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In vivo and *in vitro* Cleavage of Glucoamylase-TNFα Fusion Protein Secreted from *Aspergillus niger*

Nada Kraševec[1]*, *Monika Svetina*[1,2], *Vladka Gaberc-Porekar*[1], *Viktor Menart*[1,2] *and Radovan Komel*[1]

[1]National Institute of Chemistry, POB 660, SI-1001 Ljubljana, Slovenia
[2]Lek Pharmaceuticals d.d., Verovškova 57, SI-1526 Ljubljana, Slovenia

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Summary

The most common expression strategy for secreting heterologous proteins from filamentous fungus *Aspergillus niger* is based on fusion with glucoamylase gene which contains cleavage site for kexin protease (KEX2). However, secretion of recombinant proteins in the form of a fusion-protein without a host-specific cleavage site is usually higher than secretion of the mature protein obtained after *in vivo* cleavage. We tried to take advantage of such a higher production by cleaving the fusion protein *in vitro* after fermentation, instead of *in vivo* during secretion. Similar level of production as after *in vivo* cleavage was found when human tumor necrosis factor α (TNFα) was produced as a fusion protein with glucoamylase having the enterokinase cleavage site. In addition to the correctly processed TNFα, some non-specific cleavage was observed, which resulted in a shortened N-terminus. This was still better than *in vivo* cleavage where only truncated forms of TNFα were obtained. Although the fusion protein was cleaved by enterokinase directly in the medium before purification, this shorter N-terminus was probably a consequence of aberrant enterokinase cleavage. Isolation of fusion protein with His-tag by affinity chromatography with immobilized metal chelate (although normally fast and easy) was not possible because the sequence of five consecutive histidines attached to the N-terminus of the glucoamylase fusion partner was completely cleaved off by proteolysis.

Key words: filamentous fungi, *Aspergillus niger*, heterologous protein secretion, human tumor necrosis factor α (TNFα), KEX2 cleavage site, enterokinase cleavage site, His-tag

Introduction

The most successful strategies for achieving secretion of heterologous proteins from filamentous fungi include transformation of a host strain with low intra- and extracellular protease activity and production in optimised media; introduction of multiple copies of genetic insert and use of *amdS* (*A. nidulans* acetamidase gene) selection marker; use of strong fungal signals for transcription control and for efficient secretion; gene fusion with a strongly expressed fungal protein cleaved *in vivo* by incorporation of a KEX2 cleavage site (*1*). By a combination of these strategies, usually several milligrams of a heterologous protein per litre of culture could be obtained. This is rather low in comparison with grams per litre in production of a homologous protein, but it is also dependent on the target protein and can be one or two magnitudes higher as well, even for some non-fun-

* Corresponding author; Fax: ++386 (0)1 4760 300; E-mail: nada.krasevec@ki.si

gal proteins. It is believed that strategies to decrease the gap should be found at the transcriptional as well as (post)translational level (*1–7*).

In the fungus *Aspergillus niger* the most commonly applied strategy for heterologous protein secretion is glucoamylase (GLA) gene fusion using the KEX2 cleavage site for cleaving the fusion protein. However, the production of recombinant protein in a fusion protein form without a host-specific cleavage site is usually at least ten times higher than production of a protein obtained after cleavage *in vivo* (*8*). In addition, kexin cleavage is not always accurate, the recognition site consisting of a pair of amino acids only. The fidelity of processing at the N-terminus of a target protein is an important consideration in assessing the value of a translational fusion strategy and it is an imperative, for example in proteins where the N-terminal sequence affects stability, enzymatic activity or therapeutic efficacy (*9*). Another important fact is that, when *in vitro* rather than *in vivo* cleavage of the fusion protein is applied, the N-terminus of a heterologous protein stays protected from exopeptidase cleavage for a longer time during secretion and during at least a part of the isolation process.

