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Case Studies Illustrating a Science and Risk-Based Approach to Ensuring Drug Quality When Using Enzymes in the Manufacture of Active Pharmaceuticals Ingredients for Oral Dosage Form

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ABSTRACT: Biocatalysis is essentially the use of enzymes to perform chemical transformations on organic compounds and has been exploited for applications in various industries including food, fine chemicals, agrochemicals, and pharmaceuticals. Due to their selectivity and ability to operate under mild conditions, enzymes offer clear advantages for efficient sustainable manufacturing processes. Rapid development of enabling technologies including gene mining, molecular biology, biocatalyst evolution, and bioprocess engineering, has created opportunities to use biocatalysis more broadly for the manufacture of small molecule intermediates and APIs (active pharmaceutical ingredients). To facilitate the adoption of biocatalysis for API manufacture and address a perceived lack of regulatory clarity, several of the current authors published a science and risk based approach to ensuring patient safety and drug quality when using biocatalysis (*Org. Process Res. Dev.* **2012**, *16*, 1986−1993). Since this publication, consultation of multiple users and stakeholders in the biocatalysis community has expanded on key guidance and specific approaches, as well as providing real examples of regulatory review and approval in this article. Our ultimate goal as highlighted in this publication is to provide a clear path and knowledge base to enable a robust and sound science and risk-based approach for utilizing biocatalysis whenever appropriate for the manufacture of small molecule pharmaceuticals.

■ INTRODUCTION

Synthetic Chemistry Routes to APIs Using Biocatalysis. The increasing complexity of drug targets requires ever more sophisticated and selective processes, especially in the realm of asymmetric synthesis. The mild neutral conditions coupled with the high specificity and low relative cost inherent with the use of enzymes has led to an emergence of the use of biocatalysts in the synthesis of medicinal compounds. The increased availability of a broad range of biocatalysts that can be applied in organic synthesis has brought into focus the need to rethink the way in which target molecules are synthesized. For decades, biocatalysis has been applied to numerous industrial processes but overall was considered a fairly narrow/niche tool for synthetic chemistry.[2] In the past 10 years, the application of biocatalysis in API synthesis has increasingly become a method of choice in many industrial processes.[3] Initially the focus was on the application of hydrolytic enzymes (e.g., lipases, proteases, esterases) and biocatalysts for asymmetric reduction of ketones (e.g., alcohol dehydrogenases, ketoreductases). This early development phase was also accompanied by advances in cofactor recycling methods on scale and also an exploration of the effect of using certain biocatalysts in low-water environments. Improved gene mining and heterologous expression/production techniques also dramatically enhanced the availability and cost-effectiveness of biocatalyst development. Recently the scope of biocatalysis has broadened significantly with new reaction platforms coming on stream, including transaminases, oxidases, nitrilases, aldolases, and ammonia lyases. Increasingly biocatalysts are available commercially

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"off-the-shelf" in forms that are readily adaptable to synthetic organic chemistry operations, which enable chemists to rapidly identify applications of enzymes using specific target intermediates of interest.[4]

Underpinning this recent uptake of biocatalysis in the pharmaceutical industry has been the introduction of some key enabling technologies which are responsible for driving forward the discovery, development, and application of enzymes and whole cells in chemical processes. Importantly, the idea of improving biocatalysts using directed evolution approaches, which was first reported and discussed in the literature in the early 1980s, is now viewed as a standard methodology for enhancing the characteristics of a biocatalyst in a targeted manner.[5]

Engineering an enzyme (catalyst) at the protein level has led to the generation of biocatalysts that are "fit-for-purpose" for application from small through pilot to large scale in chemical processes.[6] Attractively, the investment in time and money in developing the biocatalyst can be tailored to the development phase of each individual project. The impact of emerging technologies in bioinformatics, next generation sequencing, and high-throughput screening have advanced sufficiently to move into an exciting era in which the rate at which we can discover new enzymes is far outstripping the rate at which we can characterize them and find new applications in chemical synthesis.[7] Alongside these advances in biocatalyst discovery and evolution capabilities, which allows improved process efficiency, is a corresponding increasing need for industry to develop sustainable manufacturing processes for chemical production.[8] Sustainable manufacturing is one of the motivators for increased adoption for biocatalysis and provides many real challenges and opportunities for future developments in this field. In addition, improved process safety and simplified operating considerations are also providing increased motivations for adopting biocatalysis.[9]

