

Expression Systems for Production of Recombinant Allergens

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Allergen · Prokaryotic systems · Eukaryotic systems · Baculovirus · Yeast · Plant · Recombinant allergens

Abstract

Recombinant allergenic proteins have been produced in a variety of different expression systems. This review gives examples of and compares prokaryotic expression systems, such as *Escherichia coli*, and eukaryotic systems including the yeasts, *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus-insect cell systems, mammalian cell systems and plant systems. Important factors to consider in choosing an expression system are discussed and examples of applications are given.

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Introduction

The characterization of many important allergens marked an important milestone in the field of allergy. The quality of natural allergen extracts has improved over the past two decades with increased standardization and characterization; however, products from natural sources are often heterogeneous and may contain many nonallergenic molecules, which can vary in composition and quantity [1, 2]. The presence of bioactive molecules can be an important factor in stability; for example, vaccines may contain proteolytic enzymes that degrade allergens or

they may contain contaminating allergens from other sources [3, 4]. Recombinant technologies can be used to circumvent these problems. Recombinant allergens similar to their native counterparts in structure, function and immune reactivity have been produced by many investigators. In-depth knowledge of molecular characteristics including three-dimensional structure and allergenic epitopes can be more readily obtained using proteins produced by recombinant technology. In the past decade a variety of recombinant allergens from plants, mites, molds, mammals and insects have been expressed using various systems, both prokaryotic and eukaryotic [5–7]. The clinical use of these products may lead to improved diagnostic specificity and sensitivity [8] and to safer and more effective immunotherapy [9]. The three-dimensional structures of recombinant allergens have been determined using NMR and X-ray crystallography including those of Ara h 1, Bet v 1, Bla g 2, Bos d 2, Der p 2, Equ c 1, Phl p 2, Ves v 5, birch pollen profilin and honey bee hyaluronidase [10–19].

The first step in producing recombinant allergens can utilize any of several strategies including IgE immunoscreening of expression cDNA libraries [20, 21], phage display with immunoscreening [22–26] or DNA-based techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) which requires the determination of a partial amino acid sequence of the natural allergen or closely related protein [27–30]. RT-PCR methods are particularly advantageous if the IgE-binding activity is dependent on posttranslational modifications, or if only a

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limited supply of specific IgE is available, and it also allows the isolation of multiple isoforms simultaneously [29, 31–35]. These are important criteria to be considered when choosing an expression system. Many recombinant allergens have been produced in bacterial expression systems, and have been shown to be correctly folded and biologically active, but many other recombinant allergens have required elaborate low-yield renaturation procedures. A variety of allergens have been produced in eukaryotic expression systems including yeast, insect/virus, mammalian and plant. The advantages of eukaryotic expression are that high-level expression is common, molecules are usually folded correctly with disulfide bonds formed, and purification can often be simplified to a single step procedure, such as affinity chromatography. This review analyzes and compares various expression systems used for production of recombinant allergens.

Prokaryotic Expression Systems

Escherichia coli is the oldest and most commonly utilized system for production of recombinant proteins. Although a large amount of experience has been accumulated, the method development for each allergen is still empirical and may present individual challenges. Many articles on the development and optimization of specific methods and overcoming a variety of problems have been published [36–38]. The bacterial expression system is easy to handle, cost-effective, and can produce a high yield. A large number of expression vectors are commercially available and offer a broad selection of fusion protein, tag and non-fusion protein production. Overproduction of foreign proteins in the cytoplasm of *E. coli* often goes together with misfolding and segregation into insoluble aggregates as inclusion bodies, which can lead to production of biologically inactive protein. In vitro refolding may be necessary and may be only partially effective and/or produce a low yield and is certainly time-consuming. One approach to overcome this problem is the use of molecular chaperones, which help the proper folding and cellular targeting of synthesized proteins [36, 39]. In addition misfolded proteins are highly susceptible to proteolytic degradation, since catabolism is an efficient way to conserve cellular resources by recycling improperly folded proteins into their constituent amino acids. The selection of an expression system that secretes eukaryotic proteins into the periplasmic space improves solubility. The periplasmic space is an oxidizing environment that contains the enzymes that catalyze formation of disulfide bonds

