Cationic antimicrobial peptides in humans

Antimicrobial peptides

Introduction

Cationic antimicrobial peptides (AMPs) are gene-encoded peptides of the host defence system made up of 12-50 amino acids, with at least 2 positive charges conferred by lysine and arginine residues and about 50% hydrophobic amino acids (Hancock and Scott 2000). They are produced from gene transcription and ribosomal translation and often, further proteolytically processed (Zhoa 2003). The peptides are folded so that non-polar amino acid side-chains form a hydrophobic face and polar, positively charged residues form a hydrophilic face (Robert and Hancock 1997). Expression of antimicrobial peptides can be constitutive or inducible by infectious or inflammatory stimuli like cytokines, bacteria and lipopolysaccharides (LPS) (Cunliffe and Mahida 2004). They have diverse structures to effectively kill a wide range of microbes at prone sites e. g the skin and lungs, and in secretions such as sweat and saliva (Yeaman and Yount 2004; Santamaria 2005). Many mammalian antimicrobial peptides rouse the host’s innate immune system (Jenssen et al 2006) instead of directly killing the host. Peptides which are found in living organisms from bacteria to plants, insects, fish, amphibians to mammals including humans (Kamysz 2005) are recorded in numerous existing databases e. g. AMSDb (Eukaryotic peptides) (Tossi and Sandri 2002), BAPDb (bacterial peptides), ANTIMIC (natural antimicrobial peptides) (Brahmachary et al 2004) and APPDb. Currently, 1831 peptides are hosted by the Antimicrobial peptide database with 99 antiviral, 453 antifungal, 100 anticancer and 1179 antibacterial peptides (The Antimicrobial Peptide database 2010).

In humans, antimicrobial peptides are produced by granulocytes, macrophages and most epithelial and endothelial cells. They boost the immune system, have anti-neoplastic properties and help in regulating cell signalling and multiplication.

Amphibian AMPs have been discovered from the skin of frogs from families ranging from Iomedusa, Pipidae, Hyperoliidae, Ranidae, Hylidae, Discoglossidae, Agalychnis and Litoria. The structure of these peptides as unravelled by CD spectroscopy, NMR spectroscopy and molecular modeling (Suh et al 1996) have been found to be generally 10-46 amino acid residues long (Rollins-Smith et al 2005), mostly linear and simple-structured, (Conlon et al 2004) the majority being hydrophobic, cationic and possessing an amphipathic a-helix in nature. Following production, they are stored in the granular glands (poison glands) of skin dermal layer to be secreted in response to injury (Bovbjerg 1963), or as defence against pathogenic bacteria, fungi, viruses and parasites. Biologically active molecules including antimicrobial peptides are produced as large proteins harbouring a signal and an acidic propiece which get cut off to give an active peptide prior to or at secretion from the poison glands (Amiche et al 1999). Cationic peptides are also expressed in the gastric mucosa cells and in the intestinal tract (Kamysz 2005). The best-known peptides isolated from frogs are caeruleins, tachykinins, bradykinins, thyrotropin- releasing hormone (Barra and Simmaco 1995), brevinins, esculentins, magainins, ranatuerins and temporins (Conlon et al 2004).

In the past, peptides were extracted using solvents like methanol or acid from the skins of amphibians after sun-drying but with concomitant

dwindling of many frog species, other alternative techniques have emerged, one of which comprises stimulating the frog using mild electricity and collecting the skin secretion; 2-4 weeks after, the secretion can be re-collected after replenishment of the glands (Barra and Simmaco 1995). Large amounts of small peptides and their analogues which are resistant to protease cleavage and contain D-amino acids can be chemically synthesised while larger peptides can be expressed in a prokaryotic host from cloned cDNAs coding for a fusion protein (Piers et al 1993). An efficient means of producing therapeutic peptides in transgenic mice red blood cells has been explained by Sharma et al (1994) whereby the required peptide is collected from proteolytic cleavage from the fusion protein where the peptide is at the C-terminal end of human a-globin.