Independent of any potential advantages from these promising technical advances, the timelines and perceived risks to implement biocatalytic process need to be acceptable—including all aspects of route scouting, development, process engineering, regulatory, etc. To aid synthetic chemists in identifying where biocatalysts might be usefully applied, a recent publication highlighted the need for rules and guidelines for "biocatalytic retrosynthesis".[10] The main objective of "biocatalytic retrosynthesis" is to encourage synthetic organic chemists to include consideration of biocatalytic step(s) when planning a synthetic route to a target molecule. This new approach will require a greater understanding by synthetic chemists regarding which possible transformations and suitable intermediates might be considered using biocatalysis, and importantly how they might impact the overall efficiency, sustainability, and safety of the final manufacturing process. The generation of searchable, structure-based, databases will undoubtedly encourage further uptake of biocatalysis, particularly if such databases contain information concerning safety and scalability of biocatalytic reactions as well as data on substrate scope, conversions, enantioselectivity, productivity, etc. In parallel with these developments the creation of new biocatalyst platforms is likely to continue apace with future developments anticipated in the use of transferases, halogenases, carboxylic acid reductases, and imine reductases, to name but a few. Based on the advances described above, the timelines required to progress from discovery of a new enzyme to practical application will undoubtedly shorten; further enabling process chemists to more rapidly integrate emerging biocatalysts into their portfolio of catalysts for consideration during route selection. These biocatalysts will not only be employed to catalyze individual synthetic transformations, but increasingly combined in cascade processes, particularly by engineering standardized host cells which possess the required overexpressed genes.[11] Advances in synthetic biology are likely to impact increasingly on the development of sustainable and cost-effective methods for the production of platform starting materials and raw materials which will in turn feed into the supply chains for API synthesis.

To streamline the increased adoption of this growing technology, increased regulatory examples and dialogue for evaluation and acceptance of biocatalysis in pharmaceutical manufacturing is key—to accelerate and lower the risk of development of these biocatalytic steps. In the first paper of this series,[1] we surveyed/outlined a comprehensive list of considerations (both real and perceived) that could arise during the development and regulatory validation of a biocatalytic process, as well as suitable ways to address each consideration. In this paper, we provide several more specific case studies that more clearly illustrate the successful development, preparation for regulatory submission, and commercialization of biocatalytic processes, representing an array of common scenarios.

Thus, the use of biocatalysts can impart significant advantages for the synthesis of APIs through all stages of development from discovery to the final launch process. With the increasing availability of diverse kits of enzyme classes, screening of biocatalysts can be initiated early in development, even in the discovery support stage, in the same way as traditional screening of metal catalysts is done. If an effective enzyme is found during this screening process, the enzyme could be employed to supply necessary API for development even in early clinical trials. Generally, the initial enzyme selected often lacks the ideal properties for a commercial process and requires further refinement to increase selectivity, reactivity, or stability. As this evolution of an enzyme can be a costly and resource intensive exercise, it is often reserved for later development where the likelihood of return on the investment is higher. At this point, the final enzyme with the necessary attributes is integrated into the process for late clinical trial API supply or even registration and launch batches.

The phase appropriate testing of potential residual enzyme should be consistent with testing for normal organic impurities. Early in development, the synthesis of preclinical batches should not require specific residual enzyme testing based on our earlier risk assessment which indicated that enzymes and potential enzyme residues such as amino acids and peptides are not associated with toxicity.[1] Therefore, considerations of the overall purity of the API should be sufficient to support preclinical studies. As the drug candidate advances in development and API is being produced for early clinical trials, the residual enzyme content as a potential impurity in the drug substance should be considered. The placement of the enzymatic process within the overall synthesis and its proximity to the final API would need to be considered for potential control strategies. As the API advances to later stage clinical trials and approaching the registration batches, the final enzyme would have been developed with appropriate residual enzyme control strategies to ensure API quality.

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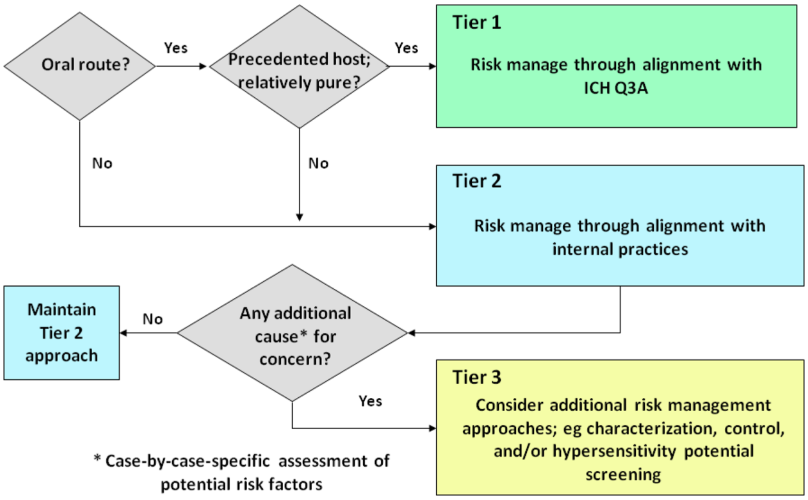

Figure 1. Basic tiered risk assessment reproduced from ref 1. Copyright 2012 American Chemical Society.

