High Level Secretion by *Saccharomyces cerevisiae* of Human Apolipoprotein E as a Fusion to *Rhizomucor* Rennin

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As the first step for production of human apolipoprotein E (hApoE) in *Saccharomyces cerevisiae*, the hApoE cDNA was cloned in *Escherichia coli*, on the basis of the nucleotide sequence reported previously. When the hApoE cDNA including its pre-sequence-encoding region was expressed under the control of the GAL7 promoter, no protein immunoreactive with anti-hApoE antibody was detected either in the culture medium or inside the cells. For efficient production and secretion of hApoE in *S. cerevisiae*, the mature hApoE-encoding region was fused to the prepro-sequence region of *Rhizomucor* rennin (MPR) and to the whole MPR gene including its prepro- and mature-MPR regions. When the fusion gene consisting of the prepro-sequence-encoding region and hApoE regions was expressed in *S. cerevisiae*, no protein reactive with the anti-hApoE antibody was detected in any fraction of the yeast cells, probably due to rapid degradation of the hApoE protein by yeast proteases. On the other hand, when hApoE was expressed as a fusion to the whole MPR protein, a considerable amount of the fused protein was secreted into the medium. The prepro-sequence of MPR was correctly processed from the fused protein in the medium by autocatalytic activity of MPR and by a protease(s) of the host cell. Integration of the fusion gene into the chromosome at a copy number of eight led to secretion of the fused protein in a larger amount than the case when the fusion gene was carried on a 2-μm plasmid with its copy number of a few hundreds, because the 2-μm derived plasmid containing the fusion gene was very unstable in the yeast cells. The secretion level was also improved by changing the culture conditions. A maximum yield of hApoE part in the secreted fused protein was estimated to be 23.7 mg per liter and the amount of the fused protein was calculated to be 53.0 mg per liter.

*Saccharomyces cerevisiae* is an efficient host for expression and secretion of heterologous proteins. Secretion of a protein usually requires a leader peptide at the amino terminus of the translation product. Successful secretion of heterologous proteins in yeast cells by using the leader peptides of yeast secretory proteins, such as α-factor and invertase, has been reported.1,2 Futhermore, several genes from filamentous fungi encoding their secretory proteins were expressed and efficiently secreted by yeast cells.3,4

We previously showed that a fungal aspartic proteinase, *Rhizomucor pusillus* rennin (MPR), was efficiently secreted from *S. cerevisiae* transformants carrying the MPR gene under the control of the GAL7 promoter.4 The prepro-sequence consisted of the N-terminal 22 amino acids as the signal peptide for secretion and the following 44 amino acids as the pro-peptide. We also showed that the pre- and prepro-sequences of MPR were useful for secretion of human growth hormone and human urokinase.5,6

We had used the MPR gene for secretion of rat apolipoprotein E (rApoE) as a fused protein.7 In this study, we cloned the human apolipoprotein E (hApoE) gene and intended to express and secrete the protein as a fusion to MPR. hApoE is a plasma lipoprotein that serves as a ligand for low density lipoprotein receptors, and through its interaction with these receptors, participates in the redistribution of cholesterol and other lipids between hepatic and extrahepatic tissues.8 When the whole MPR sequence including the prepro and mature parts was fused to the hApoE sequence, the fused protein was secreted into the medium, as was observed for the secretion of rApoE.7 For improvement of the production of MPR–hApoE, the use of an integration vector was useful because of its stability. We also established improved culture conditions. Under these culture conditions, the yield of the MPR–hApoE fused protein secreted into the medium reached approximately 53.0 μg/ml.

Materials and Methods

Strains and plasmids. *S. cerevisiae* strain MC16 (α, leu2 his4 ade2) was used as the host for production of hApoE. *Escherichia coli* strain JM105 [Δlac pro thi rpsL endA aecB15 hsdR4 F′ traD36 proAB lacIq lacZ ΔM15] was used as the host for DNA manipulation. Plasmid pJG5, a yeast expression vector that contained the GAL7 promoter and GAL10 transcriptional terminator sequences, was described previously.5 Plasmid JPI containing the MPR gene and plasmid JPI38 containing the mutant MPR gene in which one of the catalytic residues, Asp-38, was replaced by Gly, were reported previously.9 Plasmid YEp13 was used as a source of the *LEU2* gene. Plasmid pUC19 was used for cloning of the hApoE cDNA.

Cloning of the hApoE cDNA from human liver. Standard DNA manipulation was done as described by Sambrook et al.10 Restriction endonucleases, T4 DNA ligase, Taq polymerase, and the Klenow fragment were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan).