Structure of antimicrobials

In spite of sharing similar features, sequence homology between antimicrobial peptides is low and secondary structure diversity is wide (Jenssen 2006). Secondary structures based on sequence homologies, 3-dimensional structures and functions can be grouped into 5 classes:

1. Linear, mostly a-helical peptides lacking cysteine, with or without a hinge region (cecropins, magainins).
2. Antimicrobial peptides with one disulphide bond that form a loop structure with a tail (bactenecins, esculentins).
3. Antimicrobial peptides with at least two disulfide bonds yielding primarily or solely a B-sheet structure (defensins, protegrins).
4. Linear peptides comprising an unusual composition of regular amino acids with the absence of cysteine (histatins, indolicidin, temporins).

5. Antimicrobial peptides derived from larger peptides or proteins with other known functions (lactoferricins, MUC7, Casocidin1).

Mode of action of peptides

Unlike numerous antibiotics or secondary metabolites that halt microorganisms over a number of days by hindering the action of key enzymes, most of the vertebrate antimicrobial peptides neutralise microbes quickly by disrupting the membrane or permeating it and targeting anabolic reactions (Barra and Simmaco 1995). The antimicrobial properties of most peptides rely on the formation of a-helical (Oren et al 2002) or B-sheet-like tubular (Fernandez-Lopez 2001) structures when interacting with negative charges on cell surfaces or when forming a-helical bundles following self-association in solution (Avrahami and Shai 2002).

The mechanism of action of antibacterial peptides is thus twofold: membrane acting (membrane permeabilisation) or non-membrane acting (affect vital intracellular processes) as depicted in Figure 3. Different models of membrane permeabilisation exist; in one model, peptides reorient to cross the phospholipid bilayer of the membrane as a cluster without assuming any particular direction, resulting in a micelle-like complex of peptides and lipids. The “ toroidal pore” model suggests that peptides enter the bilayer at 90 degrees to it, the hydrophilic regions interacting with phospholipid head groups and the hydrophobic regions interacting with the lipid core. Additionally, the membrane curves inward so that the bilayer lines the pore too. The “ barrel-stave” model involves insertion of peptides perpendicularly to the bilayer, forming the “ staves” in a “ barrel”-shaped cluster, such that hydrophilic regions of the peptides project into the pore lumen and the

hydrophobic regions contact the lipid portion of the bilayer membrane. According to the “ carpet” model, peptides group together parallel to the lipid bilayer and local areas are coated like a “ carpet” (Lai and Gallo 2003). Buforin II in frogs crosses the bacterial membrane without causing permeabilisation and binds DNA and RNA in the cytoplasm of E. coli (Park et al 1998).

Antifungal peptides have been found to function in different ways one of which is fungal cell lysis e. g. frog Magainin targeting C. albicans (Tytler et al 1995; Zasloff et al 1987) and frog Brevinin-1 acting on Batrachochytrium dendrobatidis as described by Rollins-Smith et al (2002). Defensin from mammals permeabilises the membrane of C. albicans (Lehrer et al 1985; Patterson-Delafield 1980); bovine Indolicidin has been found to act similarly on T. beigelii (Lee et al 2003). Another strategy of antifungal peptides is the disruption of synthesis of fungal cell wall (De Lucca and Walsh 1999). Cecropin from insects as studied by De Lucca et al (1998) binds to membrane cholesterol or ergosterol and affects the fungus Aspergillus fumigates. Bovine lactoferricin and the hybrid peptide of Helicobacter pylori ribosomal protein L1 and magainin-2, HP(2-9)-MA(1-12), have been shown by Bellamy et al (1993) to cause extensive damage to the ultra structure of the cell wall of C. albicans . Yet another mode of action has been documented which involves depolymerisation of the actin cytoskeleton illustrated by Pn-AMP 1 from plants which attacks S. Cerevisiae and C. albicans actin. In contrast, as reported by Helmerhorst et al (1999) and Kavanagh and Dowd (2004), Histatin from human primates acts on mitochondria in C. albicans. Rollins-Smith et al (2002b) found that the peptides magainin I and II, PGLa,

and X. Laevis CPF, Phyllomedusa sauvagii dermaseptin and R. catesbeiana ranalexin were more potent at the zoospore transmission period of the chytrid fungus life cycle than against mature stages. It has been found that peptides with primarily antifungal activity particularly abound in polar and neutral amino acids, pointing to a structure-activity link (Lustig et al 1996).

