Transgalactosylation/Hydrolysis Ratios of Various β-Galactosidases Catalyzing Alkyl-β-Galactoside Synthesis in Single-Phased Alcohol Media

Irina Mladenoska*, Eleonora Winkelhausen and Slobodanka Kuzmanova

Ss. Cyril and Methodius University, Faculty of Technology and Metallurgy, Department of Biotechnology and Food Technology, Rudjer Boskovic 16, MK-1000 Skopje, Republic of Macedonia

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Summary

Three microbial galactosidases, Aspergillus oryzae, Escherichia coli and Kluyveromyces marxianus β-galactosidase, were used as catalysts for transgalactosylation synthesis of alkyl-β-galactosides in single-phased alcohol media. Their selectivity towards different alcohol nucleophiles was quantified by determining the transgalactosylation/hydrolysis ratio in the water/alcohol mixtures containing water in concentrations below the level of saturation. p-Nitrophenyl-β-galactoside was used as a glycosyl donor at a concentration of 10 mM. Both the total reaction rate (transgalactosylation+hydrolysis) and the ratio between the transgalactosylation (alcoholysis) and hydrolysis increased with the increase of water activity. Although the A. oryzae β-galactosidase showed relatively low total activity (3.13 μmol/(min·mg protein)), it exhibited the highest selectivity towards the hexanol nucleophile among the examined enzymes (0.65). The selectivity values in all the examined cases were below one, which implies that the hydrolysis, and not the synthesis, was the dominating reaction. The total reaction rate (transgalactosylation+hydrolysis) was strongly affected by the water activity, and for the specific water activity in the different alcohols, it increased in the following order: n-octanol<n-hexanol<n-butanol.

Key words: transgalactosylation/hydrolysis ratios, β-galactosidases, alkyl-β-galactoside synthesis, single-phased media, selectivity factors

Introduction

Alkyl-glycosides are non-ionic surfactants distinguished by unique properties that provide a wide range of possibilities for usage. Their application in food, chemical and pharmaceutical industry as surface active agents seems very promising, since they possess high surface activity, low toxicity and good biocompatibility. It is especially their biocompatibility that makes those emulsifiers so suitable for use in food industry, particularly in 'emulsified products' such as mayonnaise and margarine. Alkyl-glycosides are also known to have good dermatological properties, which opens a range of possibilities for their application in pharmaceutical industry for personal care products.

Enzymatic production of alkyl-glycosides, no doubt, offers certain advantages over the chemical synthesis. Mild reaction conditions and possibilities for selective production of specific compounds are among them (1). Enzymes employed in alkyl-glycoside production are glycosidases. This group of enzymes belongs to hydrolases (EC 3.2.1), enzymes that normally catalyze the hydrolysis of glycosidic bonds. However, in media with low water content, these enzymes catalyze reactions that are not hydrolytic, but are either reversed hydrolysis or...
transglycosylation. Both types of reactions are widely used for enzymatic synthesis of alkyl-glycosides (2-5). Nevertheless, the transglycosylation is preferred among the researchers because of the possibilities that this process offers (6–8). The transglycosylation reaction is kinetically controlled, and as a consequence it becomes possible to overshoot the equilibrium conversion of the reactant into product, which is not possible when using the reverse hydrolysis process.

Biocatalysis in non-conventional media has been performed in both two-phased and single-phased reaction systems, although in the last decade, the number of publications covering bioorganic synthesis in single-phased reaction media has increased more rapidly than of those dealing with biocatalysis in two-phased reaction systems (9–11). In the research area dealing with the application of enzymes in organic media, a lot of articles concerning lipase application in reversed hydrolysis and transesterification processes have been reported so far (12–15). However, reports about the application of glycosidases in bioorganic synthesis reactions are less numerous, mainly due to the lack of knowledge how to use those enzymes in organic media.

One of the most valuable characteristics that glycosidases possess when being applied in non-aqueous media is that they become capable of producing selective compounds with very specific properties. Alkyl-β-glycosides also belong to this group of compounds possessing unique properties that arise from the intrinsic selectivity of the enzymes. In order to quantify the intrinsic enzyme selectivity, the introduction of the selectivity factors (S and S5) as a function of the transglycosylation/hydrolysis ratio has been proposed (16,17). There are very few studies concerning the effect of water activity on the selectivity of glycosidases (17,18). In this work, the selectivity of three glycosidases towards different alcohol nucleophiles that serve as aglycon donors for the alkyl glycoside production is investigated.

