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Construction of a low-serine-type-carboxypeptidase-producing mutant of *Aspergillus oryzae* by the expression of antisense RNA and its use as a host for heterologous protein secretion

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Abstract Using an antisense control strategy, we isolated an *Aspergillus oryzae* mutant that produced low levels of carboxypeptidases (CPases). The mutant TF_{C-1} expressed the antisense RNA of the structural gene of CPase O and showed about 30% of the CPase activity in the parent strain. Gel filtration analysis indicated that this mutant decreased the CPase activities not only of CPase O but also of CPase O-1 and O-2. This result indicated that the antisense RNA was able to control the expression of the CPase genes as a group. Using the mutant as a heterologous protein expression host that produced the low levels of CPases, a stable and higher level of lysozyme expression could be obtained compared with the wild-type. *In vitro* proteolytic degradation assay also demonstrated that human lysozyme was degraded by purified CPase O.

Introduction

There have been some attempts to obtain high amounts of eukaryotic proteins secreted from *Aspergillus oryzae* (Tsuchiya et al. 1992, 1994; Davies 1994; Stewart et al. 1996); however, the yields of heterologous proteins have generally been low. An important contributing factor may be the degradation of heterologous proteins by native fungal proteases present in the culture medium (Cohen 1977; Thompson 1991; Archer et al. 1992). The proteases produced by *A. oryzae* are therefore a barrier to using this fungus as a host for production of heterologous protein products. To overcome this problem, it would be extremely useful to establish mutant strains that are specifically deficient in extracellular protease activities.

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The availability of molecular genetic technology offers the possibility for novel approaches to the modification of *A. oryzae* strains for that purpose (Berka et al. 1990; Mattern et al. 1992; Archer et al. 1994; Hintz et al. 1995; Verdoes et al. 1995; Van den Hombergh et al. 1995). Using an in vitro gene replacement strategy, Berka et al. (1990) deleted the gene coding for aspergillopepsin A, an aspartic protease produced by *A. awamori*. Mattern et al. (1992) also obtained mutants of *A. niger* deficient in extracellular proteases. In addition, antisense control strategy has been adapted by researchers to inhibit gene expression in eukaryotic cells. In this approach, vectors are constructed to express a high level of the antisense RNA complementary to the RNA transcript of a target gene to inactivate that gene.

According to earlier studies, *A. oryzae* produces two main kinds of protease, serine-type carboxypeptidase (CPase) and aspartic endopeptidase under acidic conditions. The studies of Takeuchi and co-workers (Takeuchi et al. 1982; Takeuchi and Ichishima 1986; Takeuchi 1990) showed that there are several forms of CPase produced by *A. oryzae* at acidic pH. The major CPase responsible for 85%–90% of the total activity is a high-molecular-mass carboxypeptidase (designated CPase O), a protein of 155 kDa that is much less sensitive to inhibition by monoiodoacetic acid. Another form of CPase, the 63-kDa low-molecular-mass CPase (designated CPase O-1 and CPase O-2), is completely inhibited by iodoacetate. The CPases release most amino acid residues, including proline, from the C-terminal of peptides and proteins at acidic pH. We selected *A. oryzae* M-2-3 as a parent strain, which had no detectable APase activity. The purpose of this study was to construct a low-CPase-producing strain and to examine the level of heterologous protein produced by the mutant.

We describe here for the first time the construction of an antisense controlled vector and its use to decrease specifically the expression of the chromosomal serine-type carboxypeptidase gene group. We used this mutant as a host to produce heterologous protein HLY (human lysozyme) and found that it expressed a more

stable and higher level of HLY activity than that of wild-type.

Materials and methods

Strains, plasmids and media

A. oryzae M-2-3, in which *argB* was disrupted, was provided by Dr. K. Gomi (1987) and used as the recipient for transformation. A selected mutant with low CPase activity was used as a host for the expression of HLY, and *Escherichia coli* JM105 was used for DNA manipulation.

