Characterization of Alternatives to Animal-Derived Raw Materials

"Animal-Free" May Not Mean "Problem-Free"

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Raw material selection, testing, and control strategies are an integral part of the preparation of a well-characterized biological product (**1**, **2**). Animal-derived raw materials may be used in cell culture, fermentation, purification, and/or formulation of biopharmaceuticals. Because any animal-derived component could carry a theoretical risk of prion (**3**) or other adventitious agent transmission (**4–6**), it is desirable to replace those components with equivalent materials that are not derived from animal sources. Such raw materials can be derived from bacteria, yeast, fungi, or various types of plants. Often it is possible to find a microbial or plant-derived component that meets or exceeds the desired functionality of the original animal component.

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However, because the alternatives to animal-derived raw materials come from various sources, they may have very different impurity profiles, and their impurities could present additional risks (**7**). Therefore, it is incumbent upon users of such materials to understand product- and process-related impurities associated with the new component and the potential impact those impurities may have on the safety, purity, and efficacy profile of a biopharmaceutical product.

The first step is to thoroughly understand the vendor's manufacturing process by performing a vendor audit. Next, a strategy should be developed for characterizing each component biochemically and functionally to ensure that it does not affect the approved process. The biochemical and functional assays used depend upon the source of the component and what perceived risks related to it are found during searches of the scientific literature. The final step may require demonstrating clearance of the component through the process (during the course of process validation) and developing a scheme for routine monitoring of cleaning effectiveness. All raw materials must be appropriately characterized and qualified for the process to be in compliance with cGMPs. A number of regulatory guidance documents are available (**8–21**) that discuss how the characterization/qualification should be performed and documented.

Why Consider Alternatives?

Using animal-derived raw materials involves many drawbacks. For example, animal-derived raw materials may increase the risk of transmitting prions or other adventitious agents that are difficult to control (**22, 23**). Although the risk is small, eliminating that risk is preferable. Several regulatory guidance documents and reviews have discussed theoretical risks associated with components derived from different animal sources. Most documents identify the animal tissues associated with the greatest risk of prion transmission (with neural tissue, such as brain and spinal cord, at the top of the list) and suggest ways to minimize that risk. In addition, although methods to inactivate prions and many viral pathogens without altering the integrity of therapeutic proteins have been described (**24**), currently no reliable test for prions is available (**25**), nor is there a validated method for inactivating or removing them. It is therefore difficult to be completely confident that a particular animal-derived component is truly prion-free.

Animals are also vulnerable to a large number of infectious agents, some of which are transmissible to humans. In addition to prions, some animals may be host to viral, mycoplasma, bacterial, or mycological infections. Some of these adventitious agents may generate waste and other byproducts that induce adverse reactions in humans such as endotoxins, mycotoxins, or other "biological effectors." Such agents may not be easily destroyed or removed by the raw material manufacturing process.

Mammalian-derived components can be considerably more complex biochemically than similar materials sourced from simpler organisms. For example, mammalian glycoproteins may have more complex, heterogeneous carbohydrate attachments. In addition, the impurities found in mammalian components can be diverse and difficult to remove. Some typical raw materials found in a recombinant protein process are listed in Table 1, along with possible alternative sources for their replacement.

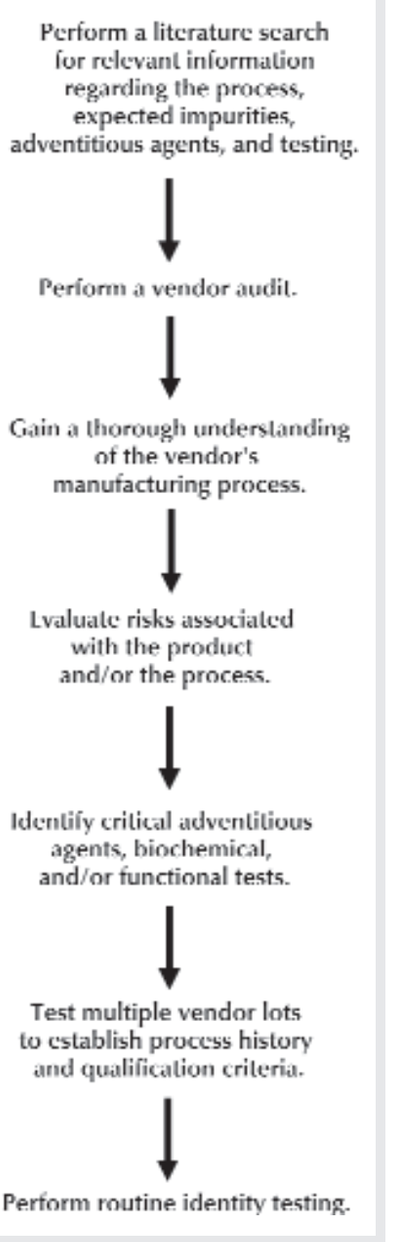
Types of Alternatives

Alternatives to raw materials derived from mammalian sources are available from commercial vendors and can be evaluated for performance comparability. Microbial sources are the most common, and typical raw materials can be obtained from bacteria, yeast, and fungi. Plant sources are also appropriate, and many components can be isolated from plant parts including grains and other seeds, fruits, wood pulp, and bark. Raw materials are also derived from aquatic sources such as fish, shellfish, algae, or insects, but those are less frequently used because of their limited commercial availability.

