Expanding the applicability of Baeyer-Villiger Monooxygenases
van Beek, Hugo

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
van Beek, H. (2014). Expanding the applicability of Baeyer-Villiger Monooxygenases [S.I.]: [S.n.]
Lyophilization conditions for the storage of monooxygenases

Hugo L. van Beek, Nina Beyer, Dick B. Janssen, Marco W. Fraaije

Molecular Enzymology Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, the Netherlands.

This chapter has been submitted.
Lyophilization has been used for more than 60 years to increase the storage stability of food, pharmaceuticals and enzymes. We chose cyclohexanone monooxygenase (CHMO) as a model enzyme to find suitable freeze-drying conditions for long-term storage of an isolated monooxygenase. CHMO is a NADPH-dependent FAD-containing Baeyer-Villiger monooxygenase (BVMO) known for its ability to catalyze a large number of oxidation reactions. Additives were tested, with a focus on establishing the optimal concentration of sugars and salts for enzyme stabilization during and after lyophilization. The results were successfully transferred to a larger scale and to two other monooxygenases, namely the BVMO cyclopentadecanone monooxygenase (CPDMO) and the heme-containing cytochrome P450 monooxygenase, P450 BM3. In the absence of a lyoprotectant, lyophilized P450 BM3 was almost completely inactivated while the lyophilized BVMOs quickly lost activity when stored at 50 °C. Lyophilization in the presence of 2% (w/v) sucrose gave the best results, with preservation of 80% of the activity of P450 BM3 upon lyophilization and almost complete protection against inactivation of all tested monooxygenases when stored as lyophilizate at 50 °C.

Abstract

Lyophilization has been used for more than 60 years to increase the storage stability of food, pharmaceuticals and enzymes. We chose cyclohexanone monooxygenase (CHMO) as a model enzyme to find suitable freeze-drying conditions for long-term storage of an isolated monooxygenase. CHMO is a NADPH-dependent FAD-containing Baeyer-Villiger monooxygenase (BVMO) known for its ability to catalyze a large number of oxidation reactions. Additives were tested, with a focus on establishing the optimal concentration of sugars and salts for enzyme stabilization during and after lyophilization. The results were successfully transferred to a larger scale and to two other monooxygenases, namely the BVMO cyclopentadecanone monooxygenase (CPDMO) and the heme-containing cytochrome P450 monooxygenase, P450 BM3. In the absence of a lyoprotectant, lyophilized P450 BM3 was almost completely inactivated while the lyophilized BVMOs quickly lost activity when stored at 50 °C. Lyophilization in the presence of 2% (w/v) sucrose gave the best results, with preservation of 80% of the activity of P450 BM3 upon lyophilization and almost complete protection against inactivation of all tested monooxygenases when stored as lyophilizate at 50 °C.
Monooxygenases catalyze the insertion of a single oxygen atom from O\(_2\) into an organic substrate. Because they often possess high regio- and stereoselectivity, monooxygenases can be employed for the biocatalytic production of high-value chemicals such as active pharmaceutical ingredients or metabolic intermediates (Torres Pazmiño et al., 2010). Two monooxygenase classes are of special commercial interest: cytochrome P\(_{450}\) monooxygenases (P\(_{450}\)s) and Baeyer-Villiger monooxygenases (BVMOs).

BVMOs catalyze the conversion of ketones into esters, often with high regio- and enantioselectivity (Kadow et al., 2012; Leisch et al., 2011). BVMOs utilize a flavin cofactor to catalyze Baeyer-Villiger oxidations. The Baeyer-Villiger oxidation of 2-butanone to produce the polymer precursor methyl propanoate is an industrially relevant example (Eastham et al., 2013). Besides Baeyer-Villiger oxidations, these enzymes are also able to oxidize heteroatoms, such as the enantioselective oxidation of sulfides (Bong et al., 2013; Riva et al., 2007). Two BVMOs were chosen for this study. The first is the extensively studied cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB9871 (Chen et al., 1988; Ryerson et al., 1982), because it has been used as the prototypical BVMO for many years. Yet, CHMO is a rather labile enzyme (van Beek et al., 2014). The second is cyclopentadecanone monooxygenase (CPDMO) from *Pseudomonas* sp. strain HI-70 which was chosen because it is complements the biocatalytic potential of CHMO and can be expressed as recombinant protein (Beneventi et al., 2009; Fink et al., 2011; Iwaki et al., 2006).