Numerous studies point to different modes of action of antiviral peptides which interfere with adsorption, entry of the virus into the cell (Belaid et al 2002) or act on the viral envelope (Robinson et al 1998). Targeted viruses are notably enveloped RNA and DNA viruses except non-enveloped adenoviruses (Bastian and Schafer 2001), echovirus 6 (Pietrantoni et al 2006) and feline. Sinha et al (2003) found that rabbit a-defensin NP-1 acts by inhibiting HSV movement from cell to cell. By interacting with the CXCR4 chemokine receptor functional as a coreceptor for HIV-1 entry into T cells (Murakami et al 1991), a polyphemusin analogue, T22 reportedly inhibits the HIV strains using that chemokine receptor (Tamamura et al 1999). Antivirals can also hamper viral entry through interaction with viral glycoproteins as in the case of ?-Defensin (retrocyclin 2) claimed by Yasin et al (2004) to protect cells from HSV-2 infection by acting on HSV-2 glycoprotein B. Viral antimicrobial peptides can act in the cell as well, stimulating genes or proteins, thereby affecting host cell antiviral mechanisms (Bowdish et al 2004); viral gene and protein expression (Wachinger et al 1998) can be hampered too.

Antimicrobial activity of peptides

Features of antimicrobial peptides

The emergence of resistant bacterial strains worldwide necessitates (Bonomo 2000) development of new families of antibiotics. Antimicrobial peptides have properties which render them attractive for development of new antimicrobial therapies. Antibiotic-resistant bacteria are often vulnerable to antimicrobial peptides (Jacob and Zasloff 1994; Tencza et al 1997); minimum inhibitory concentrations (MIC) of 1-8 μg/mL competitive with the most powerful antibiotics against resistant organisms have been recorded with peptides (Isogai et al 2009). Lui et al (2009) observed strong effectiveness of peptide nanoparticles against bacteria, Streptococcus haemolyticus (gram-positive bacteria), Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis and also against drug-resistant bacteria methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant Enterococcus, yeast tropicalis and fungi, Candida albicans, Cryptococcus neoformans and Stachybotrys chartarum. Antimicrobial peptides not only eradicate bacteria rapidly and neutralize endotoxins but are synergistic with antibiotics and active in animal models (Hancock and Scott 2000). Furthermore, they exhibit a broad spectrum of activity by virtue of their relatively non-specific mode of action. In addition, mutations leading to classical antibiotic resistance do not affect them and they do not easily select antibiotic resistant variants (Shai 2002; Huang 2000). However, peptides have drawbacks limiting their use e. g. they are cleaved by proteases notably trypsin-like proteases and researchers still need to probe further into uptake mechanisms into different tissues (Boman 1995).

Activity of Frog peptides

Numerous studies aimed at understanding further antimicrobial expression of antimicrobial peptides have been conducted. In line with a first line of defence to the frog, release of antimicrobial peptides are stimulated in response to an environment conducive to microbial proliferation; it has been observed that while the freeze-tolerant wood frog, Rana sylvatica, shows no signs of any antimicrobial peptide activity in winter, it produces and secretes an active peptide upon exposure to warmer conditions (Matutte et al 2000). Rollins-Smith et al (2002a) found that effectiveness is enhanced when mixtures of peptides rather than single ones are released; magainin II and PGLa, synergistically hamper growth of B. dendrobatidis and B. ranarum on the skin of X. Laevis.