Having these clues in mind, we tried to take advantage of the higher production of a protein in the fusion protein form, but in the absence of a host-specific proteolytic cleavage site. We studied production of TNF α in filamentous fungus *A. niger* by cleaving it *in vitro*, after the secretion process was completed, instead of cleaving it *in vivo* during secretion. Thus, besides protecting the N-terminus of the heterologous protein against proteases, we also expected greater cleavage fidelity. In this paper we will present a comparison of *in vitro* versus *in vivo* cleavage of the TNFα fusion protein as produced in *A. niger*.

Methods and Materials

Construction of A. niger expression plasmids

Escherichia coli DH5α was used as a host for molecular cloning. **pAN56-2M** plasmid is a modified pAN56-2 (TNO, Zeist, NL) (*1*) containing an additional *Hin*dIII cloning site (*10*). **pAN56-MH** plasmid (Fig. 1) was constructed from pAN56-2M with insertion of a His-tag into the *Bss*HII restriction site downstream to the *preproglaA* signal sequence. The linker was annealed from two partially overlapping oligonucleotides: 5'-CGC GCT CAC CAC CAC CAC CAC and 5'-CGC GGT GGT GGT GGT GGT GAG. To get this construct, it was necessary to precede it with the insertion of a 1053 bp long *Sac*I fragment, containing a single *Bss*HII restriction site, into the *Sac*I restriction site of pUC19. Insertion of the His-tag was confirmed by DNA sequencing. **pANXTNFH** is the expression plasmid for the GLA-TNFα fusion protein with the site for *in vivo* cleavage by kexin protease (GLA:$_{KEX}$:TNFα). **pANTNFH** is the plasmid for expressing the fusion protein without this cleavage site (GLA::TNFα) (Kraševec, in preparation). **pANETNFH** is the expression plasmid for the GLA-TNFα fusion protein with a site for *in vitro* cleavage by enterokinase (GLA:$_{EK}$:TNFα). It was constructed in the same way as pANXTNFH, but another 5'-overhanging primer (5'-CG ACG GCG CCG GAT GAC GAT GAC AAG GTC AGA TCA TCT TCT CGA), containing the *Nar*I restriction site and enterokinase cleavage site was used in PCR amplification of the natural human TNFα cDNA from plasmid pE4 (ATCC). **pANHETNFH** is a plasmid for expressing the fusion protein GLA-TNFα and harbouring the His-tag and a site for *in vitro* cleavage by enterokinase (5HisGLA:$_{EK}$:TNFα). The human TNFα cDNA was inserted into plasmid pAN56-2MH. In all constructs, selection marker *amdS* was introduced into the *Not*I re-

