Organic Process Research & Development

Concept Article

pubs.acs.org/OPRD

Use of Enzymes in the Manufacture of Active Pharmaceutical Ingredients—A Science and Safety-Based Approach To Ensure Patient Safety and Drug Quality

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ABSTRACT: A significant number of marketed pharmaceuticals contain active pharmaceutical ingredients that are manufactured in part using biocatalysis as a key enabling technology. The utilization of biocatalysis is growing due to significant advances in technologies for enzyme discovery, supply, and improvement, as well as an increased focus on applications for chiral drugs and green chemistry. Nevertheless, there still remains a lack of clarity around quality and regulatory expectations when using biotransformations in research and manufacturing, and this lack of clarity can be a barrier to the uptake and adoption of biocatalysis. This commentary will explore and offer some rational, coherent, and achievable strategies for the use of biocatalysis in the manufacture of small molecule active pharmaceutical ingredients (APIs) based on a scientific, risk-based approach to drug quality and patient safety. We also seek to invite other interested parties to contact us with their views to add to the topics discussed here with the goal of expanding these thoughts into an industry-based white paper.

■ INTRODUCTION

It is often thought that enzyme transformations (or biocatalysis) is a new and emerging technology in the manufacture of small molecule active pharmaceutical ingredients (APIs). In fact, a relatively large number of pharmaceuticals already on the market contain intermediates produced by biocatalysis. These include many historically important examples,[1] such as pseudoephedrine from nearly a century ago, to more recent APIs, such as rosuvastin, atorvastatin, pregabalin, sitagliptin, aliskiren, amoxicillin, cephalexin, and paclitaxel, and a number of steroid-based anti-inflammatory and female contraceptive agents. Other drugs that currently may contain biocatalysed transformations or are being considered for generic switching to synthetic routes using biocatalysis are clopidogrel, valsartan, montelukast, and the human immunodeficiency virus (HIV) and hepatitis C protease inhibitors.[2] It should be noted that this is not an exclusive list by any means, and some of the older drugs listed above (antibiotics, steroids) could have only been accessed in clinically useful quantities at reasonable cost (and in a sustainable fashion) using biotechnology. Indeed, the use of biocatalysis in the production of specific pharmaceuticals and intermediates may be kept as propriety information and never reported.

An explosion in the number and quantities of enzymes available to the synthetic organic chemist has made biocatalysis an increasingly attractive and viable manufacturing option. This in turn has been driven by significant scientific advances in genomics, molecular biology, cloning and heterologous expression, and bioinformatics.[3] As always, the cost of goods and process productivity are key drivers of adoption. In addition, sustainability and the adoption of greener and safer technologies are also clear factors influencing manufacturing route selection today and going forward.[4] Biocatalysis is a green technology, and life cycle analysis shows that the use of recombinant technologies plays a major part in maximising the sustainability benefit of a biocatalysed process compared to a traditional chemical process.[5]

Two excellent examples are the Merck & Co./Codexis synthesis of sitagliptin using a recombinant ω-transaminase[6] and the Pfizer synthesis of pregabalin.[7,8] In the first example, the evolved enzyme had a compounded improvement in biocatalytic activity of more than 25,000-fold, with complete selectivity for the (*R*)-enantiomer of sitagliptin, the active ingredient in Januvia. The new biocatalytic process eliminated the hazardous high-pressure hydrogenation, all metals (rhodium and iron), and wasteful metals removal and chiral upgrade unit operations. The benefits of the new process include a 56% improvement in productivity with the existing equipment, a 10−13% overall increase in yield, and a 19% reduction in overall waste generation.[6] This new process won the "Greener Reaction Conditions" Presidential Green Chemistry Challenge award in 2010.[9] In the case of pregabalin, the active ingredient in Lyrica, the switch to a lipase-catalysed hydrolytic process from a classical salt resolution realized calculated reductions in usage of 185,000 tonnes of organic solvent (92% reduction), 1,890 tonnes of Raney nickel (87% reduction), and 10,000 tonnes of starting material (39% reduction) and elimination of 4,800 tonnes of mandelic acid throughout the manufacturing lifespan of the product. These improvements reduced the E factor (ratio of the mass of waste per unit of product) for pregabalin from 86 to 17.[8] Through these, and many other examples, the benefits of

Special Issue: Safety of Chemical Processes 12

Received: June 11, 2012
Published: July 20, 2012

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biocatalysis as an important component of process improvement and the green chemistry tool box are clearly established.

