ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 21 (2003) 31–34

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Enzyme inactivation due to Maillard reactions during oligosaccharide synthesis by a hyperthermophilic glycosidase: influence of enzyme immobilisation

Marieke E. Bruins*, Antoine J.H. Thewessen, Anja E.M. Janssen, Remko M. Boom

Department of Food Technology and Nutritional Sciences, Division of Food and Bioprocess Engineering, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Abstract

The extremely thermostable β-glycosidase from *Pyrococcus furiosus* was used for the production of oligosaccharides with lactose as a substrate. Using a thermozyme made it possible to operate at higher reaction temperatures, and thus to increase the substrate concentration. This increased the substrate concentration and the subsequent lower water concentration suppressed hydrolysis and therefore improved the oligosaccharide yield.

During the reaction, brown pigments were formed, caused by Maillard reactions. This changes the structure of the enzyme and causes faster inactivation of the enzyme, compared to normal inactivation by temperature.

This faster inactivation is the main design criterion for the reaction system. Reduction of Maillard reactions can be done by altering the process conditions or through modification of the enzyme, either chemically or by altering the enzyme structure through genetic modifications.

In this work, chemical modification of the enzyme was chosen by covalent immobilisation on Eupergit. Unfortunately, the immobilisation did not reduce Maillard reactivity.

Keywords: Maillard; Enzyme; Inactivation; Oligosaccharides; Glycosidase; *Pyrococcus furiosus*; Thermophilic; Immobilisation

1. Thermostable enzymes

Recent developments have shown that thermophiles are a good source of novel catalysts that can be of great industrial interest. These “thermozymes” not only show high thermostability, but also improved chemical, solvent and pH stability. They can be used in several industrial processes where they replace mesophilic enzymes or chemicals.

The application of thermozymes is often found when the enzymatic process is compatible with existing (high temperature) process conditions. Further important advantages of performing processes at higher temperatures are the reduced risk of contamination, lower viscosity, improved transfer rates and improved solubility of the substrates. However, co-factors, substrates or products might be unstable or other side reactions may occur; therefore, the application will only be feasible in selected processes [1].

2. β-Glycosidase from *Pyrococcus furiosus*

The extremely thermostable β-glycosidase from *P. furiosus* was used for the production of oligosaccharides with lactose as a substrate. By using a

* Corresponding author.
E-mail address: marieke.bruins@algemeen.pk.wan.nl (M.E. Bruins).

1381-1177/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
PII: S1381-1177(02)00131-5

thermozyme, we were able to increase the substrate concentration. This increased substrate concentration and the subsequent lower water concentration suppressed hydrolysis and therefore improved the oligosaccharide yield.

In our experiments, tri- and tetra- and pentasaccharides were formed with lactose as a substrate. These oligosaccharides have future use as prebiotics in functional foods. Our experiments, furthermore, showed that higher temperatures are beneficial for oligosaccharide production and that the absolute and relative yield of oligosaccharides increases with temperature. It was also found, however, that at high temperatures enzyme inactivation was much faster under production conditions than in the absence of sugars [2].

3. Maillard reactions

During enzymatic oligosaccharide production at higher temperatures, brown pigments are formed due to Maillard reactions. The Maillard reaction is a non-enzymatic, chemical condensation reaction between amino acids and carbohydrates. In an enzyme–sugar mixture, this means that, e.g. ε-amino acids from lysine in the enzymes react with the reducing end of the sugar, changing the structure of the enzyme. The total number of lysines in our enzyme is 34 out of a total of 472 amino acids [3], which makes is susceptible to this type of modification. They appear on the outside of the enzyme and not in the active centre. The change in primary enzyme structure causes increased inactivation of the enzyme, relative to normal inactivation by temperature.

We studied inactivation kinetics of the enzyme with and without sugars. In applied reaction mixtures containing sugars, such as lactose, the occurrence of Maillard reactions was followed by measuring the development of the brown colour at 420 and 470 nm. The occurrence of the Maillard reactions between the sugar and the enzyme was related to the increased enzyme inactivation at higher temperature [2].

4. Possible solutions

It has become clear that for enzymatic reactions at elevated temperatures for the conversion of small sugars, it is important to solve the problems caused by Maillard reactions. Reduction of the extent of Maillard inactivation can be achieved by changing the process conditions or by modification of the enzyme.