Frog peptides are seen as interesting and potentially useful molecules that could be effective against a range of human pathogens (VanCompernolle et al 2005), viral, bacterial or fungal. In the 1960s, a 24 amino acids long antibiotic peptide bombinin secreted from the skin of the frog Bombina variegata was isolated but discouraging high haemolytic activity restricted applicability (Csordas and Michl 1970). Since then, large numbers of various peptides have been discovered with antimicrobial potential; Gaegurin, for instance, from a Korean frog is described as having a considerable spectrum of activity with mild haemolytic activity, rendering it a potential antibiotic (Suh et al 1996). Recently, high amounts of peptides were discovered in Litoria chloris, the Australian red-eyed tree frog which blocked HIV without harming T cells; the peptides appeared to target the HIV virus probably by insertion into its outer membrane envelope and punching holes in it

(VanCompernolle et al 2005). Some frog peptides have aroused interest as potential agents for treatment of Type 2 diabetes as exemplified by a peptide isolated from the skin secretions from the leaf frog, Hylomantis lemur with the ability to release insulin from the rat BRIN-BD11 clonal B cell line; A synthetic version of the peptide, phylloseptin-L2 was remarkably good at that. Other peptides from phyllomedusid frogs are a 13 amino-acid-residue peptide from Agalychnis calcarifer (Abdel-Wahab et al 2005) and peptides from Agalychnis litodryas (Marenah et al 2004a) and Phyllomedusa trinatis (Marenah et al 2004b) with a structure similar to dermaseptins.

Therapeutic applications

Antimicrobial peptides are appealing for therapeutics since they are rapidly produced at low metabolic expenses, stored easily in abundance and readily available shortly following an infection, to rapidly counteract a wide range of microbes (Zhoa 2003). The 20 existing amino acids confer tremendous diversity in sequence and structure of peptides, presenting opportunities in creating a whole range of novel drugs (Hancock and Scott 2000)

Peptides can be used for battling antibiotic-resistant bacterial infections or septic shock (Finlay and Hancock 2004). Other potential applications include topical applications for preventing sexually transmitted diseases (Rana et al 2006) including HIV/HSV (Reddy et al 2004), Meningococcal meningitis, diabetic wounds e. g. foot ulcers, gastric helicobacter infections, impetigo (Gunaratna et al 2002; Reddy et al 2004), treating eye infections (Migenix 1998). Creams with snail’s mucin containing antimicrobial peptides are currently marketed for topical applications treating skin infections and acne inflammation (Cottage 2007). Efforts have also been directed at developing

magainin analogs into anticancer drugs (Boman 1995). Furthermore, as attempts persevere to alter the immune system of the vectors or their symbionts to confer to the vectors the ability to eradicate the parasites (Ham et al 1994; Gwadz et al 1989), peptide antibiotics are seen as a potential weapon in fighting insect-borne diseases like malaria, trypanosomiasis, and filariasis. Besides using AMP as proteins, genes encoding AMP can be delivered as gene therapy. Genetically altered bacteria making the antimicrobial in situ can be used for targeting pathogens, which is particularly relevant to the treatment of dental caries, Crohn disease, and other disorders in which disturbances in natural microflora play a role and host-microbe balance must be preserved (Palffy 2009). Peptides are also used for food preservation as exemplified by Nisin, produced by certain strains of Lactococcus lactis subsp. Lactis (Joerger 2003).

Engineering peptides for enhanced activity

Natural peptides do not always possess all the features required to make them suitable therapeutics, validating the need to engineer their primary structure to confer those properties to them (Sarah et al 1999) e. g. stability, reduced toxicity (Won and Ianoul 2009); existing desirable properties e. g. potency, selectivity or specificity of antimicrobial activity can also be strengthened.

