The Journal of Microbiology, June 2001, p.95-101
Vol. 39, No. 2
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Controlled Expression and Secretion of *Aspergillus oryzae* Alkaline Protease in *Aspergillus nidulans*

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(Received April 13, 2001 / Accepted June 9, 2001)

In an effort to develop an efficient expression and secretion system for heterologous proteins in *Aspergillus nidulans*, the PCR-amplified coding sequence for alkaline protease (AlpA) of *A. oryzae* was cloned into a fungal expression vector downstream of *A. nidulans alcA* (alcohol dehydrogenase) promoter to yield pRAAlp. Transformation of *A. nidulans* with pRAAlp gave stable transformants harboring various copy numbers (3 to 10) of integrated *alpA* gene, from among which 6 representatives were selected. On a medium containing 0.8% ammonium sulfate that represses the expression of the host's own protease, the *alcA* promoter-controlled AlpA expression was strongly induced by threonine but repressed by glucose. The level of AlpA secretion was highest (approximately 666 mU/ml) in transformant ALP6 containing the largest copy number integrated *alpA*. However, the level of AlpA secretion was not necessarily proportional to the copy numbers of the integrated *alpA* genes. The N-terminal sequence of the secreted mature AlpA was determined to be Gly-Leu-Thr-Thr-Gln-Lys-Ser and its molecular mass to be approximately 34 kDa, indicating that AlpA is properly processed by the removal of 121 N-terminal amino acids.

Key words: *Aspergillus nidulans, Aspergillus oryzae,* akaline protease, expression, secretion

Members of genus *Aspergillus* are recently being considered as attractive hosts for heterologous gene expression and foreign protein production by recombinant DNA technology. As well as some microbial heterologous proteins such as pectate lyase of *Erwinia crotovora* (1), restrictocin of *Aspergillus restrictus* (3) and cellobiohydrolase II of *Trichoderma reesei* (26), quite a few animal proteins such as interleukin-6 (7), interferon α2 (19), lactoferrin (36), lysozyme (32) and bovine chymosin (8, 30) have been expressed and secreted in *Aspergillus* strains.

Out of the elements which compose the expression and secretion systems for heterologous protein production in *Aspergillus*, efficient promoters are the most important. A few *Aspergillus* promoters including *glaA* (23) and *aphA* promoters (19) of *A. niger*; *amyB* (32) and *glaA* promoters (31) of *A. oryzae*; and *gpdA* (4), *amdS* (33), *pgk* (27) and *alcA* promoters (35) of *A. nidulans* have been utilized for the expression of heterologous proteins in *Aspergillus*.

Another important factor for the efficient protein secretion is signal peptide sequence. Although a few heterologous proteins such as restrictocin (2) and human lactoferrin (35) have been reported to be secreted by the aid of their own signal sequences, the leader sequence of glucoamylase (*glaA*) has been mainly used to direct the secretion of heterologous proteins in *Aspergillus* species (4, 7, 23). However, it is thought that different heterologous proteins need different leader sequences to optimize secretion levels, *i.e.*, no one leader sequence is likely to prove universal in its utility (2).

Although *Aspergillus* species are considered to be attractive host systems for heterologous protein secretion, in the majority of cases, the yields have been much lower than the commercially acceptable gram-per-litre level, with the exceptions of bovine chymosin (8) and human lactoferrin (36). Several factors including the low stability of heterologous mRNA (10), the misfolding of heterologous protein followed by intracellular degradation aided by the action of BiP (16), the improper match of secretion signals with heterologous proteins (2), and the extracellular proteolytic cleavage by the action of fungal proteases (34) can contribute to the low yields of heterologous protein production. Therefore to manipulate high levels of heterologous protein production in *Aspergillus* requires development of a variety of strong promoters and leader sequences to test for suitability with different types of protein and a more detailed understanding of the molecular

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basis of the secretion process. It is also important to construct protease-deficient *Aspergillus* strains to protect the secreted heterologous proteins from proteolytic cleavage in the culture medium (14). An alternative to using the protease-deficient mutants is to develop an expression system in which the host's own protease that affects the stability of the secreted heterologous protein is minimized.

A. nidulans has offered a wealth of information on the classical genetics of various genes as well as an efficient DNA transformation system, which is thought to be quite beneficial to the development of an efficient secretion system of heterologous proteins in this fungus by using molecular biological techniques. We are developing an efficient overexpression system for heterologous proteins in *A. nidulans* using *A. oryzae alpA* encoding an alkaline protease (AlpA) as an example. Alkaline proteases of *Aspergillus* are widely used in therapeutics such as anti-inflammatory agents and in the preparation of immunizing agents, collagen derivatives, and various proteolysates. The alkaline protease of *A. oryzae*, which plays a most important role in producing the taste of soy sauce by hydrolyzing the raw materials (27), is used as a drug in the therapy of digestive disorders together with other proteases of this fungus (12). In this report, we describe the construction of *A. nidulans* transformants harboring variable numbers of the coding sequence for *A. oryzae* AlpA which was fused with *alcA* promoter (35), and the inducible heterologous expression and secretion of AlpA under conditions that minimize the expression of the host's own protease.

