Formation of native disulfide bond patterns in small antifungal proteins and their γ-core optimized analogs

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Introduction

The increasing number of fungal infections makes the development of new antifungal agents essential. Small proteins from filamentous fungi can be promising lead compounds. The *Penicillium chrysogenum* antifungal protein (PAF) and the *Neosartorya fischeri* antifungal protein 2 (NFAP2) are representatives of this family. These proteins have different locations for the evolutionary-conserved γ-core motif.

PAF AKYTGKCTKSKNECKYKNDAGKDTFIKCPKFDNKKCTKDNNKCTVDTYNNAVDCD

NFAP2 IATSPYYACNCPNNCKHKKGSGCKYHSGPSDKSKVISGKCEWQGGQLNCIAT

Our previous studies showed that substitutions in small peptides representing the γ-cores of PAF [1] and NFAP2 [2] led to more effective antifungal agents. Therefore, the γ-cores of the proteins were replaced by an optimized peptide sequence, and the modified protein analogs were attempted to be prepared by chemical methods. In the case of NFAP2, knowledge of the native disulfide bond pattern was the prerequisite for the synthesis. The sequences of the modified proteins are the following:

PAF$^{imp\gamma}$ AKYTGKCKTKKNKCKYKNDAGKDTFIKCPKFDNKKCTKDNNKCTVDTYNNAVDCD

NFAP2$^{imp\gamma}$ IATSPYYACNCPNNCKHKKGSGCKYHSGPSDKSKVISGKCKTKKNKCIAT

Aims

- Development of synthetic strategies for the preparation of PAF and NFAP2 analogs containing optimized γ-cores and having native disulfide bridge patterns.
- Determination of native disulfide connectivity of NFAP2.
- Investigation of the antifungal effects of modified PAF and NFAP2.

NFAP2 - determination of the disulfide pattern

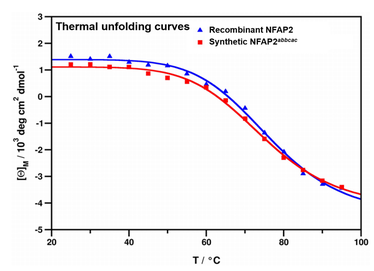
Since there are no tryptic or chymotryptic cleavage sites between Cys[9] and Cys[15], common mass spectrometry-based methods cannot be used to determine the disulfide bridge pattern of NFAP2. *In silico* predictions based on NMR results suggested two possible disulfide connectivities: *abbcac* and *abbacc* [4]. Both proteins were synthesized by chemical methods applying orthogonal protection to the cysteine thiols. Cleavage from the resins resulted in the following proteins:

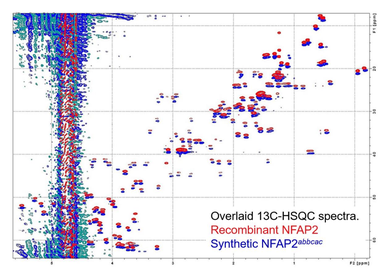
NFAP2^{abbcac} IATSPYYA(SH)C(Mob)NC(Mob)PNNCKHKKGSGC(Acm)KYHSGPSDKSKVISGKC(SH)EWQGGQLNC(Acm)IAT

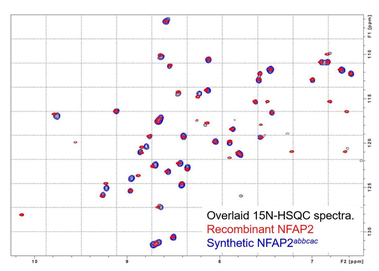
NFAP2^{abbacc} IATSPYYA(SH)C(Mob)NC(Mob)PNNCKHKKGSGC(SH)KYHSGPSDKSKVISGKC(Acm)EWQGGQLNC(Acm)IAT

The pair of free sulfhydryl groups were oxidized by air oxygen in a pH 7.5 NH_4OAc buffer. Acetamidomethyl (Acm) groups were removed, and the formed thiol functions were oxidized by iodine in an acidic solution. Cleavage of 4-methoxybenzyl (Mob) groups with trifluoromethanesulfonic acid (TFMSA) in the presence of trifluoroacetic acid (TFA) and anisole was followed by air oxidation in a pH 7.5 NH_4OAc buffer.

RP-HPLC analysis, ECD measurements, NMR investigations, and antifungal susceptibility tests against *Candida albicans* proved the identity of NFAP2^{abbcac} with recombinant NFAP2. Consequently, the disulfide bridge pattern of NFAP2 is ***abbcac***.