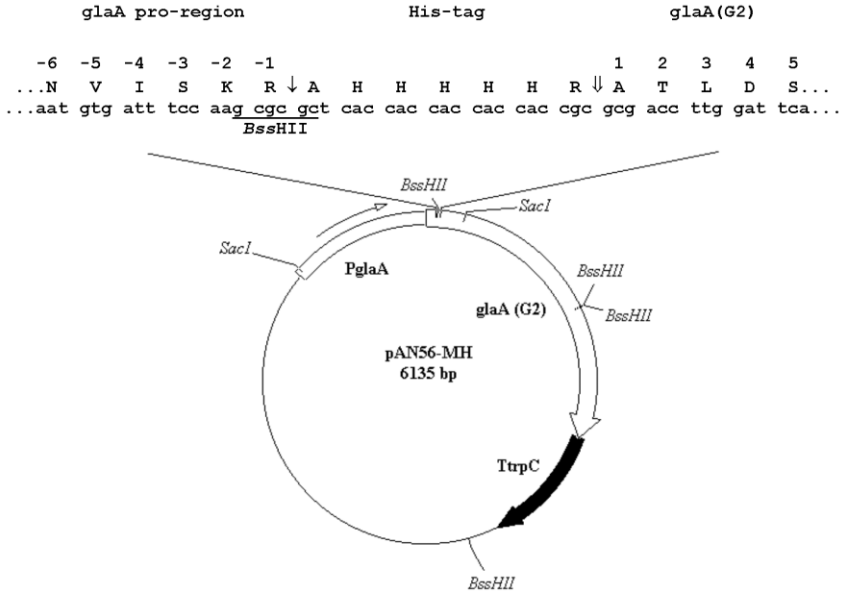

Fig. 1. pAN56-2MH expression plasmid. Pgla – the promoter of *A. niger* glucoamylase gene; glaA(G2) – the shorter coding region (G2) of *A. niger* glucoamylase gene; TtrpC – the terminator of *A. nidulans* tryptophan synthase gene. Expected cleavage site of the His-tagged fusion protein (5HisGLA:$_{EK}$:TNFα) after the glucoamylase pro-region ↓; actual cleavage site detected by N-terminal analysis ⇓

striction site, which is reflected by A in the name of plasmids. Transformant strain harbouring pANXTNFHA was named **XTNFHA**, with pANTNFHA – **TNFHA**, with pANETNFHA – **ETNFHA** and the strain with pANHETNFHA – **HETNFHA**.

Strains, transformation procedure and culture conditions

A protease-deficient strain *A. niger* AB 1.13 (*cspA1*, *prt-13*, *pyrG1*) (TNO, Zeist, NL) (*11*), a vacuolar protease deficient strain *A. niger* AB6.4Δ*pepE* (*fwnA*, *pyrG1*, *glaA::ble*, Δ*pepE*) (TNO, Zeist, NL) (*12*) and a strain lacking a functional *kexB* gene *A. niger* NW266 (Δ*argB*, *pyrA6*, *nicA1*, *leuA1*, *cspA1*, *argB*::Δ*kexB*) (Wageningen University, NL) have been used as recipients for transformation. Protoplast formation and transformation were carried out as described by Punt *et al.* (*13*). Before transformation, *A. niger* strains were cultivated in enriched minimal medium (MM) (*14*), supplemented with 0.5 % of yeast extract and 0.1 % of casamino acids. *A. niger amdS*$^+$ transformants were selected on osmotically (1.2 M sorbitol) stabilized MM plates with 1 mM acetamide as N-source and supplemented with 1.5 mM CsCl and 1.5 % of bacteriological agar (Oxoid). For even more gene copies, selection was performed on plates containing 1 mM acrylamide. For routine production the mycelia were cultivated in flasks with maltodextrin medium (MM with 5 % of maltodextrin as a carbon source) for 39 h at 30 °C and 180 rpm. Plates were supplemented with 2 mM and liquid media with 5 mM uridine, 1.5 mM leucin and 8 μM nicotinamid were added when necessary.

Analysis of proteins

SDS polyacrylamide gel electrophoresis (SDS--PAGE) was performed with a Mini Protean II Electrophoresis Cell (BioRad) on 15 % polyacrylamide gel (*15*). Proteins were stained with Coomassie blue. For Western blot proteins were electroblotted onto a nitrocellulose membrane (Sartorius) using a semi-dry blotting transfer (LKB). The membrane was immunostained using a primary rabbit anti-TNFα polyclonal antibody serum (ZTK, Ljubljana, SI) and goat anti-rabbit horseradish peroxidase conjugated secondary antibody (BioRad). Staining reaction of the blots was carried out with 4-Cl-1-naphtol. The yield of mature TNFα was determined after densitometric evaluation of Western blot against the TNFα calibration curve.

The fungal culture medium was separated from mycelium by filtration through Whatman paper (black ribbon) immediately after routine production fermentation and kept frozen at –70 °C. A sample of 16 μL (corresponding to 0.6 μg of total protein) was cleaved with 0.5–2 μL of enterokinase (corresponding to 0.05–2 U) (*10*) in 50 mM Tris-HCl, pH=8.0, 1 mM $CaCl_2$, 0.1 % of Tween 20 (addition of 1/10 reaction volume of 10x buffer to the sample) at 23 °C for 24 h. The reaction was stopped by the addition of 1/3 volume of 4x SDS sample buffer and heated for 5 min at 95 °C. Two control reactions were set up, the first with and the second without incubation, both without the addition of the enzyme.

