

# 细菌诱导对昆虫细胞系NIH-SaPe-4生长与 抗菌蛋白活性的影响

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**摘要** 离体昆虫细胞系在昆虫免疫、抗菌肽及蛋白研究和药物开发方面具有较好的应用前景。该文对双翅目麻蝇科麻蝇成虫卵巢胚细胞系NIH-SaPe-4在藤黄微球菌诱导和非诱导条件下, 细胞密度和活力的变化、诱导对细胞生长的影响、抗菌活性及其活性随时间的变化关系等进行了研究, 并对所得抗菌蛋白进行了初步分离纯化和稳定性评估。结果表明, 诱导使得细胞密度增长减缓, 活力变弱。诱导和非诱导组细胞均可产生对3种革兰氏阳性菌具有抑菌活性的抗菌蛋白, 其中对藤黄微球菌的抑菌活性最明显; 诱导组细胞抗菌蛋白活性出现时间、稳定期抑菌活性均大于非诱导组, 诱导菌消失一段时间后抗菌活性恢复到同等水平。抗菌蛋白具有酸碱稳定性和热稳定性。2组抗菌蛋白粗提液经凝胶、反相分离纯化后均得到一种60 kDa左右的抗菌蛋白, 诱导组电泳后条带亮度大于非诱导组。该研究为昆虫细胞抗菌蛋白性质、分离纯化等研究奠定了科学基础。

**关键词** 昆虫细胞系; 诱导; NIH-SaPe-4; 抗菌蛋白; 藤黄微球菌

昆虫是地球上种类最多、数量最大的生物类群, 研究其免疫功能与高等动物免疫的内在联系可促进人类对免疫问题的总体认识, 从而为人类健康带来益处, 因此, 昆虫免疫研究已迅速发展为一个新的研究领域<sup>[1-4]</sup>。目前, 已经从昆虫中分离到近80多种抗菌肽和抗菌蛋白<sup>[5]</sup>。抗菌肽和抗菌蛋白是生物体内存在的一类天然抗菌活性物质, 其自身携带有大量的正电荷, 具有广谱抗菌活性并且本身无毒、无害<sup>[1-2, 6-9]</sup>, 对原核生物有极强的杀灭作用而对真核细胞无伤害<sup>[7]</sup>。昆虫抗菌肽通常分子量较小, 除了具有明显的抑菌活性外, 还对癌细胞具有杀伤作用<sup>[8]</sup>; 亦有报道称昆虫抗菌肽Cecropin及其衍生物具有抗疟原虫和锥虫的活性, 且对宿主细胞没有伤害<sup>[10-11]</sup>; 还有一些昆虫抗菌肽对真菌具有很强的杀灭作用<sup>[9, 12]</sup>。另有研究报道, 多种具有与免疫功能相关的蛋白, 本身也具有较强的杀灭细菌的作用, 如转铁蛋白、血蓝蛋白、血红蛋白、溶酶素、酪蛋白、组蛋白、传递信号过程蛋白、早期的免疫发育蛋白等, 一般分子量在10 kDa~100 kDa, 这类蛋白的部分结构或整体对细菌具有较强的杀伤作用<sup>[13-19]</sup>。因此, 抗菌肽和抗菌蛋白在未来临床和商业方面具有很好的应用前景<sup>[20]</sup>。

现有的昆虫抗菌肽及蛋白研究主要集中在体

型较大的昆虫活体中进行, 但大量活体昆虫的获得受季节和养殖技术的影响, 同时由于虫体的蛋白含量较低, 从虫体内提取分离和纯化较困难, 因此较难大量获得抗菌肽及蛋白, 也很难进行分子机制方面的研究。离体昆虫细胞由于在人工培养液中生长, 可不受季节限制, 且从培养液中分离纯化抗菌肽及蛋白相对较容易, 在昆虫抗菌肽和蛋白研究方面显示出很好的应用价值。有研究报道, 在昆虫传代细胞系中发现了转铁蛋白<sup>[21]</sup>及其它新的抗菌肽<sup>[9, 22-24]</sup>; 大肠杆菌诱导的菜青虫离体细胞系能够产生对大肠杆菌起杀灭作用的抗菌肽<sup>[24]</sup>; 草地贪夜蛾细胞系SF-9、斜纹夜蛾细胞系SL-1、粉纹夜蛾细胞系BTI-Tn-5BI经诱导后的培养液也分别对大肠杆菌K12D31和德氏卑沙门氏菌有较强的杀灭作用<sup>[25-26]</sup>。研究结果为从离体昆虫细胞系中获得抗菌肽及蛋白提供了可能, 同时, 昆虫细胞系还为免疫反应的研究提供有用的体外模型和手段。