P\(_{450}\)s contain a heme cofactor as prosthetic group which is essential for catalysis. P\(_{450}\)s are known for their role in human steroid metabolism and xenobiotic detoxification (Munro et al., 2013). Therefore they are a prime target for drug development in the pharmaceutical industry (Guengerich, 2002). P\(_{450}\)s are also considered interesting biocatalysts, because the scope of catalyzed reactions is broad (Mansuy, 1998) and numerous products of commercial interest are conceivable. Here we focus on P\(_{450}\) BM3 (CYP102A1), derived from *Bacillus megaterium*, which *in vivo* catalyzes the hydroxylation of fatty acids (Boddupalli et al., 1990). P\(_{450}\) BM3 has become the model enzyme for many studies on P\(_{450}\) systems (Munro et al., 2002). Both storage stability and operational stability are highly relevant for biotechnological applications that depend on the use of the above-mentioned biocatalysts. For long-term storage of proteins, lyophilization is an attractive approach. In the case of P\(_{450}\)s, the optimal conditions for lyophilization of several specific P\(_{450}\)s have been investigated (Bauer et al., 1994; Blanquet et al., 2005; Chefson et al., 2007; Zhao and Auclair, 2009). For BVMOs, most effort was put into protein engineering to increase the thermostability and thereby increase the 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. Except for extending storage time, lyophilization allows the screening of new substrates without extensive knowledge of molecular biology and facilitates the use of enzymes in non-aqueous media (Serdakowski and Dordick, 2008). While lyophilization has been used in the past to prepare formulations of isolated BVMOs or of cell-free extract containing a BVMO, no optimization of the lyophilization conditions has been done.

Denaturation of an enzyme can occur by heating, freezing, agitation or by chemical degradation in an aqueous formulation. Lyophilization protects against these effects. Water-mediated processes such as
hydrolysis and deamidation are limited by the removal of water. Protein unfolding, aggregation and chemical modifications are diffusion controlled reactions. Drying an enzyme results in the formation of an amorphous phase. Below the glass transition temperature ($T_g$) of this amorphous state the rate of diffusion-controlled reactions is greatly reduced. However, additives might still be required because storage below the $T_g$ is not always sufficient to keep the protein from degrading, especially at longer timescales. Additives might also be required to prevent aggregation during the lyophilization process or rehydration. Additives may also compensate for the loss of essential waters during lyophilization by forming hydrogen bonds with the protein (Allison et al., 1999). Sugars are widely used as lyoprotectants during freeze-drying, with sucrose, trehalose and mannitol being most commonly used (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 different applications of the enzyme the use of sugars might not be preferred, and alternatives are required. Various compounds were tested that could also have beneficial effects: salts, reducing compounds, wetting agents, amino acids and phosphate, the substrate for the fused coenzyme regenerating enzyme.

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, several stabilizing additives were discovered which might be useful in specific applications, specifically magnesium sulfate and serine.

**Materials and methods**

**Chemicals, equipment and vectors**

Chemicals were acquired from Sigma-Aldrich, Oriental Yeast Co., TCI Europe and Riedel-de Haën. An Alpha 2-4 LD plus freeze dryer (Christ) was used for lyophilization and a Synergy MX (BioTek) was used for all spectrophotometric work. An Äkta purifier (GE Healthcare) was used for purification. The gene coding for P450 BM3 was kindly provided by prof. B. Hauer (Stuttgart) on a pJoe vector and was subcloned into a pBAD vector using 5'-ttgggctaaccaggggaatcatactgacaataaagaaaatgcac-3' as the forward and 5'aactcaatgatgatgatgatgatggtcgaccccagcccacagtctttt-3' as the reverse primer.

**Enzyme purification**

PTDH-fused CHMO and CPDMO were expressed and purified as previously reported (Torres Pazmiño, et al. 2009), with the exception that no glycerol and DTT were added to the buffers. *E. coli* Top10 cells containing pBAD P450 BM3 were grown in 3 × 400 mL TB$_{Amp}$ in shaking flasks at 37 °C. Expression was induced by the addition of 0.02% L-arabinose at an OD$_{600}$ of 0.7-0.8 and 0.5 mM δ-aminolevulinic acid was added. The cells were incubated for 48 h (17 °C at 180 rpm) after induction before they were harvested (2880 × g, 30 min) and washed once with ice cold sodium phosphate buffer (50 mM, pH 7.5). The cell pellet was stored at -20 °C. For purification all steps were carried out on ice to prevent inactivation of the enzyme. Cells were resuspended in 30 mL of the sodium phosphate buffer and lysed using sonication. The resulting crude extract was centrifuged (3645 × g, 40 min) and the supernatant was recovered. For ammonium sulfate precipitation the cell free extract was diluted to a protein concentration of 20 mg/mL. P450 BM3 was precipitated at an ammonium sulfate concentration of 50% and
collected by centrifugation (17000 × g, 15 min) and resuspended in sodium phosphate buffer (50 mM, pH 7.5). The enzyme was further purified by affinity chromatography using a 5 mL HF His trap column. The imidazole in the elution buffer was removed by a second step of ammonium sulfate precipitation with 90% ammonium sulfate. The protein pellet was resuspended in Tris/HCl buffer (50 mM pH 7.5).