Materials and Methods

Materials

Three types of β-galactosidase were used, one from a fungal source (Aspergillus oryzae), one of the yeast origin (Klyveromyces marxianus) and the third enzyme was from a bacterial source, an Escherichia coli β-galactosidase. All of them were purchased from Sigma Chemicals (St. Louis, USA). The organic solvents, butanol, hexanol and octanol were received from Sigma Chemicals (St. Louis, USA), while acetonitrile was a Merck (Darmstadt, Germany) product. Other chemicals, such as sugar substrates, p-nitrophenyl-β-D-galactoside and galactose, as well as hexyl-β-D-glucoside and molecular sieves (UOP Type 3A) for drying solvents (diameter 0.3 nm) were also purchased from Sigma Chemicals (St. Louis, USA).

Methods

Enzymatic reactions

p-Nitrophenyl-β-D-galactopyranoside was dissolved in butanol, hexanol or octanol (dried with 0.3-nm molecular sieves) to a concentration of 10 mM. Different volumes (40–280 μL) of aqueous buffer containing the desired amount of enzyme were added. The buffers used were: 50 mM citrate buffer (pH=5.0) for A. oryzae β-galactosidase, or 50 mM phosphate buffer (pH=7.0) containing 1.0 mM MgCl₂ for E. coli and K. marxianus β-galactosidases. The final reaction volume was 2 mL. The reactions were carried out in closed glass vials on an orbital shaker at 50 °C with vigorous shaking. Water content of the reaction medium was measured by Karl-Fisher titration method. Corresponding water activity (a_w) was calculated by using UNIFAC Activity Coefficient Calculator 3.0 (17).

Analytical methods

Two methods for analysis of the substrate and the product content in the reaction medium were applied, the HPLC and the spectrophotometric method. The HPLC system used was equipped with Knauer K-1001 pump and RID differential refractive index detector Shodex RI-71. The column used was LiChrospher 100 RP-18 (4×250 mm, 5 μm) from Merck, Germany. The analyses proceeded under the following conditions: temperature 25 °C, flow rate of 1 mL/min and the composition of the mobile phase of acetonitrile/water 25:75. A spectrophotometric method for measuring the total enzyme activity (hydrolytic+transglycosylation) was used (17). The quantity of depleted p-nitrophenyl-β-D-galactoside was calculated from the amount of liberated p-nitrophenol. The reaction was followed by measuring the increase of absorbance at 405 nm. The spectrophotometric system used was Varian Cary 50 Scan UV-Visible. The molar absorption coefficient for p-nitrophenol at pH=7.0 and t=50 °C was ε₄₀₅=9.022 mM⁻¹.cm⁻¹.

Determination of the kinetic parameters

The kinetic parameters K_m and v_max and the specificity constant v_max/K_m for the glycosidases used were derived from the initial reaction rates (μmol/(min·mg)) of the total enzyme activity as a sum of the hydrolysis and the transgalactosylation. The reaction media were supplemented with the substrate, p-nitrophenyl-β-D-galactoside in a concentration that varied in the range of 10–100 mM. The water activity of the reaction mixture had a value of a_w=0.92. The K_m and v_max constants were calculated using non-linear regression in GraphPad Prism 5 (GraphPad Software, Inc., San Diego, USA).

Protein concentration assay

The concentration of protein was measured by the method of Lowry using BSA (bovine serum albumin) as a standard (19).

Results and Discussion

The conversion of p-nitrophenyl-β-D-galactoside into alkyl-β-D-galactoside (transgalactosylation) and galactose (hydrolysis) proceeds via a two-step reaction (Fig. 1). In the first step, the enzyme cleaves the p-nitrophenyl-β-D-galactoside, followed by liberation of the p-nitrophenol, and forms the galactosyl-enzyme complex. In the degalactosylation step galactose is formed as a result of hydrolysis and corresponding alkyl-β-galactoside as a pro-
duct of transgalactosylation. The yield, in the kinetically controlled period of the reaction, depends on the ratio of synthesis ($r_s$) and the hydrolysis ($r_H$). The interaction of the galactosidases with the donor substrate $p$-nitrophenyl-$\beta$-galactoside was measured as a total initial enzyme activity, which is a sum of the transgalactosylation and hydrolytic activity.