Materials and Methods

Strains, media and transformation

A. nidulans TAR17 (*yA2 methH2 argB2 trpC801 chaA1*) which was supplied by Dr. Han (Wonkwang University, Iksan, Chonbuk 570-749, Korea) and used as a recipient strain for transformation, was maintained on *Aspergillus* complete medium (CM) supplemented with 0.6 M KCl, 4 mM tryptophan and 4 mM arginine (CKWR). The CM composition was 1% glucose, 0.15% casamino acid, 0.15% yeast extract, 1% (v/v) of vitamin stock solution (0.01% riboflavin, 0.05% *p*-aminobenzoic acid, 0.05% pyridoxin-HCl, 0.05% thiamine-HCl, 2 ppm biotin, and 0.05% folic acid) and 2% (v/v) of minimal salt stock solution (15.2% $NaNO_3$, 2.6% $MgSO_4$ $7H_2O$, 15.2% KH_2PO_4, 2.6% KCl, 0.4% ammonium molybdate, 0.04% $ZnCl_2$, 8 ppm $MnCl_2$ $4H_2O$, 32 ppm $CuSO_4$ $5H_2O$ and 129.6 ppm $FeCl_3$) (11). For transformation of *A. nidulans*, 1.0×10^6 conidia of TAR17 were inoculated into 100 ml of CKWR broth and grown at 37°C for 16 to 18 h with agitation. The mycelia were harvested by filtration through a nylon net, and washed with distilled water and osmotic buffer (0.6 M KCl, 10 mM NaCl, pH7.5). Transformation mediated by polyethylene glycol (PEG) was performed as described by Yelton *et al.* (39), and the protoplasts of transformants were regenerated on minimal medium (MM) supplemented with 0.6 M KCl and 4 mM tryptophan (MKW). The MM composition was 1% glucose, 0.6% $NaNO_3$, 0.052% KCl, 0.052% $MgSO_4$ $7H_2O$, 0.152% KH_2PO_4 and 0.2% (v/v) of Hunters trace elements (2.2% $ZnSO_4$ $7H_2O$, 1.1% H_3BO_3, 0.5% $MnCl_2$ $4H_2O$, 0.5% $FeSO_4$ $7H_2O$, 0.16% $CoCl_2$ $6H_2O$, 0.16% $CuSO_4$ $5H_2O$, 0.11% $NH_4Mo_7O_{24}$ $4H_2O$ and 5% EDTA).

To support the inducible expression of AlpA in *A. nidulans* transformants, an inducing medium (MLT) whose composition was the same as MKWR except for 0.8% ammonium sulfate, 1% lactose in place of the 1% glucose, and 100 mM threonine as an inducer.

Plasmids were amplified in *Escherichia coli* DH5α [*supE44 lacU169(80lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*], and *E. coli* cells were grown in Luria-Bertani (LB) medium. *E. coli* transformation was done by the standard method (24).

Construction of the vectors for AlpA expression and secretion

Prior to the assembly of *A. oryzae* AlpA expression cassette, pRA that can facilitates the expression of heterologous proteins in *A. nidulans* was constructed as follows. A 0.5-kb fragment containing the terminator of *A. nidulans* anthranilate synthetase gene (*trpC*) was amplified from pAN7-1(22) using a pair of primers, P1s (5GGCGAATTCCCGGGGATCCGTCGACAAGAATA AAACGCGTTTTCGGG3) and P2a (5AGAAAGA-AAGCTTACCTCTTAAACAAGTGTACCT3). PCR was performed with a DNA thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer Cetus, Norwalk, Connecticut, USA) using *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany), and the reaction conditions were 33 cycles of the following: 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C. The PCR product was digested with *Eco*RI and *Hind*III, and cloned into *Eco*RI-*Hin*dIII digested pUC19 to yield pUT. Then the 0.5-kb fragment containing the promoter of *A. nidulans alcA* was amplified from pAL4 (37) using a pair of primers, P3s (5GGCGAATTCTGAA-AAGCTGATTGTGATAGT3) and P4a (5GGCGAATTC-TTTGAGGCGAGGTGATAGGATT3), digested with *Eco*RI, and inserted into the *Eco*RI site of pUT in the correct orientation to yield pAT. The 1.4-kb fragment which comprised *alcA* promoter and *trpC* terminator was excised with *Pvu*II from pAT and ligated with *Pvu*II-digested pPK1 (38) containing ornithine carbamoyl transferase gene (*argB*) of *A. nidulans*, producing pRA (Fig. 1).