Plasmid pMAR1 used for the transformation was provided by Dr. K. Kitamoto. It carried the whole *argB* gene, which was used as a selection marker. Plasmid pMARCPOGR was constructed by inserting a 3.2-kb *Bam*HI fragment containing an antisense sequence of the CPase O structural gene into the *Bam*HI site of pMARI (Fig. 1). The cloning procedure and the sequence of the CPase O gene will be published elsewhere. A plasmid, pNAHLY (Tsuchiya et al. 1992), containing the *A. oryzae niaD* gene (a selection marker) and the gene for HLY, provided by Dr. K. Gomi, was used as an expression vector in *A. oryzae*. The promoter and terminator of *amyB* were used for the expression of HLY in this plasmid.

The media for the growth of *A. oryzae* were as follows. Fungal growth minimal medium (Gomi et al. 1987) was used as the transformation medium. Potato/dextrose/agar medium supplemented with arginine was used for spore preparation of *A. oryzae* M-2-3. SP medium (soluble starch, 3.5% polypeptone 2.0%, KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$ 0.5%) was used to prepare mycelia for fungal transformation, Southern blot analysis and Northern blot analysis. Liquid medium of 5% rice bran was used to produce CPases of *A. oryzae* M-2-3 and transformants. Czapek-Dox medium (Tsuchiya et al. 1992) was used for the expression of HLY.

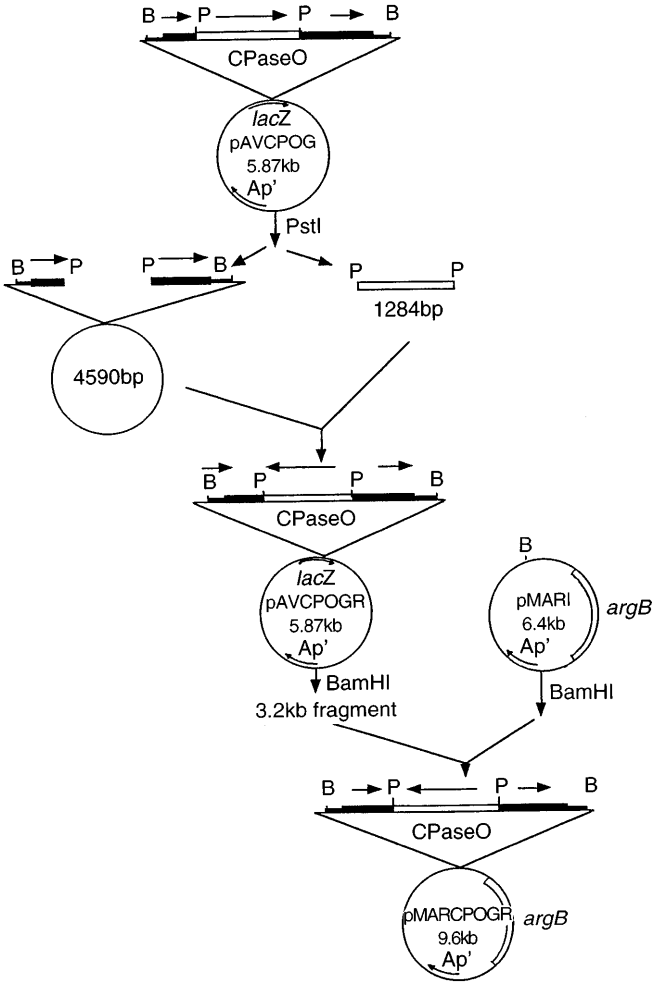

Fig. 1 Construction of the vector expressing antisense control RNA. The plasmid pMARCPOGR was constructed by inserting a 3.2-kb *Bam*HI fragment containing an antisense controlled carboxypeptidase (*CPase*) O structural gene into the *Bam*HI site of pMARI. The promoter and terminator of CPase O were used as the promoter and terminator

Transformation experiments

Transformation of *E.coli* was carried out according to the standard method (Maniatis et al. 1982). Transformation of *A. oryzae* was performed using the method described by Iimura et al. (1987).

