微生物学通报
Microbiology
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MAR 20, 2008, 35(3): 436~442

专论与综述

防御素基因工程制备研究进展

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摘　要: 本文综述了防御素在重组表达与制备方面的主要进展, 包括在原核细胞表达时宿主菌、载体、表达策略的选择, 在酵母等真核细胞中进行重组表达的优缺点, 以及重组防御素纯化研究的现状。本文还概括了当前防御素研发与临床应用面临的主要问题, 将来的研究方向和开发前景。

关键词: 防御素, 重组表达, 纯化工艺, 生物活性

Advances in Gene-engineering Production of Defensins

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Abstract: This paper reviews the recent advances in recombinant expression and purification of defensins, including the choice of host cells, vectors and expression strategies in prokaryotic and eukaryotic cell expression systems, as well as the status of purification processes. By summarizing the problems existed in the production and clinical applications of defensins, the authors here also pointed out the research directions for defensins, and conceived the prospects for its exploitation in the future.

Keywords: Defensins, Recombinant expression, Purify process, Bioactivity

防御素(Defensins)是动植物及人类表达的一大类既参与病原微生物的防御, 又参与机体免疫保护的活性肽。现已发现近千种防御素, 有关防御素的分类、氨基酸序列、空间结构与分子特征、表达调控与分布规律、遗传意义与生物活性、作用机理和潜在的医学应用价值等方面均已展开了广泛而深入的研究与总结[1−5]。在针对传统抗生素的临床耐药菌感染日益严重的今天, 因防御素具有抗菌等多种生物活性及其不易诱导产生耐药菌株的独特机理, 使其可能成为全新的抗病原微生物的药物。为了深入研究防御素的结构和生物学活性, 寻求开发与应用策略, 诸多科技工作者付出了艰辛的劳动, 取得了令人鼓舞的成果。本文综述了防御素在重组表达、分离纯化与活性等方面取得的最新进展, 探讨将来的研究方向, 并展望开发前景。

1　重组表达

防御素的获得主要有 3 条途径：从细胞或体液中提取; 化学合成; 重组表达与纯化。因防御素在组织中的表达量极少, 加之纯化工艺的难度与高成本, 多数研究所得的量甚微, 不能满足对其结构与活性等研究的需要[6,7]。目前多肽合成仍很昂贵; 应研究和开发的需要, 基因工程制备无疑是大量生产防御素的首选方法。

基金项目：国家自然科学基金(No. 30771892); 重庆市院士基金(No. 2007AB5022); 军队“十一五”科技功关项目(No. 06G076)
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收稿日期：2007-08-07; 接受日期: 2007-09-30

1.1 原核细胞表达

为寻求低成本、高效益的制备工艺，人们在原核系统中进行了大量防御素的表达与制备研究。

1.1.1 宿主菌选择：为获得高效表达，选择合适的表达宿主菌是关键步骤之一。重组基因翻译产物在细菌胞质或周质时，易被宿主酶降解，尤其象防御素样的小肽[6]。因此，选择蛋白酶缺失宿主非常有利于外源基因的稳定表达，尤其当重组产物以可溶性形式存在时。当前, Novagen等公司开发了多种蛋白酶缺失或具有特殊功能的基因改造工程菌株可供选择。其中BL21 菌株因缺失*lon*和*ompT*蛋白酶，成为防御素重组表达的首选菌种[7−10]。BL21(DE3)融合有T7 RNA聚合酶基因，特别适用于克隆在pET系列载体中的防御素基因表达[8−11]。大肠杆菌细胞质环境呈还原性，不利于外源多肽的正常折叠，因此选用*trxB*和*gor*基因突变菌株作为表达宿主菌，可能会促进防御素分子内多对二硫键的形成，从而极大地提高可溶性表达量和生物活性[11,12]。