■ DISCUSSION

Biologically derived entities like vaccines, monoclonal antibodies, and therapeutic enzymes are vital components in the treatment of many diseases. Likewise, fermentation has been used for decades to produce small molecule APIs and starting materials for semisynthetic medicines like antibiotics. There are specific regulatory guidances for these products. However, the application of these guidelines to use of enzymes/biocatalysis to produce small molecule APIs is not appropriate since in biocatalysis, the enzyme is a simple process aid and may not appear as part of the active pharmaceutical formulation. Our first publication discussed some of the high level arguments regarding a science-based tiered risk assessment. However, whether the protein residue can be treated as a standard organic impurity is still an unset issue. Many aspects such as the nature and property of the protein residues and the safety data available, should be taken into consideration during the science-based risk assessment. The toxicological profile of biocatalysts do not indicate a need for special treatment or concern around potential enzyme related residues, especially for API's delivered by the oral route, for which the Tier 1 risk management approach was proposed, as in Figure 1.[1] For Tier 2 and 3 API's, each case needs to be considered on its own merits and should have an individual risk assessment to determine acceptable levels of protein residues.

In this paper, we offer illustrative case studies to support specific strategies for process evaluation and approval for API production involving biocatalysis.

Strategies for Managing Potential Enzyme-Related Residues in API. Gardossi et al. have published a very comprehensive guide on the enzyme properties and reaction parameters that should be gathered for reporting enzyme reactions.[12] This also can act as a guide for the level of experimental detail and information required to successfully scale up and establish a reliable biocatalyzed process.

The production of API usually involves many synthetic steps and each step comprises multiple operations, e.g., reaction, pH adjustment, filtration, and extraction in organic solvents. Many of these unit operations provide opportunities for purging proteins, and therefore, pharmaceutical intermediates and thus APIs derived from biocatalytic processes are not expected to contain residual protein originating from enzyme preparations. The potential presence of residual proteins in APIs prepared using biocatalysis was discussed in the earlier publication.[1] In order to support the absence of residual proteins, batches are typically assayed for residual proteins during development stages and during process validation with inclusion of such testing in the API specification deemed not necessary. Specifically here, we describe a variety of suitable analytical techniques for the demonstration of absence of proteins in biocatalysis derived pharmaceutical intermediates. The examples discussed here are from a mixture of marketed commercial products and development projects. In order to expedite publication of this material, some structures and compound names/numbers have been excluded. We believe that the key messages and learnings are not detracted from by this strategy.

A high level review across six actual pharmaceutical projects is shown in Table 1. In all cases, the adoption of a route using a biocatalyst was driven by providing a lower cost, more sustainable supply of API to the market. This represents results for four different enzymes and two biocatalyst types—isolated enzymes and two whole cell biocatalysts. Clearly, the results detailed in Table 1 suggest that there are no issues in applying biocatalysis pre- or post registered starting material (RSM). Some testing for endotoxins (lipopolysaccharides, LPS) is done if bacterial whole cells are used for Tier 1 APIs, although the relevance to an orally dosed product is questionable since LPS are deactivated/destroyed in the gastric tract. It should be noted that all injectable API's are routinely tested for pyrogens such as LPS as part of the release specification. Testing for LPS is most commonly performed by using the *limulous amoebocyte* lysate test (LAL test).The basis of this analytical technique is to measure the binding between the LPS and an enzyme from the horseshoe crab. This LPS testing is detailed in several pharmacopeia.[13] For further discussion on enzyme/LPS residues and other cell-derived impurities, see the preceding paper to this current publication.[1]

In the following case studies, some included as examples in Table 1, we will look in more detail at analytical methodology that can be used to detect/quantitate enzyme residues in organic materials. This is not a comprehensive list of procedures available but gives an idea of what is achievable across a wide range of methods with varying degrees of technical complexity. Some analytical tests are very specific for the enzyme; others are more general for amino acids/peptides and thus will detect the enzyme, any fragments due to degradation, or other proteins introduced alongside the active enzyme. Due to various confidentiality concerns, we have not been able to provide more in depth details for Table 1 examples 3, 4, and 6.

