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Characterization of a natural larger form of the antifungal protein (AFP) from *Aspergillus giganteus*

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Abstract

Two major proteins, α-sarcin and an antifungal polypeptide (AFP), are secreted by the mould *Aspergillus giganteus* MDH 18894 when it is cultured for 70–80 h. A third major protein is also found in the extracellular medium at 48–60 h, but it disappears as the culture proceeds. This protein has been isolated and characterized in terms of apparent molecular mass, electrophoretic and chromatographic behaviour, NH_2-terminal primary structure, amino acid content, spectroscopical features, reactivity against anti-AFP antibodies, and antifungal activity. Based on the obtained results it would be an extracellular inactive precursor form of AFP, designated as the *l*arge *f*orm of AFP (lf-AFP). Its amino acid composition is identical to that of AFP but containing six extra residues. NH_2-terminal sequence analysis of the first eight amino acid residues of this polypeptide revealed that the extra residues can be perfectly accommodated within the DNA-deduced sequence of the precursor form of AFP. Its alignment with precursor sequences of different proteins, secreted by a variety of *Aspergillus* spp., reveals the existence of a common tetrapeptide at the carboxy-terminal end of their leader peptides. This sequence would be Ile/Leu-Xaa-Yaa-Arg, being mostly Xaa and Yaa an acid residue (Asp/Glu) and alanine, respectively. The presence of lf-AFP as an extracellular protein would be in perfect agreement with the existence of this tetrapeptide motif, that can be involved in the protein secretion mechanisms of filamentous fungi.

Keywords: Precursor sequence; Structural motif

1. Introduction

AFP is a highly basic and small-size *anti*f*ungal* protein secreted by the mould *Aspergillus giganteus* MDH 18894 [1]. This molecule has been recently characterized [2–4], including the elucidation of its three-dimensional structure in aqueous solution [5]. It exhibits a new folding motif for a complete protein: five highly twisted antiparallel strands, defining a small and compact β-barrel with the four disulfide bridges of the molecule remaining within the interior of this structure [5]. This protein, which has been also revealed to be extremely resistant to proteases [4], would be the first representative characterized member of a new group of polypeptides within the much larger family of toxic proteins organized around a three- or four-disulfide core, such as defensins [6] or thionins [7].

Abbreviations: AFP, antifungal protein; DTT, D,L-dithiothreitol; MIC, minimal inhibitory concentration; PAF, *Penicillium chrysogenum* antifungal protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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AFP can be purified in rather large amounts from the extracellular medium of *A. giganteus* cultures after 80–100 h of fermentation at 30°C [1,4]. However, we have observed the presence of a larger form of this protein if cultures are collected after only 48–60 h of incubation. The isolation and characterization of this new polypeptide, as well as its relationship with the *Aspergillus* spp. leader peptides, are herein reported.

2. Materials and methods

The proteins were purified from the extracellular medium of *A. giganteus* MDH 18894 incubated at 30°C for 48–60 h. The culture medium employed, as well as any other fermentation conditions, have been reported elsewhere [1,4,5]. The purification procedure was that already described for α-sarcin [8] and AFP [4] and includes retention of the proteins on Amberlite IRC 50, equilibrated in 50 mM sodium phosphate buffer (pH 7.0), followed by elution with 0.6 M NaCl. The fractions containing the selected polypeptides were pooled, dialyzed, concentrated and loaded onto a gel filtration column (3 × 120 cm) of Biogel P10, equilibrated in 50 mM Tris-HCl buffer (pH 7.0), containing 0.1 M NaCl. This second chromatography rendered electrophoretically homogeneous proteins which were further desalted on a Biogel P2 column, equilibrated in either 50 mM acetic acid or 50 mM ammonium carbonate (pH 7.0).

Antifungal activity was assayed against *Fusarium oxysporum* and *Trichoderma koningii* as described in Ref. [4]. The agar dilution method was used to measure this antimicrobial activity. Minimal inhibitory concentrations (MIC; concentration of protein that caused the complete inhibition of growth) were evaluated at 30°C after incubation for 48–72 h.

Absorbance measurements were carried out on an Uvikon 930 spectrophotometer. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter, and the results were expressed as mean residue weight ellipticities in units of degree × cm^2 × $dmol^{-1}$. The mean residue weight employed was 113, calculated from the amino acid sequence of AFP [2]. Fluorescence spectra were recorded on a SLM Aminco 8000 spectrofluorimeter. All these determinations were performed under conditions described elsewhere [9].