The coding region of *A. oryzae alpA* gene was amplified by PCR using primers P5s(5ATGCAGTCCATCAA-GCGTACCTTGCTCCTCCTCGGAGCTATCC3) and P6a (5TTAACCGTTACCGTTGTAGGCCAGCAGGTTAGG GCTACCC3). The 1.4-kb PCR fragment containing *alpA*

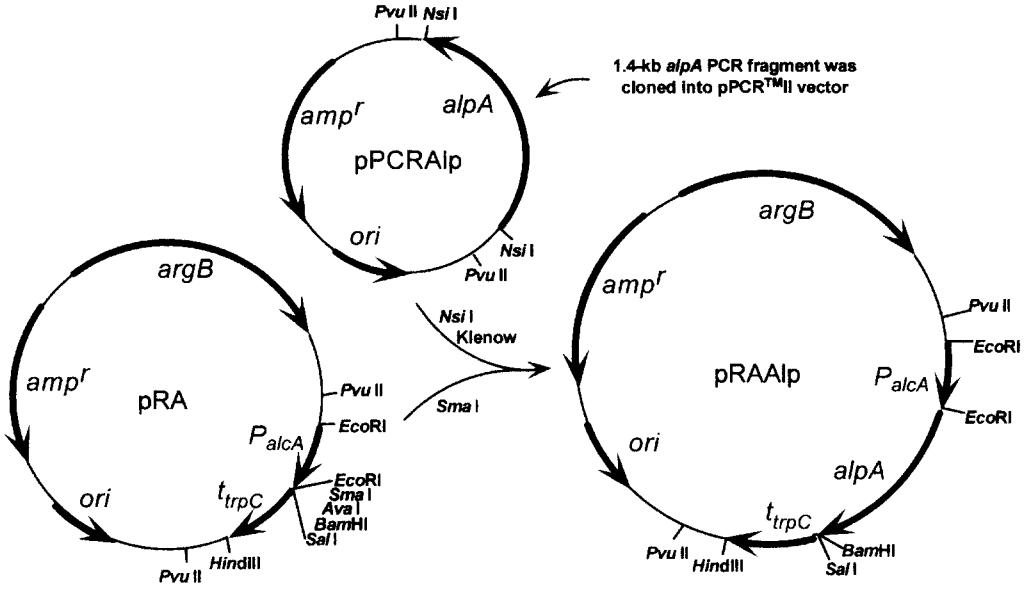

Fig. 1. Construction of pRAAlp plasmid used for the expression of *A. oryzae* AlpA in *A. nidulans*. The 1.4-kb PCR fragment containing the coding region of *A. oryzae alpA* was excised from pPCRAlp vector by digestion with *Nsi*I, blunted with Klenow enzyme, and inserted into the *Sma*I site of pRA between the *alcA* promoter and *trpC* terminator in the correct orientation yielding pRAAlp. P_{alcA}, *alcA* promoter; t_{trpC}, *trpC* terminator.

gene was cloned into a PCR cloning vector, pPCR™II (supplied by Dr. B. J. Lee at the Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea), which was derived from pBluescript II KS(−) (Stratagene, La Jolla, CA, USA). The 1.4-kb PCR fragment included in the resulting pPCRAlp vector was sequenced by the dideoxy method (25). By comparing the nucleotide sequence of the fragment with those of *A. oryzae alpA* (GenBank accession No. E03647) and *A. sojae alpA* (21), the 1.4-kb PCR fragment was confirmed to contain a full-length coding sequence for AlpA of *A. oryzae*. The 1.4-kb *alpA* fragment was then excised from pPCRAlp with *Nsi*I, blunted with Klenow enzyme (Promega, Madison, Wisconsin, USA), and cloned into the *Sma*I site of pRA between the *alcA* promoter and *trpC* terminator in the correct orientation to yield pRAAlp (Fig. 1).

DNA sequencing

DNA sequencing was performed by dideoxy chain termination (25) using the Sequenase Version 2.0 DNA sequencing Kit (United States Biochemical, Cleveland, OH, USA), universal sequencing primers, and [α-^{35}S]-dATP (Amersham).

Genomic DNA preparation and southern blot analysis

For preparation of chromosomal DNA from *A. nidulans* strains, the mycelia grown for 24 h at 37°C with agitation at 100 rpm were harvested by vacuum filtration through a filter paper and rinsed with cold deionized water. Washed mycelia were then freeze-dried and powdered in a mortar. Genomic DNA was isolated from the mycelia using a rapid extraction procedure developed for *A. nidulans* (39).

Southern blot analysis was carried out by using an ECL direct nucleic acid labeling and detection kit (Amersham, Buckinghamshire, UK). DNA samples digested with proper restriction enzymes were run on an agarose gel and blotted onto a Hybond-N$^+$ membrane. Labeling of probe DNA and subsequent hybridization was done by the procedures recommended IN the manufacturer's manual. After hybridization, the membrane was washed under low stringency conditions, *i.e.*, washed consecutively with 1×SSC and 0.5×SSC buffers at 42°C.