In cationic AMPs, antimicrobial activity has been ascribed to the net positive charge since the outermost portion of bacterial membranes is negatively charged by virtue of the negatively charged phospholipids (Brogden 2005). Substitution of acidic residues with basic ones, reportedly boosted the potency of LLP1 against S. aureus from the increase in net charge of the

peptide (Robert and Hancock 1997). Hydrophobicity (aiding in membrane insertion) and flexibility, driving the peptide's change in conformation to allow interaction with the membrane, have also been identified as important (Jenssen 2006). Antibacterial activity of peptides can be enhanced by altering their flexible secondary structures; altering the membrane-associated conformation of indolicidin to bring the N and C termini closer together increased activity against gram-negative bacteria (Ianoul 2010). Oren and Shai (2000) claim that a successful designing strategy in the synthesis of antimicrobial peptides is the incorporation of non-natural amino acids (such as Ornithine) and the utilization of D-Amino acids with the aim to sustain natural activity and confer resistance against proteases. It has been found that, often, modest alterations to ineffective antimicrobial peptides successfully confer antifungal properties as evidenced by the resulting potent action of magainin analogues coupled with undecanoic acid or palmitic acid in contrast to the native peptide, against yeast and opportunistic fungal infections (Avrahami and Shai 2003)

However, often, one roadblock in peptide production is that expression of antibacterial proteins in bacteria may be suicidal to the cells; this could be circumvented by making a fusion protein which is made to accumulate in inclusion bodies at a later stage in growth (Boman 1995). A report of this technique applied on four fusion proteins was recently published (Piers et al 1993). Use of expression vectors in hosts that are insensitive to the peptide antibiotic is also proposed as a solution that has met with success in producing an insect defensin in yeast (Reichchart et al 1992). There are reports of the baculovirus system being used to express a fusion protein

cecropin A although with rather low yields, in an insect cell line (Andersons et al 1991). Using the same virus vector in live insects, expression of cecropin about 60 times higher was recorded in the hemolymph.

Pseudin antimicrobial peptides

Introduction

Pseudin-1, Pseudin-2, Pseudin-3 and Pseudin-4 are antimicrobial peptides with structural similarity, which are secreted from the skin of the bright green and pink paradoxical frog from the Pseudidae family, Pseudis paradoxa, inhabiting Trinidad and the Amazon basin (Olson et al 2001). Pseudins, a subfamily of the Frog Secreted Active Peptides (FSAP) are cationic, amphipathic and helical (Olson et al 2001). Pseudin-2, the most abundant and powerful 2685. 4 Da peptide comprises 24 residues (GLNALKKVFQGIHEAIKLINNHVQ). In aqueous solutions pseudin-2 coils randomly while in those emulating the hydrophobicity of the cell membrane e. g. 50% trifluoroethanol/water, it assumes an a-helical conformation (Yasser et al 2008).

Antimicrobial activity of Pseudin-2

As testified by Olson et al (2001), Pseudin-2 effective against Escherichia coli (MIC= 2. 5 µM), Staphylococcus aureus (80 µM) and Candida albicans (130 µM) and has very weak haemolytic activity against human erythrocytes in contrast to other frog antimicrobial peptides (Colon 2004). Another study by Pal et al (2005) showed that strains of several pathogenic bacteria Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus epidermidis and Streptococcus were also effectively acted upon by multi-l-lysine-substituted analogues. They also demonstrated that

gradually raising the cationicity of pseudin-2 by replacement of several residues with l-lysine in the hydrophilic part of the peptide enhanced the antimicrobial property. An analogue [D-Lys3, D-Lys10, D-Lys14]pseudin-2 in particular, in contrast to others had a significantly increased antimicrobial effect against E. coli and S. aureus and, low haemolytic and cytolytic activity against human erythrocytes. Replacing Asn with Lys at codon 3 was found to double the antimicrobial activity against E. coli and S. aureus from a reduction in destabilisation of the a-helix besides greater positive charge (Pal et al 2005).

Kim et al (2007), in a study attempting to link structure to mechanical action of pseudin-2 in microorganisms and liposomes, suggested that the antimicrobial activity of Pseudin-2 is ascribed to the punching of holes in the target cell membrane through its oligomerisation according to the toroidal pore model in zwitterionic liposomes and the barrel-stave model in anionic liposomes.