NIH-SaPe-4是由双翅目麻蝇科(Sarcophagidae)麻蝇(*Sarcophaga peregrina* Rob.-Desvoidy)成虫卵巢胚建立的细胞系<sup>[27]</sup>, 其细胞在正常培养过程中就会

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产生Cecropin样的4类抗菌蛋白,其分子量为4 kDa至20 kDa,对多种微生物具有较强抑菌活性<sup>[28-33]</sup>。但缺少细菌诱导对细胞生长的影响、细菌诱导对抗菌蛋白活性影响等方面的报道。本文重点研究NIH-SaPe-4细胞系在诱导和非诱导条件下,抗菌蛋白产生随时间的变化关系、抗菌蛋白抑菌活性的强弱变化、诱导对细胞系生长的影响以及抗菌蛋白的分离纯化等,以期昆虫细胞系抗菌蛋白研究奠定科学基础。

## 1 材料与amp;方法

### 1.1 供试细胞系及其培养

NIH-SaPe-4细胞系由东京农业大学三桥淳博士馈赠,保存于本所细胞库;实验时按照常规方法复苏,复苏后的细胞用辅加10%胎牛血清的MM培养基培养,PBS漂洗2次,备用。

### 1.2 供试细菌及其处理

抑菌试验检测菌:藤黄微球菌(*Micrococcus luteus*)、金黄色葡萄球菌(*Staphylococcus aureus*)、枯草芽孢杆菌(*Bacillus subtilis*)、大肠杆菌(*Escherichia coli*)、绿色脓杆菌(*Pseudomonas aeruginosa*) (以下简称藤黄、金球、枯草、大肠、绿脓)保存于本所菌种库, LB固体培养基培养, 36 °C过夜以备用。

诱导菌:藤黄微球菌,接种于LB液体培养基, 150 r/min恒温摇床培养过夜, 121 °C、25 min灭活, PBS漂洗2~3次,以备诱导用。

### 1.3 诱导方法和NIH-SaPe-4生长状况的观测

将藤黄微球菌和NIH-SaPe-4细胞按细菌:细胞=1 000:1的比例加入NIH-SaPe-4细胞MM培养液中,对照组不加藤黄微球菌, 26 °C恒温培养,每隔12 h显微镜观察细胞生长状况并拍照,用Vi-cell活力分析仪采集细胞的生长状况参数(细胞活力、活细胞数量、总细胞数量),同时收集细胞培养液,将细胞培养液经0.22 μm微孔过滤后于-20 °C冷冻保存,共得到不同培养时段抗菌蛋白粗提液样品86个,待观测结束后所有样品一起冷冻干燥,干燥粉末样品置于-20 °C冰箱冻存备用。抗菌活性检测前用500 μL MM培养基溶解。

### 1.4 抗菌蛋白活性检测

杯碟法:采用麦式比浊法用生理盐水调节金球、枯草、大肠、绿脓、藤黄等5种菌浓度至 $1 \times 10^5$ /mL,用无菌棉棒涂匀涂在预先装于9 cm医用培养皿中的LB培养基平板上,每皿放置灭过菌的直径5 mm圆滤

纸片7个,每个滤纸片加5 μL上述处理过的样品,每个样品设置3个重复和1个培养基对照,培养皿置于36 °C恒温条件下培养16 h。游标卡尺测量抑菌圈直径,数据减去滤纸片直径。

比浊法:在96微孔板中加入处理过的样品10 μL,每个样品设置3个重复和对照,将96微孔板放入冷冻干燥机内,冻干样品,分别在每个孔中加入相同浓度的藤黄微球菌液250 μL, 36 °C恒温培养8 h。自动ELISA仪对藤黄微球菌的浓度进行测定。其中 $D_{606}=1$ 代表菌浓度为 $1 \times 10^9$ /mL; D值低,抑菌活性强, D值高,抑菌活性弱。