As well as analysis for potential bioresidues in API and intermediates, it is necessary to demonstrate that plant equipment has been cleaned after a campaign using biocatalysts. Cleaning was briefly covered in our first paper.[1] A number of the analytical techniques discussed below are also suitable for the analysis of residual cleaning solutions. Alternatively, more general techniques like total organic carbon analysis (TOC) can be performed.

Case Study 1: Pregabalin (Example 1 in Table 1). Pregabalin is the API in Lyrica and is manufactured via a chemoenzymatic process which uses lipolase to resolve diester **1** in the first step of the regulatory synthesis (Figure 2).[9] At the end of the enzymatic step, the aqueous phase containing **2** ((*S*)-CNDE sodium salt) was separated and washed with toluene. This aqueous phase was then heated to effect thermal decarboxylation of **2** to give an organic phase containing **3** ((*S*)-CNE) and an aqueous phase which also contained insoluble denatured enzyme. Separation of the organic and aqueous phases at this step effectively removed enzyme from the process, as demonstrated by testing 10 lots of **3** for residual protein with the Bradford protein assay. The results of this testing showed no residual protein in any of the samples limit of detection (LOD 0.04% w/w). This purge and fate study demonstrated control of potential biocatalyst related impurities in step 2 of the regulatory synthesis. Therefore, potential biocatalyst related impurities were not likely to be found in API, as confirmed by testing of commercial lots for total protein and amino acids as described above. The absence of potential biocatalyst related impurities in pregabalin manufactured via the chemoenzymatic route was demonstrated by testing 24 commercial lots of pregabalin for amino acids, with and without treatment to hydrolyze proteins or peptides in the samples to their constituent amino acids. Samples were analyzed by LC/MS/MS after derivatization with EZfaast. The results of this testing showed no amino acids in any of the samples above the LOD (0.05%). Twenty lots of pregabalin manufactured via the chemoenzymatic process were also tested for total proteins with the Micro BCA assay (see Table 2), and the results of this testing showed no detectable levels of residual proteins (LOD < 0.1%). Purge and fate studies were also conducted to demonstrate control of potential biocatalyst related impurities. The data from these studies clearly demonstrated the absence of potential biocatalyst related impurities in pregabalin API manufactured via the chemoenzymatic process and supported Pfizer's proposal not to test for biocatalyst related impurities in API. The upstream control strategy to not test for biocatalyst related impurities in the API was accepted by all agencies/authorities responsible for drug registration to which the regulatory dossier was filed.[15]

Case Study 2: Atorvastatin (Example 2 in Table 1). Acetonide **7** is a key intermediate in the synthesis of atorvastatin, the API in Lipitor. An enzymatic process (Figure 3) was implemented for synthesis of **7** to replace a chemical reduction which used sodium borohydride and triethylboron to reduce ketoester **5** to diol **6**. Enzymatic reduction of **5** proceeded with almost perfect *cis*-selectivity (<0.1% *trans* diol) compared to the chemical process which gave a 20:1 mixture of *cis*:*trans* and was carried out in aqueous buffer at 45 °C, eliminating the need for cryogenic conditions used in the

Table 1. Enzyme Fate Across Six API Projects (KRED = Ketone Reductase)

	Example 1: pregabalin	Example 2: atorvastatin	Example 3: API 3	Example 4: API 4	Example 5: API 5	Example 6: API 6
dosing route/risk management	oral tier 1	oral tier 1	oral tier 1	oral tier 1	oral tier 1	oral tier 1
enzymatic step	post RSM ester hydrolysis	post RSM, ketone reduction	pre RSM, C−C bond formation	pre RSM, ketone reduction	post RSM, alcohol oxidation	pre RSM ketone reduction
product phase	launched	launched	launched	launched	preclinical	preclinical and phase I
regulatory approval	yes	yes	yes	yes	not yet	not yet
stage in synthesis. analysis carried out on the intermediate stage	several steps before API	several steps before API	several steps before API	several steps before API	penultimate step	several steps before API
enzyme type	lipolase (liquid formulation of enzyme produced in *A. oryzae* fermentation)	KRED (liquid formulation of enzyme produced in *E. coli* fermentation)	enzyme (Cells from *E. coli* fermentation)	KRED (liquid formulation of enzyme produced in *E. coli* fermentation)	oxidase (whole cell formulation produced in *Gluconobacter oxidans*)	KRED (dry powder produced from *E. coli* fermentation)
control strategy	test for total proteins, amino acids, and endotoxins; demonstrate fate and purge; no enzyme residue specifications for API	test for total proteins; demonstrate fate and purge; no enzyme residue specifications for API	test for total proteins, DNA, endotoxins and microbiological residues; demonstrate fate and purge; no enzyme residue specifications for API	test for total proteins; no enzyme residue specifications for API	test for total proteins, DNA, endotoxins and microbiological residues; demonstrate fate and purge; no enzyme residue specifications for API	test for total proteins; in-process testing; demonstrate fate and purge; no enzyme residue specifications for API

