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Short communication

Lyophilization conditions for the storage of monooxygenases

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ABSTRACT

Cyclohexanone monooxygenase (CHMO) was used as a model enzyme to find suitable freeze-drying conditions for long-term storage of an isolated monooxygenase. CHMO is a Baeyer–Villiger monooxygenase (BVMO) known for its ability to catalyze a large number of oxidation reactions. With a focus on establishing the optimal formulation, additives were tested for enzyme stabilization during and after lyophilization. The results were successfully transferred to two other monooxygenases, namely the BVMO cyclopentadecanone monooxygenase (CPDMO) and a cytochrome P450 monooxygenase, P450 BM3. In the absence of a lyoprotectant, lyophilized P450 BM3 is almost completely inactivated, while the lyophilized BVMOs quickly lost activity when stored at 50 °C. Lyophilization in the presence of 2% (w/v) sucrose was found to be the best formulation to preserve activity and protect against inactivation when stored as lyophilizate at 50 °C.

1. Introduction

Monooxygenases often possess high regio- and stereoselectivity and are used for the production of high-value chemicals such as active pharmaceutical ingredients or metabolic intermediates (Torres Pazmino et al., 2010). Two monooxygenase classes are of special commercial interest: cytochrome P450 monooxygenases (P450s) and Baeyer–Villiger monooxygenases (BVMOs). In general both classes exhibit suboptimal stability and could benefit from optimized storage conditions.

BVMOs catalyze the conversion of ketones into esters and typically exhibit high chemo-, regio- and enantioselectivities (Kadow et al., 2012; Leisch et al., 2011). An industrially relevant example is the Baeyer–Villiger oxidation of 2-butanone to produce the polymer precursor methyl propanoate (Eastham et al., 2013). Besides Baeyer–Villiger oxidations, these enzymes are also able to catalyze the enantioselective oxidation of sulfides, used for the production of pharmaceuticals (Bong et al., 2013; Riva et al., 2007). Two BVMOs were chosen for this study. The first is the prototypical and rather labile (van Beek et al., 2014) cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB9871 (Chen et al., 1988; Ryerson et al., 1982). The second is cyclopentadecanone monooxygenase (CPDMO) from *Pseudomonas* sp. strain HI-70 which complements the biocatalytic potential of CHMO and which is highly expressed as recombinant protein (Beneventi et al., 2009; Fink et al., 2011; Iwaki et al., 2006) but shares only 26% sequence identity with CHMO.

P450s are known for their role in human steroid metabolism and xenobiotic detoxification (Munro et al., 2013) and are a prime target for drug development (Guengerich, 2002). P450s are also considered interesting biocatalysts because the scope of catalyzed reactions is broad (Mansuy, 1998; Munro et al., 2013). Here we focus on the model enzyme P450 BM3 (CYP102A1) from *Bacillus megaterium*, which catalyzes the hydroxylation of fatty acids and has been a prime candidate for enzyme engineering studies (Boddupalli et al., 1990; Munro et al., 2002).

Both storage stability (before use) and operational stability (during use) are highly relevant for biotechnological applications (Polizzi et al., 2007). For the storage of proteins, lyophilization is an attractive approach. Lyophilization also facilitates the use of enzymes in non-aqueous media (Serdakowski and Dordick, 2008). Optimal conditions for the lyophilization of several P450s have been investigated (Bauer et al., 1994; Blanquet et al., 2005; Chefson et al., 2007; Zhao and Auclair, 2009). For BVMOs research has focused on engineering thermostable BVMOs which would display a better operational stability (Opperman and Reetz, 2010; van Beek et al., 2012). In addition, several naturally stable BVMOs have been discovered (Beneventi et al., 2013; Fraaije et al., 2005). In the case of stable enzymes, lyophilization remains of interest because it simplifies shipping and storage. While lyophilization has been used in the past for BVMOs (Leipold et al., 2012, 2013), optimization of the lyophilization conditions has not been explored.