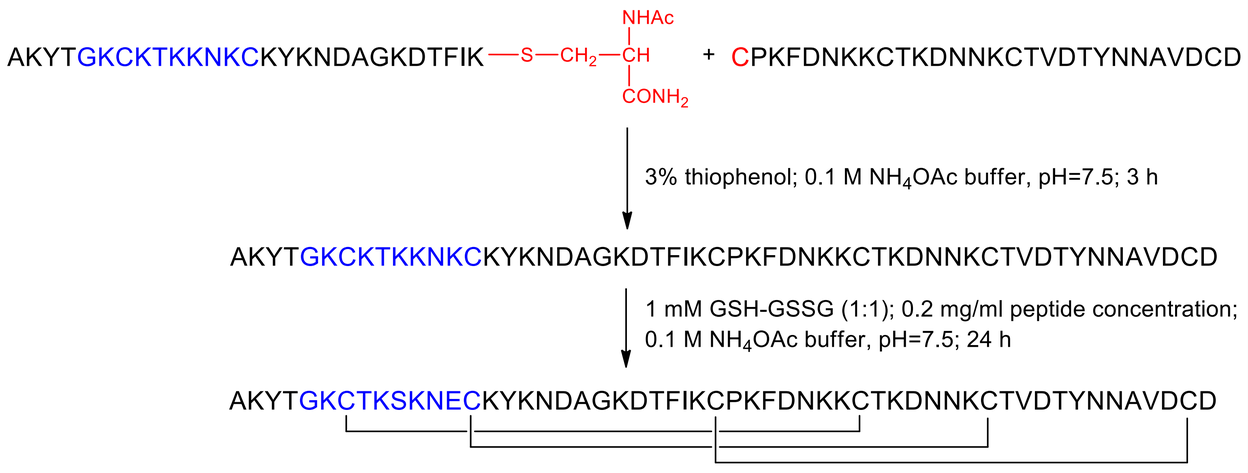


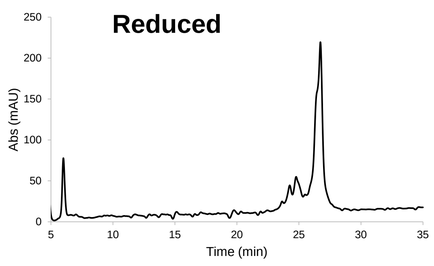
PAF$^{imp\gamma}$

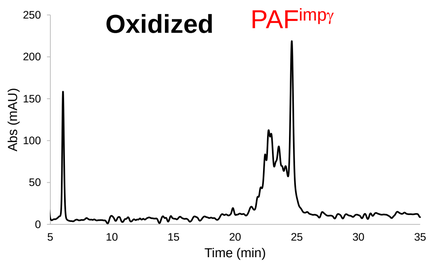
Synthesis and folding

Since stepwise solid-phase synthesis of PAF$^{imp\gamma}$ failed, native chemical ligation (NCL) was used. The thioester of the N-terminal part was prepared on a preloaded Cys-SH resin using Boc chemistry [3], while the C-terminal part of the protein was synthesized on a preloaded Wang resin using Fmoc chemistry. NCL was carried out in a pH 7.5 ammonium acetate (NH_4OAc) buffer containing 3% thiophenol.

After purification, oxidation was performed in a 1 mM reduced glutathione (GSH)-oxidized glutathione (GSSG) (1:1) buffer at pH 7.5, which resulted in the formation of a native disulfide bridge pattern.