Assay of protease activity

Protease activity was determined by monitoring the amount of tyrosine released from casein (Wako, Osaka, Japan) (13). The reaction mixture consisting of 20 μl of crude enzyme, 200 μl of 1% casein (Wako, Osaka, Japan) in 0.25 M Tris-HCl buffer (pH7.0) and 180 μl of distilled water was incubated at 37°C. Then 600 μl of 0.4 M trichloroacetic acid (TCA) was added to the mixture to stop the reaction and the resulting precipitate was removed by centrifugation. The amount of tyrosine present in the TCA-soluble supernatant was determined by measuring A_{280}. One unit of protease activity was defined as the amount which catalyzed the release of 1 μmole of tyrosine per min under the conditions described. Protein was measured as described by Lowry *et al.* (18) using bovine serum albumin as a standard.

SDS-PAGE

SDS-PAGE was carried out as described previously (17) with some modification. The proteins were stained with 0.25% Coomassie blue R250 solution in methanol/acetic

acid/water (40:10:50, by volume) mixture for 2 h. Destaining was performed in methanol/acetic acid/water (30:10:60) mixture.

N-Terminal amino acid sequencing of secreted AlpA

The *N*-terminal amino acid sequence of AlpA secreted by *A. nidulans* transformants was determined by the microsequencing method (20). The culture filtrate was first concentrated by ultrafiltration through an Amicon PM10 membrane (Millipore, Bedford, USA) and separated on an SDS-polyacrylamide gel. The proteins on the gel slab were transferred to a PVDF membrane by electroblotting using a semi-dry transfer set (Bio-Rad). After the PVDF membrane was stained with freshly prepared 0.2% Ponceau S solution in 1% acetic acid, the protein bands of interest were cut into small rectangular pieces (5×5 mm). The membrane pieces were rinsed with water, air dried, and stored at 4°C. The proteins were sequenced on an automatic sequencer (Model 477A, PE Applied Biosystems, Foster City, USA) equipped with on-line PTH analysis.

Results and Discussion

Construction of recombinant strains and genomic Southern blot analysis

A. nidulans TAR17 was transformed with the secretion vector pRAAlp to yield tens of transformants. The transformants were subjected to dot blot analysis (data not shown) and several representatives were selected for genomic Southern blot analysis (Fig. 2). While no chromosomal DNA fragment of *A. nidulans* TAR17 hybridized with the 0.5-kb *Nco*I-cut *alpA* fragment containing no *Bam*HI site, all the transformants showed multiple positive bands representing *alpA* fragments integrated at multiple loci in their chromosomes. Transformant APL1 presented three positive signals indicating that this transformant contains three copies of *alpA* fragment integrated into three distinct loci of its chromosomes. Other transformants showed even more positive signals including the 7.2-kb signal corresponding to *Bam*HI-digested pRAAlp vector itself. Relative intensities of the 7.2-kb signals, however, varied among the transformants. For instance, the 7.2-kb signal showed roughly double the intensity of the other fragments in ALP2, ALP3, and ALP4, but showed at least five-times stronger intensity than the other signals in ALP5 and ALP6 transformants. This result supports not only that the transforming vector was integrated in tandem arrays at at least one locus but also that more than two tandem copies of the vector were integrated at a single locus. Thus it was assumed that ALP2, ALP3, and ALP4 have either two double tandem or one triple tandem integration in addition to single integrations of pRAAlp at several different loci. In ALP5 and ALP6 transformants, the copy numbers of the vector involved in the multiple tandem integrations seemed to be even higher (approximately 6 in sum). The total copy number of integrated *alpA* gene fragments in each ALP transformant was estimated on the basis of this result (Table 1). In addition, this result is in accordance with the view that the integration of foreign circular DNA in the genome of *A. nidulans* can be driven not only by homol-

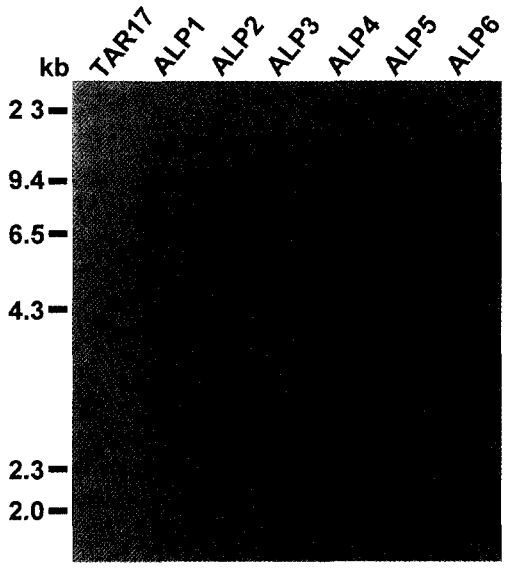

Fig. 2. Genomic Southern blot analysis of the transformants of the *A. nidulans* TAR17 formed by transformation with pRAAlp plasmid. Genomic DNAs from the parental (TAR17) and transformed strains were digested with *Bam*HI, separated on a 1.0% agarose gel, and transferred to a nylon membrane. DNA samples were probed with 0.5-kb *alpA* fragment excised from pPCRAlp with *Nco*I. Labeling of the probes and detection of the hybridized DNA bands were conducted by using an ECL direct nucleic acid labeling and detection kit.