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Denaturation of an enzyme can occur by heating, freezing, agitation or by chemical degradation in an aqueous formulation. Lyophilization can protect against some of these effects (Carpenter et al., 1997). Water-mediated processes such as hydrolysis are limited by the removal of water. Drying an enzyme also results in the formation of an amorphous phase. Below the glass transition temperature (T_g) of this amorphous state the rates of diffusion-controlled reactions such as unfolding and aggregation are greatly reduced. Additives can prevent aggregation during the lyophilization process or rehydration and can compensate for the loss of essential waters during lyophilization (Allison et al., 1999). Sugars such as sucrose and trehalose are widely used as lyoprotectants during freeze-drying (Souillac et al., 2002; Wang et al., 2009). We focused on sucrose because it has the same protective effects as trehalose in many cases (Allison et al., 1999; Chefson et al., 2007), while being significantly cheaper. For some enzyme applications the use of sugars might not be preferred, and alternative additives are required. Therefore various other compounds were also tested that could have beneficial effects: salts, reducing compounds and amino acids.

The initial optimization of sucrose concentrations and the testing of other additives were done with the prototype BVMO, CHMO. Conditions that were found to be stabilizing were transferred to CPDMO and P450 BM3 to test the general applicability of the additives for lyophilizing monooxygenases. The addition of sucrose was shown to be extremely beneficial for all three tested enzymes. Additionally, magnesium sulfate and serine were discovered to be stabilizing, which might be useful in specific applications.

To determine the stabilizing effect of different concentrations of additives, we chose to store the enzyme for a short time at an elevated temperature to amplify differences in stability caused by these additives. CHMO loses more than half of its activity upon lyophilization without any additives, and loses most of its residual activity within a few days when stored at 50 °C (Fig. S1). These initial results showed that there was room for improvement by adding lyoprotectants. The addition of sucrose had an enormous effect on the stability of lyophilized CHMO. Around 10–50 mg/mL sucrose was found to optimally preserve activity (Fig. 1). These concentrations were used in combination with 2.5 mg/mL enzyme. This indicates that the protein is embedded in a sugar-matrix, and the protecting effect is probably caused by the formation of an amorphous bulk-phase. While low sucrose concentrations

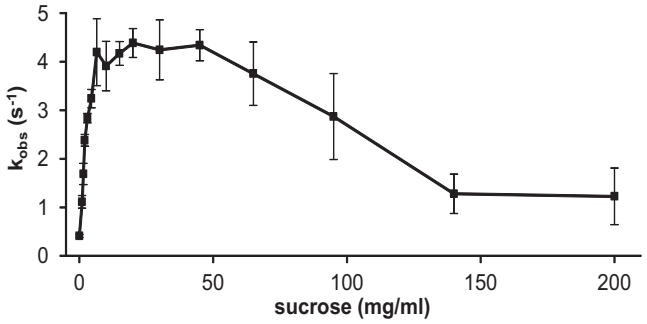

Fig. 1. Observed rates of CHMO after lyophilization, incubation at 50 °C for 72 h and reconstitution of the enzyme. Standard errors shown are from measurements of six lyophilized samples.

were not effective in stabilization, also high concentrations of sucrose (>50 mg/mL) decreased the stability of the lyophilized monooxygenase. Previously two P450s were stabilized by using high concentrations of sucrose (Chefson et al., 2007). Yet, also in this case lower activity recoveries at the highest applied concentrations of sucrose were observed. Pure amorphous sucrose has a T_g that is lower than that of the protein. At higher sucrose concentrations the T_g might be lowered below the 50 °C storage temperature, leading to inactivation. The increased stability caused by the addition of only a small amount of sucrose might result from the replacement of hydrogen bonds normally formed with water (Allison et al., 1999).