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Oligonucleotides were synthesized automatically on a Cyclone Plus DNA synthesizer. The first strand DNAs from the human liver poly(A)-containing RNA (Researchunfinished, Tokyo, Japan) were synthesized by the standard method. The hApoE cDNA was cloned by the polymerase chain reaction (PCR) method by using two primers, 1 (5'-CTAACGCTGATGTTGGCCTG-3') and 2 (5'-ATAGTATGGTGTTGGCCACAG), on the basis of the sequence of the hApoE gene reported by Paik et al. 10 The sense primer 1 contained a translational initiation codon (ATG) and a HindIII end for experimental convenience. The primer 2 contained a complementary stop codon (TCA) and an EcoRI end. PCR with Taq polymerase in a DNA thermal cycler was done at 94°C for 1 min, 40°C for 2 min, and 72°C for 3 min, in a total of 25 cycles. An amplified DNA fragment of about 970 bp was then cloned in HindIII plus EcoRI-digested pUC19 to construct pTHAE5 (Fig. 1). The EcoRI site in pTHAE5 was changed into a BamHI site with the Klenow fragment and a 10-mer BamHI linker, resulting in pTHAB. The nucleotides of the cloned fragment were sequenced by the dyeoxy chain termination method. 11

Construction of hApoE expression vector. For construction of plasmid JHAP1, the unique HindIII site located in front of the GAL7 promoter in JHGS was first changed into a SauI site, using the Klenow fragment and SauI site linker, resulting in JGHS. The GAL7 promoter sequence was synthesized by the PCR method by using JGHS as the template, and two primers, 3 (5'-GGGCTGCCACCCCATGGGAGGACATG) and 4 (5'-GCCAAGCTTGGAGATTTGGAGGT), on the basis of the sequences of the GAL7 promoter 13 and MPR gene, 14 respectively. The former and later primers contained a SauI and a HindIII site, respectively. The conditions for the reactions were: 94°C for 1 min; 40°C for 2 min; 72°C for 15 cycles. The SauI--HindIII fragment containing the GAL7 promoter and the HindIII BamHI fragment containing the whole hApoE cDNA in pTHAB5 were introduced between the SauI site and BamHI site in the expression vector JGHS by three fragments-ligation, resulting in JHAP1. To construct plasmid pTHAX5 containing the mature region of hApoE gene, a part of the mature region of hApoE was amplified from pTHAX5 using primers, 5 (5'-CCCCCTAGACAGGGTTGGACAGCGGTCG)- and 6 (5'-GCCGACGTCCGGGCTCGGCCTGTCG), the former and later primers contained artificial XbaI and Aval sites, respectively. After this PCR product had been digested with XbaI and Aval, this fragment and the Aval--BamHI fragment from pTHAB5, were introduced between the XbaI and BamHI sites in plasmid pUC19, resulting in pTHAX5. For construction of JHAP2, JHAP3, and JHAP4, the prepro-region and the whole region of MPR (MPRD38), were amplified from JPI (PJP138G) using primers, 7 (5'-CGCAAGCTTTAGCTTCTGCTCCAGATC)- and 8 (5'-CGGTCTAGACGGACGCCGCTTACCGC)-, and 9 (5'-GGGCTGGACGCTTCTGCTACCCCGG)-, respectively. The former primer 7 and the later primers 8, 9 contained artificial HindIII and XbaI sites for experimental convenience. After these PCR products had been digested with HindIII and XbaI, these fragments and the XbaI--BamHI fragment from pTHAX5, were introduced between the HindIII and BamHI sites in JHAP1, resulting in JHAP2, JHAP3, and JHAP4, respectively.

For construction of an integration vector, the EcoRI site in JHAP1 was first changed into a SauI site with the Klenow fragment and an 8-mer SauI linker, and then the SauI--XbaI fragment containing the LEU2 gene from plasmid YEP13 was introduced in the SauI site in the plasmid, resulting in the plasmid JHAP1N. Secondly, the HindIII--BamHI fragment containing the MPR-hApoE fusion gene from JHAP3 was introduced between the HindIII and BamHI sites in JHAP1N, resulting in JHAP5. This plasmid was used after digestion with EcoRI.