Table 1. Production of extracellular proteases by the parental (TAR17) and transformed strains of *A. nidulans* in inducing and non-inducing broth media

Strain	Approximate copy number of integrated *alpA*	Protease activity (mU/ml)	
		Non-inducing medium (MKWR)	Inducing medium (MLT)
TAR17	0	7.2 ± 0.1	7.5 ± 0.2
ALP1	3	8.7 ± 0.1	180.2 ± 2.3
ALP2	6	13.8 ± 0.2	475.9 ± 3.8
ALP3	7	9.9 ± 0.3	249.8 ± 4.6
ALP4	9	14.0 ± 0.2	427.5 ± 6.9
ALP5	7	14.5 ± 0.3	368.6 ± 2.5
ALP6	10	17.1 ± 0.2	665.5 ± 9.3

For induction of AlpA expression, conidia of each *A. nidulans* strain were inoculated and grown in CMWR broth for 36 h at 37°C, and the mycelium was harvested and cultured in MLT (inducing) and MKWR (non-inducing) media for 8 h at 37°C. The culture filtrate was then used as a crude enzyme preparation for protease assay.

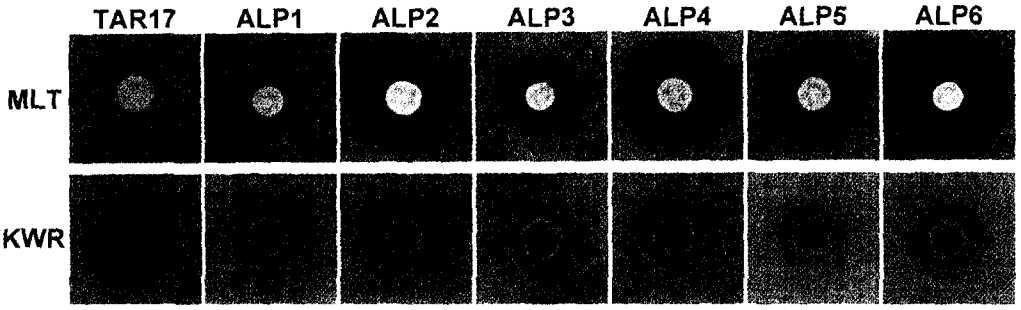

Fig. 3. Production of extracellular proteases by the parental (TAR17) and transformed strains of *A. nidulans* on agar plates. Each *A. nidulans* strain was grown on inducing (MLT) and non-inducing (MKWR) agar media for 2 days at 37°C.

ogous recombination but also by nonhomologous recombination (29).

Expression and secretion of AlpA

Secretion of *A. oryzae* AlpA expressed in *A. nidulans* transformants was first observed on inducing (MLT) and non-inducing (MKWR) plates after 2 days-cultivation at 37°C. As shown in Fig. 3, the colonies of TAR17 strain did not exhibit any visible halos on any of the plates. In contrast, all the ALP transformants showed clear hallos around the colonies on inducing plates, but no halo on non-inducing plates (Fig. 3). Among some transformants, the size of halos varied in accordance with the copy numbers of the integrated *alpA* gene; for example, ALP1 containing 3 copies of *alpA* gene formed the smallest and most turbid halos while ALP6 containing approximately 10 copies formed the largest and clearest ones. On the other hand, transformants ALP3 and ALP5, although they both contained approximately 7 copies of *alpA* gene, showed halos similar in size to ALP1.

In order to estimate the amount of secreted AlpA more accurately, the extracellular protease activity of each transformant was assayed individually. To accomplish this, washed mycelia of each *A. nidulans* strain grown in CMWR broth were cultured individually in inducing (MLT) and non-inducing (MKWR) broth media at 37°C. The culture filtrate was then used as a crude enzyme preparation for protease assay.