**Lyophilization**

50 µL aliquots containing 5-25 µM enzyme and different concentrations of additives in a 96-well PCR plate were covered with a semi-permeable AeraSeal (Sigma-Aldrich) cover and flash frozen in liquid nitrogen and subsequently freeze-dried overnight at 0.05-0.07 mbar without temperature control. To prevent the effect of the used buffer from having a large effect on the results, the concentration Tris/HCl buffer was reduced to 5 to 12 mM in the lyophilization samples.

**Activity determination**

The lyophilized cake was dissolved in a volume of buffer equal to the total volume before lyophilization. 50 mM Tris/HCl pH 8.5 was used for the BVMOs or 50 mM Tris/HCl pH 7.5 for P450 BM3. When the hydrophobicity of the material was too high to allow fast dissolution, a short centrifugation step was used to completely submerge the cake. The enzyme was diluted to an appropriate concentration (0.05-0.3 µM) in the presence of 0.5 mM cyclohexanone (for CHMO), 1.5 mM (±)-bicyclo[3.2.0]hept-2-en-6-one (for CPDMO) or 1.0 mM lauric acid (for P450 BM3). Reactions were started by the addition of NADPH to a final concentration of 150-500 µM, and initial rates at 25 °C were determined by following the absorbance at 340 nm. Reported data points are obtained from 3 to 6 samples that were taken from the same stock solution before lyophilization. Data is compared between conditions in the same batch, as the handling, freezing cycles and storage before lyophilization affect the rate of the enzyme in general.

**Incubations**

To simulate extended storage conditions, the lyophilized enzyme samples were incubated at 50 °C for 48 h (sucrose concentrations) or 72 h (other additives) in a ventilated stove, covered by the same semi-permeably filter as used during lyophilization. For the time course measurement, samples were stored at room temperature and subsequently at 50 °C in an air ventilated stove for the required number of days.

**Results and discussion**

As freeze-drying is a technique that is generally very mild to the enzyme, extended storage times or elevated temperatures are required to see the small differences in stability caused by additives. In initial experiments, the thermostable enzyme phenylacetone monooxygenase (PAMO) was used (Fraaije, et al. 2005), but we were unable to accurately observe any degradation in short time periods (data not shown). This is in line with the observation that PAMO is thermostable and tolerates a variety of organic solvents. CHMO has been shown to be more labile and was used for further experiments (van Beek, et al. 2012). CHMO lost more than half of its activity upon lyophilization without any additives, and lost most of its residual activity within days when stored at 50 °C (Fig. 1). These initial results showed that additives could play an important role in creating a stabilized formulation of a labile enzyme like CHMO.
To determine the stabilizing effect of different concentrations of additives, we chose to store the enzyme for a short time at elevated temperatures to amplify differences caused by these additives. Lyophilized samples were incubated at 50 °C before measuring the activity of the enzyme. As a starting point the inexpensive disaccharide sucrose was used as a lyoprotectant. Several concentrations of sucrose were tested to find the optimal concentration. Around 10 to 50 mg/mL sucrose in the enzyme solution was found to optimally preserve activity (Fig. 2). 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 not only caused by a few key sucrose molecules bound to the enzyme to replace water, but also because of an amorphous bulk-phase formed by sugar. While low sucrose concentrations were not effective in stabilization, also relatively high doses of sucrose (>50 mg/mL) decreased the stability of the monooxygenase upon lyophilization.

Figure 1: Percentage of residual activity of CHMO after lyophilization without additives and incubation at 50 °C. The activity of the enzyme before lyophilization is set to 100%. Standard errors shown are from 3 measurements.