The influence of water activity in hexanol on the total enzymatic activity (sum of hydrolysis and transgalactosylation) of the three different microbial galactosidases was investigated in the range of $a_w=0.6–0.92$ (Fig. 2). The galactosidases used had different properties and belonged to different families. Thus, the A. oryzae galactosidase belonged to the glycosyl hydrolase family 1, while the K. marxianus and the E. coli galactosidases belonged to family 2.

It was observed that the total initial activity increased with the increase of water activity. Thus, for the water activity of $a_w=0.92$, E. coli $\beta$-galactosidase showed the highest specific activity of 7.0 $\mu$mol/(min-mg), while the activities of K. marxianus and A. oryzae $\beta$-galactosidase differed in orders of magnitude of 0.30 and 3.13 $\mu$mol/(min-mg).

Since the transgalactosylation reaction is known to be kinetically controlled, it was interesting to determine the basic kinetic parameters $K_m$, $v_{\text{max}}$, and $v_{\text{max}}/K_m$ for the three glycosidases catalyzing the conversion of the substrate $p$-nitrophenyl-$\beta$-galactoside, and later on, to determine the competition between the transgalactosylation and the hydrolysis.

Carrying out the transgalactosylation reaction by varying the concentration of the substrate in the range between 10 and 100 mM, it has been shown that the reaction rate followed the Michaelis-Menten kinetics. The apparent kinetic constants are given in Table 1. The differences in the specific activities of the three enzymes depicted in Fig. 2 are also reflected here through the values for the maximum velocity ($v_{\text{max}}$). The E. coli $\beta$-galactosidase, having the highest specificity constant $v_{\text{max}}/K_m$ of 3.5 and $v_{\text{max}}$ value of 21.5 $\mu$mol/(min-mg), possessed the highest affinity towards $p$-nitrophenyl-$\beta$-galactoside as a substrate. These results correspond well with the data obtained for the total enzyme activity from which it is obvious that the E. coli $\beta$-galactosidase besides having the highest values for the kinetic constants also has the highest activity among the three enzymes examined. It should be noted that for the rest of the experiments, the substrate concentration of 10 mM was used.

That a correlation between the total enzyme activity and the apparent kinetic constants indeed exists has been reported earlier. In a study of glycosidase-catalyzed alcoholysis of pentyl-$\beta$-glucoside, the highly thermostable *Pyrococcus furiosus* $\beta$-glucosidase, characterized with the most pronounced total activity, had the highest values for the apparent kinetic constants (17).

The transgalactosylation/hydrolysis ratio is very important in characterizing the enzyme ability for alkyl-

![Fig. 1. Conversion of p-nitrophenyl-β-galactoside into transgalactosylation and hydrolysis products](image-url)

![Fig. 2. The effect of $a_w$ on the total activity of β-galactosidases from A. oryzae, E. coli, and K. marxianus](image-url)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Kinetic parameters</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_m$/mM</td>
</tr>
<tr>
<td>E. coli $\beta$-galactosidase</td>
<td>6.2</td>
</tr>
<tr>
<td>A. oryzae $\beta$-galactosidase</td>
<td>22.8</td>
</tr>
<tr>
<td>K. marxianus $\beta$-galactosidase</td>
<td>12.4</td>
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-β-glycoside formation. It strongly depends on the intrinsic selectivity of the enzyme. It also depends on the ratio of concentrations of alcohol and water. For describing the intrinsic selectivity of the enzymes in the transgalactosylation reaction, selectivity factor ($S_C$) was used:

$$\frac{r_S}{r_H} = S_C \left[ \frac{[\text{alcohol}]}{[\text{water}]} \right]$$

where $r_S$ and $r_H$ are the rates of transgalactosylation and hydrolysis.

As can be seen from Fig. 3, the ratio of synthesis towards hydrolysis $r_S/r_H$ depended on the water activity of the reaction medium. This dependence was more pronounced for the $A.\ oryzae$ β-galactosidase, reaching the maximal value of 0.65 for the water activity $a_w=0.92$. It seems that both the total enzyme activity and the ratio $r_S/r_H$ increase with the increase of the water activity.