As presented in Table 1, all the transformants and the TAR17 strain exhibited only basal levels of protease activity in MKWR broth. Together with the result of the plate assay (Fig. 3), this result seems to be primarily due to the repression of intrinsic protease production on non-inducing medium containing 0.8% ammonium sulfate. It is in good accordance with the observation that expression of extracellular protease is repressed in the presence of ammonium and especially sulfate in *A. nidulans* (15). In addition, the *alcA* promoter that is located upstream of the *A. oryzae alpA* in the expression cassette was assumed to be repressed by glucose in the transformants (9). In MLT broth containing threonine as an inducer of *alcA* promoter and lactose as a non-repressing carbon source instead of glucose, all the ALP transformants showed extracellular protease activities that reached their maximum values after 8 h of induction. In comparison, *A. nidulans* TAR17 showed only negligible protease activity even in the inducing medium. This result supports that the coding region of *A. oryzae alpA* gene, even though it contains three introns, is effectively expressed in *A. nidulans* by the action of *alcA* promoter and that AlpA formed in the mycelia of the transformants is secreted into the medium in an active form. The induced protease activities, however, greatly varied among the ALP transformants. In accordance with the result of the plate assay, ALP6 containing approximately 10 copies of integrated *alpA* showed the highest activity (665.5±9.3 mU/ml) and ALP1 containing 3 copies showed the lowest activity (180.2±2.3 mU/ml). In this case, the copy number of integrated *alpA* gene seems to be primarily involved in determining the amount of secreted AlpA. On the other hand, ALP3 and ALP5 transformants showed quite a bit lower levels of protease activity in spite of the relatively high copy numbers of integrated *alpA* (7 copies). Thus the level of AlpA secretion seems to be not necessarily proportional to the copy number of the integrated *alpA* gene. This phenomenon would be partly due to the integration of the expression cassette into the locus which is involved in the activation of *alcA* promoter (6).

Total proteins of the induced and uninduced culture filtrates of each strain were analyzed by SDS-PAGE. In each of the induced culture filtrates of the transformants, a 34-kDa protein which was almost absent in the corresponding uninduced culture filtrates, was detected (Fig. 4). In addition, a smaller protein with a molecular mass of 19 kDa was also visible in each of the induced cultures. The molecular mass of the larger protein was roughly matched to the predicted value of the mature AlpA of *A. oryzae* which was derived from its precursor form by removal of pre and pro regions comprising 21 N-terminal and 100 subsequent amino acids, respectively (28). The molecular mass of the smaller one (19 kDa) was also matched up to the estimated value of the polypeptide containing the pro-region of AlpA. The amount of 34-kDa protein present in the induced culture filtrates of the transformants

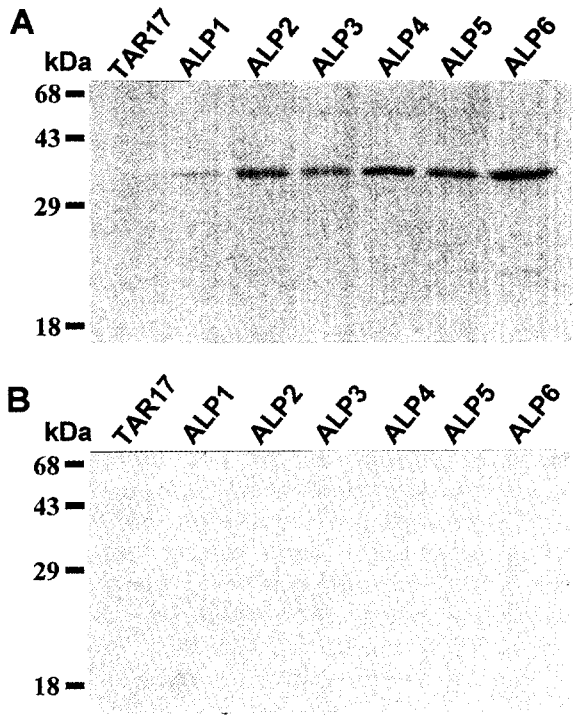

Fig. 4. SDS-PAGE of extracellular proteases from the parental (TAR17) and transformed strains. Mycelium of each *A. nidulans* strain grown in CMWR broth for 36 h at 37°C was incubated in inducing (MLT) and non-inducing (MKWR) broth media for 8 h at the same temperature, and then the proteins in the induced (A) and uninduced (B) culture filtrates (10 μl in each lane) were separated on an SDS-polyacrylamide gel. Molecular weight markers are bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and β-lactoglobulin (18 kDa).

showed a high degree of correspondence to that of the protease activity: for example, the induced culture filtrate of ALP6 contained a much larger amount of the 34-kDa protein than that of ALP1. In addition, the *N*-terminal amino acid sequence of the 34-kDa protein from the induced culture filtrate of ALP6 transformant was determined to be Gly-Leu-Thr-Thr-Gln-Lys-Ser, which is completely coincident with that of the mature form of *A. oryzae* AlpA (28). These results indicate that the extracellular protease activity of each transformant was derived from the secreted *A. oryzae* AlpA protein and that the N-terminal region of AlpA correctly functions as a secretion signal and is processed as in the original host.