Process conditions that are of importance are the type and the concentration of reducing sugars, the pH, temperature, oxygen concentration and water activity. Lowering the water activity below 0.6 will decrease Maillard reactions. This might not be possible in solely aqueous solutions, but the use of hydrophilic solvents, or ionic liquids could stabilise the enzyme against Maillard mediated inactivation. A low pH suppresses the Maillard reactions. In our experiments the pH was set at 5.0 or 5.5. Unfortunately, at lower pH the enzyme become much less active. Oxygen is one of the substrates at the end of the Maillard reaction, so lowering the concentration of dissolved oxygen can have a positive influence on enzyme stability.

It is further possible to modify the enzyme, either by chemical modification, or by altering the enzyme structure by protein engineering. Specific replacement of lysines, e.g. with arginines removes the aminogroups that are available for the Maillard reaction, and thus will suppress the extend of Maillard reactions. These aminogroups can however also be protected by chemical modification, e.g. by coupling to polyethylene glycol, by chemical crosslinking or by covalent immobilisation. For all these routes, it is however not clear whether this will influence the activity of the enzyme significantly.

5. Immobilisation

We immobilised the β-glycosidase on Eupergit, using the method according to Fischer et al. [4]. The enzyme loading after immobilisation was measured to be 15 U/(g Eupergit). The kinetics of the immobilised enzyme was compared to that of the free enzyme. Furthermore, the immobilised enzyme was re-used in three successive batches to test the enzyme stability.

Comparison of the kinetics of the immobilised enzyme to that of the free enzyme was done at 60 and 80 °C. The initial conversion and formation rates are given in Table 1. For the reactions at 80 °C, the sugar concentrations in time are shown in Fig. 1. Lactose was hydrolysed into glucose and galactose and oligosaccharides were synthesised in a kinetically controlled

Table 1
Conversion of lactose with β-glucosidase from *Pyrococcus furiosus* as a free enzyme and immobilised at 60 and 80 °C[a]

	60 °C free enzyme	60 °C immobilised	80 °C free enzyme	80 °C immobilised
V_{ini} lactose	0.25	0.30	0.84	0.90
V_{ini} glucose	0.20	0.24	0.61	0.67
V_{ini} oligo	0.07	0.08	0.32	0.24
V_{ini} galactose	0.09	0.12	0.09	0.36
Synthesis/hydrolysis[b]	0.8	0.7	3.6	0.7
Synthesis/hydrolysis[c]	0.6	0.5	1.0	0.5

[a] 0.66 U/(g solution) (pNPG test). The immobilised or free enzyme was incubated with 0.5 mol/kg lactose in a 0.2 mol/kg citrate buffer at pH 5.0. Initial conversion rates are given in mmol/(kg min).
[b] Ratio of initial galactose and oligosaccharide formation.
[c] Ratio of galactose and oligosaccharide concentration after 1500 min reaction.

reaction. The oligosaccharides consisted mainly of trisaccharides but also tetra- and pentasaccharides were formed in later stages of the reaction. Although the amount of active enzyme was equal, according to *p*-nitrophenyl-β-D-glucopyranoside (pNPG) testing [5], differences in initial conversion rates for the substrate lactose were found. At both 60 and 80 °C, the conversion rates were higher for the immobilised enzyme. This may be attributed to different diffusion rates for pNPG and lactose and their products, which leads to different concentrations of the products at the surface, and thus to different conversion rates. The absolute and relative increase in oligosaccharide production with temperature is consistent with previous results [2].

Immobilisation, however, has a negative effect on oligosaccharide formation. This can be caused by diffusion limitations caused by molecular size differences in the small unwanted by-products (monosaccharides) and the bigger oligosaccharides. At 80 °C, it even cancels out the positive effect of temperature upon the synthesis–hydrolysis ratio.

Immobilisation may protect the enzyme from Maillard reactions. The brown end-products of this reaction can be measured optically at 470 nm. At 80 °C, browning of the reaction mixture occurred and was measured in time for both the free and immobilised enzyme. Similar values of browning were found for browning of both reaction mixtures. After prolonged incubation, more browning was found for the immobilised enzyme than for the free enzyme. To test the enzyme stability, the immobilised enzyme was re-used in three successive batches. These results are shown in Fig. 2. The enzyme was incubated with 0.5 mol/kg lactose in a 0.2 mol/kg citrate buffer at pH 5.0 and 60 °C. Conversion of lactose was followed for 24 h