Protein hydrolyses were performed at 108°C in evacuated and sealed tubes for 24 h, with 5.7 N HCl, containing 0.1% (w/v) phenol and *nor*-leucine as internal standard, and analyzed on a Beckman Model 6300 automatic amino acid analyzer equipped with an IBM-based System Gold enhancement. Automatic Edman degradation of the proteins was performed on an Applied Biosystems model 477A sequencer using a model 120A on-line phenylthiohydantoin analyzer and the standard Applied Biosystems program [10]. Polyacrylamide gel electrophoresis were developed in the presence of 0.1% (w/v) SDS, according to [11]. When necessary, samples were previously reduced with 5% (v/v) 2-mercaptoethanol or 0.2% (w/v) DTT. Proteins were either stained with Coomassie Brilliant Blue R-250 or transferred to Immobilon membranes. Electrophoretic transfer was performed for 1 h at 0.9 mA/cm^2 in 48 mM Tris-HCl, 39 mM Gly, 0.037% (w/v) SDS and 20% (v/v) methanol. Immunodetection was carried out by first incubating the membrane with rabbit polyclonal antibodies raised against homogeneous AFP, and then with goat anti-rabbit IgG horseradish peroxidase conjugate. PAGE-SDS was also used to calculate the molecular mass of

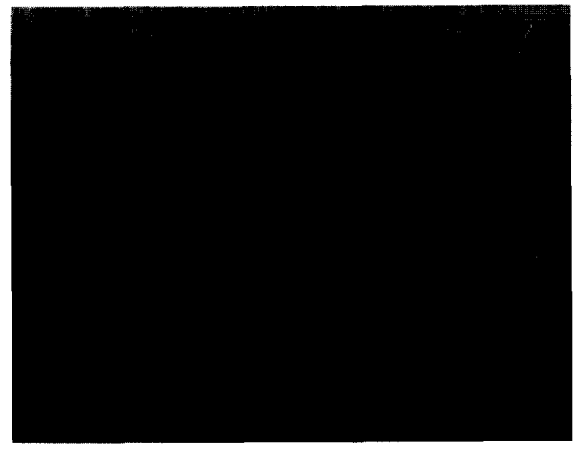

Fig. 1. 15% PAGE–0.1% SDS of *Aspergillus giganteus* MDH 18894 extracellular medium aliquots taken at different times. Lane 1: 24 h; lane 2: 48 h; lane 3: 60 h; lane 4: 75 h; lane 5: 120 h; lane 6: isolated α-sarcin; lane 7: BioRad Low Range molecular mass standards: phosphorylase b (97.4 kDa); serum albumin (66.2 kDa); ovalbumin (45.0 kDa); carbonic anhydrase (31.0 kDa); trypsin inhibitor (21.5 kDa); lysozyme (14.4 kDa). This time-pattern distribution can slightly change from one culture to another.

the purified proteins under denaturing conditions. This estimation was also performed for the native proteins by means of a gel filtration chromatography performed on a Pharmacia FPLC system. The column used was a Superdex 75 HR 10/30 (1 × 30 cm) equilibrated in 50 mM sodium phosphate buffer (pH 7.0), containing 0.2 M NaCl. The flow rate used was 0.5 ml/min.

3. Results

Aspergillus giganteus MDH 18894 grown in a culture medium composed of 2% corn starch, 1.5% beef extract, 2% peptone and 0.5% NaCl produces two major extracellular proteins [1] when incubated at 30°C for more than 70 h (Fig. 1). Both proteins have been previously characterized in great detail. The larger one (17 kDa) is α-sarcin, a ribosome-inactivating protein [1,12,13]. The smaller one, composed of 51 amino acid residues, is an antifungal protein (AFP) [2,4,5]. α-Sarcin and AFP are purified from the *Aspergillus* extracellular medium according to a well-established method (Fig. 2).