[40, 41]. It has been shown that co-overproduction of protein disulfide isomerases can significantly increase disulfide bond formation [42]. The proper folding factors such as peptidyl-prolyl *cis/trans* isomerase must also be present [43]. Other bacterial expression systems can be used to secrete heterologous proteins into the medium. For example a *Caulobacter* expression system is commercially available. To our knowledge this system has not been used to produce a recombinant allergen. Fusion proteins were originally designed to facilitate protein purification and immobilization, since the solubility of passenger proteins is improved by the normally secreted carrier protein. The main disadvantage of fusion proteins is that expensive proteases are required to cleave the recombinant protein, and that cleavage may be incomplete [36]. Problems in translation may also occur when working with prokaryotic systems. For example the arginine codons AGA and AGG are rare in prokaryotes, and are translated inefficiently, and in extreme cases even inhibit protein synthesis and cell growth [44]. Such problems can be addressed by site-directed mutagenesis to replace these codons, but this is time-consuming and requires multiple experiments, or by the selection of special strains of *E. coli* with extra copies of the necessary tRNA genes that can circumvent this problem [44]. Cell-free expression methods are being developed to replace whole cell prokaryotic expression systems. These certainly offer many advantages over the intact cell methods. They allow the production of proteins toxic to cells, focusing on the metabolic pathways for protein synthesis [38], and most provide a system that allows for a great variety of manipulations [45–47]. These systems have been used for several years, but limited studies have shown mainly low-yield production. Recent improvements have demonstrated that high-yield production is possible for some proteins [46]. Cell-free systems are very useful when only 0.05–0.1 mg of recombinant protein is needed. The limitations described above are general considerations for the production of any recombinant protein, but can be critical for recombinant allergen production, in particular because many IgE antibody responses are dependent upon the native conformation of the protein. It has been proposed by some authors that posttranslational modifications are not really necessary for structural analysis of the protein. However experiments have shown that some recombinant allergen proteins react with IgE antibodies from allergic patients, but have little or none of the enzymatic activity of the natural protein. Using such proteins to determine the structure of the protein may show the structure of the IgE-binding epitopes, but is certainly not sufficient to determine the

Table 1. Examples of recombinant allergens expressed in various systems

Expressed in	Allergen	Comments	References
<i>E. coli</i>	Api m 1	Renatured to full enzyme and IgE-binding activities	74, 75, 76
	Api m 2	Only 20–30% enzyme activity after renaturation	65
	Cyp c 1	Produced in fully active form when calcium added	77
	Ara h 1	Codon usage requires specially engineered strain	44
	Bet v 1	Isoforms with varying IgE-binding activity	32
	Cor a 1	Bet v 1 cross-reactive, isoforms	31
	Bla g 4	Low yield	53
	Fel d 1	Not glycosylated	54
	Ves v 5	Insoluble	55
<i>Pichia</i>	Cyn d 1	No IgE-binding activity	50
	Alt a 1	IgE-reactive	50
	Mus m 1	IgE-reactive, glycosylated	51
	Bla g 4	Native conformation	52
	Fel d 1	High yield	53
	Der p 1	Glycosylated	54
	Ves v 5	Hyperglycosylated precursor	56
<i>Saccharomyces</i>	Der p 1	High yield, native conformation	55
	Der p 2	Insoluble	57
	Procalin	IgE-binding activity	58
Baculovirus	Procalin	Immunoreactive	59
	Api m 2	Full enzymatic and IgE-binding activities	65
	Sol i 2	IgE-reactive, native conformation, natural cleavage of initiation sequence	62
	Fel d 1	Fully immunoreactive	67
	Lep d 2	Isoforms produced	63
	Mal f 1	Similar to that expressed in <i>E. coli</i>	66
	Cte f 1	Fully reactive with IgE antibodies	68
Mammalian	Aed a 1	Immunoreactive apyrase	64
	Per a 1	Low yield	73
Plant	Bet v 1	IgE binding, mouse antibody binding	72
	Hev b 1	IgE binding	72
	Hev b 3	IgE binding	72

native structure of the protein, since the major natural biological activity is absent. Prokaryotic expression systems are still one of the first choices for allergen expression, since they are relatively easy to use, and there are many examples of allergens successfully produced in these systems (table 1). We cannot overlook the basic simplicity of the systems and the many potential variants available, which make these systems both the most studied and most commonly used. Future understanding of prokaryotic expression systems will lead to the possibility of more systematic method development rather than the trial and error experiments now necessary for each protein that one wishes to produce.

Eukaryotic Expression Systems

The major advantage of a eukaryotic over bacterial expression systems is the ability to perform many of the posttranslational modifications including processing signal sequences, folding, disulfide bond formation, and adding lipids and carbohydrates. A variety of eukaryotic expression systems are available; the most commonly used are yeast, insect, mammalian cell and plant. Each of these systems will be discussed below.