DNA hybridization analysis

The DNA samples digested with appropriate restriction enzymes were separated on an 0.8% agarose gel and then transferred to a nylon membrane (Hybond N, Amersham, UK). Hybridization was performed according to standard procedures (Sambrook et al. 1989).

Western blotting analysis

Samples were electrophoresed on sodium dodecyl sulfate (SDS)/polyacrylamide gels using the method of Laemmli (1970), and then transferred to Hybond-C membrane (Amersham). The primary antigen-antibody complex was detected using the peroxidase-labelled goat anti-(rabbit Ig) antiserum, and the membranes were developed by reacting with peroxidase and staining with H_2O_2 3-3 diaminobenzdine development reagent.

Preparation of total RNA and Northern blot analysis

RNA was prepared from mycelia grown for 24 h at 30 °C in SP medium by a modification of the method of Robert et al. (1983). Total RNA (10 μg) was fractionated by formaldehyde/agarose gel electrophoresis, transferred to a Hybond-N nylon membrane (Amersham) and cross-linked with UV light. The cDNA of CPase O was used as the DNA probe, which was fluorescein-labelled with a random-primer fluorescein dUTP labelling kit (DuPont, USA). Hybridization was done at 42 °C in 5 × Standard saline phosphate EDTA butter (SSPE) 5 × Denhardt's solution, 50% formamide, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The hybridization membrane was washed with 2 × SSPE, 0.1% SDS and 0.1 × SSPE, 0.1% SDS solution for 5 min at 68 °C.

Gel filtration analysis

A Sephadex G-200 column (80 cm, diameter 2 cm) was used for gel filtration, and 0.01 M sodium acetate buffer (pH 5.0) containing 0.2 M NaCl was used as eluting buffer according to Takeuchi (1982).

Protein measurement

The protein content was estimated by measuring the absorbance at 280 nm ($A^{1\%}_{1cm,\ 280\ nm} = 18.8$; Takeuchi 1986) using a Hitachi UV 1100 spectrophotometer.

Isolation of *niaD* mutants and transformation with pNAHLY

Nitrate-assimilation-defective spontaneous mutants of *A. oryzae* M-2-3 and TF_{C-1} were obtained by positive selection for resistance to chlorate (Cove 1979). Spores were harvested in 2 ml 0.1% SDS. Aliquots (0.2 ml) were spread out on minimal medium containing 470 mM chlorate with 10 mM glutamate as the sole source of nitrogen. Transformation of *A. oryzae* with pNAHLY was performed by the method described above. A protoplast suspension was added to transformant selection minimal medium pre-cooled to 45 °C. The plates were incubated at 30 °C for up to 5 days.

Enzyme assay

The CPase activity was assayed routinely with Z(benzyloxycarbonyl)-Glu-Tyr in 50 mM sodium acetate buffer (pH 3.7) as substrate. One katal of the CPase was defined as the amount of enzyme required to liberate one mole of tyrosine from Z-Glu-Tyr per second at 30 °C and pH 3.7, according to the IUPAC and IUB recommendations (Florkin and Stotz 1973).

The activity of APase was checked by using casein (E. Merck, Frankfurt) as substrate according to the method of Ichishima (1970).

Lysozyme activity was measured as described by Morsky (1983) and Tsuchiya et al. (1992). Enzyme solution (200 µl) was mixed with 800 µl *Micrococcus luteus* ATCC 4698 cell suspension (Sigma, USA; 150 µg cell/ml) in 50 mM phosphate buffer, pH 6.4. A decrease in absorbance of the mixture at 450 nm, caused by the lysis of the bacterial cells, was monitored at room temperature. One unit (U) was defined as the activity reducing absorbance by 0.001 per minute.

In vitro proteolytic degradation assays

For assay of proteolysis, 100 µg HLY was added to 2 nkat purified carboxypeptidase (CPase O) in citrate buffer pH 4.0 and incubated at 30 °C. The purified CPase O had no endopeptidase activity (Takeuchi et al. 1986). Incubation was stopped by addition of the denaturing solution, and then the sample was analyzed by SDS/polyacrylamide gel electrophoresis (PAGE).