A third protein is observed in the extracellular medium at shorter periods of incubation (48–60 h) (Fig. 1). It can be chromatographically resolved from the above two proteins (Fig. 2) and it is slightly

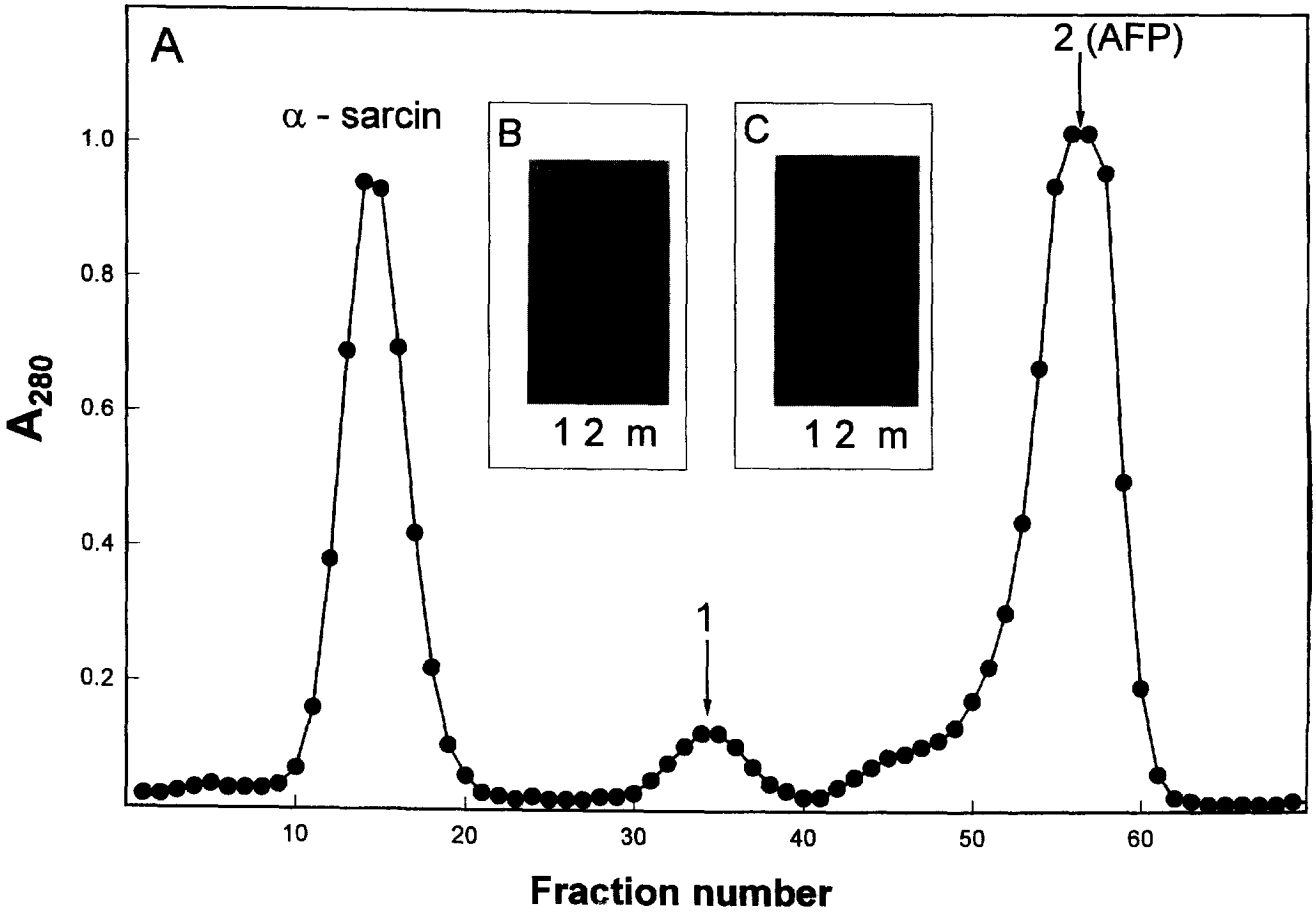

Fig. 2. (A) Elution profile of the Biogel P10 chromatography of the fractions containing α-sarcin, lf-AFP and AFP, pooled from the Amberlite IRC50 column. The proteins coelute from the ion-exchange column and, therefore, must be resolved on this size exclusion chromatography. (B) 15% PAGE–0.1% SDS of aliquots corresponding to the two peaks marked as 1 and 2. These samples were previously reduced with 0.2% (w/v) DTT. The higher molecular mass component in both lanes corresponds to the dimer resulting disulfides rearrangement. (C) Western blot analysis of part (B). The membrane was immunostained by means of antibodies raised against AFP. In both experiments, gel and Western blot, a third line (m) is also shown corresponding to BioRad prestained molecular weight standards.

larger than AFP (Figs. 1 and 2B), being recognized by anti-AFP antibodies in Western immunoblotting experiments (Fig. 2C). Taking into account these facts and the results presented below, this polypeptide is named lf-AFP, *l*arger *f*orm of AFP.