1.1.2 表达载体选择：防御素对宿主细胞具有潜在毒害作用，应尽量选择高度可抑制性和诱导性的启动子，要求很低的本底表达以减少对宿主细胞和表达量的不利影响。目前应用比较广泛的pET系统[8−10,13,15]和pQE系统[14] 调控紧密，重组基因的表达量较高。在表达较长基因时，在转录、翻译和表达量层次上*tac*启动子优于T7 启动子[17]，虽然后者启动转录的能力较强。为获得分泌型的防御素融合蛋白，选择表达载体的启动子强度应尽量与宿主细胞的转运能力保持一致，并优化蛋白表达速率，以防止大肠杆菌的转运系统达到饱和状态[18]。

1.1.3 融合表达策略：鉴于防御素对宿主菌具有毒性，人们多选用融合表达形式。融合表达具有以下优点：①避免了防御素抑/杀表达宿主细胞[19]。②目的肽与承载分子融合后可使目的肽的一些生化特性暂时不在宿主细胞内显示，从而保护防御素免受蛋白酶降解，增强表达产物稳定性[6]，提高表达量[15]。③可利用承载分子的特殊性质进行纯化。④融合蛋白中可设计承载分子与目的肽间的裂解位点，可获得不含多余氨基酸残基的天然序列，有利于活性研究。⑤融合蛋白分子量增大，可不再需要连接半抗原而直接制备目的肽的抗体[20]。因此，融合表达成为获得防御素的主要方法之一。

1) 承载分子选择：将防御素直接与短肽标签融合表达时，表达量均很低[8,19,21−23]，可能原因在于增加的短序列标签并未屏蔽防御素对宿主细胞的毒性。因此在标签序列与防御素之间插入一个承载分子可能具有重要作用。为获得稳定、高效的表达工程菌株，人们尝试了多种承载分子(表 1)，根据生化特性不同，将其分为酸性、中性和具有其它特性的蛋白。酸性承载分子有RepA[7]、Prochymosin[25]、PaP3[14]、MMIS[15]、DEAD-box[17]、Polh[26]和Thioredoxin[8−10]等等，其中后 5 种与特定防御素融合后均获得高表达。原因可能在于：①承载分子多数来源于微生物，不会因含大肠杆菌稀有密码子而妨碍翻译过程。②承载分子PI值均小于 6.0，具有较多阴离子，可以中和防御素的正电荷，避免了翻译生成的阳离子肽与mRNA和DNA相互作用后对宿主细胞产生毒性，降低表达量[15]。③阴离子承载分子与阳离子防御素在各自的正负电荷作用下形成特定的空间结构，使防御素免遭蛋白酶的降解，稳定了表达产物。中性承载分子(如F4 of PurF[15])具有

表 1 承载分子特性
Table 1 Character of carrier molecular

承载分子 (Carrier molecular)	来源 (Origin)	大小 (Size)(kD)	pI	特性 (Character)	目的肽 (Targeted peptide)	表达效率 (Expression efficiency)	文献 (Reference)
RepA	大肠杆菌	10.7	5.7	不溶	CP2600	约 20%	7
Thioredoxin	细菌	11.2	4.1	可溶、促二硫键形成	hBD-2	346 mg/L	8
PaP3	绿脓假单胞菌	17.6	5.6	可溶	hPAB-β	40%	14
MMIS	Magainin	2.55	2.6	酸性、可溶、包涵体	Buforin II	50%	15
Prochymosin	牛	40.6	4.8	不溶	P2	16%	24
Polh	杆状病毒	24.5	5.8	不溶、抗降解	Hal18	40%	25
F4 of PurF	大肠杆菌	15.2	unknown	中性、不溶	MSI-344	30%	27

不溶特性，能促进翻译产物形成包涵体，屏蔽防御素的生物活性，保护了宿主细胞，同时防御素也不会被酶降解，包涵体也利于产物纯化。因此选用中性蛋白作为融合伴侣也可能是获得高表达防御素的一种策略。人们还尝试了一些具有特殊性质的承载分子，企图找到高效稳定表达特定防御素途径。Intein与不含半胱氨酸的线性肽融合表达时不需设计裂解位点，利用其特有性质可将目的肽纯化，但表达量偏低[28]。