Results

Isolation and characterization of low-CPase-producing mutants

A. oryzae M-2-3 was used as the recipient in the transformation experiment. When protoplasts of M-2-3 were mixed with plasmid pMARCPOGR in the presence of PEG 4000 and $CaCl_2$, and then generated in soft agar overlays, about 50 transformants appeared; no colonies appeared on minimal medium without the plasmid. These transformants were able to grow on successive transfers to minimal medium without arginine. The CPase activity of all 50 transformants was checked, and most showed lower activity than that of wild-type M-2-3. One of the mutants among the transformants showed 30% of the wild-type CPase activity; this was designated TF_{C-1} and chosen for further study. TF_{C-1} showed stable activity on successive cultures.

To confirm that the low-CPase-producting phenotype was a result of the integration of plasmid into the genome of *A. oryzae*, Southern blotting analysis was carried out using a digoxigenin-labelled CPase gene as the probe. As shown in Fig. 2, only one band was observed in wild-type M-2-3, and for the transformant TF_{C-1} more than two distinct bands were seen, suggesting that the construction vectors were integrated into the genome of *A. oryzae* M-2-3. The bands corresponding to the transformant were denser than that of the wild type.

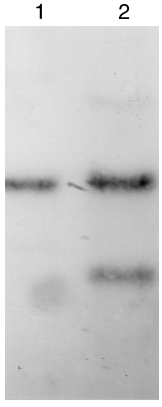

Fig. 2 Southern blotting analysis of transformants. Chromosomal DNA (2 µg) was isolated from the transformants and the recipient *Aspergillus oryzae* M-2-3, and then digested with *Eco*RI (*lane 1* wild-type *A. oryzae* M-2-3, *lane 2* TF_{C-1}), run on a 0.7% agarose gel, blotted, and hybridized to a digoxigenin-labelled probe consisting of the 3.2-kb fragment of the gene encoding CPase O

Northern blotting analysis using a fluorescein-labelled CPase cDNA as the probe, showed that mutant TF_{C-1} expressed the antisense control of the structural gene, and indicated more abundant expression than that of wild type M-2-3 (Fig. 3).

Western blotting indicated that the band of transformant TF_{C-1} was weaker than that of M-2-3 (Fig. 4).

The expression of antisense RNA not only decreased the activity of CPase O but also the activity of CPases O-1 and O-2

Forms of CPase produced by the mutant TF_{C-1} under liquid culture were characterized by gel filtration after 5 days culture in 5% rice bran pH 4.5. As shown in Fig. 5, there were two peaks in all of the samples analyzed

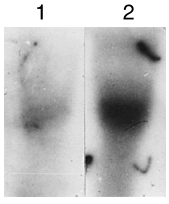

Fig. 3 Northern blot analysis of transformants. Total RNA (5 µg) of transformants isolated from mycelia was fractionated by formaldehyde/agarose gel electrophoresis and blotted onto Hybond-N nylon membrane in 20 × Standard saline phosphate EDTA butter (SSPE). The fluorescein-labelled cDNA of CPase O was used as a probe. *Lane 1* wild-type strain *A. oryzae* M-2-3, *lane 2* transformant TF_{C-1}

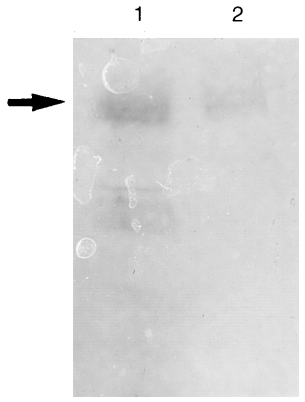

Fig. 4 Western blotting analysis of the carboxypeptidase secreted from *A. oryzae* M-2-3 and *A. oryzae* transformant. Concentrated culture supernatants were electrophoresed on sodium dodecyl sulfate polyacrylamide gel by the method of Laemmli (1970). *Lane 1 A. oryzae* M-2-3, *lane 2* concentrated culture supernatant of transformant TF_{C-1}