Several available guidances may serve as established and familiar precedent, for many of the issues related to the use of protein biocatalysts in API manufacture. For example, guidance on API purity (Food and Drug Administration Code of Federal Regulations—FDA-CFR, European Medicines Agency—EMEA, International Conference on Harmonisation—ICH) is very clear around purity, starting materials, and levels of residual solvents,[10] metals from chemical catalysts/reagents,[11] and emerging topics such as potential genotoxic impurities.[12] These levels are usually set as permissible daily exposure (PDE) which are in turn calculated from known or quantitative structure–activity relationships (QSAR) generated toxicology data. This guidance is not so explicit and specific for processes that include biocatalysis—for which several (perceived and real) issues are open to opinion, and where risk-based scientific arguments must be used to ensure the quality of the API and ultimately the safety of the patient. Biocatalysis can be used to support early and late stage development, such as for preclinical and clinical investigations, and manufacturing. For example, atorvastatin, pregabalin, and sitagliptin are all manufactured using biocatalysis in the regulated current Good Manufacturing Practice (cGMP) portion of their syntheses, including up to the last synthetic step.[6,13]

Existing guidances such as for fermentation processes or veterinary APIs[14] and for the production of biologic APIs, provide some insights for issues such as residual proteins in APIs. After all, recombinant proteins are also used as pharmaceutical agents, and of course cell-derived products—such as small molecule natural products, monoclonal antibodies, and vaccines—are increasingly used in the clinic as therapeutic agents. However, the complexity of regulations needed for the patient safety of biologics is substantially more stringent, and it is not scientifically relevant to apply to the relatively low residual levels of known proteins that could be present in chemical APIs produced using biocatalysis during some step(s) of the synthesis.

So while biologics and fermentation guidance exists, and can give some assistance, we believe that it is inappropriate to apply this directly to small molecule API manufacture where enzymes are used purely as catalysts. Thus, we have an interest in formulating more relevant, complete, and simple strategies directly suitable for small molecule processes involving enzyme-catalyzed steps, and this need not be too onerous or different than any other catalysed reaction.

In addition, biocatalysis may be applied to both preclinical and toxicology batches, and there is a general goal to ensure no negative impacts to safety arise from residuals from the biocatalyst. In our opinion—especially for organisations with little or no previous experience with the use of enzymes—there is understandably some uncertainty around quality and regulatory issues which may hinder the uptake of this technology. This is especially true for input enzyme quality and specification and potential biological residues that may be carried through to the API. Thus, a familiar fall back has been a reliance on traditional—less green—and potentially less efficient and cost-effective—chemical technology.

We feel there is a need to debate and establish industry best practice, as current regulatory guidance does not explicitly cover the use of enzymes for small molecule synthesis. As a precursor to an established best practice, we propose a tiered scientific evaluation and safety-based risk assessment approach. Ultimately, we believe the assessment will show that biocatalysis can sit comfortably in the c-GMP manufacture of small molecule APIs, with appropriate understanding and preparation.

■ DISCUSSION

Anecdotally, many potential concerns for the application of biocatalysis in pharmaceutical processes have been expressed or encountered. Our aim is to systematically discuss the range of considerations—including the rationale for each potential concern, options for risk assessment and direct measurement, and approaches for mitigating or eliminating each risk. This may help prioritize real concerns, allay unnecessary concerns, and promote current best practices. Ultimately, our goal is to promote a focus on the most informed consideration and adoption of biocatalysis, appropriate to any particular process.

We will break down the discussion into four sections and discuss each in turn, although several threads are, of course, linked. It is not our intention to provide a totally comprehensive review of each topic, but merely highlight how one might manage these issues. The key topics addressed are as follows:

- Enzyme/biocatalyst source, quality, and specification
- Processing issues
- Residues in APIs and strategies for managing potential impurities
- Comments on general toxicity and tiered risk assessment

Enzyme/Biocatalyst Source, Quality, and Specification. Microorganisms are an important source of most industrial enzymes. The Association of manufacturers and formulators of enzyme products (Amfep) maintains a list of enzymes used in food, feed, detergent, and other industries, and as of October 2009, this list contained more than 260 enzymes, of which approximately 90% were manufactured using native and recombinant microorganisms.[15] Recombinant microorganisms are an increasingly important source of enzymes, as they provide advantages in the development and manufacturing of enzymes with improved properties.[16] The food industry, which accounts for approximately half of the industrial enzyme business, uses many microbial enzymes produced by both native and recombinant strains.[17] Almost exclusively, at this time, recombinant overexpressed (produced at higher concentrations than in native systems) enzymes are used in pharmaceutical synthesis rather than natural enzymes. This decreases biocatalyst cost, maximises selectivity and efficiency, and increases standardization and security of supply when compared to enzymes obtained from natural sources.[18] Issues with biocatalyst lot reproducibility and enzyme isoforms are also avoided with recombinant biocatalysts. The application of recombinant technologies also greatly improves the life-cycle impact of the biocatalyst,[5] and even allows potential access and ready use of enzymes having sequences originally derived from mammals.[19]