Protein aminoterminal sequences were analysed by an Applied Biosystems Model 492 Pulsed Field Liquid Sequenator.

For Ni-NTA chelating chromatography a 2-mL syringe with glass wool stopper was filled with 1.5 mL of an aqueous suspension of Ni-NTA agarose (1:1) (Qiagen) and washed with 10 mL of buffer A (20 mM K-phosphate buffer, 200 mM NaCl, pH=7.1). Before applying to the column, samples of extracellular proteins of fungal cultures (10 mL) were centrifuged for 15 min at 12 500 rpm and 4 °C, and pH was then adjusted to 7.1 with NaOH or by dialysis against buffer A. The applied sample was incubated on the column for 30 min at 4 °C and then the unbound protein was eluted with 10 mL of buffer A. Bound proteins were eluted in 2-mL fractions by buffer A with increasing concentrations of imidazole (10, 50 and 100 mM, respectively).

Results and Discussion

In vivo cleavage of the glucoamylase-TNFα fusion protein

We have chosen the fusion strategy for TNFα gene expression in the filamentous fungus *A. niger*. TNFα gene was fused with the glucoamylase gene coding region (GLA:$_{KEX}$:TNFα) and free mature TNFα protein was obtained after *in vivo* cleavage by kexin protease. Kexin, which cleaves behind a dibasic amino acid pair (*16*), is encoded by *kexB* gene in *A. niger*, its specificity being more similar to KEX2-maturase from the yeast *Saccharomyces cerevisiae* than to mammalian furine (*17*). The upstream amino acid sequence of the KEX2 cleavage site (Lys-Arg) in our construct was the one found in the pro-region of glucoamylase. The yield of mature TNFα was up to 13 mg/L of maltodextrin medium culture of *A. niger* AB1.13. It possessed the expected high cytotoxic activity against cell line L-929 (10^7 IU/mg). The N-terminal sequence indicated a cleavage between Ser-5 and Arg-6 (Table 1).

In the parallel expression experiments of the fusion protein without a host-specific cleavage site (GLA::TNFα), a product partially cleaved between Ser-4 and Ser-5 appeared after 132 h long fermentation in soya medium and occasionally also in maltodextrin medium (Table 1).

In vivo release of mature protein from a fusion protein is not always accurate, which is true also in our case. MacKenzie *et al.* (*18*) reported aberrant processing of bovine pancreatic trypsin inhibitor (BPTI) from the fusion protein (GLA:$_{KEX}$:BPTI), where only 35–60 % of the protein was processed correctly. According to the study of the fidelity of processing lysozyme (HEWL) fusions (GLA:$_{KEX}$:HEWL) by *A. niger* (*9*), the sequences both upstream and downstream of the KEX2 cleavage site affect the fidelity of cleavage, but the sites of cleavage are unlikely to be due solely to the kexin protease. The temporal expression of the kexin protease activity has not been reported but it has been for many secreted proteases, thus the processing of the fusion construct can be culture-age dependent and it is likely that extracellular proteases contribute to the processing patterns. Our observations of aberrant splicing of TNFα are likely to be in accordance with these explanations.

