

Secretion of *Aspergillus oryzae* Alkaline Protease in an Osmophilic Yeast, *Zygosaccharomyces rouxii*

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To produce *Aspergillus oryzae* alkaline protease (Alp) in an osmophilic yeast *Zygosaccharomyces rouxii*, we constructed an expression plasmid consisting of the *Z. rouxii* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter, the prepro-Alp cDNA of *A. oryzae*, the whole sequence of *Z. rouxii* plasmid pSR1, and the G418 resistant gene. The resulting plasmid, when introduced into *Z. rouxii* cells, directed the secretion of a large amount (about 300 mg/l) of Alp into the culture medium. The N-terminus and specific activity of the enzyme were identical to those of *A. oryzae* Alp.

The osmophilic yeast *Zygosaccharomyces rouxii* is important in the production of both ethanol and the characteristic flavor of soy sauce during brewing. A number of physiological studies of this yeast^{1–4)} have been done, but genetic studies^{5–7)} have been few. To improve industrial strains of *Z. rouxii* by recombinant DNA technology, the host-vector system of this yeast has been studied through the isolation of the plasmid pSR1,^{8,9)} the cloning of the *Z. rouxii GAPDH* gene as a promoter,¹⁰⁾ and improvement of the transformation procedure for *Z. rouxii*.¹¹⁾ Two reports^{12,13)} have described the expression of foreign DNAs in *Z. rouxii* using this host-vector system. However, no studies have yet shown that the expressed protein is secreted into the culture medium. In the case of *S. cerevisiae* acid phosphatase,¹³⁾ the active enzyme is secreted into the periplasmic space, but not into the culture medium.

In previous reports,^{14,15)} we described the

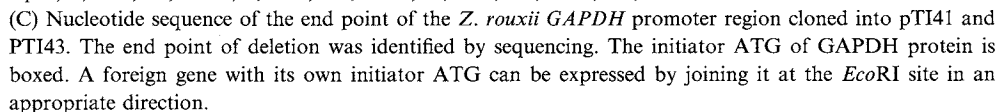
isolation and structural analysis of the alkaline protease (Alp) cDNA from *A. oryzae*, and also the secretion of enzymatically active Alp in *S. cerevisiae*.

In this study, to investigate the secretion of a foreign extracellular protein by *Z. rouxii*, we constructed plasmids designed to express the prepro-Alp cDNA of *A. oryzae* in *Z. rouxii*, and introduced them into *Z. rouxii* cells. A large amount (about 300 mg/ml) of Alp was secreted into the culture medium, and its enzymatic properties were identical to those of native Alp as far as they were examined.

Materials and Methods

Strains and plasmids. *Aspergillus oryzae* ATCC20386 was used as a source of native Alp and Alp cDNA coding for the prepro and mature regions together with the 5' and 3' non-coding regions (Fig. 1A). *Escherichia coli* strain DH1[F[−] *recA1 endA1 gryA96 thi-1 hsdR17* (*r_k[−] m_k⁺*) *supE44*] was used as a carrier for recombinant plasmids. Yeasts, *Saccharomyces cerevisiae* strain NA87-11A [(*cir*⁺)

