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Model-based characterization of operational stability of multimeric enzymes with complex deactivation behavior: An *in-silico* investigation

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HIGHLIGHTS

- Multimeric enzymes require multimeric deactivation models for stability characterization.
- The often used Lumry-Eyring deactivation models is insufficient as empiric description for multimer deactivation.
- Conversion in a continuous enzyme membrane reactor allows model discrimination and yields reliable characterizations.
- Relaxed stochastic criteria allow for identification of parameterization applicable to process design.

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ABSTRACT

A comprehensive characterization of the enzyme kinetics and stability is required in order to design biotransformation processes efficiently. In particular, the characterization of the operational stability (stability under process conditions) of biocatalysts lacks established procedures and the few reported procedures have hardly been evaluated by transfer to reactor scale. In the scope of this modeling study three potentially applicable characterization methods were tested: (i) progress curves recorded at different temperatures; (ii) isothermal continuous reactor operation at different (elevated) temperatures; and (iii) continuous reactor operation with a temperature gradient. Input data from those procedures were generated *in-silico* by introducing two virtual dimeric enzymes with defined, complex deactivation mechanisms. The applied degradation pathways account for experimentally proven phenomena, which were neglected in operational stability descriptions so far, such as multiple intermediate unfolding state and subunit dissociation. By introducing a random normal error data sets with typical experimental variance were generated. On the basis of these data a model-based experimental analysis for parameter estimation was applied which included the testing of different simplified models (with increasing complexity until over-parameterization became evident). The obtained fits were evaluated by the lack of fit variance and the significance level of the F-statistics. Both criteria indicated a dimeric model with two intermediate unfolding states as best description, which was the model with the highest similarity to the models that had been applied to produce the data in the first place. When subjecting the obtained parameterization to a virtual process design task the quality of the prediction could be easily tested by comparison with the prediction from the original mechanistic model that was used to produce the input data. The obtained results clearly indicate that the often used Lumry-Eyring type models should not be used as empiric description for potentially complex multimeric enzyme deactivation mechanisms as it is done in current operational stability investigations. Next, for the continuous reactor procedures a nice correlation between the obtained significance level of the F-statistics and the accuracy of process prediction was found demonstrating the usefulness of these experimental procedures for parameter estimation. Further, it was possible to estimate a specific threshold significance level that can be used as an indicator for reliable parameterizations with respect to process design.

1. Introduction

Biotransformations have matured into a well established, industrially applied technology due to the frequently unmatched chemo-, regio- and stereoselectivity of enzymes and their ability

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to operate under non-intensive and often environmentally friendly conditions (Straathof et al., 2002; Villadsen, 2007). In addition, sophisticated Darwinian evolution-based methods have been developed in order to optimize enzymes towards higher performance or accepting non-natural substrates, which are nowadays considered standard technologies (Arnold, 2001; Reetz, 2001). However when it comes to characterization of the respective enzyme for application in an industrial setting, the potential of model-based approaches is – besides a few notable exceptions (Berendsen et al., 2007; Braun et al., 2011; Chen et al., 2007; Schroen et al., 2002) – not adequately exploited (Gernaey and Gani, 2010; Jimenez-Gonzalez and Woodley, 2010; Sin et al., 2009; Vasic-Racki et al., 2011; Villadsen, 2007). Rational process design for a biotransformation reaction requires an accurate characterization of enzyme kinetics and operational stability under the projected process conditions, which are typically not stringently specified in the early stages of process development. In particular, the selection of an appropriate reaction temperature is crucial for efficient operation (Illanes and Wilson, 2003; Polizzi et al., 2007). While increasing the temperature typically greatly enhances the catalytic activity of an enzyme, its stability is often drastically decreased. Consequently, for a given set of process specifications (such as operating time, conversion, substrate feed, and enzyme amount) there exists a distinct temperature-dependent productivity optimum. Therefore, parameterized expressions for enzyme activation and denaturation kinetics as a function of the temperature are required to determine the most (cost-) efficient operation reliably.

Arguably, model-based approaches are required in order to obtain such a comprehensive characterization which includes a – frequently neglected – statistical analysis of the data (Gernaey et al., 2010; Marquardt, 2005) which can provide confidence intervals of the estimated parameters and support or reject the obtained fit (and the underlying model) on the basis of, for example, experimental and lack of fit variance.

Reaction mechanisms are often available for the analysis of enzyme kinetics from the literature or can be deduced from similar reactions with the same stoichiometry (Cornish-Bowden, 1995; Segel, 1975) which then can be easily translated into a mathematical kinetic expression. Furthermore, suitable experimental setups (for example, progress curves) and protocols have been validated that ensure reliable parameterization of these expressions (Berendsen et al., 2006; Buhler et al., 2006; Flores and Halling, 2002; Schroen et al., 2001; Straathof, 2001; Swarts et al., 2008). Thus, within the boundaries set by the experimentally applied conditions, enzyme performance can be comprehensively characterized, for example, as a function of pH or temperature (Sin et al., 2009). Although the applied reaction models and experiments potentially need to be refined in order to account for particular effects such as inhibitions, the design of experiments as well as the selection of models is in principle established.