Table 1. Glucoamylase-TNFα fusions

pANXTNFH	glucoamylase-G2					*KEX2 cleavage site*							TNFα				
(GLA:$_{KEX}$:TNFα)	512	513	514								1	2	3	4	5	6	7
	P	T	A	P	***N***	***V***	***I***	***S***	***K***	***R*** ↓	V	R	S	S	S ⇓	R	T
pANTNFH	glucoamylase-G2				TNFα												
(GLA::TNFα)	512	513	514		1	2	3	4	5	6	7						
	P	T	A	P	V	R	S	S ⇓	S	R	T						
pANHETNFH	glucoamylase-G2				*enterokinase cleavage site*							TNFα					
(5HisGLA:$_{EK}$:TNFα)	512	513	514							↓ 1	2	3	4	5	6	7	
	P	T	A	P	***D***	***D***	***D***	***D***	***K*** ⇓	V	R ⇓	S	S	S	R	T	

Expected cleavage site of the fusion proteins ↓; actual cleavage sites detected by N-terminal analysis ⇓

Furthermore, the N-termini extend free from the TNFα trimer, and are thus more accessible to proteases (*19*), however they do not participate in trimer interactions and are not important for biological activity. TNFα contains a quite exposed dibasic amino acid pair Arg-31-Arg-32 in the mature 17 kDa protein. With an *in vitro* degradation test of TNFα in *A. niger* maltodextrin culture medium (Fig. 2) we detected a degradation product of TNFα (only 12 % of the total TNFα after 24 h of incubation), which probably resulted from cleavage at this site. The same 14 kDa fragment of recombinant TNF α has also been detected in the bacterium *E. coli* (*20*). In this test cleavage after Ser-5 was not detected, due to the small difference in molecular size, but it cannot be excluded. Cleavage of the TNFα N-terminal sequence has also been observed when produced by different strains of *E. coli* (*21*). A few amino acids shorter TNFα N-terminus after *in vivo* release thus resulted either from the activity of other proteases or from non-specific kexin processing, or both.

In vitro cleavage site of the glucoamylase-TNFα fusion protein

Enterokinase cleavage *in vitro* and introduction of different tags for fusion protein purification have been extensively used in *E. coli* (*22*) but to our knowledge have not yet been applied in filamentous fungi. The catalytic domain of enterokinase, a serine endoprotease from bovine duodenum, has most of the characteristics of a universal cleavage reagent. It is highly active, it works over broad ranges of pH, in denaturant or detergent concentrations, and has high specificity for a pentapeptide recognition sequence Asp_4-Lys, cleaving it on its carboxy-terminal side. Recombinant enterokinase has greatly increased the utility of fused gene expression technology (*23*). This enzyme was also expressed in the filamentous fungus *A. niger* (*10*).

In order to obtain a mature protein from the fusion protein after the fermentation, we constructed a plasmid vector containing the enterokinase instead of host-specific *in vivo* KEX2 cleavage site. As expected, the level of fusion protein with the enterokinase cleavage site (GLA:$_{EK}$:TNFα) in *A. niger* AB1.13 was comparable to that of fusion protein without any cleavage site (GLA::TNFα) and it was more than ten times higher than the level of *in vivo* released TNFα as determined after immunostaining by anti-TNFα polyclonal antibody. The enterokinase cleavage of GLA:$_{EK}$:TNFα was com-

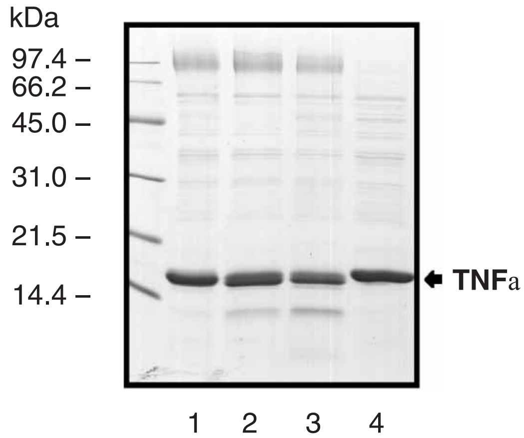

Fig. 2. *In vitro* degradation test of TNFα. Sensitivity of TNFα to degradation of *A. niger* AB1.13 host strain was determined by incubation of 70 % of pure TNFα prepared in *E. coli* (*29*) with host strain filtrate, obtained after growth under routine production conditions (in liquid maltodextrin medium for 39 h at 30 °C). The figure shows the separation of proteins on 15 % SDS-PAGE gel stained with Coomassie blue after: 1–2 h; 2–18 h; and 3–24 h of incubation at 30 °C; 4 – untreated TNFα sample (0.5 μg). Sizes of molecular weight standards in kDa are indicated on the left side of the gel