In this study, we constructed some multi-copy transformants of pRAAlp containing the coding sequence for *A. oryzae* AlpA downstream of *alcA* promoter, and showed relatively efficient inducible expression and secretion of AlpA in *A. nidulans*. Using *A. nidulans* as a host for production of heterologous proteins seems to be beneficial for a couple of reasons. First, as mentioned above, *A. nidulans* has lots of genetic information and an efficient transformation system useful for development of an efficient secretion system of heterologous proteins using molecular biological techniques. Secondly, this fungus secretes many fewer extracellular proteases that severely affect the stability of the secreted heterologous proteins than other *Aspergillus* spp. such as *A. niger* and *A. oryzae*. Furthermore, the level of extracellular protease of *A. nidulans* host can be reduced to a negligible level by addition of 0.8% ammonium sulfate in the medium. It can also be pointed out that the pre-region of *A. oryzae* AlpA deserves to be an effective secretion signal for other heterologous proteins as that of *A. oryzae* glucoamylase (GlaA) which has been mainly used to direct the secretion of various foreign proteins in *Aspergillus* species (4, 5, 7, 23, 36).

Acknowledgments

This work was supported by a grant from Korea Research Foundation (KRF-1999-015-D10080). We thank Dr. Dong Min Han of Wonkwang University and Dr. Byeong Jae Lee of Seoul National University for kindly supplying the *A. nidulans* strains and vectors used in this study.

References

1. Bartling, S., J.P. van den Hombergh, O. Olsen, D. von Wettstein, and J. Visser. 1996. Expression of an *Erwinia* pectate lyase in three species of *Aspergillus*. *Curr. Genet.* 29, 474-481.
2. Brandhorst, T. and W.R. Kenealy. 1995. Effects of leader sequences upon the heterologous expression of restrictocin in *Aspergillus nidulans* and *Aspergillus niger*. *Can. J. Microbiol.* 41, 601-611.
3. Brandhorst, T., R. Yang, and W.R. Kenealy. 1994. Heterologous expression of the cytotoxin restrictocin in *Aspergillus nidulans* and *Aspergillus niger*. *Protein. Expr. Purif.* 5, 486-497.
4. Broekhuijsen, M.P., I.E. Mattern, R. Contreras, J.R. Kinghorn, and C.A. van den Hondel. 1993. Secretion of heterologous proteins by *Aspergillus niger*: production of active human interleukin-6 in a protease-deficient mutant by KEX2-like processing of a glucoamylase-hIL6 fusion protein. *J. Biotechnol.* 31, 135-145.
5. Carrez, D., W. Janssens, P. Degrave, C.A. van den Hondel, J.R. Kinghorn, W. Fiers, and R. Contreras. 1990. Heterologous gene expression by filamentous fungi: secretion of human interleukin-6 by *Aspergillus nidulans*. *Gene* 94, 147-154.
6. Cheevadhanarak, S., D.V. Renno, G. Saunders, and G. Holt. 1991. Cloning and selective overexpression of an alkaline protease-encoding gene from *Aspergillus oryzae*. *Gene* 108, 151-155.
7. Contreras, R., D. Carrez, J.R. Kinghorn, C.A. van den Hondel, and W. Fiers. 1991. Efficient KEX2-like processing of a glucoamylase-interleukin-6 fusion protein by *Aspergillus nidulans* and secretion of mature interleukin-6. *Biotechnology.* 9, 378-381.
8. Dunn-Coleman, N.S., P. Bloebaum, R.M. Berka, E. Bodie, N. Robinson, G. Armstrong, M. Ward, M. Przetak, G.L. Carter, R. LaCost, L.J. Wilson, K.H. Kodama, E.F. Baliu, B. Bower, M.