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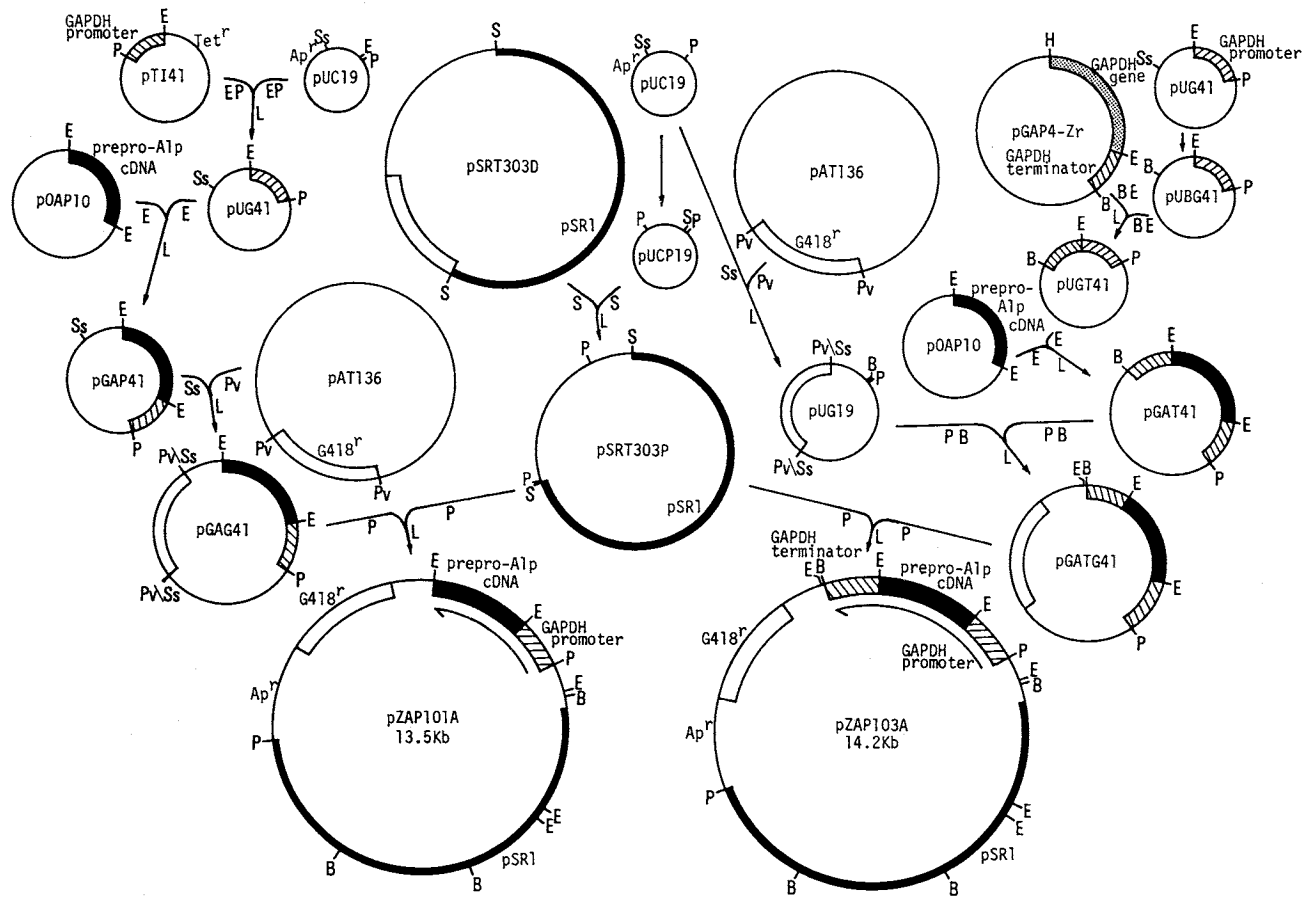


Fig. 2. Construction of Expression Plasmids Containing the Alp cDNA for *Z. rouxii*.

The 1.5-kb *EcoRI*–*EcoRI* fragment containing the Alp cDNA from pOAP10 was inserted at the *EcoRI* site of pUG41 containing the *Z. rouxii* GAPDH promoter from pTI41, resulting in pGAP41. The 1.7-kb *PvuII*–*PvuII* fragment containing the G418 resistant gene from pAT136 was inserted into the *SspI* site of pGAP41 to obtain pGAG41. A *Bam*HI site was created at the *SspI* site of pUG41, resulting in pUG41. The 0.7-kb *EcoRI*–*Bam*HI fragment containing the *Z. rouxii* GAPDH terminator region of pGAP4-Zr was inserted into the *EcoRI*–*Bam*HI gap of pUG41 to obtain pUGT41. pGAT 41 was constructed by insertion of the *EcoRI* fragment containing the Alp cDNA from pOAP10 into the *EcoRI* site of pUGT41. The *PstI*–*Bam*HI fragment containing the promoter-cDNA-terminator was separated from pGAT41 and inserted into the *PstI*–*Bam*HI gap of pUG19 to obtain pGATG41. Furthermore, the 6.4-kb *SalI*–*SalI* fragment of pSRT303D, containing the whole pSR1 sequence, and the 2.6-kb of pUCP19 with insertion of the *PstI* linker at the *SspI* site of pUC19 were ligated to obtain pSRT303P. pZAP101A was constructed by ligation of the 7-kb *PstI*–*PstI* fragment of pSRT303P, containing the whole pSR1 sequence, with pGAG41. pZAP103A was constructed by ligation of the same fragment of pSRT303P with pGATG41. Arrows show the direction of transcription. Abbreviations for the restriction sites are: B, *Bam*HI; E, *EcoRI*; P, *PstI*; Pv, *PvuII*; S, *SalI*; Ss, *SspI*; L, T4-DNA ligase.