plete, despite being carried out in a mixture of fungal extracellular proteins. However, the final yield of mature TNFα obtained by this procedure was no higher than that after kexin cleavage *in vivo* (GLA:$_{KEX}$:TNFα) (Fig. 3). This could only be explained by the presence of extracellular proteases probably acting to the free TNFα as in the case of its *in vivo* release. Moreover, the fusion protein could also be immunostained differently from a mature protein.

In addition, two different N-termini were found. The first resulted from the expected enterokinase cleavage site, but the second resulted from the cleavage between Arg-2-Ser-3 (Table 1) and it was different from that after *in vivo* release. The latter could be due to residual protease activity of *A. niger* AB1.13 because of the cleavage reaction carried out in a mixture of fungal extracellular proteins. Anyhow, a non-specific enterokinase cleavage could not be excluded. The same aberrant N-terminus is reported also in the case of a highly purified His10-TNFα cleaved by enterokinase, in which His--tag has been removed at enterokinase cleavage site (*24*). In addition, a cleavage of TNFα (no enterokinase cleavage) has been detected when exposed to enterokinase

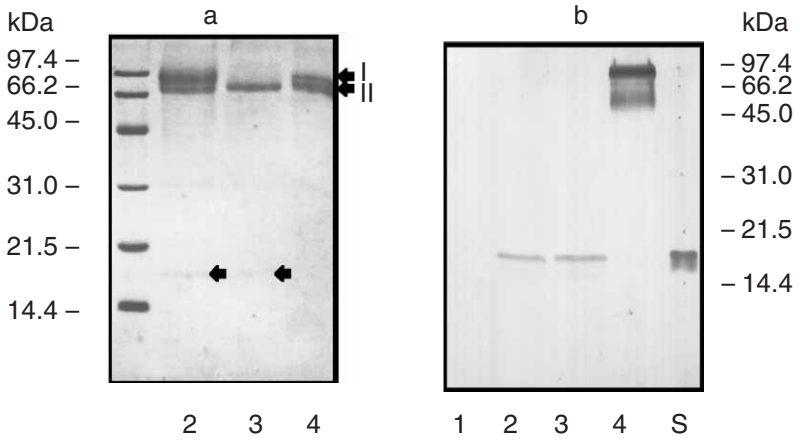

Fig. 3. Comparison of extracellular proteins from TNFα-transformants with KEX2 or enterokinase cleavage site. Proteins from strains derived from *A. niger* AB1.13 host strain were detected: a) with Coomassie blue on 15 % SDS-PAGE gel and b) with polyclonal TNFα antibodies on Western blot. 1 – from the host strain; 2 – from XTNFHA strain with *in vivo* cleavage site for glucoamylase-TNFa fusion protein; 3 and 4 – from HETNFHA strain with *in vitro* cleavage site for His-tagged glucoamylase-TNFα fusion protein after (3) and before (4) enterokinase cleavage; S- TNFα standard (0.25 μg). Sizes of molecular weight standards in kDa are indicated on the sides of the gel or the blot. Arrows indicate protein bands from which N-terminal amino acid sequence was determined; I and II show proteins from line 4 analysed for the presence or absence of His-tag

(personal communication, Maja Kenig, to be published in *J. Chromatogr. A*).

In the hope of finding a better protease deficient background we transformed a vacuolar protease deficient strain *A. niger* AB6.4Δ*pepE*, in which aspergillopepsin E and wild type *glaA* gene are missing. In this case, after enterokinase cleavage of unpurified fusion protein (GLA:$_{EK}$:TNFα), TNFα was totally degraded as is shown in Fig. 4b for 5HisGLA:$_{EK}$:TNFα fusion protein that in this case suffered identical degradation. It appears that the choice of protein deficient strain is important, but it is also very specific for each protein.