This is not the case for the characterization of biocatalyst stability, despite its long recognized central role as a potential bottleneck in industrial biocatalysis (Weijers and Van't Riet, 1992). The predominant deactivation mechanism under operational conditions is not known for most enzymes, prompting most model-based investigations to apply the simplest physically meaningful degradation mechanism, the "Lumry-Eyring" model (Lumry and Eyring, 1954). It accounts for three different protein states: (i) the correctly folded active state E; (ii) an inactive state U that enables refolding upon a change in environmental conditions; and (iii) a completely denatured state D (Klibanov, 1983; Lumry and Eyring, 1954; Tanford, 1970). The latter state is irreversibly accumulated by denaturation by various processes (Ahern and Klibanov, 1988; Manning et al., 2010; Sanchez-Ruiz, 2010) while rapid equilibration between E and U is assumed (Fig. 1a). The model was later introduced as equilibrium model in the scope of operational biocatalyst stability studies (Daniel et al., 2001; Eisenthal et al., 2006; Peterson et al., 2004) (the two models feature the same reaction scheme but differ in the formal description of the unfolded protein state U). However, a number of experimental studies have indicated that protein unfolding mechanisms can follow more complex pathways including, for example, multiple intermediate protein states (Mayne and Englander, 2000; Shortle, 2002; Vreuls et al., 2004) and subunit dissociation in case of multimeric enzymes (Mendoza-Hernandez et al., 2000; Prakash et al., 2002; Srinivasulu and Rao, 1996; Vreuls et al., 2004). For a number of proteins the latter clearly constitutes a primary degradation pathway as demonstrated by the large stabilization effects obtained when strengthening subunit interactions, for example, by protein engineering or immobilization (Bjork et al., 2004; Fernandez-Lafuente, 2009; Gribenko et al., 2009). In consequence, a formal general representation of multimeric enzyme degradation should include dissociation into the monomeric subunits, unfolding and irreversible denaturation (Shriver and Edmondson, 2009) which, for a dimeric protein includes already at least four protein states (active dimer E_2, (partially) unfolded inactive dimer U_2, monomeric state U, denatured state D (Fig.1d)). Evidently, the broadly applied three state monomeric model cannot sufficiently cover important elements of enzyme degradation. However, characterization of operational stability for the purpose of process design does – *in* sensu stricto – not require a model that accurately reflects the inactivation mechanism. In this case an empiric description that accurately reflects the active enzyme concentration in the required condition space is sufficient. Nevertheless, it remains unclear whether the application of the simple three state model is warranted in particular when multimeric enzymes are considered. Previous investigations of operational stability are not conclusive in this regard as the obtained parameterizations have hardly been properly evaluated (for example, by transfer to reactor scale) or subjected to a rigorous statistical analysis.

Obviously the selection of an appropriate deactivation model is only one step in a sequence towards proper biocatalyst stability characterization. Supplying the proper data as input for model-based parameter estimation that enables parameter determination with acceptable accuracy and (non-mechanistic) model

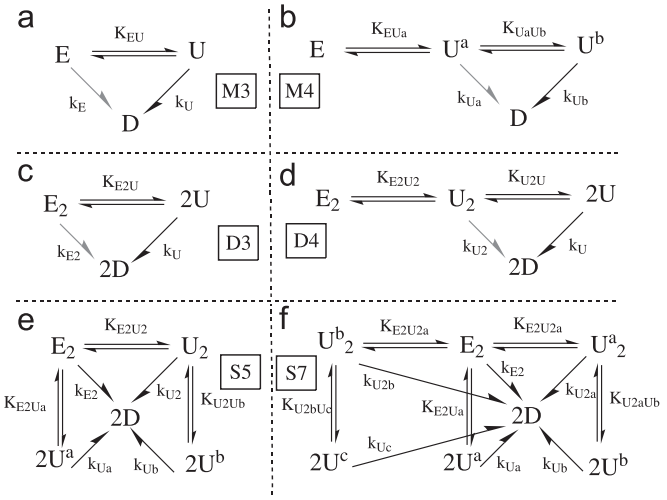

Fig. 1. Overview of deactivation mechanisms assumed in this work. The models are classified by the number of involved states. For mechanisms (a–d) two instances are considered depending on the number of irreversible deactivation pathways as indicated by the gray reaction arrows. Capital letter K refers to equilibrium constants, small letter k to reaction rate constants.