MAT α leu2-3, 112 his3 trp1 pho5-1] and *Zygosaccharomyces rouxii* ATCC13356 were used as the hosts for the expression of Alp cDNA.

Plasmids pUC19,¹⁶⁾ pUC119,¹⁷⁾ and pBR322¹⁸⁾ were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Plasmid pOAP10 containing the 1.5-kb prepro-Alp cDNA fragment (Fig. 1A) at the *EcoRI* site of pUC119 was constructed for a previous report.¹⁵⁾ Plasmid pSRT303D,¹⁹⁾ consisting of the whole sequence of *Z. rouxii* plasmid pSR1 and the G418 resistant gene, was donated by Y. Oshima (Department of Fermentation Technology, Osaka University). For the comparison of promoter activity, plasmids pTI41 and pTI43 containing different lengths of the *Z. rouxii GAPDH* promoter region constructed as shown in Fig. 1B, were used. The end points of deletion were

identified by sequencing (Fig. 1C). pGAP4-Zr contains the 3-kb *HindIII*–*BamHI* fragment carrying the *Z. rouxii GAPDH* gene from pGAP1-Zr.¹⁰⁾ The 0.7-kb *EcoRI*–*BamHI* fragment was excised from pGAP4-Zr and used as a terminator region. pAT136 was constructed by deleting the *EcoRI* fragment containing yeast DNA from pAJ50²⁰⁾ and is equivalent to pBR322::Tn601 (G418^r). The yeast expression vectors pMA56, pAAR6, and pAAH5 were obtained from the Washington Research Foundation.²¹⁾ The expression plasmid pOAP101 containing the prepro-Alp cDNA at the *EcoRI* site of pMA56, was constructed for a previous report.¹⁵⁾

Media. For cultivation of *E. coli*, L-broth containing 1% Bactotrypton (Difco), 0.5% yeast extract (Difco), and