Culture conditions. S. cerevisiae was transformed by the protoplast method. 15 YPD medium (2% Bacto-Peptone [Difco Laboratories, Detroit, U.S.A.], 1% yeast extract [Difco], 2% glucose, and 0.08% adenine sulfate) and YPGal-1 (2% Bacto-Peptone, 1% yeast extract, 0.08% adenine sulfate, and 3% galactose), YPGal-2 (6% Bacto-Peptone, 3% yeast extract, 0.08% adenine sulfate, and 3% galactose), YPGal-3 (2% Bacto-Peptone, 1% yeast extract, 0.02% adenine sulfate, 3% galactose, and 50 mM MOPS [3-N-morpholino] propane sulfonic acid), pH 7.0 media, were used for cultivation of yeast transformants. SD medium (0.67% yeast nitrogen base [Difco], 2% glucose, and 0.02% adenine sulfate) were used as the selective medium. Transformants were first cultured at 30°C in YPD medium. The cells were harvested by centrifugation and resuspended in the original culture volume of YPGal medium and cultivation was continued aerobically at 30°C.

Immunoblot analysis of hApoE produced by yeast cells. Proteins secreted into the culture medium were precipitated by the addition of cold trichloroacetic acid to final concentration of 10% and the pellet obtained by centrifugation was washed with ethanol-ether (1:1). The pellets were then dissolved in 50 mM Tris-hydrochloride, pH 8.0. The samples were collected from yeast cells harboring JHAP3, JHAP4, and JHAP5 were precipitated by the addito of 2.5 volumes of ethanol. The proteins in the intracellular fractions were analyzed as described previously. 19 These fractions were electrophoresed on SDS-polyacrylamide gel (7.5 or 10%) and analyzed by Western bluse using a rabbit anti-MPR polyclonal antibody prepared by the method of Etoh et al. 20 or an anti-hApoE polyclonal antibody (Chemicon International Inc., Temecula, U.S.A.), with anti-IgG (H+L)-horseradish peroxidase conjugate (Chemicon International Inc.). hApoE was measured by the stained intensity of proteins on nitrocellulose filters with a densitometer (CS-9000, Shimadzu, Kyoto, Japan) using hApoE (Chemicon International Inc.) as the standard.

EndoH Treatment of MPR-hApoE fusion protein. S. cerevisiae MC16 harboring JHAP3 was cultured aerobically at 30°C for 60 h in YPGal-2 medium. After the cells had been removed by centrifugation, the supernatant was diluted 8 times, adjusted to pH 8.0, and put on a DEAE-Toyopearl 650m column (4×10 cm, Tosoh Co., Ltd., Tokyo, Japan) equilibrated with 20 mM Tris-hydrochloride, 5 mM EDTA, 1 mM DTT, 0.5 mM PMSF (Sigma Chemical Co., St. Louis, U.S.A.), 1 μM pepstatin (Sigma), pH 8.0. After the column had been washed with the same buffer, adsorbed proteins were eluted with 0.2 M NaCl in the same buffer. The relative fused protein content was monitored by dot immunoblotting on a nitrocellulose filter by using the anti-MPR polyclonal antibody. The fractions containing the fused proteins were pooled and concentrated by ultrafiltration, using a CENTRICUT (Kuraray Co., Osaka, Japan). The sample was diluted twice and applied to a heparin-Sepharose column (HiTrap-Heparin 5mL, Pharmacia Biotech Inc., Tokyo, Japan) equilibrated with 20 mM Tris-hydrochloride, 5 mM EDTA, 1 mM PMSF, and 1 μM pepstatin, pH 8.0. After the column had been washed with the same buffer, adsorbed proteins were eluted with 2 M KC1 in the same buffer. The fractions containing the fused proteins were pooled and concentrated by ultrafiltration. The sample was applied to gel filtration chromatography using an FPLC system equipped with a Superose 12 column (1×30 cm, Pharmacia). The protein eluted with 20 mM Tris-hydrochloride, pH 8.0 was concentrated with a CENTRIFUG. The sample was digested with endo-β-N-acetyl glucosaminidase H (EndoH, Seikagaku Co., Tokyo, Japan) according to the method of Tarentino et al. 19

Plasmid stability. Plasmid stability was determined by checking the leucine auxotrophy. After cultivation for 10 generations in YPD medium or YPGal medium, about 1000 yeast cells were plated onto both the selective SD and the nonselective YPD plates. The percentage of colonies on the selective medium versus the nonselective medium was taken as the index of plasmid stability.