To facilitate the use of enzymes in biocatalytic applications, other classes of compounds were tested which might interfere less with specific assays or product purifications. Compounds were tested that could be already present in a protein preparation or are required for downstream applications. An obvious candidate is ammonium sulfate because this salt is extensively used to precipitate proteins. Other ammonium and sulfate salts were also tested (Fig. 2 and Figs. S3 and S4) to find out whether the anion or the cation would be responsible for the stabilization. Ammonium sulfate itself did not stabilize the enzyme upon lyophilization. However, a stabilizing effect of magnesium sulfate was discovered: 25 mM $MgSO_4$ protected the lyophilized enzyme equally well when compared with 20 mg/mL sucrose (Fig. 2). In contrast, magnesium

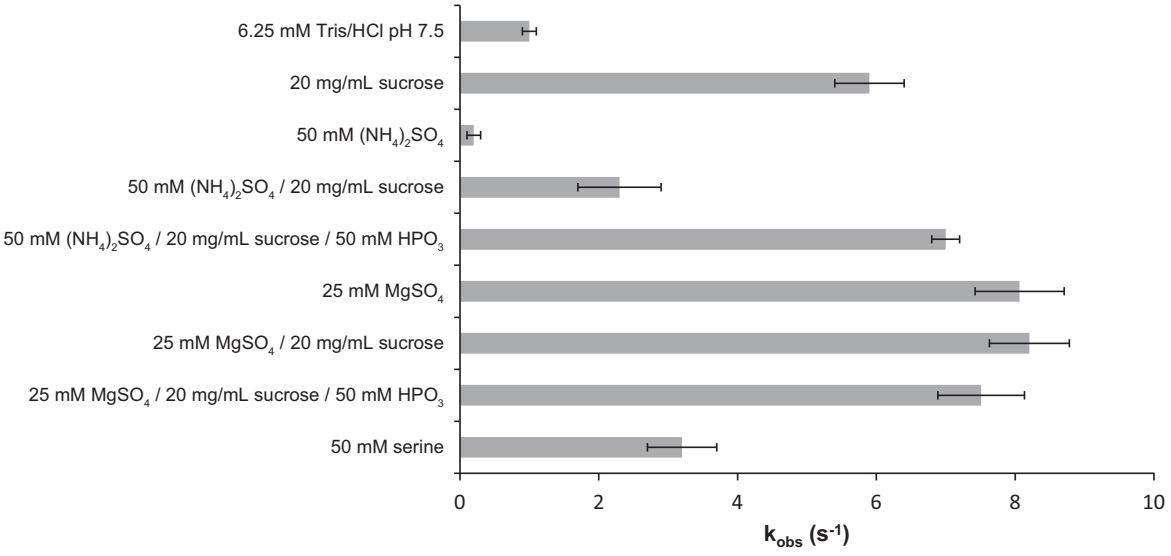

Fig. 2. Observed rates of CHMO containing various additives after lyophilization and 48 h incubation at 50 °C.

