting 20 mM *p*-nitrophenyl glucoside in hexanol at different water activities at 40°C.

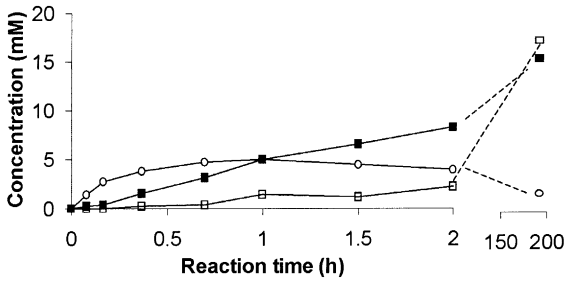

Fig. 4. Hexyl-glucoside synthesis from 20 mM *P*-nitrophenyl-glucoside in hexanol at three different water concentrations. At 17% (—○—) water and at a water activity of 0.96 (—■—) and 0.75 (—□—). All reactions consisted of 20 mg Celite preparation (50 mg almond-β-glucosidase per g Celite) in a total volume of 3 ml.

hexyl-glucoside was obtained when a separate water phase was present (i.e. 17% water). In this case, the maximum yield was thus kinetically controlled. Upon a decrease in the water concentration below the saturation level of the hexanol phase (i.e. a water activity of 0.96) the initial rate decreased 4×. The maximum yield became thermodynamically controlled the decrease in hexyl-glucoside concentration due to secondary hydrolysis that is typical for *trans* glycosylations in water or water organic two-phase systems did not occur. A further decrease in water activity to 0.75 decreased the reaction rate another 7× but increased the thermodynamic yield, see Fig. 4. A decrease in water activity was not beneficial for the kinetically controlled yield. Instead, the kinetically controlled yield was favoured by high water concentrations. On the contrary, the thermodynamic yield benefits of course from a low water concentration as long as the enzyme is not inactivated.

In order to get a fast production in the *trans* glycosylation it is favourable to have a high water content. Both high total reaction rate and high *trans* glycosylation/hydrolysis ratio is obtained at high water content, but in order to get a high yield it is important to use a low water activity. The influence of water activity on the kinetics was studied in more detail.

3.4. Water activity dependence of the kinetics with different substrates

The *V*/*K* increased with increasing water activity for all substrates. In Table 2 the resulting *V*/*K* for three different *p*-nitrophenyl glycosides (glucose, xylose and fucose) at three different water activities (0.53, 0.75 and 0.96) is shown. A variation in the glycon part of the glycosides did not influence the water activity dependence with respect to *V*/*K*. Neither did a variation in the aglycon part influence the water activity dependence. A plot of log(*V*/*K*) for seven glycosides differing in the aglycon part versus the water activity revealed more or less straight lines with similar slopes, see Fig. 5. The influence of decreasing water activity was thus the same for all glycosides tested. Engineering the glycoside substrates does not seem to alter the influence of the water activity on the reaction kinetics.

Table 2
Specificity constants (*V*/*K*) based on the *trans* glycosylation rate for three *p*-nitrophenyl glycosides[a]

Glycon	Water activity		
	0.53	0.75	0.96
D-Glucose	1.2	3.3	11
D-Fucose	0.37	4.7	16
D-Xylose	0.012	0.16	0.22

[a] Specificity constants (*V*/*K*): unit, $\mu mol\,min^{-1}\,mg^{-1}\,M^{-1}$.

Correlations between the specificity constants (*V*/*K*) and physiochemical properties of the substrates were investigated. The alkyl substituents have similar leaving group power and it was therefore of special interest to study the influence of the chain length on

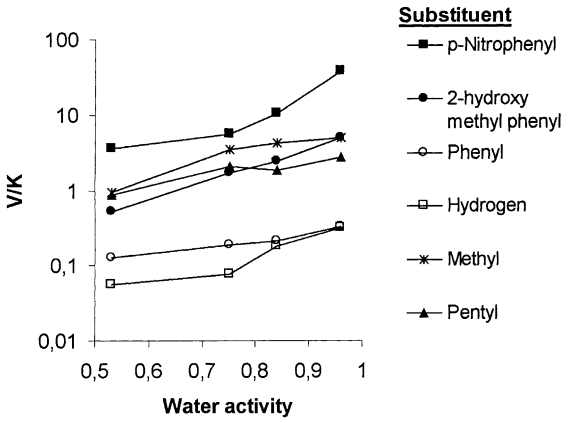

Fig. 5. Influence of water activity on log(*V*/*K*) for alkyl and aryl glucosides in hexanol at 40°C. The *V*/*K* was based on the total activity (hydrolysis and transglucosylation).