The obtained lf-AFP is a homogeneous protein according to its electrophoretic pattern (Fig. 2) and NH_2-terminal analysis (the faint bands in Fig. 2B and 2C correspond to the dimeric forms as the sequence analysis revealed). The sequence of the first eight amino acid residues of lf-AFP is: Asp-Glu-Ser-Ala-Val-Leu-Ala-Thr. This sequence matches the reported DNA-deduced primary structure of the precursor form of AFP [3], as is shown in Fig. 3. The amino acid composition of lf-AFP is given in Table 1 in comparison to that of AFP. The presence of six extra residues (Asx, Ser, Glx, Ala, Val, Leu) can be observed in lf-AFP, which account for the first six NH_2-terminal amino acid residues given above (Fig. 3). The antifungal activity of both proteins was assayed against two of the fungal species which are more sensitive to AFP [4], *Fusarium oxysporum* and *Trichoderma koningii*. The MIC values obtained for lf-AFP are, at least, 30-fold higher than for AFP, in both cases. Therefore, these results suggest the possibility of lf-AFP being an extracellular inactive precursor form of AFP.

When PAGE-SDS results are used to estimate molecular masses, 7.0 kDa and 6.0 kDa are obtained for lf-AFP and AFP, respectively. These values are in good agreement with those calculated from the amino acid sequence of AFP [2] and the six extra amino acids in lf-AFP. Similar results (data not shown) are obtained for non-reduced samples, in spite of the high number of disulfide bridges present in AFP [2]. However, the molecular mass derived from the size-exclusion behaviour of lf-AFP does not correspond to that deduced from PAGE-SDS. In fact, the values obtained from analytical gel-filtration chromatography (FPLC) on Superdex 75 (Fig. 4) are 6.0 kDa for AFP and 8.1 kDa for lf-AFP.

Finally, AFP displays a rather unique spectro-

-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	
					Asp	**Glu**	**Ser**	**Ala**	**Val**	**Leu**	**Ala**	**Thr-**	lf-AFP (NH_2-terminal amino acid sequence)
Gly	Leu	Asp	Ala	Arg	Asp	Glu	Ser	Ala	Val	Leu	**Ala**	Thr	AFP; *A.giganteus* [3]
Asp	Leu	Asp	Ala	Arg	Ala	Glu	Ala	Gly	Val	Leu	**Ala**	Lys	PAF; *Penicillium chrysogenum* [15]
Pro	Leu	Asp	Ala	Arg	**Ala**		Thr	Trp	Thr	Cys	Ile	Asn	restrictocin & mitogillin; *A.restrictus* [16,17]
Pro	Leu	Glu	Ala	Arg	**Ala**	Val	Thr	Trp	Thr	Cys	Leu	Asn	α-sarcin; *A.giganteus* [18]
Glu	Leu	Glu	Ala	Arg	**Asp**	Ser	Cys	Thr	Phe	Thr	Ser	Ala	polygalacturonase A; *A.flavus* [19]
Pro	Ile	Glu	Ala	Arg	**Asp**	Ser	Cys	Thr	Phe	Thr	Thr	Ala	polygalacturonase II; *A.niger* [20]
Pro	Ile	Glu	Ala	Arg	**Gly**	Ser	Cys	Thr	Phe	Lys	Thr	Ala	polygalacturonase II; *A.tubigensis* [21]
Glu	Leu	Glu	Ala	Arg	**Asp**	Ser	Cys	Thr	Phe	Thr	Ser	Ala	polygalacturonase; *A.parasiticus* [22]
Pro	Leu	Glu	Glu	Arg	**Ala**	Ala	Thr	Trp	Thr	Cys	Met	Asn	clavin; *A.clavatus* [23]
Pro	Leu	Asp	Lys	Arg	**Thr**	Lys	Val	Ala	Ser	Cys	Ser	Gly	24 kDa metalloproteinase; *A flavus* [24]
Pro	Ile	Glu	Pro	Arg	**Gln**	Ala	Ser	Val	Ser	Ile	Asp	Ser	endo(1,4)β-xylanase A; *A.awamori* [25]
Val	Leu	Val	Ser	Arg	**Ser**	Ala	Gly	Ile	Gln	Tyr	Val	Gln	endo(1,4)β-xylanase C; *A.awamori* [26]
Ser	Leu	Val	Glu	Arg	**Ala**	Cys	Asp	Tyr	Thr	Cys	Gly	Ser	RNase T1; *A.oryzae* [27]
Gln	Leu	Thr	Arg	Arg	**Thr**	Glu	Val	Thr	Asp	Cys	Lys	Gly	neutral protease II; *A.oryzae* [28]
Glu	Leu	Val	Glu	Arg	**Gly**	Ser	Ser	Cys	Thr	Phe	Thr	Ser	polygalacturonase B; *A.flavus* [29]
Glu	Leu	Lys	Ala	Arg	**Asp**	Ser	Cys	Thr	Phe	Thr	Ser	Ala	polygalacturonase; *A.oryzae* [30]