Southern blot analysis. The chromosomal DNAs were extracted from the yeast stains harboring JHAP1 and were digested with HindIII. The MPR gene were used as the probe. Southern hybridization was done as described previously. 10

Results

Cloning and sequencing of hApoE cDNA

The hApoE cDNA was amplified by PCR using the cDNA synthesized from the total poly(A)-containing mRNA of human liver. The primers were designed according to the sequence of the hApoE gene reported by Paik et al. 11 The amplified DNA fragment of about 970 bp with restriction enzyme linkers at both ends was cloned into pUC19, resulting in plasmid pTHAE5. The nucleotide sequence of the cloned fragment showed an open reading frame of 317 amino acids that was identical to those reported by Paik et al. 11 The hApoE cDNA on pTHAE5 obtained in this way was used for further experiments.

Construction of plasmids for expression of the hApoE gene

The prepro-region (−61 Met to −1 Phe) was shown to
be useful for secretion of human growth hormone and urokinase by *S. cerevisiae*. We therefore constructed plasmid JHAP2 in which the DNA region encoding the mature hApoE protein (+1 Lys to +299 His) was connected to the prepro-region of MPR via three amino acids, Ser-Leu-Asp, derived from an *XbaI* linker (Fig. 1b). This fused gene was placed between the GAL7 promoter and the GAL10 terminator. We also constructed plasmid JHAP1, in which the whole hApoE gene was placed between the same promoter and terminator, to examine whether the signal sequence (−18 Met to −1 Ala) of hApoE was functional in *S. cerevisiae*. Two additional plasmids we constructed were JHAP3 and JHAP4. JHAP3 contained the hApoE part encoding the mature protein downstream of the whole MPR gene (Fig. 1a). JHAP4 had the same construction as JHAP3, but contained a mutation of Asp38Gly. Asp-38 is one of the catalytic residues and this mutation completely abolishes enzyme activity.9)

We also constructed an integration plasmid, JHAP5, using JHAP1 as the starting material. The mature hApoE-encoding region in this plasmid was connected to the whole MPR gene. After introduction of JHAP5 into strain MC16, we isolated a transformant containing eight copies of the fused gene at the *LEU2* locus, as shown by Southern hybridization (data not shown).

**Production of hApoE and fusion proteins**

*S. cerevisiae* MC16 was transformed with each of the above-described plasmids by the protoplast method. The transformants were cultured first in YPD medium and then transferred to YPGal medium to induce the expression of the intact hApoE gene and the fused genes. The production was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with the anti-hApoE and anti-MPR antibodies. In the case of JHAP1 and JHAP2, however, no protein immunoreactive with the antibodies was detected at any stage of growth in any fraction (data not shown). On the other hand, some proteins immunoreactive with the anti-hApoE and anti-MPR antibodies were detected in the culture medium of *S. cerevisiae* harboring JHAP3, JHAP4, and JHAP5, only when the transformants were cultured in the galactose-containing medium (Fig. 2a). JHAP3 and JHAP5 directed the secretion of the proteins of 76 and 74 kDa, the sizes of which were in agreement with the calculated value (76 kDa) of the mature MPR (42 kDa)–hApoE (34 kDa) fused protein. The difference in 2 kDa resulted from the heterogeneity of glycosylation at two sites, as described below.

Plasmid JHAP4 containing the active site mutation of the MPR gene directed the secretion of some proteins reactive with the antibodies; two of them had the same sizes (76 and 74 kDa) as those for JHAP3 and JHAP5, and the additional one was slightly larger (Fig. 2a). A protein reactive with the antibodies that was accumulated inside the cells had the same size as this additional protein (Fig. 2a) and as the proteins accumulated inside the cells harboring JHAP3 and JHAP5 (data not shown). One of the basis of the mechanism of MPR secretion, we assume that this largest protein is the proMPR–hApoE fused protein. In the secretory pathway of MPR in *S. cerevisiae*, MPR is first secreted as proMPR, and then processed by autocatalytic proteolysis and by host-dependent proteolysis. It is well conceivable that the inability of the mutant MPR to cleave its pro-sequence leads to accumulation of the pro-form. The 76- and 74-kDa proteins are supposedly produced through the host-dependent proteolysis.