Lamsa, and H. Heinsohn. 1991. Commercial levels of chymosin production by *Aspergillus*. *Biotechnology*. 9, 976-981.
9. Felenbok, B., and H.M. Sealy-Lewis. 1994. Alcohol metabolism. *Prog. Ind. Microbiol.* 29, 141-179.
10. Gouka, R.J., P.J. Punt, J.G. Hessing, and C.A. van den Hondel. 1996. Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. *Appl. Environ. Microbiol.* 62, 1951-1957.
11. Harsanyi, Z., I.A. Granek, and D.W. Mackenzie. 1977. Genetic damage induced by ethyl alcohol in *Aspergillus nidulans*. *Mut. Res.* 48, 51-74.
12. Haussner, K., P. Hilgendorf, C. Hofbauer, J. Demeester, and A. Lauwers. 1996. New international F.I.P. method for the determination of the activity of *Aspergillus oryzae* proteases. *Pharmazie* 51, 946-950.
13. Impoolsup, A., A. Bhumiratana, and T.W. Flegel. 1981. Isolation of alkaline and neutral proteases from *Aspergillus var. columnaris*, a soy sauce koji mold. *Appl. Environ. Microbiol.* 42, 619-628.
14. Katz, M.E., P.K. Flynn, P.A. vanKuyk, and B.F. Cheetham. 1996. Mutations affecting extracellular protease production in the filamentous fungus *Aspergillus nidulans*. *Mol. Gen. Genet.* 250, 715-724.
15. Katz, M.E., R.N. Rice, and B.F. Cheetham. 1994. Isolation and characterization of an *Aspergillus nidulans* gene encoding an alkaline protease. *Gene* 150, 287-292.
16. Knittler, M.R., S. Dirks, and I.G. Haas. 1995. Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* 92, 1764-1768.
17. Lee, S.K., D.W. Lee, and P.J. Maeng. 1997. Inducible expression of yeast mitochondrial citrate synthase in *Aspergillus nidulans*. *Mol. Cells*. 7, 489-494.
18. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
19. MacRae, W.D., F.P. Buxton, D.I. Gwynne, and R.W. Davies. 1993. Heterologous protein secretion directed by a repressible acid phosphatase system of *Aspergillus niger*. *Gene* 132, 193-198.
20. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262, 10035-10038.
21. Murakami, K., Y. Ishida, A. Masaki, H. Tatsumi, S. Murakami, E. Nakano, H. Motai, H. Kawabe, and H. Arimura. 1991. Isolation and characterization of the alkaline protease gene of *Aspergillus oryzae*. *Agric. Biol. Chem.* 55, 2807-2811.
22. Punt, P.J., R.P. Oliver, M.A. Dingemanse, P.H. Pouwels, and C. A. van den Hondel. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* 56, 117-124.
23. Roberts, I.N., D.J. Jeenes, D.A. MacKenzie, A.P. Wilkinson, I. G. Sumner, and D.B. Archer. 1992. Heterologous gene expression in *Aspergillus niger*: a glucoamylase- porcine pancreatic prophospholipase A2 fusion protein is secreted and processed to yield mature enzyme. *Gene* 122, 155-161.
24. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
25. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
26. Saunders, G., T.M. Picknett, M.F. Tuite, and M. Ward. 1989. Heterologous gene expression in filamentous fungi. *Trends. Biotechnol.* 7, 283-287.
27. Streatfield, S.J., S. Toews, and C.F. Roberts. 1992. Functional analysis of the expression of the 3'-phosphoglycerate kinase *pgk* gene in *Aspergillus nidulans*. *Mol. Gen. Genet.* 233, 231-240.
28. Tatsumi, H., Y. Ogawa, S. Murakami, Y. Ishida, K. Murakami, A. Masaki, H. Kawabe, H. Arimura, E. Nakano, and H. Motai. 1989. A full length cDNA clone for the alkaline protease from *Aspergillus oryzae*: structural analysis and expression in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 219, 33-38.
29. Tilburn, J., C. Scazzocchio, G.G. Taylor, J. H. Zabicky-Zissman, R.A. Lockington, and R.W. Davies. 1983. Transformation by integration in *Aspergillus nidulans*. *Gene* 26, 205-221.
30. Tsuchiya, K., K. Gomi, K. Kitamoto, C. Kumagai, and G. Tamura. 1993. Secretion of calf chymosin from the filamentous fungus *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol.* 40, 327-332.
31. Tsuchiya, K., T. Nagashima, Y. Yamamoto, K. Gomi, K. Kitamoto, C. Kumagai, and G. Tamura. 1994. High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* 58, 895-899.
32. Tsuchiya, K., S. Tada, K. Gomi, K. Kitamoto, C. Kumagai, Y. Jigami, and G. Tamura. 1992. High level expression of the synthetic human lysozyme gene in *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol.* 38, 109-114.
33. Turnbull, I.F., D.R. Smith, P.J. Sharp, G.S. Cobon, and M.J. Hynes. 1990. Expression and secretion in *Aspergillus nidulans* and *Aspergillus niger* of a cell surface glycoprotein from the cattle tick, *Boophilus microplus*, by using the fungal *amdS* promoter system. *Appl. Environ. Microbiol.* 56, 2847-2852.
34. van den Hombergh, J.P., L. Fraissinet-Tachet, P.J. van de Vondervoort, and J. Visser. 1997. Production of the homologous pectin lyase B protein in six genetically defined protease-deficient *Aspergillus niger* mutant strains. *Curr. Genet.* 32, 73-81.
35. Ward, P.P., G.S. May, D.R. Headon, and O.M. Conneely. 1992. An inducible expression system for the production of human lactoferrin in *Aspergillus nidulans*. *Gene* 122, 219-223.
36. Ward, P.P., C.S. Piddington, G.A. Cunningham, X. Zhou, R.D. Wyatt, and O.M. Conneely. 1995. A system for production of commercial quantities of human lactoferrin: a broad spectrum natural antibiotic. *Biotechnology (NY)* 13, 498-503.
37. Waring, R.B., G.S. May, and N.R. Morris. 1989. Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. *Gene* 79, 119-130.
38. Wieser, J. and T.H. Adams. 1995. *flbD* encodes a Myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development. *Genes Dev.* 9, 491-502.
39. Yelton, M.M., J.E. Hamer, and W.E. Timberlake. 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc. Natl. Acad. Sci. USA* 81, 1470-1474.