选择合适的承载分子与目的肽进行融合是成功重组表达的关键因素之一。二者之间是否匹配主要取决于承载分子的大小、生化特性和目的肽自身的结构特性。模拟防御素体内表达的前肽结构可能是选择和设计承载分子的较好原则。设计和选择承载分子时应考虑以下因素：(1)承载分子要大于目的肽2 倍以上，便于稳定表达和纯化过程中二者的层析分离；(2)承载分子 pI 值应与目的肽相差 2 以上，从而使整个融合蛋白的等电点趋于中性，利于在宿主菌体内稳定存在，以及离子交换层析过程中的良好分离；(3)承载分子亲水性要好，以增强表达产物可溶性。(4)在承载分子和目的肽间应增加适当的裂解位点，以便于纯化过程中二者的分离。

2) 肽分子串联表达：追求目的肽的高生产效率是开发研究必须解决的问题。虽然与承载分子的融合可大幅度提高目的蛋白的产量，但由于防御素分子小，实际分子产率却较低。因此，将肽分子单体自身简单串联[34,35]、或串联多聚体与承载分子融合[36,37]、或与承载分子融合成的杂合单体再串联[15,38]、或杂合单体串联体又与承载分子融合[17]的表达策略可能是又一优选方案。利用同尾酶可构建肽分子同向串联聚合体，产物切割后能得到末端一致的单体。肽分子单体串联体很单一，理论上计算表达产物的生化特性可能偏碱(阳离子肽)或偏酸(阴离子肽)，也许得不到高效表达的工程菌株。但Rao等[34]利用此法获得高产量hPAB-β。肽分子串联后与承载分子融合表达可能是获得高效表达与高分子产率的优选方案。然而，关于串联表达能否提高多肽得率的研究结果尚不一致[15,34,35,37,38]，还受到目的基因、承载分子、表达载体和宿主菌等因素的影响。研究还发现，目的肽得率也不是简单推算的随着串联单体数越多，就可获得越高的表达量和纯化单体[34,37,38]。对于特定表达体系，高效表达的串联载体不同，有表达量较佳的二聚体[38]、三聚体[34]、四聚体[38]、六聚体[15]、九聚体[35]或十二聚体[17]等；有时随着串联数增加，表达量反而降低。产生此现象的原因可能是不同防御素的生化特性不同，多聚体构象的改变以及质粒在宿主菌中的稳定性极差导致了串联表达失败；或因富含半胱氨酸的防御素串联翻译后形成分子间交联和不正确折叠，表达产物形成难溶包涵体致使不能纯化[34]；或因串联体转录后形成较长的裸露RNA，易受酶的功击而处于不稳定状态，进而导致翻译失败。为稳定长链RNA，Lee等[17]将串联体再与DEAD-box承载分子融合获得了高效表达。 在特定防御素的小试开发研究中，应充分考虑这些因素对获得高效表达的工程菌株可能造成的影响。

1.2 酵母表达系统

近年来，已有数种防御素在酵母系统中成功表达[59,63,69]。研究表明，防御素在酿酒酵母中的表达水平较低[70−72]；选用毕赤巴斯德酵母获得了高效表达[63,64,66]，表达量高达 70 mg/L，并且重组肽具有天然肽相似的二级结构和生物活性。目前，酵母系统在防御素工业开发中尚面临的主要问题有：(1) 应用较多的pPIC9K等表达载体均应用α因子信号肽将目的肽分泌出胞内，但常出现因信号肽酶切割效率低而导致目的肽N-端多出数个氨基酸[63]。(2) 重组子表型同外源基因整合的拷贝数和高表达特性尚不一定对应，有待建立更好的挑选高效表达阳性重组子的方案[62]。(3) 防御素在表达和纯化过程中易被降解[60]。(4) 如何避免目的肽在表达中不进行可能会影响结构与活性的无关修饰等。尽管如此，随着更多、更好的表达载体、工程菌株的开发和发酵工艺的发展，酵母可能将成为防御素工业开发的表达系统。