One might expect that recently available *A. niger* strain NW266 deleted for KEX2 (Δ*kexB*) (*17*) would help to address the problem of which endoprotease(s) was causing aberrant cleavage. Unfortunately, this mutation is introduced in a wild type and not in a protease deficient strain used in our experiments, which made a comparison impossible. When we applied Δ*kexB* strain to produce either enterokinase or KEX2 cleaving site containing TNFα fusion proteins (and three other non-fungal proteins), we observed fusions only in the case of products containing enterokinase cleavage site. In addition, the 5HisGLA:$_{EK}$:TNFα fusion protein was totally degraded already in the incubated control reaction without any enterokinase added (Fig. 4c).

Isolation of a glucoamylase fusion protein with His-tag

It could be advantageous to isolate the fusion protein before cleavage. For this purpose Immobilized Metal-Affinity Chromatography (IMAC) was chosen for use with His-tagged products (*25*).

The expression plasmid with the enterokinase cleavage site was modified by inserting five histidines at the N-terminus of the glucoamylase fusion partner. However, the secreted His-tagged fusion protein (5HisGLA:$_{EK}$:TNFα) did not bind to the chelate affinity matrix as expected. Its N-terminal sequence showed the absence of the

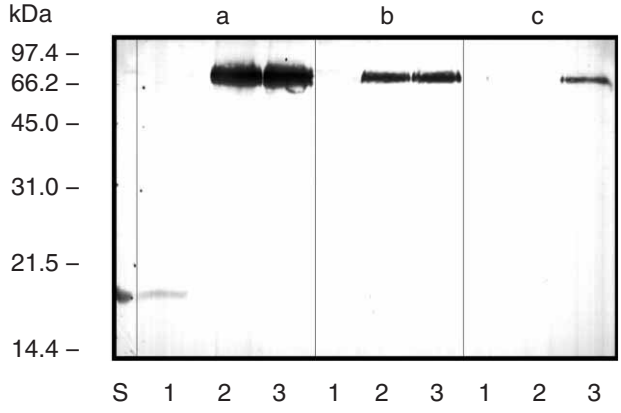

Fig. 4. Comparison of enterokinase cleavage of extracellular proteins from TNFα-transformants derived from different host strains. Proteins from HETNFHA strains derived from: a) a protease-deficient strain *A. niger* AB1.13 host strain; b) a vacuolar protease deficient strain *A. niger* AB6.4Δ*pepE* host strain and c) a strain lacking a functional *kexB* gene *A. niger* NW266 were detected with polyclonal TNFα antibodies on Western blot. S – TNFα standard (0.25 μg); 1 – enterokinase cleavage reaction; 2 and 3 – a control reaction with (2) and without (3) incubation. Sizes of molecular weight standards in kDa are indicated on the left side of the blot

His-tag, although its sequence in the plasmid was verified by DNA sequence determination (data not shown). His-tag was not present in band I (Fig. 3), which disappeared after enterokinase cleavage, nor in band II, both of which were shown by N-terminal amino acid sequence to correspond to glucoamylase.

Although the His-tag approach failed, we tried to purify the 5HisGLA:$_{EK}$:TNFα fusion protein away from proteases from the culture medium by conventional means such as ammonium sulphate precipitation, ultrafiltration and ion-exchange chromatography to confirm if the proteolysis or aberrant cleavage was associated with a fungal enzyme or the added enterokinase. It seemed that in our case proteases were copurified, as

the 5HisGLA:$_{EK}$:TNFα fusion protein from strain *A. niger* AB 1.13 was disappearing during the procedure of purification and an unspecifically cleaved-off TNFα was appearing (not presented).