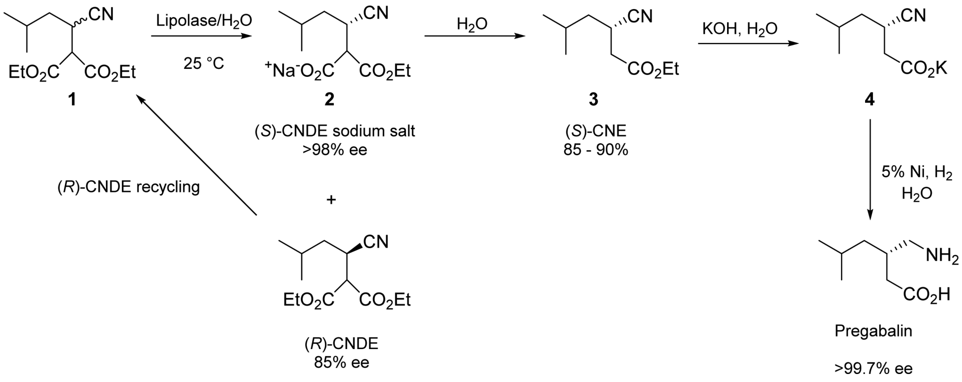

Figure 2. Chemoenzymatic synthesis of pregabalin.

Table 2. Comparison of Methods for Quantitating Proteins in Solution Using Commercially Available Colorimetric Protein Kits[19]

assay	range	comment
NanoOrange	10 ng/mL to 10 μg/mL	Low protein-to-protein signal variability. Detection not influenced by reducing agents or nucleic acids.
BCA	0.5 μg/mL to 1.5 mg/mL	Samples must be read within 10 min. Not compatible with reducing agents.
Bradford	1 μg/mL to 1.5 mg/mL	Proteins precipitate over time. High protein-to-protein signal variability. Not compatible with detergents.
Lowry	1 μg/mL to 1.5 mg/mL	Lengthy, multistep procedure. Not compatible with detergents, carbohydrates, or reducing agents.
absorbance at 280 nm	50 μg/mL to 2 mg/mL	High protein-to-protein signal variability. Detection influenced by nucleic acids and other residues.

chemical reduction process. The enzyme was produced by a recombinant *E. coli* fermentation and formulated as a lysate. Workup of the enzymatic reaction was carried out by addition of acetone which resulted in an organic layer containing diol **6** and an aqueous phase which contained the enzyme. Enzyme was effectively purged from the process by separation of the aqueous and organic phases as demonstrated by analysis of crude **6** samples from 4 lots which showed no detectable protein (Bradford protein assay, LOD < 0.01%). Colorimetric assays like Bradford are discussed in more detail below. Analysis of isolated intermediate **7** from four lots also showed no detectable protein (Bradford protein assay, LOD < 0.01%) further confirming that proteins were purged from the process. This data indicated that biocatalyst related impurities were not likely to be found in **7**, or atorvastatin API, prepared from **7** via six further chemical process steps. The upstream control strategy to not test for biocatalyst related impurities in the intermediate or the API was accepted by all agencies/authorities responsible for drug registration to which the regulatory dossier was filed.[15]

Case Study 3: Biooxidation Using Oxidase Enzyme in Whole Cell System (Example 5 in Table 1). A biooxidation was performed under GMP control on a polyol substrate using a whole cell system containing a membrane bound dehydrogenase enzyme delivering a key ketone intermediate at the penultimate step of an API synthesis. The biocatalyst was delivered as a resting cell slurry, the stability and shelf life of which were thoroughly assessed during development, alongside enzyme activity tests to be run during the biocatalyst manufacture as well as prior to use. The reaction mixture composition allowed for adjustments for variations of specific activity of the biocatalyst cell slurry, while maintaining high volumetric throughput and constant product concentration at the end of the synthesis step. Cells were removed by depth filtration, followed by a 0.2 μm sterilizing filtration. The ketone product was unstable, and all further purification activities were conducted after the subsequent reaction step forming the API. Purification involved ion exchange in catch-and-release mode, multiple carbon treatments and two crystallizations. Bioburden was detected and measured as protein content via Bradford assay, with a limit of detection of below 2 ppm in solution prior to isolating crude API from the first crystallization. This ensured a regulatory acceptable removal of bioburden well before the final isolation.