Assessing Immunogenicity: The risk factors associated with a particular raw material depend upon its source and method of manufacture. Components from different sources may have different microbial and/or viral contaminants, different product- or process-related impurities, and different potentials for adverse events. Table 2 lists some examples. Risk factors can be placed into several categories: immunogenicity, modulation of biological activity, exposure to pathogens or other adventitious agents, and impact on potency. Only the first (immunogenicity) is discussed in this manuscript. Certain relevant references (**5, 6, 26–28**) detail the other risk factors.

The potential for immunogenicity of a raw material in humans is difficult to predict because extensive testing in animal models may not provide sufficient sensitivity. Immunogenicity must be evaluated in clinical studies and may or may not pose a significant risk to the patient population depending on the application. Immunogenicity should be evaluated in the context of a patient's immune status. For example, pediatric patients (particularly neonates or infants) and those who are immuno-suppressed may mount only a minimal response compared with older children and immuno-competent adults. Peptides weighing 1,000–6,000 Da are considered "potentially immunogenic," whereas those smaller than 1,000 Da are unlikely to elicit an immune response. Chemically complex impurities can also elicit an immune response (**29**).

Figure 1: Strategy for characterization of raw materials



Strategy for Characterization: To ensure that potential risk factors are identified and appropriately evaluated, a verification strategy is

required. This strategy should include the steps shown in Figure 1.

The vendor audit is a critical part of a raw material qualification program. An audit should have four objectives: developing a good working relationship with the vendor; making a quality agreement part of the supply agreement; gaining a thorough understanding of the vendor's raw materials, process, equipment, and procedures; and ensuring adequate cGMP compliance, segregation between different products, and change control (notification of changes).

An Example: Protein Hydrolysate

A generally accepted practice is to remove raw materials used as nutritional supplements in cell culture or fermentation during the purification process, regardless of potential toxicities. Removal is demonstrated using spiking studies or sufficiently sensitive assays. For example, protein hydrolysates are frequently added to recombinant protein production processes, and such hydrolysates can be immunogenic upon parenteral administration. They may be made using autolysis, proteolysis, or pyrolysis, and are often composed of a large proportion of free amino acids with a lesser amount of proteins/peptides, polynucleotides, and lipids. Extensive hydrolysis has proven to be an effective strategy to minimize allergens in the treatment of allergic disorders and dietary management, even for infants sensitized *in utero* or early in life (**30**).

Because immunogenicity can be a risk when using protein- or peptide-containing raw materials such as protein hydrolysates, appropriate steps to mitigate that risk should be considered. One approach to minimizing risks from protein hydrolysates is to partially purify the hydrolysate by ultrafiltration, which removes most high molecular weight proteins/peptides, polynucleotides, and lipids — or using diafiltration to remove unwanted low molecular weight components. Extensive hydrolysis of proteins and peptides, which effectively removes all antigenic epitopes, is preferable for the production of biopharmaceuticals.

Depending on the hydrolysate source, it may be necessary to test for adventitious agents. These include any viral, mycoplasma or bacterial agents or byproducts that might be initially present in the raw material or introduced during its production and processing. Examples of tests for such agents or byproducts include bioburden, sterility, endotoxin, PCR, and other appropriate cell culture assays for adventitious agents.

Relevant biochemical characterization will help the user understand the prevalence of a particular risk (by determining, for example, peptide size and content). Thorough characterization is required to obtain the necessary understanding of a raw material and its production process to allow for documenting reproducibility and control. For protein hydrolysates, a critical question is, "What types of peptides are expected (that is, does the process use proteases, acid digestion, and so on)?" The most likely analytical tools for characterizing a hydrolysate are

Figure 2: Fit to an exponential function (blue line) of the total amino acid amount (circles) in an untreated protein hydrolysate sample, as a function of cycle number in a protein sequencer. By cycle 23, the measured value (in pmoles) for the total amino acid content is statistically indistinguishable from that of the sample following acid treatment (squares), which reduces the peptidic component to free amino acid, di- and tripeptides. Free amino acids are not detected in the protein sequencer and are quantitated separately by amino acid analysis.