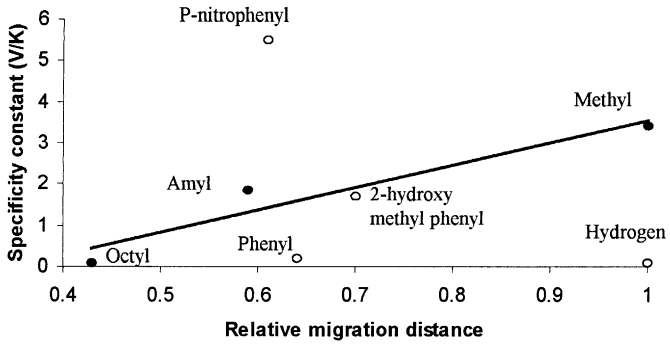

Fig. 6. The specificity constant (*V*/*K*) as a function of the polarity (defined as the relative migration distance on a reversed-phase TLC plate) of alkyl (filled circles) and aryl glucosides at a water activity of 0.75 and at 40°C.

V/*K*. A linear trend was obtained between the polarity (defined as the migration distance of the glucoside on a reversed-phase TLC plate) of the alkyl-glucosides (filled circles in Fig. 6) and the *V*/*K*, see Fig. 6. The most polar one, methyl-glucoside, revealed highest *V*/*K*. The leaving groups of 2-hydroxy methyl phenyl and *p*-nitrophenyl glycoside can form resonance stabilised molecules and thus they are very good leaving groups. It seems that this property adds up to the general trend, being that the most polar glycosides express the highest *V*/*K*. The solvation of the substrate in the reaction medium influences the K_m value and therefore the specificity constant. In order to compare the results obtained in hexanol with previously published data from aqueous solution the specificity constants were compensated for the solvation according to Halling [13] by multiplying the *V*/*K* with the partition coefficient (Table 3) of the alkyl-glucoside between hexanol and water, see Fig. 7. The *V*/*K* increased for the longer alkyl-glucosides similar to what has been reported by Dale et al. [6] for an aqueous medium

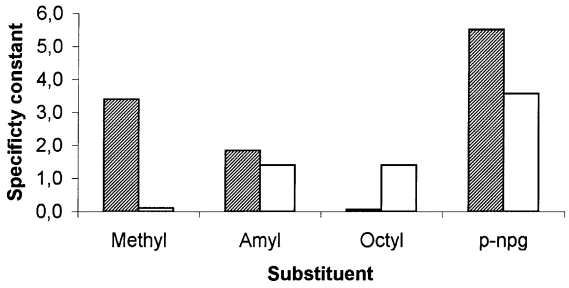

Fig. 7. The specificity constant (*V*/*K*) for three alkyl glucosides and *p*-nitrophenyl glucoside in hexanol at a water activity of 0.75 and at 40°C (filled bars) and after compensation for solvation by multiplying the *V*/*K* with the partition coefficient from Table 3 (open bars).

Table 3
Influence of water activity on the kinetic constants for different 1-substituted β-glucopyranosides

Substituent	K_p[a]	Water activity 0.53		Water activity 0.75	
		K_m (mM)	V_{max} (mU/mg)	K_m (mM)	V_{max} (mU/mg)
Hydrogen	0.01	7 ± 3	1.3 ± 0.8	9 ± 4	2.0 ± 0.6
Methyl	0.03	16 ± 5	16 ± 2	21 ± 9	108 ± 22
Amyl	0.76	7 ± 2	12 ± 1	9 ± 1	38 ± 2
Octyl[b]	23	12 ± 6	0.9 ± 0.2	47 ± 10	2.8 ± 0.4
Phenyl	0.34	2 ± 2	1.0 ± 0.2	5 ± 2	1.9 ± 0.4
p-Nitrophenyl	0.65	8 ± 6	73 ± 20	24 ± 7	186 ± 30
2-Hydroxy methyl phenyl	0.20	2 ± 2	3.6 ± 0.9	10 ± 3	29 ± 5

[a] Partition coefficients of the glucoside between the hexanol phase and the aqueous phase in a two-phase system.
[b] Based on the transglucosylation activity.