Enzyme preparations used in the food industry are regulated by the FDA and other regulatory agencies as discussed in the literature.[17,20] Enzymes are commonly found in fresh and processed food items and are considered intrinsically safe, as they are typically degraded into peptides and amino acids, such as other dietary proteins, and have not been associated with toxicity.[16] In the safety evaluation of enzyme preparations for the food industry, the toxicologic potential of the production strain is considered the primary concern, and this concern relates to the potential synthesis of orally active toxins by the production strain. These toxins include bacterial toxins, which are proteinaceous and cause food poisoning, as well as fungal toxins, that are typically small molecules (MW <1000) that are acutely toxic and may also induce chronic and developmental toxicities. The

dx.doi.org/10.1021/op300153b | Org. Process Res. Dev. 2012, 16, 1986−1993

toxicologic potential of production strains may be managed through the establishment of a safe strain lineage, which involves the use of thoroughly characterized, nonpathogenic, and nontoxicologic microorganisms that have a history of safe use as a starting point for the generation of improved strains.[17]

Toxicologic potential should also be considered in the production of enzyme preparations for the manufacture of pharmaceutical intermediates as a means of managing safety risk. Therefore, the use of microbial strains derived from nonpathogenic and nontoxic strains with a history of safe use should result in a lower theoretical cause for safety concerns. *E. coli* K12 and yeast strains such as *Pichia pastoris* and *Saccharomyces cerevisiae* and the fungal strain *Aspergillus oryzae* are nonpathogenic and nontoxic organisms that belong to risk group 1 (agents that are not associated with disease in healthy adults humans)[21] and have been used for biocatalyst production. Use of microorganisms belonging to risk group 2 or higher for biocatalyst production should be avoided or will require a thorough risk analysis.

Consideration of what should be included on a specification or certificate of analysis (CoA) will depend on the nature of the biocatalyst. A purified isolated enzyme or supported pure enzyme will require less information than a whole cell based catalyst; however, all should have bovine spongiform encephalopathy (BSE)/transmissible spongiform encephalopathy (TSE) statements. The potential presence in APIs of infectious agents such as viruses and prions, which cause BSE and TSE, is a major concern with the use of enzymes obtained from mammalian sources or manufactured using mammalian-derived input materials. A study has shown that virus particles are deactivated by standard chemical processing techniques,[22] but prions are thought to be much more resistant to chemical and standard sterilization techniques and any chemical or combined chemical and heat treatment would be difficult to validate. Therefore, the biocatalyst should be certified by the manufacturer that it is free from virus and BSE/TSE materials and no mammalian products have been used in the fermentation and downstream processing of the biocatalyst. Since it would be difficult to test biocatalysts for the definitive absence of virus or prions to a specification, a clear statement of avoidance of mammalian materials in manufacture should suffice. This is reflected in the guidance quoted below.

> *...complete elimination of risk at source is rarely possible, the measures taken to manage the risk of transmitting animal TSEs via medicinal products represent risk minimisation rather than risk elimination...the basis of regulatory compliance should be based on risk assessment...*
>
> EMEA/410/01 Revision 2 October 2003

> *The pharmaceutical industry should ideally avoid the use of bovine materials and materials from other animal species in which TSEs naturally occur. If absolutely necessary, bovine materials should be obtained from countries which have a surveillance system for BSE in place and which report either zero or only sporadic cases of BSE.*
>
> WHO, Nov 2002

Mammalian products from animals under a certain age and of certain tissue types, or from counties that have never presented cases of BSE/TSE in livestock animals, are acceptable for use.[23] However, audit trails in such cases can be very complex, and the clear-cut recommendation is that no mammalian products are used in production of the biocatalyst. Fortunately, biocatalyst production using recombinant hosts should not require the use of mammalian ingredients. Broader use of standardized hosts, grown in pre-evaluated, defined media, and even expressing synthetic genes, can help essentially eliminate potential hazards from foreign DNA or proteins. If mammalian enzymes are avoided, the most obvious point source of mammalian material is in nutrient mixtures for the fermentation broth. End users need to be aware, however, that fermentation nutrients are not the only place where mammalian products and potentially TSE/BSE can be introduced. Other potential sources are certain amino acids, some antifoaming agents that are tallow-based, and other additives added postfermentation, such as DNase enzymes. Animal charcoal can also be derived from mammals. Some enzyme suppliers may not appreciate these potential sources of contamination, so the end user should ensure focused questions are asked of the biocatalyst manufacturer/supplier. Enzymes derived from mammals, e.g., pig liver esterase and porcine pancreatic lipase, have been used for c-GMP manufacture; the user just needs to be aware of the potential regulatory issues with this type of material.[22] Where it would be beneficial to use a mammalian enzyme, synthetic biology/molecular biology can be employed; if the amino acid or gene sequence is known, a synthetic gene can be produced and the enzyme heterologously expressed in a safe nonmammalian producer strain.[19] In conclusion, concerns around BSE/TSE are best addressed in advance through appropriate sourcing, risk assessment, and certification by the enzyme manufacturer's process. The TSE statement from suppliers should at a minimum contain the name of the material, batch reference, TSE assurance statement, and supplier's details, so any queries can be addressed.

For an established c-GMP process, Table 1 contains items that would be typical in a CoA/Specification for a biocatalyst.