### 1.5 抗菌蛋白的耐酸碱性、热稳定性

(1)用盐酸、磷酸、氨水配置pH分别为1, 3, 5, 6, 7, 8, 9, 11和13的溶液,分别与等体积样品溶液混合,室温放置1 h后进行抑菌试验,每个处理设置3个重复,以同浓度的未处理抗菌蛋白粗提液样品、细胞培养液为对照,采用比浊法检测处理后的样品对藤黄微球菌的抑菌活性。

(2)分别对样品进行沸水浴,时间梯度为:20, 40, 60, 80, 100, 120 min,进行抑菌试验,每个处理设置3个重复,未沸水浴对照(0 min样品)和培养基作对照,比浊法检测处理后的样品对藤黄微球菌的抑菌活性。

### 1.6 抗菌蛋白的分离纯化及SDS-PAGE电泳

收集的样品分别按照诱导组和非诱导组合并后冷冻干燥、测定质量后用等量超纯水溶解,0.22 μm膜过滤。凝胶柱采用Sephadex G-200(1.6 cm×60 cm),洗脱液为0.05% NaCl,流速1 mL/min, 3 mL/管收集;反相柱采用Resource 15RPC(3 mL),溶液A:0.1% TFA,溶液B:80%乙腈-水溶液(含0.085% TFA, V:V)。梯度洗脱,流速2 mL/min, 0.5 mL/管收集组分。将收集的各组分冷冻干燥后与细胞系培养液一起进行Tircine-SDS-PAGE,方法参照Schagger<sup>[34]</sup>,分离胶浓度为10%,浓缩胶为4%,实验电压90 V,考马斯亮蓝染色30 min,凝胶成像仪拍照。

### 1.7 结果分析

不同时间段收集样品的细胞生长参数,抑菌活性数据应用SPSS 19.0进行配对, *t*检验分析。

## 2 结果

### 2.1 诱导对NIH-SaPe-4细胞生长的影响

诱导和非诱导条件下NIH-SaPe-4细胞的生长状况见图1,活细胞密度随时间变化关系见图2。由图

1和图2结果可知: 在培养的72 h前诱导和非诱导组细胞密度增长速度均变化不大。非诱导组细胞生长恢复期为72 h, 72 h后进入对数生长期, 细胞的倍增时间

为24 h, 细胞达到最大密度所需时间为288 h, 492 h后细胞密度开始下降。诱导组细胞恢复期为108 h, 108 h后进入对数期, 细胞的倍增时间在24~36 h之