Many types of yeast have proven to be useful for recombinant protein production. Several have been well characterized genetically and are known to perform most eukaryotic posttranslational modifications. The single-celled eukaryotic organisms grow rapidly with minimal requirements and are attractive expression systems competing with the better-established prokaryotic systems.

Pichia pastoris

During the past two decades, the methylotrophic yeast *P. pastoris* has been developed into a valuable tool for high-yield expression of proteins. The increasing popularity of this system is due to its simplicity, the capability to perform posttranslational modifications such as disulfide bond formation and glycosylation, the capability to produce high yields, and the availability as commercial kits [48]. A variety of vectors are now available. *P. pastoris* is capable of adding both *O*- and *N*-linked carbohydrates [49]; however, as a lower eukaryote it adds only mannose residues, but hyperglycosylation as has been observed in *Saccharomyces cerevisiae* seems to occur less frequently in *P. pastoris*-produced proteins. Proteins which in their native forms are nonglycosylated may be glycosylated when produced by this yeast. Foreign proteins can be expressed in *P. pastoris* in either intracellular or extracellular forms. Because of protein stability and secondary structure requirements, optimal secretion usually occurs with proteins that are secreted naturally. Standard expression cassettes for secretion are available commercially. The results may vary between proteins with natural signal sequences and those with artificial signal sequences [48].

The first allergens to be expressed in *Pichia* were Cyn d 1, Alt a 1, Mus m 1 and Bla g 4 [50–53]. A number of other allergens have been expressed using *Pichia* and some examples are listed in table 1. The expressed allergens are produced in high yields typically in the 5–200 mg/l range. Some allergens have been expressed in both *E. coli* and *P. pastoris*. Although the IgE reactivity was comparable when Bla g 4 was produced in both systems, the recombinant protein production in *E. coli* only yielded 0.25 mg/l whereas *P. pastoris* produced 50 mg/l [53]. Similarly, it was reported that *E. coli*-derived recombinant Fel d 1 performed as well in the radioallergosorbent test as did Fel d 1 produced in *P. pastoris*; however, for structural analyses, the protein produced in yeast cells was preferred, since its glycosylation was needed for the correct folding [54]. Antigen 5 from vespid venom was produced as an insoluble intracellular product in *E. coli*,

while it was secreted when produced by yeast and was shown to have the native conformation [55]. Cyn d 1, a Bermuda grass pollen allergen, showed no IgE-binding activity when produced in *E. coli* in contrast to positive IgE reactivity when produced in *P. pastoris*, probably due to the lack of native conformation of the protein produced by the prokaryotic system [50]. *P. pastoris* produced a hyperglycosylated form of mite allergen, Der p 1, which had decreased IgE-binding activity and poor induction of histamine release from basophils. It was suggested that the hyperglycosylation hindered the maturation of the protein, but the stable hypoallergenic form could have potential use in immunotherapy [56].

S. cerevisiae

Only a few allergens have been produced in the yeast *S. cerevisiae*. Although a eukaryotic expression system with the ability to perform posttranslational modifications, it is known to hyperglycosylate proteins, and therefore researchers usually tend to prefer *P. pastoris*. Chua et al. [57] report the expression of an IgE-binding recombinant Der p 1 in this yeast. However, the protein was insoluble and had to be denatured and renatured. They did not study the glycosylation of the recombinant allergen, or compare it with the recombinant produced by *P. pastoris*. Mite allergen, Der p 2, and procalin, a major allergen from *Triatoma*, a biting insect, have been successfully expressed in baker's yeast and both recombinants showed strong IgE-binding activity [58, 59].

Baculovirus

Insect cells have proven to be good hosts for the production of recombinant proteins. The baculovirus system has been used for protein overproduction since the late 1980s, and its use is increasing. It is an expression system with all the advantages of higher eukaryotic organisms. The viruses are usually derived from *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV), and the host cells are usually *Spodoptera frugiperda* ovarian cell lines Sf 9 and 21 or the *Trichoplusia ni* egg-derived cell line High Five. A variety of vectors are commercially available. Most of the vectors use the polyhedrin gene promoter. The polyhedrin gene is necessary for the formation of occlusion bodies in the cell nucleus, but is not necessary for the production of infectious viral particles and has been deleted in these vectors and is replaced with the foreign DNA. Possee [60] has written a short review of the major advantages and improvements in this system. The

glycosylation pathway in insects is different from that in other eukaryotic organisms and has been reviewed recently [61].