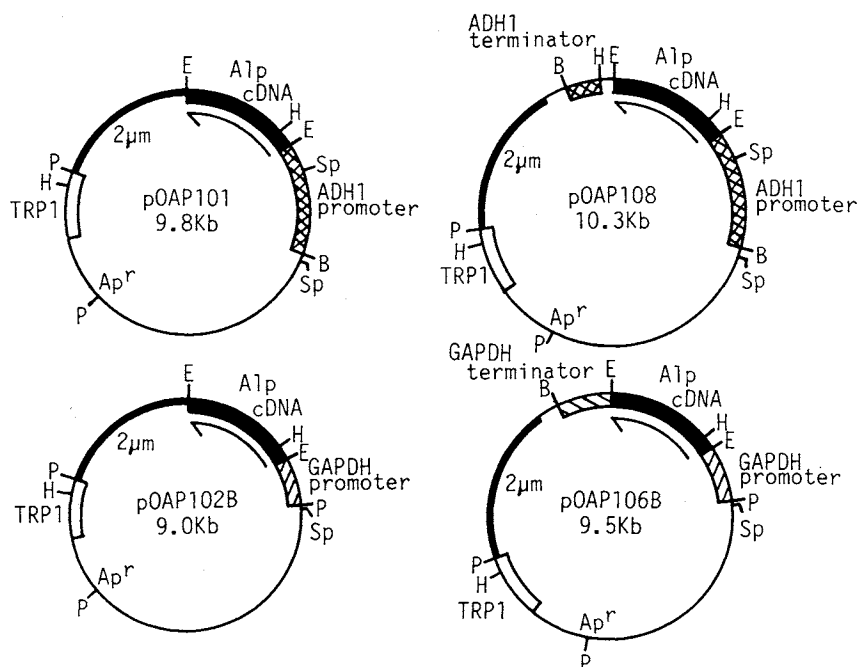


Fig. 3. Structure of Expression Plasmids Containing the Alp cDNA for a *S. cerevisiae* Host.

Four expression plasmids, pOAP101,¹⁵⁾ pOAP108, pOAP102B, and pOAP106B were constructed. The 1.5-kb *SphI*–*EcoRI* fragment containing the *S. cerevisiae ADH1* promoter of pMA56 was replaced by the 0.7-kb *SphI*–*EcoRI* fragment containing the *Z. rouxii GAPDH* promoter from pUG43 which was constructed from pTI43 and pUC19 in the same way to obtain pUG41, illustrated in Fig. 2. The resultant vector was designated pMG56. The 1.5-kb *EcoRI* fragment of the Alp cDNA was inserted at the *EcoRI* site of pMG56 in an appropriate direction to obtain pOAP102B. The 4.7-kb *EcoRI*–*PstI* fragment containing the *S. cerevisiae ADH1* promoter from pAAR6, the 1.6-kb *PstI* fragment carrying the *TRP1* gene from pMA56, the 2.1-kb *PstI*–*BamHI* fragment containing 2μm DNA from pAAH5, the 0.5-kb *BamHI*–*EcoRI* fragment containing the *S. cerevisiae ADH1* terminator region from pAAR6, and the 1.5-Kb *EcoRI* fragment of the Alp cDNA was ligated to obtain pOAP108. The 3.5-kb *SphI*–*BamHI* fragment carrying the Alp cDNA between the *S. cerevisiae ADH1* promoter and the terminator of pOAP108 was replaced with the 2.9-kb *SphI*–*BamHI* fragment carrying the Alp cDNA between the *Z. rouxii GAPDH* promoter and the terminator from pGAT43 which was constructed from pTI43 in the same way to obtain pGT41 illustrated in Fig. 2. The resultant expression plasmid was designated pOAP106B. Arrows show the direction of transcription. Abbreviations for the restriction sites are: B, *BamHI*; E, *EcoRI*; H, *HindIII*; P, *PstI*; Sp, *SphI*.

0.5% NaCl was used, and when necessary 50 µg/ml ampicillin, 50 µg/ml kanamycin, or 10 µg/ml tetracyclin was added. SD medium supplemented with 60 µg/ml L-histidine and 60 µg/ml L-leucine was used for cultivation of *S. cerevisiae* harboring plasmids, and contained 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco). The nutrient medium YPD, used for cultivation of *S. cerevisiae* and *Z. rouxii*, contained 2% glucose, 2% Polypeptone (Nihon Seiyaku), and 1% yeast extract (Difco). G418 (100 µg/ml) and 1% milk casein were added to YPD medium when appropriate.

Recombinant DNA techniques. All restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo and used according to the manufacturer's instructions. *E. coli*, *S. cerevisiae*, and *Z. rouxii* were transformed by the methods of Hanahan,²²⁾ Beggs,²³⁾ and Ito *et al.*,²⁴⁾ respectively. Preparation of DNA from *E. coli* was performed according to standard procedures.²⁵⁾

Western blotting. SDS-PAGE was done with a 10–20% gradient gel (Daiichi Pure Chemicals Co., Ltd.). Proteins in the gel were transferred to a PVDF membrane (Millipore) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membrane was then immunostained using anti-Alp antiserum raised in rabbits, and a blotting detection kit (Bio-Rad).

Assay of enzyme activity. Protease activity was measured using azocasein as a substrate by the method of Fukushima *et al.*²⁶⁾ One unit of protease activity (PU) was defined as the amount which catalyzed the release of 1 µg of tyrosine per minute under the conditions described.