Table 1. Suggested CoA/Specification for a Biocatalyst

test	specification
prion impurities	TSE/BSE free certification
appearance	liquid, solid, colour, etc.
pH	actual or acceptable range
activity	a measure of specific activity (units/weight or volume)[a]
	protein concentration (or cell dry weight)
contamination	certificate no living genetically modified organisms—GMOs—(microbial count)[b]
	certificate for no contamination during fermentation
additives	organic solvents, stabilisers, preservatives—identity and % level
identity	
batch number	

[a]Comment on activity units. It is advisible that the enzyme supplier assays of the enzyme vs the substrate are to be used rather than a simple "test" compound normally used to quantify activity. This may not be feasible in early development but is worth exploring moving toward established manufacture. [b]Food grade enzymes are assayed for contamination for pathogenic organisms such as *Salmonella*. Depending on what the biocatalyst is used for, a science-based approach can be used to assess this risk and if such assays are required.

In setting a specification of a biocatalyst, an understanding of the purity of the enzyme preparation with regard to other proteins that may be catalytically active may be needed. Small changes in the protein sequence around the N terminus may lead to mixtures of closely related enzymes with different catalytic activities.[24]

In the early stages of a development program, it is a precarious strategy to accept and use a biocatalyst on CoA. Positive

Identification that the desired protein is present at the anticipated levels is no guarantee of activity, and a pass for use test is needed to ensure that the reaction shows the expected initial rate of conversion and the desired enantiomer/regioisomer/product is produced. It is also recommended that impurities in feed stocks/solvents are given some consideration as potential enzyme inhibitors.

Further considerations need to be applied to supported biocatalysts, where the enzyme is attached to a solid support through either absorption or covalent immobilization. Whole cells can be likewise used encapsulated. Immobilization may facilitate enzyme removal after reaction, allow for recycling of expensive/scarce enzymes, or enable chemistry in predominantly organic solvent. Catalyst immobilization is not an issue with biocatalysis per se, but with any processing unit operation that uses synthetic polymeric substances that are generally regarded as insoluble but which do have the possibility of leaching organic compounds into the process stream and, hence, potentially compromise the quality of the product. Of particular concern are impurities/leachates that are known or suspected carcinogens, such as divinylbenzene, or whose structures would give a positive alert in potential genotoxic impurity screening.[12] It is anticipated that facilities involved with c-GMP manufacture would have standard operating procedures to assess the risk of plastics/polymers in contact with process streams.

It is always worthwhile to run a few simple tests for enzyme leakage. Although some loss will not be a c-GMP quality issue, this will affect the usable life span of a supported biocatalyst and overall process economics. It is also recommended that any potential loss of enzyme into a process stream is investigated to determine if the enzyme remains active, especially in aqueous streams. For certain enzymes, such as lipases and proteases, this can give rise to process deviations if contact is later made with hydrolysable substances or solvents.

It can be expected that, in stirred tank reactors, there could be some attrition of enzyme resins; however, normal polishing filtration will remove any insoluble particulates. Generally, attrition or grinding of enzyme resins will shorten their usable lifespan and consideration should be given to this aspect when designing the agitator/stirring regime for scale-up in stirred tank reactors. Whilst most polymeric supports are chemically stable under normal process conditions, it should be recognized that some polymer-based catalysts, generally regarded as being very stable, such as Novozym 435, can degrade under harsh conditions and the soluble degradants contaminate the process stream.[25]

Processing Issues. With regard to processing, apart from the enzyme, other additives can be used in biotransformations.

Other materials typically added or introduced to enable biotransformation reactions are generally benign, including cofactors such as nicotinamide adenine dinucleotide and the corresponding phosphate (NADH, NADPH), and pyridoxal-phosphate, and other materials, such as glucose and buffer salts. Generally, these cofactors will be removed using normal purge and control methods and further downstream processing.[26] Metal ions are required by some enzymes and may be added to biotransformation reactions separately or as part of the biocatalyst. Therefore, these metal cofactors, which can include copper, iron, manganese, magnesium, molybdenum, nickel, selenium, and zinc, may require monitoring to determine their fate.

With regard to storage and reuse of biocatalysts, enzymes, whole cells, and related preparations need to be stored under conditions that are known to retain enzyme activity. If stored cold or frozen, then due care needs to be taken when scaling-up, since warm up and hold times may be significantly different from those in the lab or pilot plant, and the lengths of freeze–thaw cycles can impact biocatalyst performance. The effect of longer cycle times on enzyme activity needs to be known before a batch of biocatalyst is charged to a reaction.

It is possible to recover and reuse supported biocatalysts. This is probably not viable or desirable with soluble isolated enzymes or whole cells, unless the whole cells are supported or encapsulated. In an ideal situation, the biocatalyst will be retained and used in some kind of continuous or flow process until spent. In batch operation, if storage is needed between campaigns, the biocatalyst needs to be stored under conditions that retain activity and prevent microbial growth.