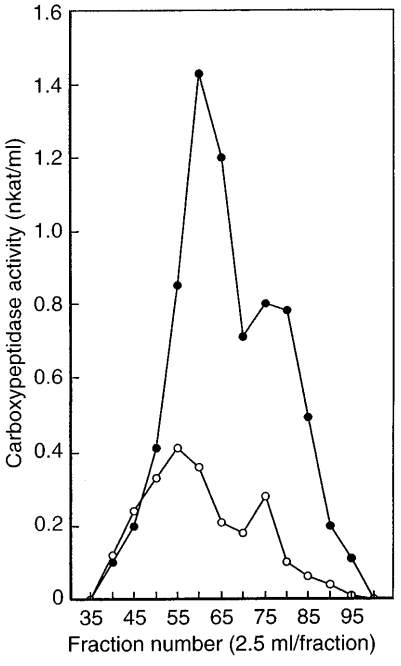

Fig. 5 Elution profiles of carboxypeptidases based on Sephadex G-200 gel filtration. Comparison of carboxypeptidase activity between *A. oryzae* M-2-3 (●) and TF_{C-1} (○). The first peak represents the activity of CPase O and the second peak the activity of CPases O-1 and O-2

(*A. oryzae* M-2-3 and TF_{C-1}). The activity of the CPase was also checked by adding 50 mM iodoacetic acid. The activity of the first peak was not inhibited by iodoacetate, while 90%–100% of the activity of the second peak was (data not shown). This suggested that the first peak represented the high-molecular-mass enzyme CPase O, while the second peak represented the low-molecular-mass CPases O-1 and O-2. In addition, in mutant TF_{C-1}, besides the decrease in the activity of the high-molecular-mass enzyme CPase O, the activity of the low-molecular-mass CPases O-1 and O-2 was also lowered. Thus, the expression of the antisense RNA decreased the activity of both CPases.

Construction of transformants for the expression of the human lysozyme gene

Several *niaD* mutants were isolated as described in Materials and methods. The transformants with pNAHLY of *A. oryzae* M-2-3 and the TF_{C-1} mutant were obtained and designated MHLY-1 and HLY-1 respectively.

The lysozyme activity of the transformants was assessed in 250-ml Sakaguchi flasks containing 100 ml cultures. No detectable lysozyme activity was found in the culture filtrate containing glucose as a carbon source, but was detected in those supernatants containing maltose as a carbon source.

Southern blot analysis of the HLY transformants

Genomic Southern blot analysis was performed to determine whether the *niaD* gene and the gene for HLY were integrated into the genome of the recipient strain TF_{C-1}-15. A 1.4-kb *Bam*HI fragment of the *niaD* gene and a 0.5-kb *Eco*RI fragment of the HLY gene were used as probes respectively. As shown in Fig. 6A, when the *niaD* probe was used, only two bands were detectable in wild-type M-2-3, but there were several distinct bands in transformants HLY-1 and MHLY-1. With the HLY probe (Fig. 6B), the signal corresponding to the gene for HLY was observed at about 500 bp, suggesting that the HLY expression plasmid was integrated into the DNA of the recipient strain.