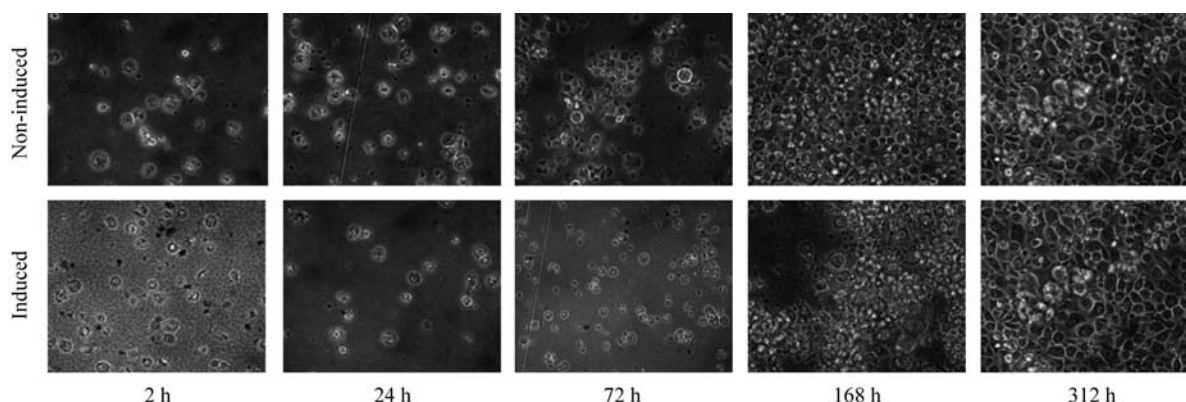


图1 诱导组和非诱导组细胞生长状态随时间变化图

Fig.1 The imagines of cell growth in the induced and non-induced conditions

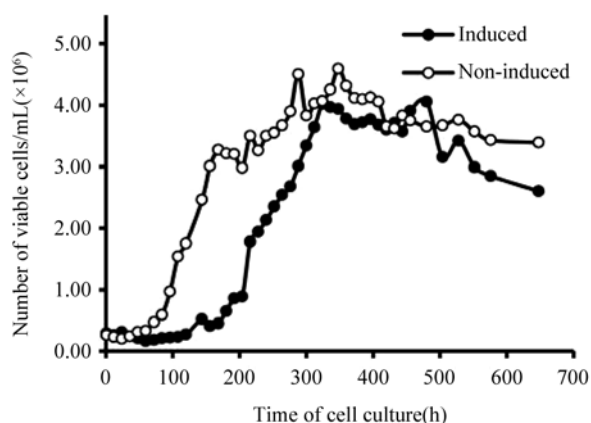


图2 诱导和非诱导组活细胞数量随时间变化图

Fig.2 The viable cell numbers of induced and non-induced groups according to time order

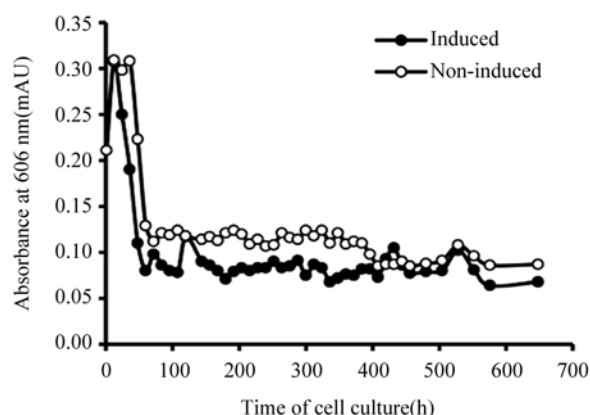


图3 诱导组和非诱导组抑菌活性对比

Fig.3 The comparison of antibacterial ability between induced and non-induced groups

表1 诱导组和非诱导组随时间变化的抑菌活性

Table 1 The Amp's antibacterial activity of induced and non-induced groups according to time order

时间(h) Time(h)	藤黄微球菌 <i>Micrococcus luteus</i>		枯草芽孢杆菌 <i>Bacillus subtilis</i>		金黄色葡萄球菌 <i>Staphylococcus aureus</i>		大肠杆菌 <i>Escherichia coli</i>		绿色脓杆菌 <i>Pseudomonas aeruginosa</i>	
	In	Non	In	Non	In	Non	In	Non	In	Non
0	0	0	0	0	0	0	0	0	0	0
12	2.7	0	0	0	0	0	0	0	0	0
24	9.8	5.9	0	0	0	0	0	0	0	0
36	12.1	8.1	2.8	0	0	0	0	0	0	0
48	15.5	10.2	4.4	2.2	3.5	0	0	0	0	0
60	19.3	13.5	4.2	3.7	3.6	0	0	0	0	0
72	17.7	15.1	4.8	3.4	3.4	0	0	0	0	0
324	18.0	14.4	4.7	3.5	3.3	1.5	0	0	0	0
480	14.6	13.8	4.9	3.8	3.8	1.9	0	0	0	0

表中抗菌蛋白的活性数据已减去滤纸片直径。单位: mm; In: 诱导组; Non: 非诱导组。

The dates of AMPs activity have removed the diameter of filter paper. Unit: mm; In: induced group; Non: non-induced group.