With regard to the cleaning of c-GMP facilities, denaturing conditions are recommended, such as aqueous acid/base or heat, to ensure any residual enzyme activity is removed. If viable GMO cells have been used, local regulations need to be followed regarding deactivation and disposal. Reactors can then be washed/cleaned down. If supported biocatalysts have been added, confirmation of removal will be required. If possible, supported catalysts are best used in contained vessels/packed beds. Residual protein in cleaning solutions can be determined by simple colorimetric tests such as BCA or Bradford protein determination, but be aware that many API molecules and intermediates will give positive tests with these reagents. Therefore, cleaning/testing for residual organics is best done before cleaning/testing for residual protein. Depending on what analytical capability is available, more sophisticated testing can be used—SDS-PAGE gels or HPLC/LC-MS analysis for specific proteins. A very simple way to deal with both proteins and organics is to clean down to a total organic carbon (TOC) limit. Related to cleaning issues and plant utilisation, the general characteristics of biocatalytic processes may also open up additional options for using disposable equipment, as used in some biopharma production processes.[27]

Residues in API and Strategies for Managing Potential Impurities. Potential impurities associated with small molecule APIs manufactured using enzymes include the enzymes themselves, other host cell proteins, DNA, endotoxins, cell wall debris, and antibiotics derived from the fermentation and downstream processing of the biocatalyst. Degradation of these potential impurities may result in the formation of additional impurities such as peptides, amino acids, and polynucleotides. With regard to managing potential contaminants, we will introduce the purge and control strategy and the consideration that these sensitive biomolecules would not survive standard chemical processing unit operations. Typically, residual protein levels of 5–100 ppm are found in intermediates directly isolated from the biocatalysis stage.[28,29] If this is several stages from the API, and many chemical processing steps are involved, reactions, solvents, filtrations, salt formations, etc., then potential impurities in the API will likely be negligible, although this should be demonstrated by analysis in the development phase. Most testing does not reveal detectable levels of DNA. Typically, batches can be assayed for DNA or residual protein in the development stage, but it is not recommended to build such tests into release specifications for the API. Typically, for oral products, we would propose that proteins be regarded as other organic impurities under ICH guidelines which would equate to 0.1% for identification and qualification, although they are rarely found at levels above ~50 ppm, or less even in intermediate

stages, and higher levels could be reasonable. Testing can be directed at specific proteins, such as the enzyme being employed as the catalyst, especially techniques that will detect both protein and any smaller peptide fragments, which are the most likely scenario for contamination. Techniques such as MALDI-TOF (which could give sequence confirmation) or LC/LC/MS have been used, but most commonly, digestion and amino acid analysis[30] are suitable for this purpose. This type of testing should detect 20 ppm or less of residual protein. Likewise, DNA can be assayed by specific techniques, such as threshold DNA analysis,[1] or via digestion and nucleotide base analysis.

Lipopolysaccharides (LPS; also referred to as endotoxins) are another potential concern, particularly in the case of injectable or inhaled drugs, or potentially for early batches for preclinical, animal toxicology. Metrics for acceptable limits of LPS for valid in vivo animal studies have been reported.[31] Considering the use of *E. coli* hosts for the production of an increasing number of commercial biocatalysts, LPS are a potential concern in APIs manufactured via biocatalysis. Fortunately, standard, sensitive tests for LPS are established, and a robust purge and control strategy should be suitable for controlling lipopolysaccharides, as they are highly water-soluble and sensitive to decomposition under typical organic chemistry processing conditions.[32] Occasionally antibiotics such as kanamycin are used in the fermentation process to produce enzymes for biocatalysis. Owing to the low levels employed, and the high instability of this material, we do not consider this to be an area of high concern.

In the synthesis of small molecule APIs using enzymes, the location of the enzymatic reaction(s) in the overall synthetic scheme should be considered in the risk assessment as a factor in regard to potential enzyme-related impurities. A synthesis which contains an enzymatic reaction in the final step should raise a higher level of scrutiny than one in which one or more chemical steps lie between the enzymatic reaction and the API. This is based on the consideration that additional steps provide opportunities for purging of potential enzyme-related impurities. Typically, enzyme-related impurities can be systematically purged from small molecule APIs based on fundamental physical differences, as discussed below.

Biologically derived materials differ fundamentally from small molecule APIs in their chemical structure and physical properties. One of the main features that differentiate small molecules from biologically derived impurities is molecular weight. The molecular weights of small molecule APIs are typically less than 1000 while protein molecules range from 10K to over one million Da, and DNA molecules range from 200K to substantially more than one million Da. LPS also range in MW from 10K to more than one million Da. These fundamental differences between small molecules and biologically derived molecules can be exploited for their separation. Therefore, techniques used for the purification of biological products (e.g., filtration, chromatography, precipitation) and those used in chemical processing (e.g., precipitation, filtration, liquid−liquid extraction, distillation, and crystallization) could be used to remove biological impurities based on differences in molecular weight, solubility, vapor pressure, and other properties.