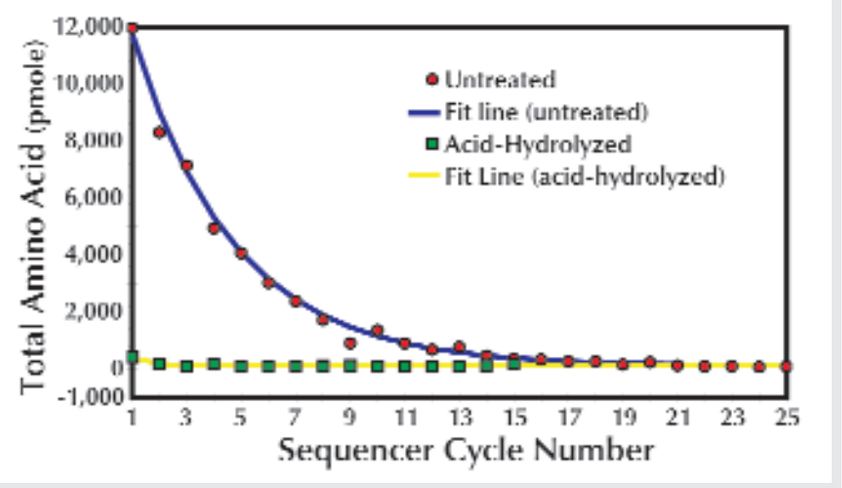


Table 1: Typical raw materials found in a recombinant protein process

Raw Material	Animal Source	Alternative Source
Albumin	Bovine	Recombinant albumin (yeast) Other microbial proteins Other nonprotein substitutes
Transferrin	Bovine	Other small-molecule iron chelating agents
Insulin	Porcine, Bovine	Human recombinant insulin
Tween-80	Bovine	Plant
Lipoproteins	Bovine	Plant Microbial Cholesterol (synthetic)
Fetal bovine serum	Bovine	Recombinant albumin (yeast) Other microbial proteins
Gelatin	Bovine	Fish Other nonprotein substitutes

Table 2: Nonmammalian source risk factors

Source	Microbial/Viral Contaminants	Impurity Profile	Biological Effectors
Bacteria	Cross-contamination with other bacteria, fungi, or bacteriophages	Endotoxins Cell wall constituents	Cell wall constituents Endotoxins Exotoxins
Yeast/Fungi	Contamination with bacteria	Cell wall constituents Fungal proteins Carbohydrates Nucleic acids	Cell wall constituents Mycotoxins
Protozoans	Contamination with bacteria	Cell wall consituents Protozoan proteins Carbohydrates Nucleic acids	Toxins
Fish/Shellfish	Contamination with bacteria	Fish proteins Carbohydrates Nucleic acids	Toxins
Aquatic plants	Contamination with bacteria	Plant proteins Carbohydrates Nucleic acids	Toxins Cell wall constituents
Grains/seeds	Contamination with bacteria	Plant proteins Carbohydrates Nucleic acids	Mycotoxins (aflatoxin) Lectins
Other plant byproducts	Contamination with bacteria/fungi	Plant proteins Carbohydrates Nucleic acids	Mycotoxins (aflatoxin) Lectins Phenolic resins
Insects	Contamination with bacteria, fungi and/or viruses	Insect proteins Carbohydrates Nucleic acids	Exoskeleton constituents

amino acid analysis (AAA) and N-terminal sequence analysis using Edman degradation.

The first method (AAA) can be used to evaluate the content of amino acids in a protein hydrolysate preparation following acid hydrolysis. The proportion of "peptidic" amino acids can be quantified and compared to free amino acids by performing AAA with and without acid hydrolysis of the sample. AAA can also be done after ultrafiltration using filters of various pore sizes for a rapid assessment of the size distribution of the peptidic fraction.

N-terminal sequencing will yield multiple N-termini for each unblocked peptide during each sequencer cycle. If a Hewlett-Packard automated N-terminal sequencing system is used, free amino acids are eliminated before the first cycle. The total amino acid amount from each cycle can be collated and plotted against the cycle number. As peptides are fully sequenced, their contribution to the total amino acid content of a sample will cease in subsequent cycles. If proteolytic cleavage is a stochastic process independent of the substrate peptide length, the total amino acid amount will decrease monotonically with increasing sequencer cycle number. Results can then be fitted to an exponential function and the number of cycles that yield significant signals above background correlated to the size and relative proportion of peptides in the original mixture. Although comparable recovery of amino acids is assumed, a possible limitation of this approach is that peptide N-termini that are "blocked" will not show a sequencing signal. It should be noted that some amino acids are destroyed by the technique.

In the example of a protein hydrolysate shown in Figure 2, the total area under the curve was calculated to give a molar size distribution. For this study, only peptides between nine and 23 amino acids long were considered, because those smaller than nine amino acids are not thought to be potentially immunogenic and those larger than 23 amino acids do not show a signal above background by the final cycle. In analysis of this protein hydrolysate, the median distribution of peptides was 11 amino acids. We calculated the concentration of potentially immunogenic peptides from the percentage of potentially immunogenic peptides and the total peptide concentration.