1.3 其它表达系统

杆状病毒表达系统利用瞬时感染、裂解受感染的昆虫细胞，从而获得可能正确修饰的重组蛋白。目前每年有数千个基因利用该系统进行表达和功能分析，昆虫正日益成为一个非常有用的生物技术表达宿主。结构和活性需要酰胺化的防御素选用该系统进行重组表达可能是明智之举[60]。然而，可能由于病毒感染昆虫细胞的效率和表达量低[74,75]等原因，未见有利用该系统大量表达防御素的报道。由于哺乳动物细胞的培养条件严格，加之其表达目的蛋白

表 2 融合蛋白裂解方法
Table 2 Cleavage method of fusion protein

化合物/酶 (Compound/enzyme)	切割位点 (Site of cleavage)	特性 (Character)	目的肽 (Targeted peptide)	切割效率 (Efficiency of cleavage)	文献 (Reference)
溴化氰(CNBr)	---M┆---	剧毒、平端	hPAB-β Buforin Cg-Def CEMA HBD2	~15% – ~80%	14、15、13、7、76
羟胺(Hydroxylamine)	---N┆G---	粘端	Hal18、hPAB-β、MSI-344	~75%	26、34、27
凝血酶(Thrombin)	---LVPR┆GS---	粘端	cecropin	~100%	54

的水平较低, 目前仅有少数研究者用来探索特定防御素的表达规律、生理功能与作用机制, 尚不可能用于开发生产。

2 重组防御素的纯化

多肽纯化过程主要包括以下步骤：①捕获阶段, 主要从细菌破胞后的上清、包涵体裂解液等体系中收集目的蛋白, 去除部分杂蛋白。对于不带标签的蛋白可选用离子交换层析, 带标签的可直接采用亲和层析或选用离子交换层析后再用亲和层析[14,17]; ②粗纯阶段, 从酶解或化学裂解融合蛋白体系、酵母发酵上清、动物细胞培养上清等溶液中回收目的肽。此步骤主要采用阳离子交换层析或凝胶层析[14,61,62,64]。将目的肽从融合蛋白中解离是纯化的关键步骤(表 2)。③精制阶段, 主要采用反相层析技术[64], 以期获得高纯度目的肽。

对于不同来源的重组防御素应根据体系中各种蛋白的生物化学特性, 设计出步骤少、成本低的纯化策略, 以获得高回收率。目前尚未见重组防御素纯化工艺的报道, 纯化工艺的开发也是防御素重组生产亟待解决的问题。

3 开发前景

防御素的药用价值已受到科技界和企业界的广泛关注, 其可能成为新一代抗微生物药物[80−83]。尽管如此, 防御素的开发和临床应用却不得不面临挑战[84]：①生产成本太高; ②未开发出稳定的剂型; ③缺乏系统的药效与代谢动力学和毒理学研究; ④缺乏快速有效地筛选高效低毒的防御素突变体策略等问题。然而, 国外数家制药公司在防御素开发中已取得了很多可喜成果。达托霉素已获美国FDA批准应用于皮肤感染的临床治疗; 近十种防御素制剂已进入临床Ⅰ、Ⅱ或Ⅲ期试验; 并且有数十种防御素正在进行临床前的研发阶段[80−84]。

基因组学与生物信息学的发展给防御素的研究注入了新的活力, 不断有新防御素被发现。防御素构效关系和重组制备已成为当前研究的热点之一。首先, 研究者们希望模拟分子体内进化原理, 基于已知多肽的功效关系所获得的知识, 应用生物信息技术对防御素分子施行体外设计和分子进化研究, 试图获得结构更为简单、生物活性更强、无溶血副作用等毒性的突变体[86]; 其次探索稳定高效的基因重组制备策略, 建立成本低廉、可行性强的纯化工艺[87], 以期大量生产防御素。

可以说, 随着科学技术的进步, 制约防御素重组表达与制备和临床应用的一些难题必将解决, 防御素可能在不久的将来会成为感染患者的福音[88−90]。

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