Protein was measured as described by Lowry *et al.*,²⁷⁾ using bovine serum albumin as a standard.

Purification of Alp and sequencing of its N-terminus. For the preparation of Alp secreted by *Z. rouxii*, *Z. rouxii* ATCC13356 harboring pZAP101A was cultured in 10 ml of YPD medium containing 100 µg/ml G418 at 30°C with shaking at 120 rpm for 72 hr. This culture was then inoculated into 2 l of the same medium and cultured at 30°C for 72 hr in a 3-l MB-C jar fermenter (Iwashiya Co., Ltd., Tokyo, Japan) with 1 vvm of aeration and agitation at 600 rpm. The culture was centrifuged to obtain the supernatant. The further purification procedure was described by Tatsumi *et al.*¹⁵⁾

For the preparation of native Alp, *A. oryzae* ATCC20386 grown on wheat bran was soaked with distilled water overnight at 4°C, and then filtered through cheesecloth to obtain a crude extract. Further purification and sequencing of the N-terminus of Alp were done as described previously.¹⁵⁾

Construction of expression plasmids containing the Alp cDNA. To produce Alp in *Z. rouxii*, we constructed the expression plasmid pZAP101A (Fig. 2) consisting of the

Z. rouxii GAPDH promoter, the prepro-Alp cDNA, the whole sequence of *Z. rouxii* plasmid pSR1, the G418 resistant gene, and pUC19. The terminator region of the *Z. rouxii GAPDH* gene was connected with a downstream sequence of prepro-Alp cDNA on pZAP101A to obtain pZAP103A (Fig. 2).

To investigate the effects of the *Z. rouxii GAPDH* promoter and terminator on Alp production in *S. cerevisiae*, we also constructed three other expression plasmids, pOAP108, pOAP102B, and pOAP106B from pOAP101¹⁵⁾ (Fig. 3). The *S. cerevisiae ADH1* promoter of pOAP101 was replaced with the *Z. rouxii GAPDH* promoter from pTI43 to obtain pOAP102B. The terminator regions of *S. cerevisiae ADH1* and *Z. rouxii GAPDH* were connected with a downstream sequence of the prepro-Alp cDNA of pOAP101 and pOAP102B to obtain pOAP108 and pOAP106B, respectively.

Results

Expression and secretion of alkaline protease in Z. rouxii

The expression plasmids pZAP101A, pZAP103A, and pSRT303D (as a control) were introduced into *Z. rouxii* strain ATCC13356 by the lithium acetate method,²⁴⁾ and each G418-resistant transformant was selected on YPD plate medium containing 100 µg/ml G418 and 1% milk casein. After incubation at 30°C for 3 days, G418-resistant *Z. rouxii* harboring pZAP101A and pZAP103A formed a halo around the cells, but the cells harboring pSRT303D did not.

Each kind of cell harboring pZAP101A and pSRT303D was cultured in YPD liquid medium containing 100 µg/ml G418. After three days, the cells were collected by centrifugation, and washed twice with distilled water, and the culture broth was filtered through a membrane filter (0.45 µm). The cell suspension and broth were analyzed by SDS-PAGE and Western blotting (Fig. 4). As in the case of *S. cerevisiae*,¹⁵⁾ a protein band reacting with anti-Alp antiserum was detected in both the intracellular sample and the broth sample of cells harboring pZAP101A (Fig. 4B, lanes 3 and 5), and the mobilities of these bands were the same as that of the purified native Alp from *A. oryzae* (control). A minor band under the Alp band may correspond to a fragment of Alp generated by boiling with SDS.