Denaturation of biological molecules typically results in precipitation and, therefore, provides an opportunity for removal by physical methods. Proteins are denatured by a variety of treatments, including organic solvents, strong acids or bases, salts, and heat. DNA can also be precipitated by treatment with organic solvents and removed. Filtration is a useful technique for the removal of biological materials that have been precipitated. Filtration is also useful for the removal of high molecular weight materials that are in solution. Microfiltration removes particles ranging from 0.02 to 10 μm while ultrafiltration can be used to remove dissolved macromolecules ranging from 1000 to 500,000 MW. Filtration with activated carbon is also effective for the removal of biological material, especially endotoxins.

Proteins, DNA, and endotoxins and their potential degradants have very low solubility in most organic solvents. Therefore, liquid−liquid extraction with an organic solvent provides an effective method to purge biological impurities following an enzymatic reaction step. Distillation is useful for the purification of small volatile molecules as proteins, DNA, and endotoxins have negligible vapor pressure. This technique may be useful for early intermediates derived from enzymatic processes but is not generally applicable to later intermediates and APIs, as these are usually solids.

FDA and EMEA guidance on fermentation products and semisynthetics states that concern overprotein contamination is minimal if the product has been through standard chemical operations such as solvent extractions, washing, and crystallization.[33]

Although the possible activity of any trace residual enzyme in API is highly unlikely, this subject does seem to be a concern. Clearly, as with the levels of potential contaminates, the concern will differ with route of administration of the API in question and the enzyme used. A science-based argument could be used that testing of API for residual enzyme activity is not valid if the protein level is 10−50 ppm. This science-based argument should also include a risk assessment that considers the type of enzyme activity and whether there has been any report of toxicity related specifically to the enzyme's activity. Most enzymes will denature to some extent during processing. Lipases and proteases, and enzymes engineered to be stable to solvent and pH extremes, and enzymes derived from thermophiles or engineered to be thermostable could be more problematic. Here some simple tests and a science based argument can be employed. A sample or solution of the enzyme could be exposed to one or more of the chemical processing steps and then assayed using an activity test to demonstrate that deactivation had occurred. If following risk analysis, then additional processing steps such as heat deactivation or ultrafiltration may be introduced.

Comments on General Toxicity of Enzymes and Tiered Risk Assessment. The toxicology of industrial enzymes has been described as unremarkable[34] and safety evaluations of enzymes used in the food[17,20] and detergent[34] industries have shown that the large majority of these enzymes do not cause systemic toxicity. Ingested proteins are usually digested into peptides, which are poorly absorbed in the GI tract, and amino acids, which have low oral toxicity. Since enzymes may be derived from biotechnological processes, ICH S6 (Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals)[35] may have some relevance even though this guidance is intended for therapeutic large molecules. ICH S6 notes that genotoxicity and carcinogenicity evaluations are generally not warranted for biotechnology-derived products as they are not expected to interact directly with DNA. Further to this point, Pariza and Johnson[17] concluded that it was unnecessary to include genotoxicity testing in safety evaluations of enzyme preparations used for food processing.

Enzymes may also contain DNA from the producing organism as a potential impurity. The risk of ingesting DNA has been considered in connection with the safety of genetically modified

foods and is discussed in a position paper (Society of Toxicology, 2002)[36] and a Royal Society report (Royal Society, 2002).[37] These reports conclude that the risk of adverse effects from ingesting DNA in foods is minimal and cite several key points, including the following: (1) ingestion of significant amounts of DNA in the typical human diet (0.1−1 g/day), (2) no evidence for direct toxicity of dietary DNA, and (3) extensive breakdown of DNA in the digestive system. Based on these reports, the risk of adverse effects from potential DNA impurities in orally administered APIs is very unlikely.

Occupational asthma and allergy are the main adverse events associated with exposure to enzymes, and they became prominent with the introduction of alkaline and heat stable proteases into detergent products in the 1960s.[34,38] These adverse events are due to an immune mediated response, with Type 1 hypersensitivity being the most common. Exposure to enzymes either through contact, ingestion, or inoculation can result in allergen-specific IgE that elicits symptoms of hypersensitivity in certain individuals.

Irritation of skin, eye, and other mucosal sites has been reported as a potential adverse effect of enzymes used in the detergent industry;[34] this is most relevant to Operator/Process Safety. Studies reported irritation effects on skin after prolonged contact with high concentrations of proteases and not with other detergent enzymes, such as cellulases, amylases, and lipases. The effects were mainly attributed to proteolytic action of proteases on skin and were mild and reversible. Proteases are used in biocatalysis and could therefore present a risk for skin and eye irritation with APIs administered nonorally. However, this risk could be mitigated by demonstrating purging of intact enzyme or deactivation during processing.