Pseudin-2 as an anti-diabetic peptide

Besides antimicrobial features, Pseudin is seen as promising, prospective insulinotropic agent for treating type 2 diabetes as an incretin mimetic (Abdel-Wahab et al 2008). Yasser et al (2008) demonstrated that Pseudin-2 and derivatives, particularly a [Lys18]-pseudin-2 derivative, enhanced release of insulin from a clonal B-cell line, BRIN-BD11 via Ca2+ independent pathways. The insulin- releasing features of [Lys18]-pseudin-2 are very similar to the gut hormones GLP-1(7-36) amide and GIP (McClenaghan and Flatt 1999)

Mutagenesis

Site-directed mutagenesis

Initially, DNA mutation was based on generation of random mutations in chromosomal DNA using X-rays and chemicals or error-prone PCR which suffered from the major drawback of not targeting the mutation to a specific gene, leading to the emergence of site-directed mutagenesis. This technique has become instrumental for altering DNA sequences in molecular biology and genetic engineering, and for investigating how protein structure relates to function (Zheng et al 2004). In vitro approaches to site-directed mutagenesis can be generally grouped into three categories (Botstein and Shortle 1985):(i) localized random mutagenesis (ii) oligonucleotide-directed mutagenesis (Cosby and Lesley 1997) and (ii) techniques restructuring DNA fragments e. g cassette mutagenesis (Lo et al 1984).

Single amino acid substitutions in proteins can be produced by oligonucleotide-directed mutagenesis. One or more amino acids at specific desired locations in the polypeptide chain can be substituted with no length change. Any residue, chemically reactive or inert, buried or exposed can be targeted, and size can be replaced without altering polarity, or polarity without altering size (e. g. aspartate to asparagine). Often, different multiple substitutions are introduced at one specific position or at several locations to study their effect on protein function (Kegler et al 1994).

Saturation mutagenesis

Using saturation mutagenesis, which often involves oligonucleotide-directed mutagenesis (Zoller and Smith 1982), a library of mutants with all possible mutations at one or more pre-determined key positions in a gene can be

created. In conjunction with high-throughput screening, saturation mutagenesis has numerous applications. For example, it has been used in enhancing the stability of the Rhizopus oryzae prolipase enzyme towards lipid oxidation products such as aldehydes (Lorenzo et al 2007) and to increase enantioselectivity of Pseudomonas fluorescens esterase (Park et al 2005). The ligand-binding specificity of the human oestrogen receptor for a resveratrol-like synthetic compound has also been successfully enhanced by saturation mutagenesis (Islam et al 2009). Randomised gene libraries are usually created by replacing part of the parental gene with a synthetic DNA cassette with specific randomised codons (Hine et al 2003).

Cassette Mutagenesis is a means of creating a library by targeting a specific site or sites of any length or sequence and replacing them with new sequences; degenerate oligonucleotides are used for inserting the predetermined degeneracy into the peptide.

Cassette Mutagenesis involves cutting the original gene contained in a plasmid with two endonucleases at identified, appropriate restriction sites to remove a small section from the plasmid. To make the sequence more amenable to mutations at any region along it, unique restriction sites around 40 nucleotides apart can be inserted into the plasmid (Stryer 2002).

The gap is subsequently replaced with a synthetic double-stranded oligonucleotide (the cassette) with sticky ends which is then ligated to the plasmid which now has the required mutation.

The new mutated insert allows protein structures or nucleic acid sequences to be studied. Bakir et al (1993) found cassette mutagenesis to be useful for

mutating nine single amino acids in the active site of Aspergillus awamori glucoamylase to modify pH dependence of the enzyme and investigate the functions of the mutated residues. Xiang and Sampson (2004) used cassette mutagenesis to screen libraries for establishing specificity of the substrate in a cholesterol oxidase-catalyzed reaction.