Clearance Studies

An ability to show that potentially immunogenic peptides are cleared during purification is important to demonstrate that a raw material is suitable for use in a manufacturing process. Clearance of such peptides down to submicrogram or nanogram levels can pose great challenges in terms of assay sensitivity. For these studies, some means is needed for distinguishing a peptide hydrolysate impurity from the protein product despite often similar physical and chemical properties. In many cases, the purification process must be shown to reduce an impurity to a level far below the limits of detection for that impurity, and therefore it is physically impossible to demonstrate sufficiently low levels in the final product by simple assay.

Clearance studies offer a powerful tool to demonstrate impurity removal by the purification process. In such studies, each major purification step of the overall process is examined separately. The impurity in question can be spiked to a high, detectable level in the normal starting material for a processing step. Comparison of the amount in the spiked starting material versus the amount remaining indicates the clearance ability of that step for that impurity. Generally, clearance values are reported in terms of $\log_{10}$ clearance — a purification step that reduces

impurity levels in the product by a factor of 100 (meaning that the step product has 1% of the amount of impurity as was in the step starting material) would mean the step gives "two logs" of clearance.

Note that performing a spiking clearance study on each individual step allows for the demonstration of a much greater clearance for an overall process. Whereas it might be possible to show at most three logs of clearance when comparing impurity levels in the initial starting material and final product of a process due to detection and background limitations, showing one to three logs of clearance on each of four steps of a process can demonstrate a total process clearance of greater than six logs. It is implicitly assumed in this calculation that the clearance yield is independent of the amount of impurity present in the material.

Our approach in demonstrating clearance of a protein hydrolysate through a purification process was to make use of the peptide's intrinsic oxidized tryptophan fluorescence properties, which provide a sensitive and selective means of distinguishing the hydrolysate from other process components. Although radiolabeling or derivatization of the peptide hydrolysate may be an option, chemical changes introduced during derivatization may alter a raw material's clearance properties during purification. The fluorescence method enabled demonstration of over seven logs of clearance for the protein hydrolysate through the purification process (Table 3).

Clearance studies were performed on each major purification process step. Impurities contained in the initial step starting material were sufficient so that spiking was unnecessary. Because the first process step removed >95% of the impurity, it was necessary to spike the normal starting material for each subsequent step with protein hydrolysate to demonstrate significant levels of clearance.

A spike of impurity must be prepared as appropriate for the needs of a given clearance study. In our example, the protein hydrolysate impurity was first fractionated using filtration membranes with a 1,000-Da MW cutoff pore size. That pore size membrane was chosen such that the portion of hydrolysate retained on the membrane was made of peptides nine amino acids long, or longer. Thus the immunogenic peptide fraction of the protein hydroysate was retained, and this was the fraction of interest for the purposes of our clearance study. Smaller peptides and free amino acids present in the hydrolysate passed through the membrane and were removed. It was essential to remove that smaller-sized fraction to get an accurate assessment of the clearance behavior of the immunologically relevant larger peptide fraction. In addition to size fractionation, the membranes were also used to concentrate the >1,000-Da peptide fraction such that the spike used for each process step was <10% of the volume of normal starting material for the step.

Knowledge Is Control

Alternatives to animal-derived components should be considered because they can eliminate the risk of transmitting prions or other adventitious agents. In evaluating substitutes for animal-derived raw materials, a thorough knowledge of the vendor and its manufacturing process (including audits) can assure that the constituents are defined and controlled. As part of an overall production strategy, it is important to assess any potential risks from raw materials: immunogenicity, adventitious agents, toxicity, and modulation of biological activity. Determination of the risks involved will dictate the analytical methods that may be most useful for characterization.

A thorough understanding and characterization of the biochemical properties of added components is essential even when they are not animal derived. Spiking clearance studies on individual purification steps can be used to show removal of added components to levels far below those achievable by direct assay of final products — as well as below the levels at which toxic, immunogenic, or other biological effects are a concern. And finally, it is important to qualify appropriate component lots based on relevant biochemical and microbial/viral tests. Taken together, these guidelines can provide strategies to

Table 3: Protein hydrolysate clearance study

Process Step	LOAD FRACTION Fluorescence	LOAD FRACTION Log_{10}	PRODUCT FRACTION Fluorescence	PRODUCT FRACTION Log_{10}	Logs Clearance
1	284,458	5.45	12,289	4.09	1.36
2	2,590,849	6.41	1,397	3.15	3.27
3	288,536	5.46	25,480	4.41	1.05
4	228,055	5.36	6,519	3.81	1.54
Total					*7.23*

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evaluate alternative sources for animal-derived components used in cell culture or fermentation.

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