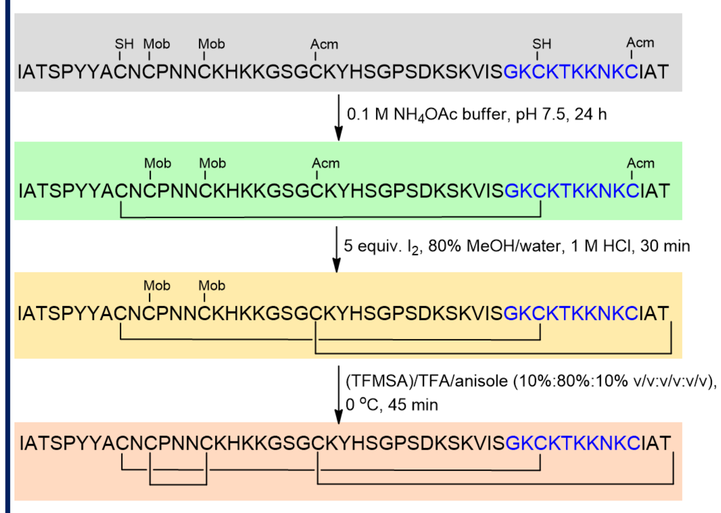


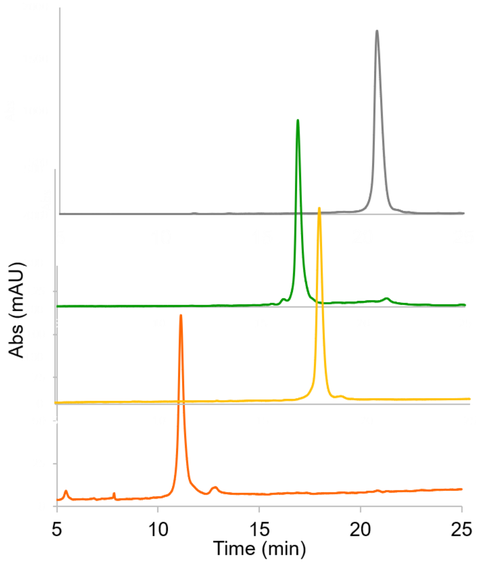
Antifungal activity

The minimum inhibitory concentration (MIC) determined on *Candida albicans* with a broth microdilution assay was found to be 5.0 μM for PAF and 1.3 μM for PAF$^{imp\gamma}$. That is, in the case of PAF, appropriate modification of the γ-core increased the antifungal effect.

NFAP2$^{imp\gamma}$ - selective protection of cysteines

With the disulfide bridge pattern of NFAP2 in hand, the γ-core modified analog of the protein was synthesized. The same procedure was followed as for NFAP2^{abbcac}.





ECD analysis of NFAP2$^{imp\gamma}$ showed an unordered structure. Although, the applied modification improved the antifungal activity of the 14-mer peptide representing the γ-core against *Candida albicans*, the MIC of the modified protein was found to be two-fold higher (12.5 μg/mL) than NFAP2 (6.25 μg/mL). This proves that in the case of NFAP2, the γ-core does not affect the antifungal effect, but rather the formation of the structure.

NFAP2$^{imp\gamma}$ - uniform protection of cysteines

Synthesis and folding

NFAP2$^{imp\gamma}$ was prepared by microwave-assisted solid-phase peptide synthesis and Fmoc chemistry.

The following methods were tested for the oxidation of cysteine thiols:

- Air oxygen in a 0.1 M NH_4OAc buffer at pH 7.5.
- 1 mM GSH-GSSG (1:1) in 0.1 M NH_4OAc buffer at pH 7.5.

None of these methods resulted in the formation of a native disulfide bond pattern.

Discussion

The γ-cores of the PAF and NFAP2 antifungal proteins were replaced by an optimized peptide sequence, and the modified analogs were synthesized by chemical methods.

PAF$^{imp\gamma}$ could only be prepared by NCL from two fragments, and oxidation in a GSH-GSSG buffer led to native disulfide connectivity. The PAF$^{imp\gamma}$ protein showed stronger inhibitory activity against *Candida albicans* compared to PAF, proving the role of γ-core in the antifungal effect.

NFAP2$^{imp\gamma}$ was prepared by stepwise solid-phase synthesis, applying orthogonal thiol protection. For this, the native disulfide bond pattern of NFAP2 was determined first, and it was found to be *abbcac*. The disulfide bridges were formed regioselectively. The produced NFAP2$^{imp\gamma}$ was less effective against *Candida albicans* than NFAP2, pointing to the importance of the γ-core in stabilizing the structure and not in the antifungal activity.

References

[1] Sonderegger C, Váradi G, Galgóczy L, Kocsubé S, Posch W, Borics A, Dubrac S, Tóth GK, Wilflingseder D, and Marx F, *Front. Microbiol*., 2018, **9**, Article 1655.
[2] Tóth L, Poór P, Ördög A, Váradi G, Farkas A, Papp C, Bende G, Tóth GK, Rákhely G, Marx F, and Galgóczy L, *BioControl*, 2022, **67** (2), 249-262.
[3] Váradi G, Tóth GK, Kele Z, Galgóczy L, Fizil Á, Batta G, *Chem. Eur. J.*, 2013, **19**, 12684-12692.
[4] Váradi G, Kele Z, Czajlik A, Borics A, Bende G, Papp C, Rákhely G, Tóth GK, Batta G, Galgóczy L, *Protein Sci.*, 2023, **32**, e4692

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