A key factor for a science-based tiered-risk assessment is the route of administration of the API. A variety of reports are available in the literature regarding allergenicity (or lack thereof) from proteins, peptides, and enzymes introduced via the oral, inhalation, dermal, ocular, or parenteral routes including IV and IM injection. Hammond and Cockburn have reviewed the available literature regarding proteins developed through biotechnology and introduced into crops.[39] Importantly, they conclude that the oral bioavailability of such proteins is negligible, although an exception is ovalbumin, a protein stable to digestion. The authors further reviewed the available data describing the safety of a variety of proteins and enzymes in feeding studies using experimental animals, and they concluded that there is negligible concern for adverse health effects from these commonly used proteins, other than the potential for immunogenicity.

The literature reports hypersensitivity and immune reactions to some proteins via inhalation or dermal exposure at manufacturing sites or by administration of a therapeutic enzyme. Heat- and alkaline-stable enzymes were introduced into detergents in the 1960s, and the development of enzyme-specific occupational asthma (OA) and other allergic sequelae in the detergent manufacturing industry was described in the literature soon thereafter.[38] Efforts to reduce exposure and the adoption of exposure guidelines led to a reduction in cases, although outbreaks of OA were described in industrial settings where adherence to exposure guidelines was poor,[40] as cited by Sarlo and Kirschner.[38]

The observation of OA and other allergic responses to enzymes has not been limited to the detergent industry. Lactase-hypersensitivity contact and allergic rhinoconjunctivitis, with individual generated IgE antilactase antibodies due to exposure to lactase, were reported in a single individual at a pharmaceutical product manufacturing plant.[41] Baur reviewed the use of enzymes across various industries and concluded that in the occupational setting (1) all the surveyed enzymes can behave as respiratory sensitizers in a dose-dependent manner, (2) dermal contact may also be a factor in the induction of IgE-mediated cutaneous and respiratory allergy, and (3) the preventative measure of most importance is to reduce the potential for inhalation exposure.[42]

The induction of hypersensitivity and/or antibody production has also been evaluated in well controlled experimental animal studies. A study in mice evaluated the immunogenicity of two enzymatically produced antibiotics produced using either chemical or enzymatic synthesis. The presence of residual enzyme-derived protein at a level of 35 ppm did not confer increased anti-IgE antibodies by the preparation.[28] Malley and Baecher showed that repeated intracutaneous injections of the bacterial enzymes alcalase and Monsanto DA-10 caused wheal and flare skin reactions in rhesus monkeys.[43] Coate et al. chronically exposed cynomolgus monkeys by inhalation to an enzyme mixture of alcalase and Milenzyme 8X, and induced an antibody response[44] that was subsequently shown to result in precipitating antibody responses in the sera, but not the lungs.[45]

Relevant Risk Assessments. A review of regulatory sites and other external information does not show any specific regulatory guidance regarding risk assessment practices for residual levels of enzyme-associated impurities in APIs. The most relevant risk assessment information appears to come from (1) the use of enzymes in the food industry and (2) occupational hygiene settings in which enzymes are used in manufactured products, such as detergents. Enzymes are ubiquitous in nature and when orally ingested are degraded and metabolized into smaller peptide fragments and individual amino acids. Enzymes naturally present in the human diet have not been associated with toxicity and are considered intrinsically safe.[16]

Risk assessments of the use of microbial enzymes in food processing have been reviewed.[17,20] Pariza and Johnson provide a comprehensive decision-tree assessment in taking into account the varying factors that could impact on human health risk assessment.[17] These authors describe a strategy employing a well-characterized, nonpathogenic, nontoxic "safe strain lineage" of microbes and describe characterization of the safety of the production strain as being the primary consideration in safety evaluation. Spök noted that the majority of industrial enzymes are presently used in the food industry.[20] The author describes potential safety concerns as being allergenic, irritative, or otherwise toxic in nature and notes that the allergenic and irritative risks are primarily issues of occupational health in the industrial production of enzymes. Olempska-Beer[16] stresses many of the same points and further notes that, since 1977, the FDA has reviewed more than 35 Generally Recognized As Safe (GRAS) notices for enzyme preparations.[46] A review of this site shows several additional enzyme preparations listed since the time of the Olempska-Beer review.

The general conceptual framework of a recent risk-based classification system for genetically modified foods[47] is comparable in several ways to our approach for biocatalysis safety evaluation. The stepwise assessment is based on a variety of factors relevant to either toxic or antinutritional effects of modified foods or allergenic effects. Factors related to potentially toxic/antinutritional effects include direct exposure to the gene product(s) in the food product, the nature of the gene products, the expected change in total dietary exposure, prior knowledge of

mode of action, structure−activity characterization, digestion/degradation products, interactions, and prior history using the gene factors. The safety of donor organisms was also discussed. Graded approaches to testing genetically modified foods were proposed in a companion paper.[48]