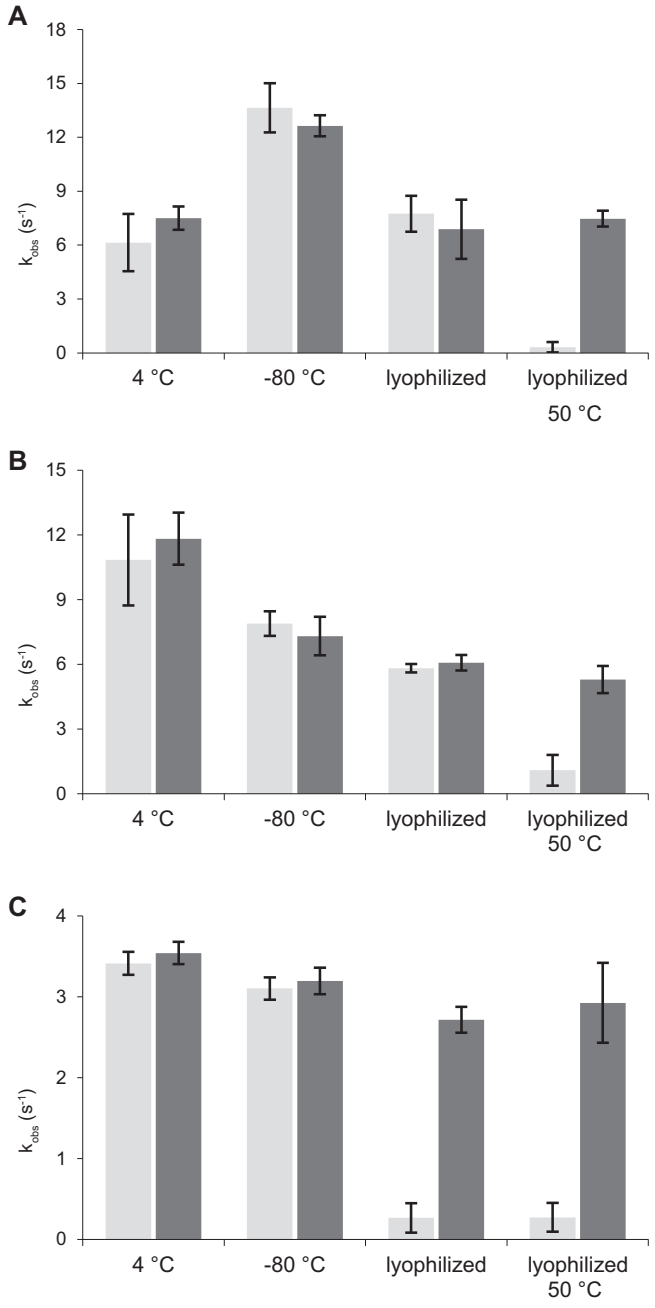

Fig. 3. Observed rates of CHMO (A), CPDMO (B) and P450 BM3 (C) after incubation for 72 h as lyophilized samples at different conditions. Lyophilized samples were stored at room temperature or at 50 °C. Activities in the absence of sucrose as lyoprotectant in light gray, activities in the presence of 20 mg/mL sucrose in dark gray. Standard errors are determined from three lyophilization samples.

chloride completely destabilizes the enzyme when used as the only additive. However, it does have a beneficial effect when adding it in combination with sucrose. This might be caused by the ability of the salt to form salt bridges with the enzyme which are not formed when only sucrose is present. When sucrose and stabilizing salts were present, phosphite could be added to the enzyme mix without significantly affecting the activity (Fig. 2). Phosphite was tested because of its use as sacrificial substrate for NADPH regeneration (Torres Pazmiño et al., 2009). The addition of both phosphite and a small amount of $NADP^+$ in the formulation would make the enzyme ready to use for small scale conversions. Serine and cysteine were also used to see if amino acids could take over the role of sugars, salts or wetting agents. Serine stabilized CHMO (Fig. 2), but the similar amino acid cysteine was not found to stabilize the enzyme, even if though it could also have a protective effect by acting as a reducing agent.

A lyoprotectant 20 mg/mL of sucrose was also used in the lyophilization of two other monooxygenases, CPDMO and P450 BM3. The optimal conditions for preserving enzyme activity varied for the different enzymes (Fig. 3). CHMO is more stable when stored at −80 °C compared to 4 °C and lyophilization results in the loss of some activity. The reverse is the case for CPDMO; this enzyme loses activity when frozen and storage at 4 °C preserves most activity. This agrees with previous results (unpublished) indicating that CPDMO should not be frozen. P450 BMO3 is relatively unaffected by freezing and lyophilization in the presence of sucrose. In constrast to the BVMOs, the activity after lyophilization in the absence of sucrose results in the loss of most activity. Gratifyingly, it was found that, as for CHMO, sucrose stabilized CPDMO and P450 BM3 upon lyophilization.

These data reinforce the proof that sucrose can be used as a stabilizing additive during lyophilization, and shows that the concentration of sucrose plays a delicate role. Alternative additives were also identified. For example, serine was found to stabilize CHMO upon lyophilization. We have also shown a (co-)substrate can be used as an additive during lyophilization. These findings might facilitate the easier distribution of more stable and applicable lyophilized formulations of both BVMOs and P450s. These findings will be helpful for future biocatalytic applications that involve monooxygenases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.03.010.