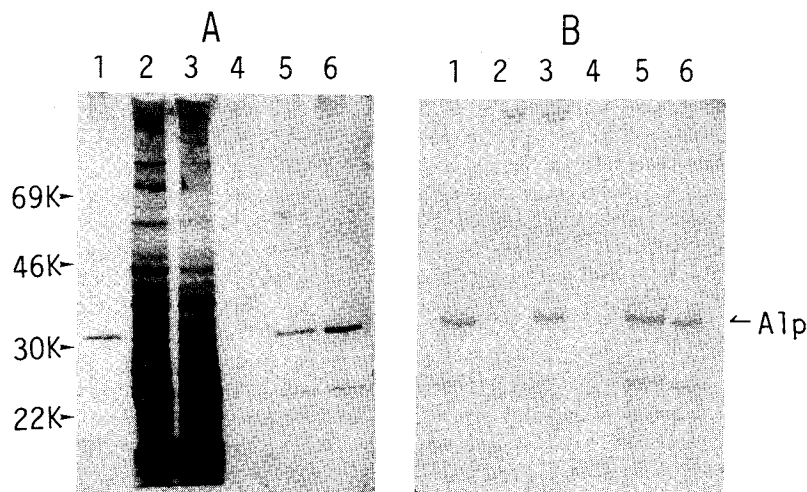


Fig. 4. SDS-PAGE and Western Blot Analysis of the Alp Produced by *Z. rouxii*.

After cultivation for 3 days, culture broth of *Z. rouxii* strain ATCC13356 harboring pSRT303D or pZAP101A was centrifuged, and each precipitate (lanes 2 and 3, respectively) and supernatant (lanes 4 and 5, respectively) from 10 μ l of culture was run on SDS-PAGE. (A) Silver staining, (B) Western blot analysis. Lanes 1 and 6 contain 1 μ g of native Alp purified from *A. oryzae* as a control. The arrow indicates the position of native Alp (34k). Molecular weight markers are bovine serum albumin (69k), ovalbumin (46k), carbonic anhydrase (30k), and trypsin inhibitor (22k).

Table I. PRODUCTION OF ALKALINE PROTEASE OF *A. oryzae* BY *Z. rouxii* AND *S. cerevisiae* HARBORING VARIOUS EXPRESSION PLASMIDS

Host	Plasmid	Origin	Promoter	Terminator	Alp production (mg/l)
<i>Z. rouxii</i> ATCC13356	pZAP101A	pSR1	<i>GAPDH</i>	—	117 (275) ^a
	pZAP103A	pSR1	<i>GAPDH</i>	<i>GAPDH</i>	110
<i>S. cerevisiae</i> NA87-11A	pOAP101	2 μ m	<i>ADHI</i>	—	2.5
	pOAP102B	2 μ m	<i>GAPDH</i>	—	11.8
	pOAP108	2 μ m	<i>ADHI</i>	<i>ADHI</i>	7.8
	pOAP106B	2 μ m	<i>GAPDH</i>	<i>GAPDH</i>	17.3

^a *Z. rouxii* harboring pZAP101A was cultivated on the SOY medium containing 7% glucose and 15% soy sauce for 7 days.

On the other hand, no band was detected in any samples of cells harboring pSRT303D, which does not contain Alp cDNA (Fig. 4B, lanes 2 and 4). In the medium in which the transformant harboring pZAP101A was cultured, only one band corresponding to native Alp was detected by SDS-PAGE analysis (Fig. 4A, lane 5).

When the cells harboring pZAP101A or pZAP103A were cultured in YPD liquid medium containing 100 μ g/ml G418, 66 and

70 PU/ml of Alp activity were detected, respectively. Since the specific activity of Alp secreted by *Z. rouxii* (Z-Alp) was 600 PU/mg (described later), the amounts of Z-Alp were 110 and 117 mg/l, respectively (Table I). Moreover, when the cells harboring pZAP101A were grown in SOY medium containing 7% glucose and 15% (v/v) soy sauce for 7 days, the maximal level of secreted Z-Alp was 275 mg/l (Table I).

Effects of NaCl concentration on growth and Alp production

Z. rouxii harboring pZAP101A was cultured in YPD liquid medium containing 100 μ g/ml G418 at 30°C for 3 days. Cells in 10 ml of cultured medium were inoculated into a 3-l jar fermenter containing 2 l of the same medium supplemented with 5% or 10% NaCl (Fig. 5). In YPD medium containing 100 μ g/ml G418, the growth reached the maximum at 36 hr and the number of Klett units did not increase even after 36 hr. The level of secreted Alp increased in proportion to cell growth until 36 hr, but thereafter increased gradually to reach 63 PU/ml at 96 hr. In the presence of 5% or 10% NaCl, the growth rate became slower, but the

final number of Klett units and final amount of secreted Alp remained unchanged. In the presence of 15% NaCl, about 180 hr was needed for maximum growth to be attained. However, the final number of Klett units and amount of secreted Alp were also unchanged (data not shown).