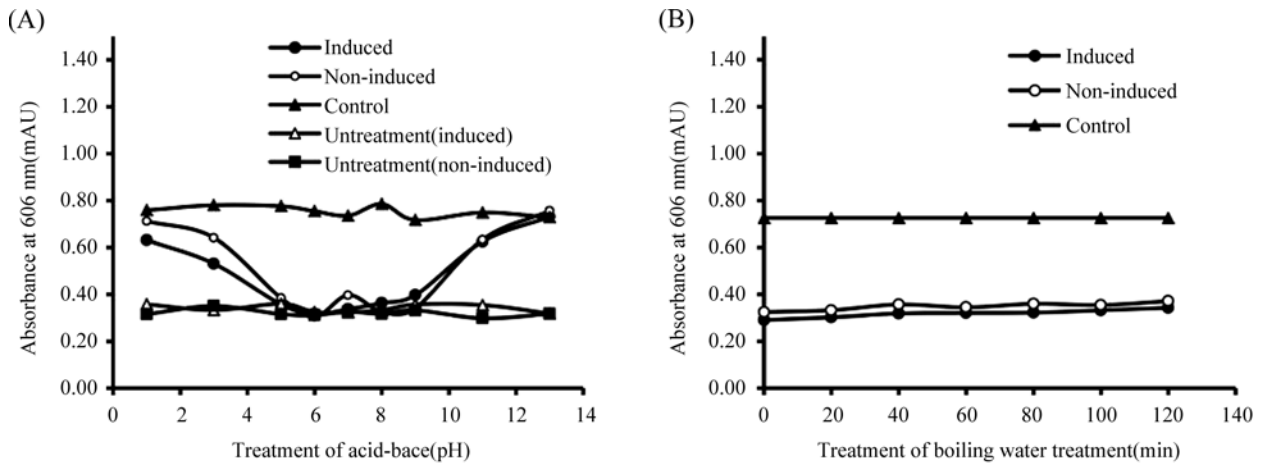
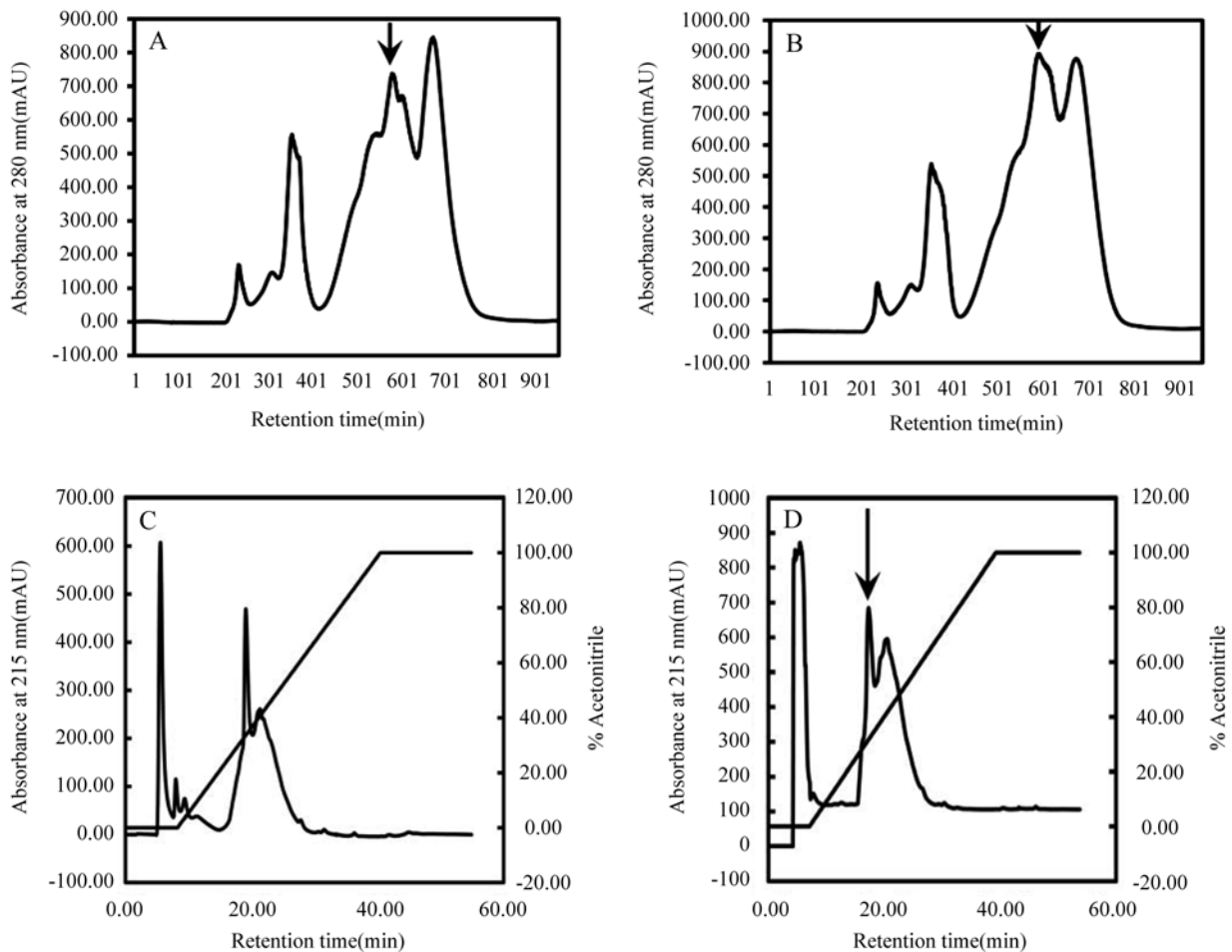