While cloning the His-tag into the construct the amino acid sequence...His-Arg-Ala-Thr... (Fig. 1) was engineered. The sequence His-Arg does not appear to be recognised by the *S. cerevisiae* Kex2p (*26*) but in a survey of the amino acid sequences of several secreted and intracellular filamentous fungal proteins (*27*) it was found that His-Arg was one of the amino acid doublets that was rarer in secreted proteins, suggesting that this might be cleaved by KEX2 or some other endoprotease. Alternatively, a modified His-tag could be created with a different amino acid sequence, but a proteolytic attack may still occur. His-tag could be placed on C-terminal of glucoamylase, but it may not bind IMAC. If placed on C-terminal of TNFα, it would disturb its structure. However, His-tag did not enable easy product recovery of tissue plasminogen activator (t-PA) expressed in *A. niger*. Purification of t-PA based on binding of the His-tag at its C-terminus to Ni-NTA was not efficient and maximal recovery of t-PA was only 14 % (*28*). Histidines, carefully inserted in the glucoamylase structure, as in case of TNFα studies (*21*), would be the optimal solution for separations.

Conclusions

A number of problems arising in the expression of TNFα in the filamentous fungus *A. niger* have been identified. The yield, when expressed as a fusion protein without a host-specific cleavage site, was higher than of the mature protein obtained after *in vivo* cleavage of the fusion protein. We tried to take advantage of this by cleaving the fusion protein *in vitro*, after fermentation. A new plasmid vector was therefore constructed with an enterokinase instead of a KEX2 cleavage site. However, although the enterokinase cleavage of the fusion protein in the culture medium was successful, the yield of TNFα was no higher than in the case of kexin cleavage. In addition, the N-terminus of the heterologous mature protein was only partially correct. It seemed necessary to isolate the fusion protein from the mixture of secreted proteins prior to cleavage, in order to avoid the action of fungal proteases. Isolation of the fusion protein by means of immobilizied metal chelate affinity chromatography failed because of proteolytic degradation of His-tag in the fermentation medium. Proteolytic activity of the host strain seemed to be still one of the main problems to be solved in obtaining secretion of heterologous proteins at high levels. Before application, a mature protein itself should be tested for susceptibility to degradation of the host strain and of enterokinase, if it is going to be applied for the cleavage of fusion protein.

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Cijepanje *in vivo* i *in vitro* fuzijskog proteina glukoamilaza-TNFα dobivenog iz *A. niger*

Sažetak

Najčešće korištena strategija za sekreciju heterolognih proteina iz plijesni *A. niger* zasniva se na fuziji s genom za glukoamilazu koji sadrži mjesto cijepanja za keksin proteazu (KEX2). Međutim, sekrecija rekombinantnih proteina u obliku fuzijskog proteina bez specifičnog mjesta cijepanja obično je veća nego sekrecija gotovog proteina dobivenog nakon cijepanja *in vivo*. Autori su pokušali iskoristiti prednost tako povećane proizvodnje, cijepajući fuzijski protein *in vitro*, nakon fermentacije, umjesto tijekom sekrecije *in vivo*. Međutim, opažena je slična razina proizvodnje humanog faktora nekroze tumora α (TNFα) kao fuzijskog proteina (s glukoamilazom koja ima enterokinazno mjesto cijepanja), kao i prilikom cijepanja *in vivo*. Osim ispravno procesuiranog TNFα opažena su i nespecifična cijepanja koja su dovela do skraćenog N-terminala, dok su pri cijepanju *in vivo* dobiveni samo okrnjeni oblici TNFα. Iako je fuzijski protein bio cijepan s enterokinazom u mediju prije purifikacije, dobiveni kraći N-terminal vjerojatno je posljedica poremećenog cijepanja enterokinazom. Izolacija fuzijskog proteina s histidinskim privjeskom pomoću afinitetne kromatografije s imobiliziranim metalnim helatom nije bila moguća, jer je sekvencija od 5 uzastopnih histidina, vezanih na N-terminal glukoamilaznog fuzijskog partnera, bila potpuno odcijepljena proteolizom.