Effects of NaCl concentration on cell growth and Alp production were further examined by halo formation on YPD plates containing 1% milk casein and 0%, 5% or 10% NaCl (Fig. 6). Since the growth rate of *S. cerevisiae* was faster than that of *Z. rouxii* on the 0% NaCl plate, a larger halo was detected around *S. cerevisiae* harboring pOAP106B. At 10% NaCl, however, no halo was detected around

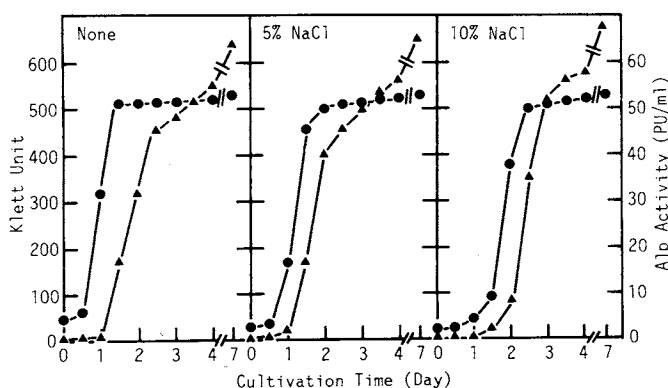


Fig. 5. Effects of NaCl Concentration on Cell Growth and Alp Production of *Z. rouxii*.

Cultivation was done in a 3-l jar fermenter. Cells harboring pZAP101A (10 ml) were inoculated into 2 l of YPD medium containing 100 μ g/ml G418 and NaCl concentrations indicated in the figure. Cultivation conditions were: agitation, 600 rpm; aeration, 1 vvm; temperature, 30°C. Alp activity was measured as described in Materials and Methods. Symbols: ●, Klett units; ▲, Alp activity in the broth.

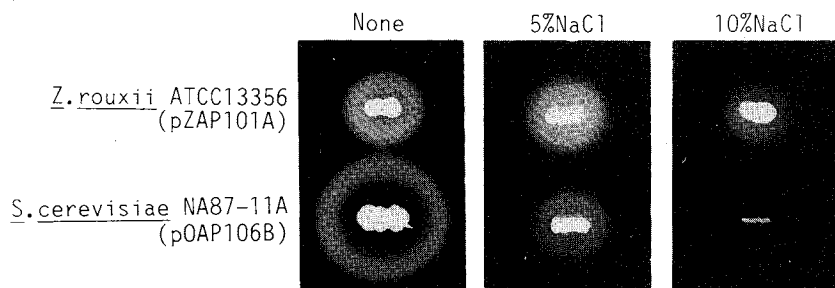


Fig. 6. Effects of NaCl Concentration on the Cell Growth and Halo Formation.

Z. rouxii strain ATCC13356 harboring pZAP101A and *S. cerevisiae* strain NA87-11A [*cir*⁺] harboring pOAP106B were inoculated on YPD plates containing 1% milk casein and NaCl concentrations indicated in the figure, and incubated at 30°C for 4 days.

the *S. cerevisiae* host because of growth inhibition by the high concentration of NaCl, but a halo was detected around *Z. rouxii* harboring pZAP101A. The *Z. rouxii* recombinant formed a halo even at 15% NaCl concentration (data not shown).

Purification and characterization of secreted Z-Alp

Z-Alp was purified from the medium, and its specific activity was 604 PU/mg protein, which was almost identical to that of purified native Alp from *A. oryzae* (615 PU/mg).¹⁵⁾

The amino acid sequence of the N-terminus of Z-Alp was Gly-Leu-Thr-Thr-Gln-Lys-Ser-Ala-Pro-Trp, which was the same as that of native Alp from *A. oryzae*.¹⁵⁾

The effects of pH on the activities of native Alp from *A. oryzae* and Z-Alp were examined using milk casein as a substrate. The same pH patterns were found between native Alp from *A. oryzae* and Z-Alp, and the maximum activities of both Alp were observed at pH 10 (data not shown).