图4 抗菌蛋白的酸碱(A)和温度(B)耐受性

Fig.4 Amps' tolerance of acid-base (A) and stability of thermal (B)

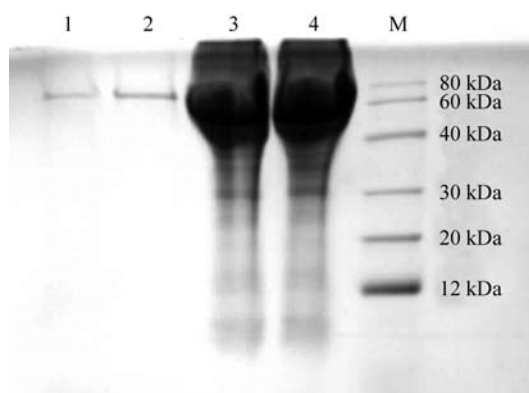


A: 非诱导组的SephadexG-200纯化; B: 诱导组SephadexG-200纯化; C: 非诱导组的Resource 15RPC纯化; D:诱导组Resource 15RPC纯化。箭头所示为活性组分所在峰。

A: purification of non-induced group by SephadexG-200; B: purification of induced group by SephadexG-200; C: purification of non-induced group Resource 15RPC; D: purification of induced group Resource 15RPC. The peaks with antimicrobial activity was marked by the arrows.

图5 诱导组和非诱导组抗菌蛋白的分离纯化

Fig.5 Isolation and purification of Induced and non-induced AMPs



1: 非诱导组; 2: 诱导组; 3: 非诱导组(纯化前); 4: 诱导组(纯化前); M: marker。

1: non-induced group; 2: induced group; 3: non-induced group before purification; 4: induced group before purification; M: marker.

图6 Tricine-SDS-PAGE电泳

Fig.6 Tricine-SDS-Polyacrylamide Gel Electrophoresis

间, 324 h细胞数量达到最大值, 468 h后细胞数量开始减少。诱导组和非诱导组在324~468 h之间细胞密度基本保持一致, 但是诱导组提前进入衰亡期。诱导组培养液中加入的藤黄微球菌随培养时间延长而减少, 72 h消失大半, 168 h显微镜下已观察不到细菌存在。非诱导组未加入菌, 为正常细胞培养, 无此现象。

诱导组和非诱导组细胞活力配对 $t$ 检验结果为 $P=0.013<0.05(T=-2.589, df=43)$ , 诱导组和非诱导组活力之间存在显著差异。分析结果表明, 藤黄微球菌的加入使得NIH-SaPe-4细胞恢复期延长、对数期减缓、稳定期缩短、衰亡期提前, 影响了细胞的生长。

## 2.2 诱导和非诱导条件下抗菌蛋白活性分析

杯碟法所得不同时间点诱导组和非诱导组抗菌蛋白对3种革兰氏阳性菌和2种革兰氏阴性菌的抑菌结果见表1, 比浊法所得2组细胞产生抗菌蛋白的时间关系和抑菌强度结果见图3, 由试验结果可知: NIH-SaPe-4细胞抗菌蛋白粗提液对3种革兰氏阳性菌(藤黄、金球、枯草)均具有较强抑菌作用, 对2种革兰氏阴性菌(大肠、绿脓)无抑菌活性。诱导组细胞分泌抗菌蛋白时间早于非诱导组。2组抗菌蛋白抑菌活性配对 $t$ 检验结果为 $P=0.000<0.05(T=-7.662, df=43)$ , 诱导组和非诱导组存在显著差异, 细菌诱导使NIH-SaPe-4细胞产生抗菌蛋白的时间提前, 活力加强。