Fig. 3. NH_2-terminal amino acid sequence obtained for lf-AFP, and primary structure at the mature protein-leader peptide boundaries for different polypeptides secreted by filamentous fungi, according to their reported DNA sequences. The length and amino acid sequence of these propeptides is very different in some cases, apart from the portion shown. The first residue of each mature extracellular protein is underlined in all cases. The conserved residues at the tetrapeptide region (see the text) are boxed. Numbering corresponds to the amino acids position in lf-AFP. (See refs. [3,15–30].)

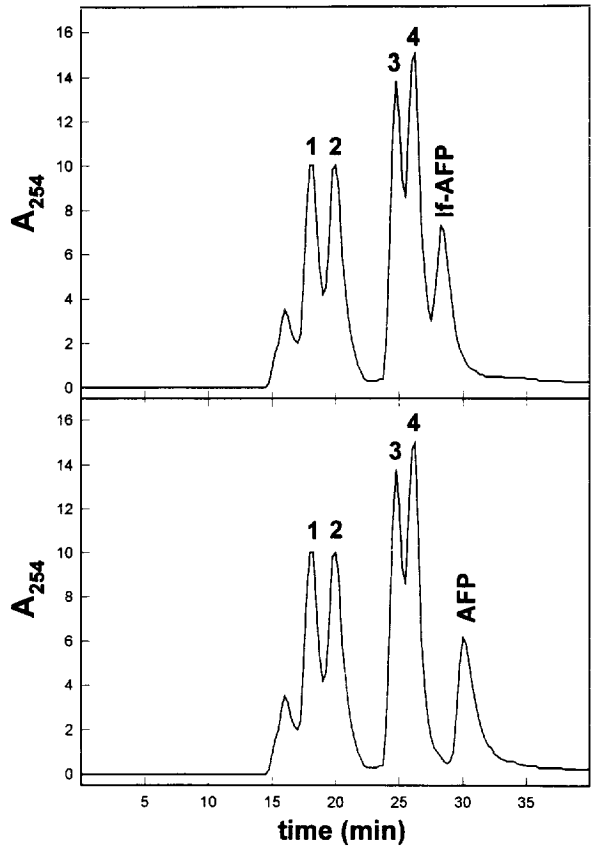

Fig. 4. Molecular weight estimation of native lf-AFP and AFP by gel filtration chromatography on Superdex 75 HR column. Two different chromatograms showing molecular weight standards and either AFP (lower) or lf-AFP (upper) are shown. The standards used were: 1, bovine serum albumin (66.2 kDa); 2, ovalbumin (45.0 kDa); 3, myoglobin (17.8 kDa); and 4, horse cytochrome C (12.3 kDa).

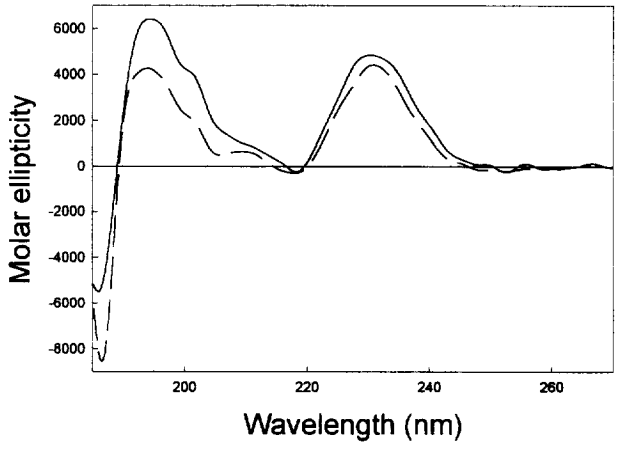

Fig. 5. Far-UV circular dichroism spectra of AFP (continuous line) and lf-AFP (dashed line). Spectra were obtained at 0.1 mg/ml protein concentration.