Alp production in S. cerevisiae using the Z. rouxii GAPDH promoter

We have described how 2.8 mg/l Alp was secreted by *S. cerevisiae* using the *S. cerevisiae ADHI* promoter.¹⁵⁾ As described above, *Z. rouxii* harboring pZAP101A produced more than 100 mg/l Alp. To examine whether the efficient expression in the *Z. rouxii* recombinant resulted from the *Z. rouxii GAPDH* promoter in pZAP101A, we constructed various expression plasmids such as pOAP102B, pOAP108, and pOAP106B (Fig. 3) and introduced each of them into *S. cerevisiae* strain NA87-11A. As shown in Table I, *S. cerevisiae* harboring pOAP102B, which carries the Alp cDNA under the *Z. rouxii GAPDH* promoter, produced 11.8 mg/l Alp. The cells harboring pOAP106B, which carries the Alp cDNA between the *Z. rouxii GAPDH* promoter and terminator, produced 17.3 mg/l Alp, and a transformant harboring pOAP108, which carries the Alp cDNA between the *S. cerevisiae ADHI* promoter and terminator, produced 7.8 mg/l

Alp.

Discussion

This report is the first one which describes the secretion and correct processing of a heterologous enzyme in the *Z. rouxii* host-vector system. Although we confirmed that the secreted Alp by *Z. rouxii* cultured has a correct N-terminus, the precise mechanism by which the pre-pro region of Alp cleaved is not known: the processing may be done either by a putative processing enzyme of *Z. rouxii* or by the autoproteolytic activity of the Alp itself. The fact that a high molecular weight band corresponding to a precursor Alp was not detected in the *Z. rouxii* cell extract by Western blot analysis (Fig. 4) suggests that the rate of processing is rapid or that processing is coupled with translocation across the ER lumen, as observed for subtilisin.²⁸⁾

The *Z. rouxii* host harboring pZAP101A or pZAP103A secreted more than 100 mg/l Alp, which is seven times larger than that produced by the *S. cerevisiae* host harboring pOAP106B, the most efficient plasmid among those we constructed for Alp production in the *S. cerevisiae* host (Table I). Even in the *S. cerevisiae* host, the *Z. rouxii GAPDH* promoter (pOAP102B and pOAP106B) indicated stronger activity than the *S. cerevisiae ADHI* promoter (pOAP101 and pOAP108). Moreover, Alp production in the *S. cerevisiae* host was increased by addition of the *Z. rouxii GAPDH* terminator region (pOAP106B), and the *Z. rouxii GAPDH* promoter from pTI43 was more efficient than that from pTI41 (data not shown). Therefore, in the case of the *Z. rouxii* host, Alp expression driven by the *Z. rouxii GAPDH* promoter may be more efficient than in the *S. cerevisiae* host. However, Alp production in *Z. rouxii* was not increase by addition of the terminator region (pZAP103A), or by using the promoter from pTI43 (data not shown). This result, along with data shown in Fig. 6, implies some differences in mRNA metabolism between *S. cerevisiae* and *Z. rouxii*. Further work is necessary on this.

Many studies have investigated the expression of foreign DNA and its secretion in yeast. In these studies, *Rhizopus oryzae* glucoamylase²⁹⁾ and *Mucor pusillus* rennin³⁰⁾ were secreted into the medium and accumulated at a considerable level, above 300 mg/l and 150 mg/l, respectively using a strain of *S. cerevisiae* as host. A comparable amount of Alp was secreted into the medium by the *Z. rouxii* host. Another advantage of the *Z. rouxii* host-vector system is salt tolerance. This expression system can be used as a screening system for salt-tolerant enzymes and for the expression system of enzymes stabilized with a high concentration of NaCl, such as *Archaeobacterium* protease.³¹⁾

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