The following examples are additional case studies not summarized in Table 1.

Case Study 4: Assay by Sodium Docecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). In one case, resolution of a racemic ester by a commercially available protease enzyme resulted in the hydrolysis of the undesired enantiomer to the acid leaving the desired ester enantiomer. The desired ester enantiomer was hydrolyzed by base, and a salt of the desired enantiomer of the acid was isolated. The acid was used for the synthesis of a starting material for an API for an orally active drug product. The protease enzyme was purchased from a commercial source and was obtained by microbial fermentation. SDS-PAGE followed by Coomassie

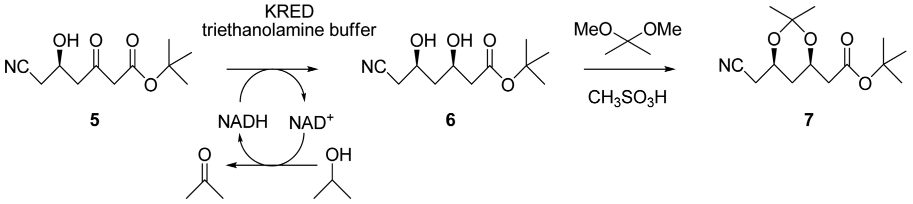

Figure 3. KRED route to atorvastatin intermediate.

blue staining was used to visualize the protein bands on the gel. The protease enzyme solution contained other microbial proteins in addition to the protease enzyme responsible for catalyzing the hydrolysis reaction. The protein corresponding to the protease enzyme was the most prominent band seen in the SDS-PAGE analysis of the enzyme solution. The amount of protein in protease enzyme solution was determined by Bradford protein assay. Three batches of biocatalysis process derived chiral acid salt, and the same three batches spiked with 0.05% enzyme solution protein were analyzed by SDS-PAGE. None of the acid batches showed any protein band, while the protein bands were clearly visible in all 0.05% protease spiked batches. Serial dilution of protease enzyme solution also confirmed that 0.05% enzyme protein was easily detectable on SDS-PAGE.

In another case, a keto acid was converted to the desired chiral amino acid by an amino acid dehydrogenase enzyme and a formate dehydrogenase enzyme, the latter enzyme was necessary to regenerate the NADH cofactor. The chiral amino acid was converted to its Boc-derivative and isolated. The chiral Boc-amino acid is a starting material for the synthesis of an API for an orally active drug candidate. The amino acid dehydrogenase and formate dehydrogenase enzymes were cloned and expressed in a recombinant *Escherichia coli* strain and produced by fermentation. SDS-PAGE analysis of the enzyme solution showed the amino acid dehydrogenase enzyme as the most prominent band followed by the formate dehydrogenase enzyme band and faint bands for other *E. coli* proteins. Five batches of the Boc-amino acid, same five batches spiked with 0.05% enzyme solution protein, and 0.05% enzyme protein solution alone were analyzed by SDS-PAGE. The protein bands were not seen in the Boc-amino acid batches, while they were clearly visible in the enzyme spiked batches and in the enzyme solution. The residual protein was shown to be below 0.05% in the biocatalysis derived starting materials for APIs which is below the reporting threshold for impurities in new drug substances.[14] SDS PAGE method of analysis has been accepted by regulatory authorities.

Case Study 5: Analysis by Amino Acid Content. A very sensitive analytical technique is to digest a sample (normally 100−200 mg) with 6 N HCl. This breaks down residual enzyme and peptide fragments into constituent amino acids, which can then be analyzed.[16] The individual amino acids are then measured and quantitated using HPLC with UV−visible or fluorescence detection with pre or postcolumn derivatization for individual amino acids. This is a very sensitive technique that will detect the presence of enzyme(s) used and any other protein material present as breakdown peptides or extraneous protein from whole cells etc. This analytical technique measures total burden of protein content, but not quantitated levels of individual enzyme/protein components due to the loss of certain amino acids. Due to the harsh sample preparation technique, oxygen-sensitive amino acids like cysteine, methionine, and tryptophan are often destroyed, so are rarely detected. Nonproteinogenic amino acids may be added to act as internal standards for quantitation. One benefit of this technique is that it can confirm the presence of proteins/polypeptide breakdown products other than the enzyme used as the biocatalyst. Techniques used to test for the presence and quantitate specific proteins can sometimes miss other enzymes and breakdown products.

