



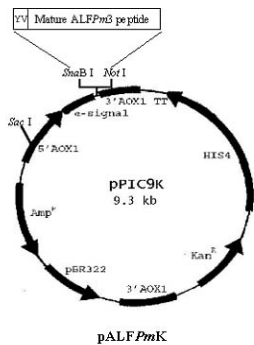
Anti-lipopolysaccharide factor (ALF) in shrimp species: from characterization to biological activities and gene expression analyses

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Anti-lipopolysaccharide factors (ALFs), originally characterized from horseshoe crabs, have been recently identified from hemocytes of the black tiger shrimp, *Penaeus monodon*, by a genomic approach. This molecule is a small basic protein located in large granule hemocytes of horseshoe crab. It binds and neutralised the toxic lipid A moiety of LPS leading to inhibition of the endotoxin-mediated activation of the coagulation cascade. In order to characterize the properties and biological activities of this immune effector, ALFPm3, was expressed in the yeast *Pichia pastoris*. In addition, to approach the function of this effector, the expression of ALFPm3 encoding gene has been studied in shrimp in response to microbial challenge.

1- Construction of *Pichia pastoris* strain producing ALFPm3.



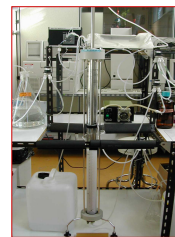
The region coding for ALF mature peptide was amplified and cut with SnaBI and NotI in order to ligated to the pPIC9K plasmid. The SacI-linearized pALFPmK plasmid was then transformed to *P. pastoris* KM71 to generate the recombinant ALFPm3 (rALFPm3). ALFK9 *Pichia* transformant was selected by G418-sulfate resistance screening.

2- Large-scale production in fermentor provided around 200-300mg/L of recombinant ALF that was purified to homogeneity.



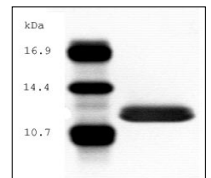
Culture conditions

- Volume: 5 l
- Synthetic medium (Boze et al., 2000)
- Batch culture on glycerol (biomass production)
- Fed-batch culture on methanol (induction phase)
- Theoretical growth rate: 0.01 h⁻¹



Purification of recombinant protein

- Ion exchange chromatography on streamline SP (Pharmacia)
- Matrix equilibrated in 20 mM Tris-HCl buffer, pH7 + 200 mM NaCl
- Elution of protein with 1M NaCl



3- The spectrum of antimicrobial activity of rALFPm3 was established *in vitro* against a range of microorganisms.

Microorganisms	Minimum inhibitory concentration (MIC, μM)
Gram (+) bacteria	
<i>Bacillus megaterium</i>	0.08-0.12
<i>Micrococcus luteus</i>	3.07-4.61
<i>Staphylococcus aureus</i>	>35
Gram (-) bacteria	
Enterobacter cloacae	3.07-4.61
<i>Erwinia carotovora</i>	1.37-2.05
<i>Escherichia coli</i> 363	0.08-0.12
<i>Klebsiella pneumoniae</i>	2.05-3.07
<i>Salmonella thyphimurium</i>	6.91-10.37
<i>Vibrio alginolyticus</i>	0.27-0.40
<i>Vibrio anguillarum</i>	0.91-1.37
<i>Vibrio harveyi</i>	0.91-1.37
<i>Vibrio penaeicida</i>	>23.3
Filamentous fungi	
<i>Fusarium oxysporum</i>	2.07-3.05

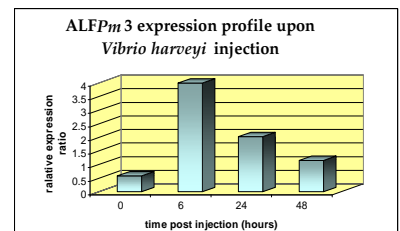
MIC are expressed as the interval a-b, where a is the highest concentration tested at which the growth of the microorganism is not inhibited and b the lowest concentration that causes the 100% growth inhibition

4- The toxicity of rALFPm3 towards the live food *Artemia* was investigated and showed that the peptide is not toxic for *Artemia*.

rALFPm3 Concentration (μM)	Artemia nauplii (instar I)	Artemia nauplii (instar II-III)
100	100 ± 0 ^a	90 ± 17 ^a
10	97 ± 8 ^a	93 ± 6 ^a
1	100 ± 0 ^a	93 ± 8 ^a
0.1	97 ± 8 ^a	90 ± 10 ^a
0	100 ± 0 ^a	93 ± 12 ^a

Survival (%) of *Artemia* nauplii after exposure during 24h to recombinant ALF (mean ± stdev, n=3, values within the same column with different superscript are statistically different, P<0.05).

5- Expression profile of ALFPm3 in hemocytes after microbial challenge.



Quantitative expression of ALFPm3 gene relative to Elongation factor 1 alpha in hemocytes of 10⁵ CFU of live-*Vibrio harveyi* infected *P. monodon* at various time after injection. This experiment used shrimps injected with 0.85% NaCl as control.

Pichia pastoris expression system provided large amount of functional rALFPm3 which allowed us to characterize its antimicrobial activity spectrum as well as its *in vivo* effect on larvae. Broad antimicrobial spectrum and activity against some pathogenic microorganisms revealed that rALFPm3 is a potentially candidate of a clinically applicable antimicrobial agent.

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