A number of recombinant allergens have been produced using the baculovirus system [62–68]. All of these recombinant allergens show IgE-binding capacity and behave with similar biological activities to the native proteins. Fel d 1, when produced in *E. coli*, had to be refolded in vitro to be immunoreactive, but was fully reactive when expressed in insect cells [67]. Production of honeybee venom hyaluronidase showed a comparable IgE reactivity when produced in *E. coli* and the baculovirus systems; however, full enzymatic activity was found in recombinant protein produced in insect cells [65]. In the case of the mite allergen Lep d 2 and the yeast allergen Mal f 1 no functional differences were observed between protein produced in prokaryotic and baculovirus expression systems. Both systems produced comparable amounts of immunoreactive recombinant proteins [63, 66]. The cat flea allergen, Cte f 1, was produced in *E. coli* and had to be refolded in vitro, only regaining partial IgE-binding activity. The recombinant protein produced in yeast and insect cells was fully IgE antibody-reactive [68]. The insect cell culture systems have an advantage for use in vitro studies using mammalian cell cultures such as T cell proliferation assays. The supernatants from the insect cell cultures can be used without problems from interfering yeast antigen contamination. The yield achieved with the baculovirus expression systems is generally not as high as that achieved with optimized *P. pastoris* production, and yields can be very low for some proteins. More sophisticated laboratory facilities and skill are required for insect cell culture compared with yeast culture, and additives required for insect cell culture serum-free media can make recombinant protein purification challenging.

Plants

Plants can be utilized as biomanufacturing systems to produce recombinant proteins. Both intact plants and cell lines can be used. A recombinant protein may be expressed by either transgenic technology or by infection with chimeric virus. Plant viruses offer a system for rapid production of substantial amounts of protein. The standard method to introduce foreign genes into plants is the well-characterized single-stranded RNA virus, tobacco mosaic virus. Chimeric tobacco mosaic virus replicates extra chromosomally and redirects high-expression protein synthesis in the host cells throughout the organism,

Nicotiana benthamiana [69]. Plants can perform most posttranslational modifications, and correct folding has been reported for heterologous protein production. Proteins can build up to high levels in plant parts and agricultural scale production is possible [70]. Plant cell cultures can be used similarly to other eukaryotic expression cell lines. There may be a number of technical difficulties in developing plant cell line production [71]. Production of allergen in whole plants may require elaborate purification methods. Proteins are trapped in plant tissues and extraction procedures may have low yields [71]. Recently, the successful production of immunoreactive recombinant Bet v 1 and two latex allergens, Hev b 1 and Hev b 3, have been reported using tobacco plants; however, the yield achieved was not reported [72]. Major advantages of plant expression are safety and ease of production without the potential of contamination by dangerous pathogens and technically demanding sterile cultures [71].

Mammalian Cells

Although several mammalian expression systems are well established, they have only rarely been used to produce recombinant allergens. One report on successful production of the cockroach allergen Per a 1 using Cos-1 cells was published in 2000 [73]. This system, a eukaryotic system with all its advantages, does not produce as high a yield as either yeast or baculovirus. It requires more sophisticated equipment and skill for cell cultures than the baculovirus system.

Conclusion

To determine which system is the best choice for expression for a particular allergen, it is necessary to ask several questions based upon available time, resources, experience available in the laboratory, and the nature of the allergen. Protein expression is still a very empirical process. Experience in working with expression systems is a very important consideration. The availability of commercial expression kits is important and most companies provide extensive user assistance. Almost all allergens are derived from eukaryotic sources. If the native conformation and/or posttranslational modification are necessary for the activity of the recombinant protein, eukaryotic expression systems are preferred. For prokaryotic expression systems, there is a large amount of experience with *E. coli*. The expression systems commercially available

are diverse, rapid and inexpensive. Prokaryotic systems do not perform the posttranslational modifications of many eukaryotic proteins, and many proteins do not fold properly. In addition there might be codon usage problems in *E. coli*. At present it is not possible to predict the ease of renaturation of proteins with multiple disulfide

bonds. Yeast expression probably requires the least specialized laboratory equipment and skills, and usually produces recombinant protein in high yield. Virus-induced expression in whole plants may become the method of choice for the large-scale production of recombinant allergen proteins in the future.

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