A racemic alcohol was resolved (50 kg scale) with Lipase PS-D using succinic anhydride as the acyl donor. After completion, the supported enzyme was removed and the undesired hemisuccinate extracted into aqueous Na_2CO_3. The TBME solvent was stripped off, and the desired (*R*)- alcohol product distilled in vacuum. Amino acid analysis typically showed values of 0.15−2.5 total ppm amino acids in the distilled alcohol. While it is highly unlikely that any protein or fragments would codistill, it is recommended to still demonstrate the absence of residues going into the API preparation.

A methyl ketone was reduced by an alcohol dehydrogenase enzyme in buffer−isopropanol (IPA). IPA was used both as solvent and the sacrificial hydride source for cofactor recycle with added NADH. The enzyme was cloned and overexpressed in *E. coli* and presented as a crude whole cell paste along with added NADH. After reaction the product was extracted into an organic solvent. The organic phase was washed with water and filtered through a mixture of Celite and activated carbon. The mixture of Celite and carbon was optimal to remove color and traces of protein. The product was crystallized following a solvent swap by distillation. Residual protein as judged by the amino acid content of products isolated using the above amino acid analysis procedure were typically 60−100 ppm. Conversion of this intermediate through several standard chemical transformations and isolation steps led to no protein residues detected in the API (<2 ppm). Analysis for LPS showed no detectable presence of LPS.

Case Study 6: Protein Measurement by Enzyme-Linked Immunosorbent Assay (ELISA). The enzyme-linked immunosorbent assay (ELISA) antibody test is a highly sensitive immunoassay for the detection of specific proteins and protein fragments.[17] Antisera raised against the enzyme is used to develop an ELISA residual protein assay specific to the enzyme of interest. The recognition of relatively small epitopes enables the antiserum to detect all forms of the enzyme, including native, denatured, and fragmented enzyme, although quantitation of fragments is often not too accurate. The antibody−antigen interaction is at such a high affinity that the detection limit is commonly as low as several ppm. ELISA is also advantageous as a result of minimal matrix interference compared with other protein detection methods.

An isolated, engineered ketoreductase (KRED) in the form of a moderately purified lyophilized preparation was used to reduce a ketone to a chiral alcohol with IPA meditated NADPH cofactor recycling. No formal extraction of the product API into an organic solvent took place; the crude alcohol was isolated by a distillation and replacement of IPA (see Figure 4). The crude alcohol was then dissolved in hot IPA, clarified, and then recrystallized by addition of water to give the API in high chemical and stereochemical purity.

Antiserum raised against the KRED enzyme described above was used to develop a residual protein assay specific to the KRED enzyme powder with an LOD of 2 ppm. More than 20 lots of API for an oral drug product have been tested to date, and all lots had residual protein below the LOD. This historical data coupled with the purge rationale of a high aqueous content crystallization, a clarification step, and high aqueous content recrystallization give a significant body of evidence to support removing residual protein testing from future lots of API.

Additional Comments on Residual Protein Assays: Colorimetric/Fluorescence Assays. A number of highly sensitive colorimetric/fluorescence based assays exist for proteins.[18−20] In most cases, reagent kits are available commercially, as in Table 2. These typically work by detecting the presence of amide bonds and therefore in many cases may

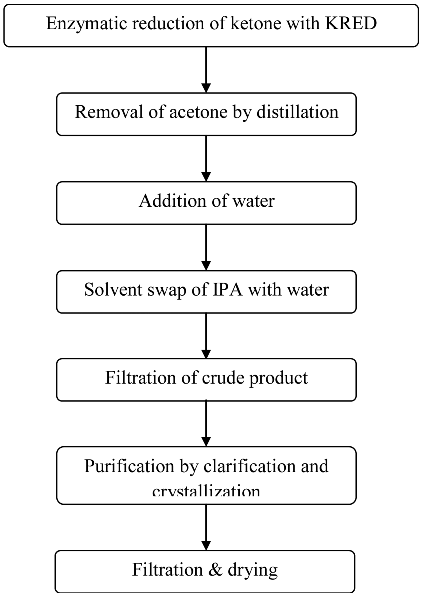

Figure 4. Process flow diagram for case study 6.

be subject to matrix effects from pharmaceuticals/intermediates and certain organic solvents. These assays are typically designed to quantitate protein in aqueous/buffer solution. These colorimetric methods could also be applied to APIs or intermediates with low aqueous solubility by partitioning a sample of the test material between an organic solvent and an aqueous buffer and then testing the aqueous fraction. These colorimetric assays can be used in some cases to measure protein content in API—some examples have been described above, but also for cleaning testing. These assay techniques may not be suitable for highly colored solutions.