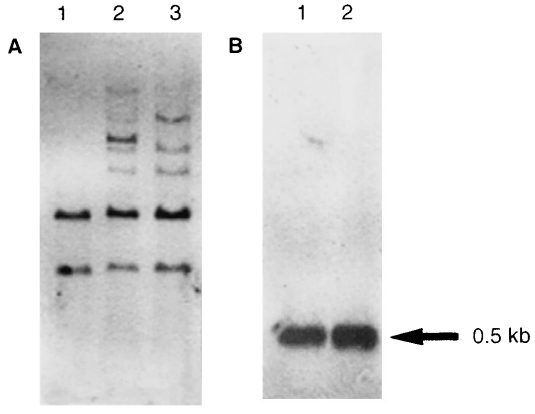

Fig. 6A, B Southern blotting analysis of human lysozyme (HLY) transformants. Chromosomal DNAs (5 μg) were isolated from transformants, then digested with *Bam*HI(*A*) or *Eco*RI(*B*), run on a 0.7% agarose gel, blotted, and hybridized to a digoxigenin-labelled probe consisting of the 1.4-kb *Bam*HI fragment of *niaD* (**A**. *lane 1 A. oryzae* M-2-3, *lane 2* MHLY-1, *lane 3* HLY-1), or a 0.5-kb fragment of the HLY gene (**B** *lane 1* MHLY-1, *lane 2* HLY-1)

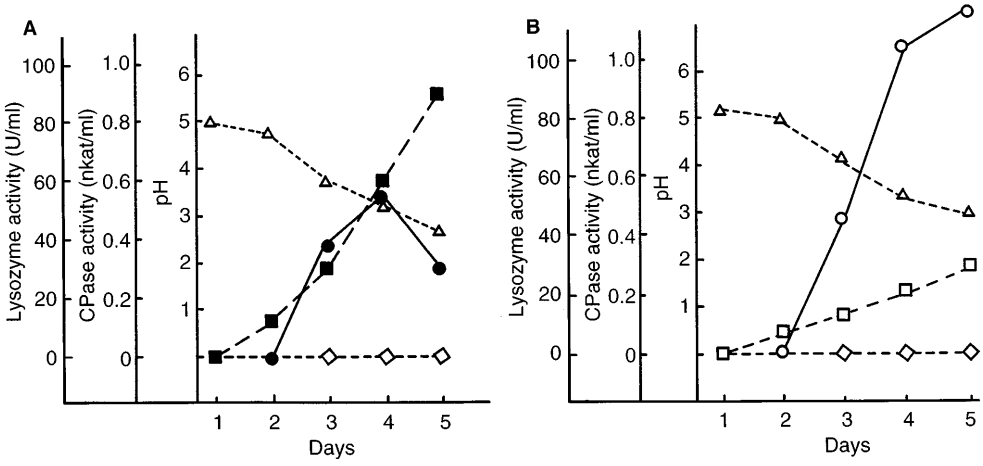

Fig. 7A, B The effects of pH and the presence of CPase on the activity of human lysozyme produced by transformants MHLY-1 and HLY-1 Transformants MHLY-1 (**A**) and HLY-1 (**B**) were grown in CD medium containing 2% maltose at 30 °C with shaking. The time course of HLY activity of MHLY-1 (–●–) and HLY-1 (–○–), the CPase activity of MHLY-1 (- - ■ - -), and HLY-1 (- - □ - -), the pH of the medium (- - △ - -) and aspartic endopeptidase activity (- - ◇ - -) were analyzed

Correlations among pH, acid CPase activity and lysozyme activity

The change of lysozyme activity and CPase activity with the change of pH in Czapek-Dox medium is shown in Fig. 7. The initial pH of the medium was adjusted to 5.0; during the culture process it decreased gradually and by the end of the culture had dropped to about 2.7. During this period, the activity of the CPase increased continuously. On the other hand, the activity of lysozyme in the control strain MHLY-1 reached a maximum at day 4 in culture, and then declined sharply. Compared with this control strain, the low-CPase-producing transformant HLY-1 showed a high level of lysozyme activity, which did not decrease even after 5 days. This decrease of lysozyme activity was followed by the increase of CPase activity, strongly suggesting that, with the decrease of pH, the proteolytic degradation of lysozyme was caused by CPases.