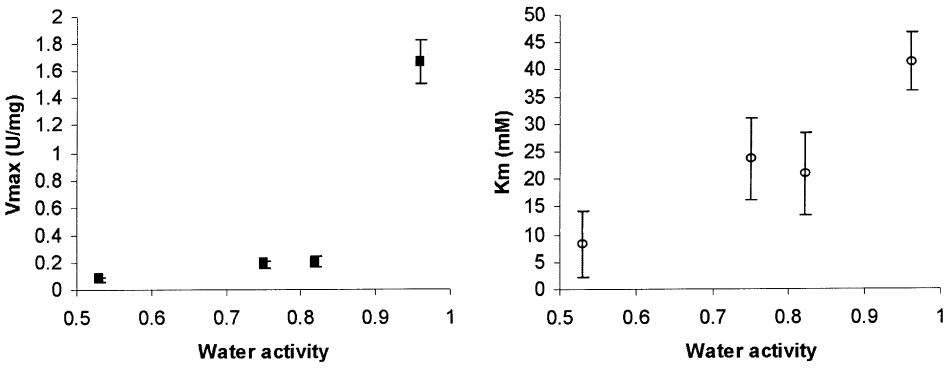

Fig. 8. The kinetic constants obtained for *p*-nitrophenyl glucoside in hexanol at 40°C and 200 rpm as a function of water activity. The calculation of the kinetic constants K_m (—○—) and V_{max} (—■—) was based on the total enzyme activity (i.e. both hydrolysis and transglucosylation).

in which the longer alkyl-glucosides were better substrates. This is thus a good example of a change in apparent substrate specificity when exchanging an aqueous medium for an organic one caused by solvation effects. The best substrate even after compensation for the solvation was *p*-nitrophenyl glucoside.

3.5. Water activity dependence of K_m and V_{max} values

The separate determination of K_m and V_{max} values was attempted using several different substrates at low water activities (0.53 and 0.75), see Table 3. Although, the uncertainty in the results is large especially for the K_m values, some significant trends were observed. The general trend for all glycosides was that the K_m was higher at a water activity of 0.75 than at 0.53. The increase in K_m was approximately 30% for all alkyl-glucosides whereas the K_m for the aryl-glucosides increased between 150 and 400%. The V_{max} was even more affected by the increase in water activity and increased 6–7× for methyl and 2-hydroxy methyl phenyl glucosides. The specificity constant V/K increased thus with increasing water activity for all glycosides tested. The substrates 2-hydroxy methyl phenyl glucoside and methyl glucoside were more influenced by the water activity than the others in Table 3. On the other hand, the kinetic constants obtained for glucose were less influenced by the water activity than the alkyl and aryl glucosides in Table 3.

The kinetics of the best substrate, *p*-nitrophenyl glucoside, were studied in a wide range of water activities. The apparent K_m increased with increasing water activity, which can be explained by a better solvation of the glucoside and the increase in V_{max} should be due to an increased hydration of the enzyme (Fig. 8). The K_m increased 5× upon increasing the water activity from 0.53 to 0.96 and the V_{max} increased 22×. The major part of the increase in V_{max} (92%) occurred between a_w 0.82 and 0.96.

4. Conclusions

High water content was favourable for the kinetics of almond-β-glucosidase catalysed hexyl-glycoside synthesis in hexanol. The kinetic constants K_m, V_{max} and V/K for the tested alkyl and aryl glycosides all increased with increasing water activity. The selectivity (*trans*-glycosylation/hydrolysis) also increased with increasing water activity. The thermodynamically controlled yield was however favoured by a low water activity. The use of activated substrates made it possible to reach high activity even at low water activities and *p*-nitrophenyl glucoside was found to be the best substrate in both hexanol and in water.

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