References

Allison, S.D., Chang, B., Randolph, T.W., Carpenter, J.F., 1999. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch. Biochem. Biophys. 365, 289–298.
Bauer, C., Corsi, C., Paolini, M., 1994. Stability of microsomal monooxygenases in murine liver S9 fractions derived from phenobarbital and β-naphthoflavone induced animals under various long-term conditions of storage. Teratog. Carcinog. Mutagen. 14, 13–22.
Beneventi, E., Niero, M., Motterle, R., Fraaije, M., Bergantino, E., 2013. Discovery of Baeyer–Villiger monooxygenases from photosynthetic eukaryotes. J. Mol. Catal. B: Enzym. 98, 145–154.
Beneventi, E., Ottolina, G., Carrea, G., Panzeri, W., Fronza, G., Lau, P.C.K., 2009. Enzymatic Baeyer–Villiger oxidation of steroids with cyclopentadecanone monooxygenase. J. Mol. Catal. B: Enzym. 58, 164–168.
Blanquet, S., Garrait, G., Beyssac, E., Perrier, C., Denis, S., Hébrard, G., Alric, M., 2005. Effects of cryoprotectants on the viability and activity of freeze dried recombinant yeasts as novel oral drug delivery systems assessed by an artificial digestive system. Eur. J. Pharm. Biopharm. 61, 32–39.
Boddupalli, S.S., Estabrook, R.W., Peterson, J.A., 1990. Fatty acid monooxygenation by cytochrome P-450 BM-3. J. Biol. Chem. 265, 4233–4239.
Bong, Y.K., Clay, M.D., Collier, S.J., Mijts, B., Vogel, M., Zhang, X., Zhu, J., Nazor, J., Smith, D., & Song, S., 2013. Synthesis of prazole compounds EP2010836590.
Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W., 1997. Rational design of stable lyophilized protein formulations: some practical advice. Pharm. Res. 14, 969–975.
Chefson, A., Zhao, J., Auclair, K., 2007. Sugar-mediated lyoprotection of purified human CYP3A4 and CYP2D6. J. Biotechnol. 130, 436–440.
Chen, Y., Peoples, O., Walsh, C., 1988. Acinetobacter cyclohexanone monooxygenase: gene cloning and sequence determination. J. Bacteriol. 170, 781–789.
Eastham, G.R., Johnson, D.W., Straathof, A.J.J., Fraaije, M.W., & Winter, R.T., 2013. Process for the production of methyl methacrylate WO 2013179005 A1.

Fink, M.J., Fischer, T.C., Rudroff, F., Dudek, H., Fraaije, M.W., Mihovilovic, M.D., 2011. Extensive substrate profiling of cyclopentadecanone monooxygenase as Baeyer–Villiger biocatalyst reveals novel regiodivergent oxidations. J. Mol. Catal. B: Enzym. 73, 9–16.

Fraaije, M.W., Wu, J., Heuts, D.P., van Hellemond, E.W., Spelberg, J.H., Janssen, D.B., 2005. Discovery of a thermostable Baeyer–Villiger monooxygenase by genome mining. Appl. Microbiol. Biotechnol. 66, 393–400.

Guengerich, F.P., 2002. Cytochrome P450 enzymes in the generation of commercial products. Nat. Rev. Drug Discov. 1, 359–366.

Iwaki, H., Wang, S., Grosse, S., Bergeron, H., Nagahashi, A., Lertvorachon, J., Yang, J., Konishi, Y., Hasegawa, Y., Lau, P.C.K., 2006. Pseudomonad cyclopentadecanone monooxygenase displaying an uncommon spectrum of Baeyer–Villiger oxidations of cyclic ketones. Appl. Environ. Microbiol. 72, 2707–2720.

Kadow, M., Loschinski, K., Mallin, H., Saß, S., Bornscheuer, U., 2012. Discovery, application and protein engineering of Baeyer–Villiger monooxygenases for organic synthesis. Org. Biomol. Chem. 10, 6249–6265.