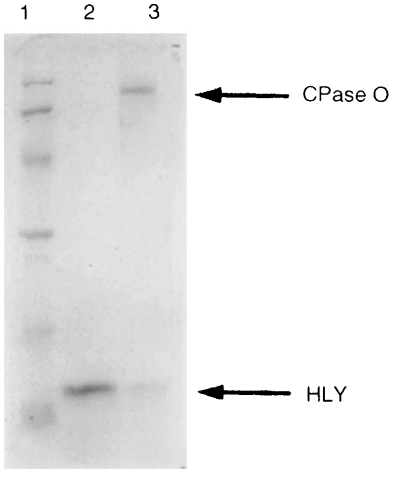

Fig. 8 In vitro proteolytic degradation of lysozyme by purified carboxypeptidase (*CPase O*). Samples were electrophoresed on a slab of 12.5% polyacrylamide gel by the method of Laemmli (1970). *Lane 1* molecular mass marker, *lane 2* lysozyme standard (10 μg), *lane 3* lysozyme (10 μg) incubated with purified CPase O (0.2 nkat)

The result of the in vitro protein degradation assay also confirmed this assumption. After incubation at 30 °C for 30 h, the proteolytic degradation was determined by SDS-PAGE (Fig. 8). Most of the HLY was degraded by purified CPase O at pH 4.0, and the sensitivity of HLY to this degradation was a major obstacle in its achieving stable production as a secreted heterologous protein in *A. oryzae*.

Discussion

According to previous studies of Takeuchi et al. (1982, 1986), *A. oryzae* secretes several CPases, all forms being inhibited by serine protease inhibitors, suggesting that these enzymes have similar active-site residues. The N-terminal amino acid sequences of high-molecular-mass CPase and low-molecular-mass CPase differed from each other, however. These CPases may be encoded by different genes and have a similar encoding region for the active site. This work was the first to use an antisense control strategy to generate strains of *A. oryzae* that produced low levels of CPases. Southern hybridization analysis revealed that multiple copies of the antisense-controlled plasmids were integrated into the genome of *A. oryzae* M-2-3 in a tandem array. Integration of multiple copies of a gene was successful in transformation experiments in other *Aspergillus* species (Yelton et al. 1984). Results of Northern blotting showed that the amount of expression of the corresponding RNA in the mutant strain was higher than that of the wild-type M-2-3. The overexpression of antisense RNA suggested that the transformant showed low CPase activity. Meanwhile, results of Western blotting confirmed that, in accordance with this low CPase activity, the expression of CPase in the transformant was also lower than that of the wild type.

Gel filtration experiments showed that two types of carboxypeptidase were produced in *A. oryzae* M-2-3. The results of gel filtration indicated that the mutant decreased the activity of both CPase O and CPases O-1 and O-2, meaning that the expressed antisense RNA of

the structural gene, including the active site region, suppressed the expression of genes encoding all serine-type CPases. This suggested that the antisense RNA corresponding to the active site acted effectively and resulted in the decrement of the expression of the CPase gene group.

We investigated whether the low-CPase-producing strain produced a higher level of heterologous protein than did the wild-type strain. Because a mutant that produced about 10% CPase activity of the wild-type strain grew poorly, we used the mutant TF_{C-1}, which had 30% of the CPase activity of the wild-type strain. This mutant grew as well as the wild-type and was suitable for use as a host to produce heterologous human lysozyme (HLY).

Tsuchiya et al. (1992) reported that when the gene for HLY was expressed from *A. oryzae* in Czapek-Dox P medium, the lysozyme activity increased for 4 days and then decreased, but in Czapek-Dox N medium it did not drop. The decrease was presumably caused by proteolysis with protease(s) secreted from the host strain. In our study, the activity of lysozyme produced by the TF_{C-1} strain was higher than that of the wild-type strain. Moreover, with the increase in level of CPase, in contrast to the wild type, in which the HLY activity decreased sharply after 4 days, the activity of strain TF_{C-1} did not decrease even after 5 days. In vitro proteolytic degradation assays also showed that HLY was sensitive to CPase. These results suggested that CPases have become an obstacle to the production of heterologous protein HLY under acidic conditions. The low-CPase-producing strain TF_{C-1} could produce more stable heterologous proteins than could the wild-type strain. Therefore, the low-CPase-producing strain may be more useful for the production of heterologous proteins.

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