Oligonucleotide synthesis or screening can be costly for cassette mutagenesis, especially if several parts of a gene are targeted for saturation mutagenesis, since for each region to be mutated, a different batch of oligonucleotide cassettes containing the mutated codon must be produced (Kegler-Ebo et al 1994).

Codon randomisation

High throughput substitution can be accomplished through randomisation of codons, resulting in a randomised gene library including all possible amino acid substitutions for the target amino acid residue. Conventional codon randomization uses oligonucleotides containing NNN-, NNB-, NNK- or NNS (N: A/C/G/T; B: C/G/T; K: G/T; S: G/C) since each of these combinations code for all 20 amino acids (Patrick and Firth 2003).

Creating NNN libraries from oligonucleotides by employing an equimolar mixture of the four nucleotides at each position represents the easiest approach to codon randomisation (Bosley et al 2005) but a sizeable portion of the library contains premature stop codons especially in the event of multiple codon-randomisations. Additionally, the most common protein mutants with Arg, Leu and Ser (with six degenerate codons) are immensely

over-represented in contrast to the rarest mutant with Met or Trp at each randomized position (Patrick and Firth 2003).

By employing reduced codon sets, the quality and diversity of the protein library can be enhanced. NNB codons have the lowest likelihood of 1 in 48 of coding for stop codon. NNK and NNS codons reduce the over-representation of the commonly-occurring variants (Patrick and Firth 2003). Although NNK and NNS give rise to a similar distribution of amino acids, NNK is preferred for libraries hosted by E. coli and S. cerevisiae by virtue of their favourite codon use but the NNK codon distributes the 20 amino acids unequally and generates 3% termination codons.

Neuner et al (1998) mutated codons with dinucleotide phosphoramidite which has the advantage of requiring only seven dinucleotide building blocks to code for all 20 amino acids. Besides being flexible, the technique can be used applied to various hosts with different codon usage patterns. Virnekas et al (1994) have reported use of trinucleotide phosphoramidites as well in random mutagenesis involving chemical DNA synthesis with a yield above 98%.

MAX randomisation

The MAX codon randomisation method designed and tested on three residues on a synthetic zing finger protein recently by Hine et al (2005) is a superior technique using simple primers efficiently to curtail library size.

Using this technique specific synthetic oligonucleotides anneal to a template – containing the codons targeted for mutagenesis which are each fully randomised conventionally (NNN)-to select the particular set of desired

codons for E. coli. 20 oligonucleotides, each made up of a complementary region and a MAX codon encoding an amino acid, base-pair at each randomised codon.

Specific selection primers are designed to cover the 5′ region of each target codon and terminate with each specific codon whose presence in the library is necessary; a set of primers is synthesised for each targeted codon. These are hybridised to the template and ligated to give a single strand. 2 extra unique oligonucleotides are needed for serving as primer-binding and restriction sites at the cassette extremities, also ensuring that PCR amplification of only the selection strand occurs. The resulting double-stranded DNA can be subjected to restriction digestion, dephosphorylation followed by ligation into an expression construct or extended to regenerate the complete gene.

One advantage of the MAX technique is that, despite the requirement for a large number of primers, their maximum number does not exceed 20 times the randomized codon-number, resulting in a library comprising 8000 mutants with the absence of codon bias and premature termination codons. While randomising NNN and NNG/T codons generates exponentially-rising redundant gene libraries with increasing randomised codons, MAX randomisation generates non-redundant libraries in essence. Amplification bias likely to arise from using PCR to generate the complementary strand can be minimised by synthesising a second strand instead of using PCR. MAX also allows circumvention of the use of a DNA synthesizer and is helpful when medium to large (103-106 variants) libraries have to be screened efficiently (Neylon 2004).

Drawbacks

MAX is not practical if only one codon needs to be randomized and cannot be used if the number of adjacent codons to be randomized exceeds two. Furthermore, it is a complicated technique (Hine et al 2003).

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