Figure 2: 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 6 lyophilized samples.
Previously two P450s were stabilized by a much higher concentrations of sucrose. But also in those cases lower activities at relatively high concentrations of sucrose are observed (Chefson et al., 2007). No explanation was given for the reduction of activity by added sucrose. A possible explanation is that 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 formation of hydrogen bonds with the protein in the place of the removed water (Allison et al., 1999).

A sugar that is often used to stabilize proteins is trehalose. No additional stabilization could be observed as compared to sucrose by the addition of 10 mg/mL trehalose (Fig. S1). The often superior stabilizing effect of trehalose is attributed to its higher hydration radius, allowing the retention of more water in the dried protein state. This effect could also be achieved by using larger quantities of sucrose. No other sugars were tested because the sucrose-lyophilized enzyme already performed as good as enzyme frozen in liquid N$_2$ and stored at -80 °C (Fig. S1). Improving the stability more would be improbable.

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. Some compounds were also tested because they could be already present in the 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. 3 and Fig. S2 and S3) to find out whether the ion or the cation would be responsible for the stabilization. Ammonium sulfate itself did not stabilize the enzyme upon lyophilization. We also observed that ammonium sulfate is interfering with the stabilizing effect of sucrose. The combined sucrose and ammonium sulfate formulation performed worse than the formulation containing only sucrose (Fig. 3). However, a stabilizing effect of magnesium sulfate was discovered. 25 mM MgSO$_4$ protected the lyophilized enzyme as well as 20 mg/mL sucrose. Magnesium 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.

![Figure 3: Observed rates of CHMO containing various additives after lyophilization and 48 h incubation at 50 °C.](image-url)
When sucrose and stabilizing salts were present, phosphite could be added to the enzyme mix without significantly affecting the activity. In the case of sucrose and ammonium sulfate the activity is even increased by the addition of phosphite (Fig. 3). Phosphite was tested because of its use as sacrificial substrate for NADPH regeneration (Torres Pazmiño et al., 2009). The addition of phosphite could be useful for small lab-scale conversions. In the current formulation 50 mM was used, resulting in a highest possible value for the total turnover number of only 2000 (the concentration of phosphite is only 2000 fold the concentration of enzyme). This makes it not suitable for any large-scale process because this leads to excessive enzyme loading (Rogers and Bommarius, 2010). The addition of both phosphite and NADP⁺ in the formulation would make the enzyme ready to use for smaller scale conversions.

Tween 20 was tested for its potential to help with the re-solubilization step by acting as a wetting agent. The wetting of the protein foam formed upon lyophilization was problematic in some formulations because it appeared to be very hydrophobic. Serine and cysteine were also used to see if amino acids could take over the role of sugars, salts or wetting agents. Tween 20 was not very effective by itself, but serine stabilized CHMO. The similar amino acid cysteine was not found to stabilize the enzyme, even if it could have protective effect by acting as a reducing agent.

![Figure 4](image-url)

**Figure 4:** 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.

The optimal concentration of sucrose (20 mg/mL) was also used in the lyophilization of two other monooxygenases, to see if the stabilizing effect was specific for CHMO or applicable to a wider range of enzymes. The sequence-related and relatively labile CPDMO, and the widely used P₄₅₀ BM₃ were chosen. The optimal conditions for stabilizing enzyme activity varied for the different enzymes (Fig. 4).
CHMO is clearly more stable when stored at -80 °C compared to 4 °C. Lyophilization also results in the loss of significant activity. The reverse is the case for CPDMO; this enzyme loses activity when frozen while storage at 4 °C preserves most activity. This agrees with previous results (unpublished) that indicate that CPDMO should not be frozen. P450 BM03 is relatively unaffected by freezing and lyophilization in the presence of sucrose. In contrast to the other monooxygenases, 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 both enzymes upon lyophilization.

Conclusions

Conditions that improved the stability of lyophilized BVMO CHMO were determined and transferred to the homologous CPDMO and P450 BM3. The use of 6.5-45 mg/mL sucrose was highly effective in obtaining a stable lyophilized monooxygenase samples. Also alternative additives were identified. For example, serine was also 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.

Acknowledgements

HL van Beek was supported by the EU 7th framework programme Oxygreen and N Beyer was supported by the P4FIFTY FP7 funded European Marie Curie Training network.
References


van Beek HL, Wijma HJ, Fromont L, Janssen DB, Fraaije MW. 2014. Stabilization of cyclohexanone monooxygenase by a computationally designed disulfide bond spanning only one residue. FEBS Open Bio. 4:168-174


Chapter 6
Lyophilization conditions for the storage of monooxygenases