Table 1
Amino acid composition obtained for AFP and lf-AFP

	AFP	lf-AFP	AFP [2]	lf-AFP-AFP
Asx	5.1	6.2	5	1.1
Thr	1.9	1.9	2	0.0
Ser	1.9	2.9	2	1.0
Glx	2.1	3.2	2	1.1
Pro	1.4	1.1	1	−0.3
Gly	4.3	4.5	4	0.2
Ala	3.9	5.2	4	1.3
Cys	n.d.	n.d.	8	–
Val	1.0	1.9	1	0.9
Met	0.0	0.0	0	0.0
Ile	1.9	1.9	2	0.0
Leu	0.1	1.1	0	1.0
Tyr	5.7	5.9	6	0.2
Phe	1.0	1.1	1	0.1
His	0.3	0.2	0	−0.1
Lys	11.6	12.0	12	0.4
Arg	1.0	1.0	1	0.0

The values expected for AFP, according to its reported primary structure, are also included [2]. The fourth column shows the result of subtracting both experimental amino acid compositions. n.d., not determined.

scopic behaviour due to its relative high content of aromatic amino acid residues [4]. lf-AFP shows identical UV-absorbance and fluorescence emission spectra to those of AFP. Only minor differences are observed in the far-UV CD spectrum (Fig. 5).

4. Discussion

Many potential reasons might be invoked to explain the presence of both lf-AFP and AFP in the extracellular medium from cultures of *A. giganteus*. However, since lf-AFP disappears as the culture of the mould proceeds while AFP remains, it is conceivable that the larger polypeptide is the naturally secreted inactive form, which is further extracellularly hydrolyzed to antifungically active AFP. The biological significance of this result remains to be elucidated; but we have obtained mature AFP by treatment of lf-AFP with the protease subtilisin (unpublished results), which may suggest the involvement of an extracellular fungal protease in the maturation process.

We have also compared the deduced partial se-

quences of the precursor forms of different proteins secreted by a variety of *Aspergillus* species (Fig. 3; only sequence portions around the mature protein-leader peptide boundaries are shown). It can be observed that the primary structures of all the mature proteins start after the tetrapeptide-Leu-Asp-Ala-Arg or a similar one resulting from a conservative substitution (Ile/Leu-Asp/Glu-Ala-Arg is mostly observed). Certainly, there are also exceptions, but the consensus sequence Ile/Leu-Xaa-Yaa-Arg is observed (Fig. 3). This motif may be of relevance for extracellular secretion of proteins in *Aspergillus* spp. and related filamentous fungi, although the leader peptides compared are very different in length and amino acid sequence. Indeed, the amino acid and DNA sequences of AFP are already known [2,3]. The mRNA of AFP contains an open reading frame coding for a precursor of 94 amino acid residues, 51 of which correspond to the primary structure of AFP. Inspection of these data reveals that the sequence of lf-AFP is also preceded by the tetrapeptide Leu-Asp-Ala-Arg. This also occurs for α-sarcin (Fig. 3), the other major protein that *A. giganteus* secretes when producing AFP and lf-AFP.

PAF (*Pc* antifungal protein) is an abundantly secreted, highly basic polypeptide, purified from the culture medium of *Penicillium chrysogenum* [14,15]. PAF is also translated as a 92 amino acid residues precursor and appears to be processed to a mature product of 55 residues, which shows 42.6% identity to AFP [14,15]. Alignment of AFP and PAF precursor sequences, as deduced from their coding DNAs [3,15], also reveals the presence of the tetrapeptide -Leu-Asp-Ala-Arg- lying at the lf-PAF-leader peptide boundary (Fig. 3). But this lf-PAF has not been described yet.

Finally, the six amino acid residues at the NH_2-terminal end of lf-AFP are predicted to be under α-helical conformation (data not shown). AFP is a compact molecule adopting a β-barrel structure, highly resistant to proteolysis. Therefore, the extra residues in lf-AFP may represent a helical segment disrupting the compactness of the AFP structure. The anomalous hydrodynamic behaviour of lf-AFP on the gel filtration chromatography, in comparison with AFP and the globular standards, would support this possibility. The accessibility of such a segment might explain the extracellular hydrolysis of lf-AFP to AFP.

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