Leipold, F., Wardenga, R., Bornscheuer, U.T., 2012. Cloning, expression and characterization of a eukaryotic cycloalkanone monooxygenase from *Cylindrocarpon radicicola* ATCC 11011. Appl. Microbiol. Biotechnol. 94, 705–717.

Leipold, F., Rudroff, F., Mihovilovic, M.D., Bornscheuer, U.T., 2013. The steroid monooxygenase from *Rhodococcus rhodochrous*; a versatile biocatalyst. Tetrahedron Asymmetry 24, 1620–1624.

Leisch, H., Morley, K., Lau, P.C.K., 2011. Baeyer–Villiger monooxygenases: more than just green chemistry. Chem. Rev. 111, 4165–4222.

Mansuy, D., 1998. The great diversity of reactions catalyzed by cytochromes P450. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 121, 5–14.

Munro, A.W., Girvan, H.M., Mason, A.E., Dunford, A.J., McLean, K.J., 2013. What makes a P450 tick? Trends Biochem. Sci. 38, 140–150.

Munro, A.W., Leys, D.G., McLean, K.J., Marshall, K.R., Ost, T.W.B., Daff, S., Miles, C.S., Chapman, S.K., Lysek, D.A., Moser, C.C., Page, C.C., Dutton, P.L., 2002. P450 BM3: the very model of a modern flavocytochrome. Trends Biochem. Sci. 27, 250–257.

Opperman, D.J., Reetz, M.T., 2010. Towards practical Baeyer–Villiger monooxygenases: design of cyclohexanone monooxygenase mutants with enhanced oxidative stability. ChemBioChem 11, 2589–2596.

Polizzi, K.M., Bommarius, A.S., Broering, J.M., Chaparro-Riggers, J.F., 2007. Stability of biocatalysts. Curr. Opin. Chem. Biol. 11, 220–225.

Riva, S., Fassi, P., Allegrini, P., & Razzetti, G., 2007. A process for the preparation of (−)-modafinil EP1777295A2.

Ryerson, C.C., Ballou, D.P., Walsh, C., 1982. Mechanistic studies on cyclohexanone oxygenase. Biochemistry 21, 2644–2655.

Serdakowski, A.L., Dordick, J.S., 2008. Enzyme activation for organic solvents made easy. Trends Biotechnol. 26, 48–54.

Souillac, P.O., Costantino, H.R., Middaugh, C.R., Rytting, J.H., 2002. Investigation of protein/carbohydrate interactions in the dried state. 1. Calorimetric studies. J. Pharm. Sci. 91, 206–216.

Torres Pazmiño, D.E., Riebel, A., de Lange, J., Rudroff, F., Mihovilovic, M.D., Fraaije, M.W., 2009. Efficient biooxidations catalyzed by a new generation of self-sufficient Baeyer–Villiger monooxygenases. ChemBioChem 10, 2595–2598.

Torres Pazmino, D., Winkler, M., Glieder, A., Fraaije, M., 2010. Monooxygenases as biocatalysts: classification, mechanistic aspects and biotechnological applications. J. Biotechnol. 146, 9–24.

van Beek, H.L., de Gonzalo, G., Fraaije, M.W., 2012. Blending Baeyer–Villiger monooxygenases: using a robust BVMO as a scaffold for creating chimeric enzymes with novel catalytic properties. Chem. Commun. 48, 3288–3290.

van Beek, H.L., Wijma, H.J., Fromont, L., Janssen, D.B., Fraaije, M.W., 2014. Stabilization of cyclohexanone monooxygenase by a computationally designed disulfide bond spanning only one residue. FEBS Open Bio 4, 168–174.

Wang, B., Tchessalov, S., Warne, N.W., Pikal, M.J., 2009. Impact of sucrose level on storage stability of proteins in freeze-dried solids. I. Correlation of protein-sugar interaction with native structure preservation. J. Pharm. Sci. 98, 3131–3144.

Zhao, J., Auclair, K., 2009. The activity of human CYP2D6 in low water organic solvents. Biotechnol. Bioeng. 102, 1268–1272.