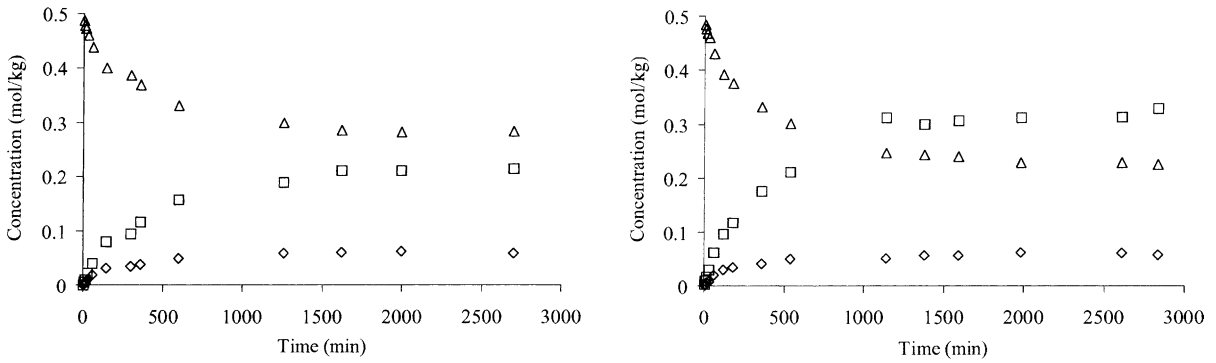

Fig. 1. Conversion of lactose with β-glucosidase from *Pyrococcus furiosus* as a free enzyme (left graph) and immobilised (right graph). For both the immobilised and the free enzyme 0.66 U/(g solution) was incubated with 0.5 mol/kg lactose in a 0.2 mol/kg citrate buffer at pH 5.0 and 80 °C; ((△) lactose; (□) monosaccharides; (◇) oligosaccharides).

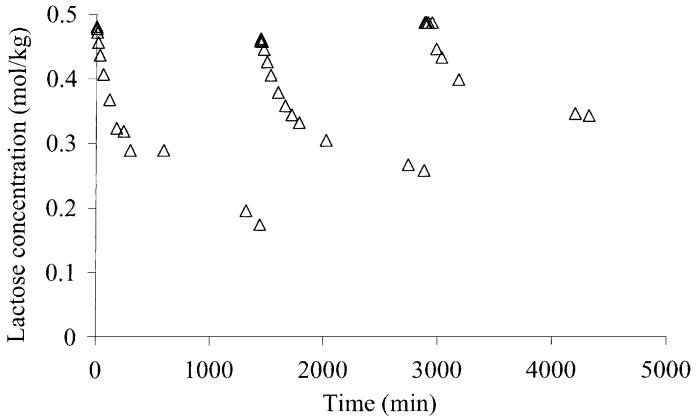

Fig. 2. Three successive batches with immobilised enzyme from *Pyrococcus furiosus* at 60 °C, pH 5.0, with 0.5 mol/kg lactose as a substrate.

after which the immobilised enzyme was removed from the reaction mixture and put into a fresh lactose solution. This was repeated twice. The initial rate of lactose conversion decreased from 1.31 mmol/(kg mm) in the first batch to 0.59 mmol/(kg min) in the second and to 0.37 mmol/(kg min) in the third batch. This decrease in enzyme activity is considerable, since the free enzyme only shows a 10% decrease in activity after 5 days of use at 60 °C.

6. Conclusions

Immobilisation of the thermostable β-glycosidase from *P. furiosus* was successfully achieved on Eupergit. Unfortunately, the immobilised enzyme remained at least as sensitive to Maillard inactivation. Further, the inactivated enzyme exhibited a reduced synthesis/hydrolysis ratio, which indicates that transfer limitation of the substrates and products plays a role in such a system.

The results indicate that a reduction of the sensitivity to Maillard reactions has to be found in adaptation of process circumstances, or in the biochemical engineering of the enzyme.

Acknowledgements

This research is supported by the Dutch Technology Foundation STW, the applied science division of the Dutch Organisation for Scientific Research, NWO, and the technology programme of the Ministry of Economic Affairs.

References

[1] M.E. Bruins, A.E.M. Janssen, R.M. Boom, Appl. Biochem. Biotechnol. 90 (2001) 155–186.
[2] M.E. Bruins, E.W. van Hellemond, A.E.M. Janssen, R.M. Boom, Biotechnol. Bioeng. (in press).
[3] M.W. Bauer, B.J. Bylina, R.V. Swanson, R.M. Kelly, J. Biol. Chem. 271 (1996) 23749–23755.
[4] L. Fischer, R. Bromann, S.W.M. Kengen, W.M. De Vos, F. Wagner, Biotechnology 14 (1996) 88–91.
[5] S.W.M. Kengen, E.J. Luesink, A.J.M. Stams, A.J.B. Zehnder, Eur. J. Biochem. 213 (1993) 305–312.