**Glycosylation by extracellular MPR-hApoE fused protein**

The two major proteins excreted from the yeast strain harboring JHAP5 were approximately 76 and 74 kDa. The
calculated size of the mature MPR (42 kDa)–hApoE (34 kDa) fused protein was 76 kDa. The fused protein was expected to be glycosylated at two sites in the MPR part, as was MPR produced in *S. cerevisiae*. The major two bands from the culture supernatant were purified by successive rounds of chromatography and electrophoresis as described in Materials and Methods. The fused proteins bound to heparin, as does native ApoE, which facilitated purification of the fused protein. The EndoH treatment of the fused proteins decreased the molecular size and yielded a single band of about 72 kDa (Fig. 2b). Since the hApoE protein contains no possible N-glycosylation sites and since the MPR part contains two N-glycosylation sites, we concluded that the 76 and 74-kDa proteins contained two and one carbohydrate chains, respectively, each of which showed a electrophoretic mobility corresponding to 2 kDa.

**Effects of the cultivation conditions on secretion of hApoE fused protein**

To characterize in more detail the MPR–hApoE secretion by *S. cerevisiae* harboring JHAP5, we examined the course of the secretion after induction by galactose. The strain harboring JHAP5 was first cultured in YPD medium and then transferred to three different media. The culture
supernatants were taken at appropriate intervals, and the amounts of both the 76 and 74-kDa fused proteins reactive with the anti-hApoE antibody were measured on the basis of the amounts of hApoE. Although the MPR–hApoE proteins were secreted efficiently after galactose induction, the fused protein was degraded during prolonged cultivation (Fig. 4). Tsuttiya et al.20) has reported that the use of ammonium chloride as a nitrogen source repressed the proteolysis of human lysozyme secreted into the culture medium from Aspergillus oryzae. On the basis of these observations, to avoid proteolysis of the MPR–hApoE proteins, we examined the production of MPR–hApoE in media containing different amounts of nitrogen sources. When the transformant was cultured in the medium consisting of three times the usual concentrations of yeast extract and Bacto-Peptone in YPGal-1 medium (YPGal-2), the proteolysis of MPR–hApoE proteins was repressed (Fig. 3a). As the cultivation was prolonged, the pH of the culture medium increased above seven (Fig. 4). On the other hand, when the transformant was cultured in YPGal-1 medium, the pH of culture medium decreased below six (Fig. 4). To investigate the possible relationship between the extracellular proteolysis and the pH of the medium in more detail, we examined the production of MPR–hApoE in YPGal medium the pH of which was kept around at pH 7 with MOPS as a buffering agent (YPGal-3). As expected, this medium repressed the extracellular degradation of the fused proteins (Fig. 3b). These results suggest that the control of the pH of the culture medium is important for protecting heterologous proteins from extracellular proteolysis. The maximum amount of the hApoE part in the fused protein was 23.7 mg per liter at 72 h after the medium shift and the amount of the fused protein secreted was calculated to be 53.0 mg per liter on the basis of the relative molecular weight ratio (hApoE/fused protein = 3474 kDa).

Effects of plasmid stability on the secretion of MPR–hApoE fused protein

S. cerevisiae harboring 8 copies of the MPR–hApoE fused genes secreted a larger amount of the fused protein than that containing the same fused gene on the 2-μm plasmid, the copy number of which is a few hundreds (Table). Because the integrated genes were thought to be stably maintained, we supposed that the smaller amount of fusion protein secreted by the strains harboring JHAP3 was due to the instability of the plasmid. We therefore measured the stability of plasmid JHAP3 in the transformants during cultivation. When S. cerevisiae harboring JHAP3 was cultured in YPGal medium, 25% of the cells retained the plasmid after 10 generations, as measured by the index of leucine auxotrophy. When cultured in YPD medium, more than 90% of the cells contained the plasmid and when S. cerevisiae harboring JPI containing the GAL7 promoter and MPR gene were cultured in YPGal medium, the cells retaining the plasmid were more than 70%.5) It is thus concluded that the expression of the MPR–hApoE fused gene leads to the elimination of the plasmid, decreasing the amount of the secreted MPR–hApoE protein.

Discussion

Plasmids JHAP1 and JHAP2 did not allow secretion of the proteins immunoreactive with the anti-hApoE or anti-MPR antibodies in the culture medium. This suggests a possibility that the signal sequence of hApoE and the prepro-sequence of MPR were not effective for secretion of hApoE, although the prepro-sequence of MPR was shown to be useful for secretion of human growth hormone and human urokinase.5,6) However, we assume that the failure to detect any protein immunoreactive with the anti-hApoE antibody in either the medium or the intracellular fraction is explained in terms of the instability of hApoE due to its high sensitivity to proteases of the yeast cell. In fact, Sturley et al.22) reported that fairly efficient production of hApoE in S. cerevisiae cells was observed when a protease-deficient mutant was used as the host. In addition, the instability of the hApoE gene in S. cerevisiae harboring JHAP1 and JHAP2 may also be explained in terms of the properties of hApoE; hApoE seems to be difficult to secrete from yeast cells, since Sturley et al.22) observed that a host mutation (upc2) with the phenotype of enhanced uptake and intracellular esterification of exogeneous cholesterol is essential for secretion of hApoE. Even from the upc2