Supported Enzymes. There may be occasions where a supported enzyme is the preferred biocatalyst. This may be due to high enzyme cost necessitating multiple reuse, ease of downstream processing, the use of packed bed flow systems, and/or working in predominantly organic solvent. The stability of an enzyme on the supporting matrix is an important parameter for process robustness and economics. Enzyme leaching from the support will impact productivity but also could impact product quality, as could organic materials leaching from the support, especially if such materials are potential genotoxic impurities (PGIs) or have functional groups that would give rise to a positive PGI alert.[21] A risk assessment needs to be carried out for both enzyme and resin and respective potential carryover of each. Risk assessment for the resin could possibly be backed by analytical testing if sufficient data is not available from suppliers and especially if the use of the resin is under conditions outside of the normal operating scope of the resin/material defined by the supplier.

Typical parameters that should be considered are listed below.

- Loss of enzyme from resin under operational conditions.
- Loss of monomers and cross-linking agents used in construction of the polymer/support material.
- Analysis for any undesirable solvents and porogens (volatile materials used to create macroporous structures within growing polymer beads) used in the manufacture of the polymer.

All companies dealing with c-GMP manufacture of APIs and intermediates should have policies and procedures around organic process aids in contact with solutions. A lot of guidance is given for polymeric materials used in food processing or as additives. Further details can be found under FDA 21 CFR 173.25.[22] For an example of the analysis of extractables from resins, see Tou et al., who published a study on organic compounds leeched from a Dowex 1 × 2 resin.[23]

CONCLUSIONS

In this commentary we have described a range of analytical techniques supported with examples to support the development and registration strategy for an API made via biocatalysts. This publication is by no means presented as a comprehensive review of all analytical methodology available to analyze proteins, protein fragments and other biomolecules. For instance, other techniques such as HPLC and HPLC-MS can also be employed.[24]

Our collective experiences are summarized below:

(1) Biocatalysis is an important technology for the more sustainable manufacturing of pharmaceuticals, with several major marketed medicines having successfully been registered with enzyme-catalyzed processes. For a discussion on suitable safe host microorganisms for the production of biocatalysts for pharma use and the avoidance of mammalian derived enzymes and mammalian products in the enzyme production to gain transmissible spongiform encephalopathies (TSE) free certification see our first paper.[1]

(2) Standard organic chemical reactions and workup unit operations including water-solvent extraction, crystallization, distillation and salt formation effectively remove enzyme residues and other biological materials, and these act as effective control and purge points.

Stage	Scale API	Enzyme Development	Phase Appropiate Residual Enzyme Testing
Discovery Support	<1-10's g	Initial Development Hit from kits/screens	No specific testing:
Pre-Phase I Support	~0.1-1 kg	Further refinement of process using initial hit enzyme	Analytical methods for testing of residual enzyme /protein established
Phase I	~1-10's kg	Scale-up of Optimized Screen hit	Preliminary whole enzyme or residual protein testing underway
Phase II	~10-100's kg	Scale-up of Optimized Screen hit: Start to optimize enzyme	Justification strategy specifications developed
Phase III	~100-1000 kg	Futher optimization and selection of Final Enzyme (possible evolultion)	Final enzyme selected:Evidence to support purging and fate strategy in place
Registration/launch	>1000 kg	Final enzyme	

Figure 5. Development timeline for developing an enzyme-catalyzed reaction.

(3) The absence of any enzyme residue in the intermediate/API may be adequately demonstrated in the development phase and/or process validation phase. In addition, routine testing of enzyme residues for release in the API specification maybe or may not be needed, depending on the nature and property of the enzyme residues, risk assessment, and commercial data supported.

(4) In all cases, testing showed the absence or minimal traces of enzyme in the intermediate/API, and in most cases, a range of analytical testing protocols can be used to quantitate low ppm levels of residual enzyme in products.

(5) Use of the appropriate analytical testing supports the above purging and fate strategies. A range of different analytical techniques can be used to quantitate enzyme residues, the choice of which would depend on the analytical capabilities available. Many of the techniques described here can also be run by analytical contract research organizations.

We anticipate the number of examples of API's produced using biocatalysis will increase in the coming years.

Finally, we would recommend enzyme development and residual enzyme testing for a biocatalysed reaction aligned with pharmaceutical development as given in Figure 5.

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Notes

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