The soap and detergent industries (SDA) have continued to follow the issues around respiratory allergies in workers in detergent manufacturing plants and most recently have published their "Risk Assessment Guidance for Enzyme-Containing Products".[34] Other entities have addressed this as well; for example, the Finnish Institute of Occupational Health issued a report entitled "Exposure, Sensitization and Allergy to Industrial Enzymes".[49] These assessments are oriented toward inhalation exposures, and key elements of the approaches include management of enzyme use through hazard identification, detailed exposure assessment in the workplace, characterization of risk, and then application of risk management steps.[34] Human and Environmental Risk Assessment (HERA) recently risk assessed subtilisins (proteolytic enzymes) that have their primary use in detergents and household cleaning products.[50] Subtilisins are of bacterial origin and are produced using a fermentation process. It was concluded that the tested products were of a low order of oral toxicity, and it was further noted that the toxicological potential is even further reduced when the enzymes are inactivated. The key human health concern identified by HERA, as for our assessment, is the potential for allergenic responses upon inhalation exposure. The HERA assessment developed a benchmark approach to quantitative risk assessment and concluded that allergic symptoms can be excluded in consumers using subtilisin-containing products when such exposures do not exceed an air concentration of 1 ng/m^3; these authors therefore reached the same conclusion as the SDA as discussed above. Other routes of exposure were also considered.[50] It was concluded that a skin and eye irritation No Observed Effect Concentration of 0.07% for humans was justified on the basis of available data. For ocular exposure, it was concluded that subtilisins in consumer products were not expected to cause more than a mild transient irritation. Overall, this risk assessment concluded that *"the use of Subtilisin in laundry and cleaning products represents no safety concerns for consumers."* and noted that occupational risk has been addressed elsewhere (as described above).

Proposed Approach to Risk Assessment and Recommendations. Based on the previous sections, it is clear that each case involving biocatalysis in the pharmaceutical industry will present a different set of circumstances. As such, a case-by-case approach to the evaluation and assessment of potential risk is needed. We describe here a tiered approach to the risk assessment. The decision tree approach is intended to assist the reader in determining the degree to which further consideration of the various points in this paper should be considered in applying these concepts to the specific application being proposed.

The first two questions to consider are whether the drug is administered via the oral route and whether the proposed enzymes are relatively pure and are derived from a precedented host expression system (see Figure 1). If the answers are yes to both of these questions, it is proposed that this presents the lowest level of concern (Tier 1) and that the sponsor can default to guidance contained in ICH Q3A to evaluate the product. If the answer to either of these questions is no, however, this would elevate the level of potential concern (Tier 2), and the sponsor would have to consider whether other control strategies beyond those in Q3A would be appropriate. Such a strategy might be

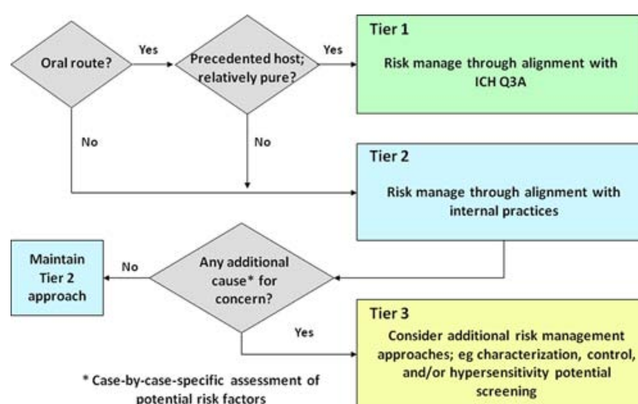

Figure 1. Schematic flow diagram illustrating various factors contributing to the level of concern in a risk assessment for the use of biocatalysis in small molecule pharmaceutical manufacturing.

based, at least in part, on considerations related to the manufacture of biopharmaceutical agents. In conjunction with those considerations, it would be necessary to consider whether other potential risk factors might exist for the case under consideration. For example, one such factor would be for an inhaled product, and given the issues related to hypersensitivity discussed in previous sections, this would add to the level of concern. A Tier 3 designation would be assigned in this case, and this would trigger additional risk mitigation activities. These could include, for example, additional characterization of the potential residual impurities, additional control measures, or increased safety monitoring and/or pharmacovigilence.

■ SUMMARY AND CONCLUSIONS

In conclusion, we believe that we have established a framework for some sensible and rational strategies that, if followed, lower any hurdles in the application of biocatalysis in the manufacture of preclinical and clinical material. Hopefully, these help to remove potential barriers in this area for some new users. In our opinion, the key issues to consider are (1) biocatalyst quality and specification; (2) process issues—purge and control strategies/residual levels of relevant biomolecules in the API—for oral products protein can be regarded as a standard impurity under ICH guidelines; and (3) tiered risk assessment.

A key driver to publish this paper was to stimulate interest from other companies working in this exciting and rapidly emerging field. The authors would ideally like to expand this forum and work toward an industry white paper, the value of which will be greatly enhanced with wider representation from all interested parties working, or planning to work, in this sector. We aim to have this work complete by mid 2013.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Deborah Finco, Stuart Goodall, Ned Mozier, Ron Ogilvie, Tim Watson, Bob Sato, and Chris Savile for helpful discussions.

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