## 2.3 抗菌蛋白的耐酸碱能力及温度稳定性

抗菌蛋白粗提液经酸碱和高温处理后的抑菌活性结果见图4。从图4A结果可见, pH5~7范围内抗

菌蛋白粗提液的抑菌活性与未处理组差异不大, 但在pH小于3和pH大于11的极端环境下, 样品的活性会受到很大的影响甚至消失。从图4B结果可知, 沸水浴2 h抗菌肽仍然保持较高活性。结果表明, NIH-SaPe-4细胞的抗菌蛋白具有一般抗菌蛋白的性质, 即酸碱相对稳定性和高温稳定性。

## 2.4 抗菌蛋白的分离纯化及SDS-PAGE

细胞系抗菌蛋白凝胶分离纯化见图5。诱导和非诱导抗菌蛋白粗提液过凝胶柱后均得到4个大致相同的峰, 利用藤黄菌做指示菌、杯碟法检测均发现在第3个峰(箭头所示)具有抑菌活性。收集凝胶柱第3活性峰组分, 冷冻干燥、0.05% NaCl溶解后, 过反相柱均可得到3个峰(图5), 抑菌活性检测第2个峰均具有抑菌活性。收集反相柱活性峰、浓缩后做小分子量SDS-PAGE, 得到图6, 由电泳图和分离过程可知, 诱导和非诱导条件下NIH-SaPe-4细胞均可产生分子量约为60 kDa抗菌蛋白, 且诱导组分泌量高于非诱导组。结果表明, 藤黄诱导和非诱导NIH-SaPe-4细胞在常规培养条件下具有组成型表达60 kDa的抗菌蛋白的能力, 在外源物质(细菌)刺激的情况下, 其抑菌活性增强。

## 3 讨论

NIH-SaPe-4细胞在藤黄微球菌的刺激下细胞恢复期延长、对数期减缓、稳定期缩短并提前进入衰亡期。诱导和非诱导组均可产生一60 kDa左右的抗菌蛋白, 经诱导的细胞抗菌蛋白产生早于非诱导组, 其抑菌活性的得到加强。诱导组和非诱导组样品过凝胶和反相柱后, 得到分离峰的个数相同, 诱导组活性峰的吸收值有所增加, 经Tricine-SDS-PAGE也显示条带亮度有所增加。分析表明, NIH-SaPe-4细胞抗菌蛋白的产生可不需要外界刺激就具有低水平表达, 在接受外源刺激后, 调控了细胞生长, 同时加强了抗菌蛋白的表达来清除不利因素。但外源刺激对细胞的生长产生了不利的影 响, 提前了细胞的衰亡。

一般认为高等动物的免疫系统表现为低水平组成型表达免疫抗体以快速应对再次感染, 以最经济的方式维持共生异己之间的平衡<sup>[35-37]</sup>; 而昆虫抗菌肽的产生和外界刺激具有直接的关系<sup>[4,13,37-38]</sup>, 诱导产生的抗菌肽对革兰氏阳性菌或者阴性菌的一种具有较强抑制作用而对另一类则抑制作用相对较弱<sup>[40]</sup>。但也有研究表明, 在没有外界刺激条件下, 麻蝇、黄粉

虫和天蚕等昆虫虫体中均有少量的抗菌肽表达<sup>[41-43]</sup>, 昆虫抗菌肽低水平的表达特性具有双重功能<sup>[13,33,37]</sup>, 麻蝇抗菌肽(sapcein)和麻蝇抗菌肽B(sapcein B)在免疫和发育两方面分别发挥功能<sup>[28-33]</sup>。另有报道在未诱导条件下NIH-SaPe-4细胞系的4种抗菌肽在麻蝇虫体早期发育、正常生长和刺激条件下都有所表达, 它们由相同mRNA编码<sup>[33]</sup>。本文研究发现, NIH-SaPe-4细胞系经藤黄诱导后和未诱导基础表达的抗菌肽对革兰氏菌中的阳性菌具有较强的抑制作用, 诱导后活性有所加强, 表明昆虫免疫系统虽然不具有专一性, 但在外界刺激的条件之下依然能够加强一类抗菌肽基因的表达, 至少在麻蝇、黄粉虫和天蚕如此<sup>[28-32]</sup>, 推测昆虫免疫特点可能和高等动物免疫具有相似之处。