![Fig. 3. The ratio of $r_S/r_H$ as a function of water activity in the transgalactosylation reaction catalyzed by β-galactosidase from Aspergillus oryzae (■), E. coli (▲), and K. marxianus (O), using p-nitrophenyl-β-galactoside as a substrate](image)

These results suggest that the enzyme having highest affinity towards the glycosyl donor does not necessarily have the highest transgalactosylation/hydrolysis ratio. Thus, although the $E.\ coli$ galactosidase showed the highest values for the apparent kinetic constants, the $A.\ oryzae$ was the enzyme that expressed the highest $r_S/r_H$ ratio and consequently, the highest selectivity towards the alcohol nucleophile. These results are very similar to the data obtained by Hansson et al. (17), where the highly thermostable β-glucosidase from Pyrococcus furiosus expressed the highest affinity towards the pentyl-β-glucoside, but the Sulfolobus solfataricus β-galactosidase was the enzyme characterized by the highest $r_S/r_H$ ratio and showed the highest selectivity towards the hexanol nucleophile.

From the results shown in Fig. 4, it is obvious that the higher water activities favour alcohols as nucleophiles. If this competition between the two reactions depended only on the nucleophile concentration, the transgalactosylation/hydrolysis ratio would decrease with increased water activity. Yet, it seems that other mechanisms stand behind this phenomenon. It can be suggested that as a result of increased enzyme flexibility at higher water activities, the enzyme becomes more open to the larger alcohol molecule. In other words, the enzyme becomes more selective when more water is added (Fig. 4). However, this behaviour holds if the system is single-phased. If the water content in the system is high enough to make a two-phased system, the total activity of the enzyme increases, but the ratio of transgalactosylation/hydrolysis sharply decreases. It should be stressed that the same behaviour of increased activity at high water activities show galactosidases that belong to both glycosidase families, 1 and 2, which have some similarity in the active site structure (20). It seems that the enzyme selectivity is closely related to its flexibility and that it increases at higher water contents reaching a maximum value for the water activity close to 1. At lower water contents the enzyme is rigid and it rather makes complexes with a smaller water molecule than with the larger alcohol molecule.

Glycosydases, unlike lipases, are known as enzymes that require high amounts of water for their catalytic activity (21). Ljunger et al. (2) used the almond β-glucosidase for octyl-β-glucose synthesis and discovered that the minimum water activity at which the enzyme showed any kind of activity (synthetic or hydrolytic) was $a_w=0.6$. They also found out that the enzyme activity sharply increased when the water activity increased from 0.6 to close to 1. Ducret et al. (22) noticed that glycosidases are very sensitive to dehydration and that they have pronounced activity only at high water activities. Having the primary idea to increase the enzyme activity at low water activities by using thermostable enzymes, the authors concluded that the behaviour of the glycosidases remained unchanged no matter how high reaction temperatures were used.

The selectivity factors of several glycosidases from both mesophilic and hyperthermophilic microorganisms, as well as of one plant glycosidase, measured in hexanol/water reaction mixture, are presented in Table 2. Both the selectivity factors and the ratios of transgalactosylation/hydrolysis ($r_S/r_H$) exhibited by the enzymes examined in this work had values below 1. This practi-


Table 2. The \( r_{\text{H}} \) and the \( S_c \) values of the different \( \beta \)-glycosidases towards hexanol as an alcohol nucleophile at \( a_w=0.92 \)

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<td><em>A. oryzae</em> ( \beta )-galactosidase</td>
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<td><em>P. furius</em> ( \beta )-glucosidase*</td>
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Conclusions

Water activity is a factor of crucial importance for the competition between the transgalactosylation and the hydrolysis. Both the total enzyme activity and the selectivity increased with the increase of the water activity, reaching highest values at water activities close to 1. In order to avoid the formation of two-phased system and thereby lowering the \( r_{\text{H}}/r_{\text{H}} \) ratio, the water content should be kept below the level of saturation. The selectivity factors of the three glycosidases in all examined cases were below 1, which means that the hydrolysis, and not the synthesis, was the predominant reaction. If the aim is to obtain high yields of alkyl-\( \beta \)-galactoside, the reaction should be carried out in short-chained alcohol media at high water activity. This work is another contribution to the studies that confirm general behaviour of the glycosidases belonging to families 1 and 2. A search for glycosidases with higher affinity towards alcohol nucleophile, which would be active at lower \( a_w \) values at which the hydrolysis would be less pronounced, could be an objective of our future research.

Acknowledgement

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References