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Table: Secretion of MPR–hApoE by Yeast Strains Harboring Each Plasmid in Different Media

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Type</th>
<th>Fused a.a</th>
<th>Cultivation</th>
<th>ApoE Secreted (μg/ml)</th>
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<tr>
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<td>MPR</td>
<td>YPGal-2</td>
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<tr>
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</tr>
<tr>
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<td>I</td>
<td>MPR</td>
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<td>16.2</td>
</tr>
</tbody>
</table>

S. cerevisiae MC16 cells were transformed with the integration (I) or episomal (E) plasmids containing MPR (D38G)–hApoE gene and cultured in each YPGal medium. Each transformant was cultured for 12 h in YPGal-1, 72 h in YPGal-2, and 60 h in YPGal-3 medium after the medium shift. The amount of the hApoE part in the excreted MPR–hApoE was measured as described in Materials and Methods. The values are the means of those obtained by three independent experiments.
mutant, the secretion level was only 40 μg per liter. The relationship between the hApoE secretion and the upc2 mutation is still unclear.

On the other hand, S. cerevisiae harboring JHAP3 and JHAP5 efficiently secreted the MPR–hApoE fused protein. This implies that the pre-sequence of MPR serves as a signal peptide for the export of the fused protein into the endoplasmic reticulum and, after this event, the mature MPR part served as a protector or a carrier to prevent the proteolysis of the hApoE part in the secretory pathway of the fused protein into the medium. After the fused protein in the form of proMPR–hApoE had been excreted into the medium, the pro-sequence is cleaved autocatalytically and by a protease(s) of the host cell, as judged by comparison of the sizes of the proteins secreted from S. cerevisiae harboring JHAP3 and JHAP4.

The fused protein secreted into the medium was rapidly degraded in the late stage. Similar degradation was also observed when the fused protein between hApoE and the active site mutant of MPR was secreted from yeast transformants (data not shown). These results indicated that this degradation depended mainly on the proteolytic activity of the host strain, but not on that of MPR itself.

When the transformant were cultured in YPGal medium in which the concentration of nitrogen sources (yeast extract and Bacto-Peptone) were high, the degradation of the secreted fused proteins was apparently repressed. During cultivation of the transformants under these conditions, the pH of the medium did not decrease in comparison with that when cultured in the standard YPGal medium. In addition, the maintenance of the pH with a buffering agent at about 7 led to considerable repression of the degradation. These observations show that it is important to control the pH of the culture medium to repress the degradation of recombinant proteins secreted from S. cerevisiae.

We previously reported that the degradation of MPR–rApoE excreted from recombinant S. cerevisiae was repressed in a pep4-3 mutant strain deficient in the PEP4 gene encoding a vacuolar aspartic proteinase, proteinase A. It is believed that protease A is not excreted into the culture medium. Consistent with this, we could not detect any proteolytic activity in the culture broth S. cerevisiae when assayed with a skim milk-containing plate. Concerning the degradation of MPR–hApoE, we therefore speculate that a trace of protease A leaks into the medium, or alternatively some unknown extracellular protease activated by protease A is responsible for the degradation. In fact, protease A does activate a number ofzymogens. To find which protease degrades the fusion protein, it is necessary to investigate the effects of various proteinase inhibitors.

The secretion level of MPR–hApoE was improved when the fused gene was integrated in the chromosome. We showed that the low level of secretion of MPR–hApoE by S. cerevisiae containing the fused gene on the multicopy plasmid was caused by the instability of the plasmid. We believe that the integration of the expression units into the yeast genome is useful, especially when the expression units contain a gene whose product has a deleterious effect on the host cell, like the MPR–hApoE protein.

In this study, we showed that MPR–hApoE bound to heparin, one of the biological activities of hApoE. This means that the MPR–hApoE fused protein easily prepared from the cultivation medium may be useful for studying biological activities of hApoE, although the cleavage of the fused protein at the junction to obtain native hApoE has not yet been done, it may be done by introduction of an artificial process site, such as Ile-Glu-Gly-Arg, recognized and cleaved by the blood coagulation factor Xa, just before the hApoE sequence.

References