抗菌肽或抗菌蛋白按功能一般分为 $\alpha$ -Helical抗菌肽、 $\beta$ -Sheet抗菌肽、extended抗菌肽和抗菌蛋白等。前3种抗菌肽通常分子量较小, 抗菌机制均与其阳离子和亲水基团有关。部分抗菌蛋白的局部序列与抗菌肽具有同源性, 经酶解后具有抗菌肽活性; 部分抗菌蛋白通过隔离微生物生长源和激发免疫功能起到抑菌作用<sup>[43]</sup>。如在埃及伊蚊(*Aedes aegypti*)血淋巴中发现, 参与免疫的蛋白(p44)同时具有诱导细胞程序性死亡的作用<sup>[44]</sup>, 甜菜夜蛾(*Spodoptera exigua* Hübner)血淋巴中获得的抗菌肽Attacin在维持渗透压和抑制细菌生长方面均有重要作用<sup>[45]</sup>, cDNA研究预测其分子量为28 kDa<sup>[47]</sup>。*Aedes aegypti*和白纹伊蚊(*Aedes albopictus*)细胞系在热灭活菌诱导的条件下, 转铁蛋白(分子量为66 kDa)以及其他3种蛋白(分子分别为111 kDa、53 kDa、32 kDa)的组成型表达得到了加强, 其中转铁蛋白具有抑菌活性, 其作者认为转铁蛋白除在铁代谢方面发挥作用外, 还可通过隔离铁离子抑制外源微生物的生长<sup>[13]</sup>, 人类乳铁蛋白也具有相似的功能<sup>[47]</sup>。昆虫血蓝蛋白<sup>[48]</sup>、18w蛋白<sup>[15]</sup>、免疫信号途径参与蛋白<sup>[42-43]</sup>、果蝇霉素等组成型表达蛋白, 对虫体的供氧、输铁及其他生理功能方面均发挥重要作用<sup>[13,48]</sup>, 同时也具有杀伤细菌的作用<sup>[49]</sup>。本文研究发现, NIH-SaPe-4细胞系经藤黄诱导后和未诱导基础表达的抗菌蛋白对革兰氏菌中的阳性菌具有较强的抑制作用, 诱导后活性有所加强, 其分子量为60 kDa左右, 而*Aedes aegypti*细胞系、*Aedes albopictus*细胞系和NIH-SaPe-4均来源于双翅目昆虫, 推测本文所发现的60 kDa左右蛋白质有可

能为转铁蛋白p66。但该蛋白是否如已报道的转铁蛋白p66一样在昆虫细胞内具有参与铁代谢和抑菌活性双重功效, 以及该蛋白的氨基酸序列、结构预测等尚待进一步研究。

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## Influence of Bacterial Induction on Cell Growth and Antimicrobial Peptide Activity in Insect Cell Lines NIH-SaPe-4

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**Abstract** Insect cell lines can be applied in the studies of antimicrobial peptides, protein (Amps) and drug development with multiple advantages. In the present study we evaluated the changes of cell density and cellular vitality in the insect cell lines NIH-SaPe-4, which originated from ovarian germ cell of adult flesh fly (*Diptera: Sarcophagidae*), after inducing by *Micrococcus luteus*. We also assayed the activity of antimicrobial protein and the changes of activity over time. Finally, the antimicrobial protein was isolated and purified and the stability of the protein was assessed. Our result demonstrated that the inducing by bacteria caused the insect cell growth slowed and impaired the cellular vitality. Both the induced group and control group could produce antimicrobial proteins with inhibitory activity against 3 types of Gram-positive bacteria. The protein showed the strongest antibacterial activity against *Micrococcus luteus*. The antimicrobial activity appeared earlier in the induced cells group, and the inhibitory activity of antimicrobial protein in induced cells group was higher than in the control group. Furthermore, the antibacterial activity in the induced group returned to the same level as in the control group after the disappearance of inducing bacteria. A kind of 60 kDa antimicrobial protein was obtained, after electrophoresis and reversed-phase purification, from the crude extracts of both induced and control groups. The electrophoresis band intensity from induced group was greater than which from the control group. The obtained antimicrobial protein showed pH stability and thermal stability. Our result could provide basic information for future studies which focus on purification and characterization of antimicrobial peptides and proteins from insect cell.

**Key words** insect cell; induction; NIH-SaPe-4; antimicrobial proteins; *Micrococcus luteus*

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