Rapid Paper
Thiamine Increases Expression of Yeast Gene
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We found that CPY production in *Saccharomyces cerevisiae* KSS8-2D/pCY303 was increased by the addition of thiamine into the medium, while the addition of thiamine had no effect on cell growth. It became clear that the positive effect of thiamine was due to transcriptional increase, because the levels of CPY mRNA were increased according to the amount of thiamine added. Furthermore, it was suggested that thiamine generally increases the expression of yeast genes, since the expression of the luciferase gene that was artificially constructed was also increased to some extent by thiamine in *S. cerevisiae*.

Key words: thiamine; carboxypeptidase Y; *Saccharomyces cerevisiae*; transcriptional activation

Carboxypeptidase Y (CPY; EC 3.4.16.1) is a yeast intracellular protease. In addition to its peptidase activity, CPY has the ability to catalyze aminolyis of C-terminal peptide esters, resulting in the amidation of the peptide. The enzyme has therefore attracted the attention of industrial chemists as a possible producer of biologically active proteins such as calcitonin.

The yeast *S. cerevisiae* has become important as a host for biologically active protein secretion. In a previous paper, we reported the transformation of *S. cerevisiae* strain KSS8-2D, a vacuolar protease missorting mutant, with pCY303 plasmid, which contains the structural gene encoding pre-proCPY fused to an inducible GAL10 promoter. The mutant strain KSS8-2D/pCY303 secreted CPY into the culture medium at concentrations as high as 50 mg per liter of culture medium. For the industrial production of CPY, however, it is necessary to improve the culture conditions, for example, by altering the composition of the medium components. Therefore, the effects of the addition of various vitamins on CPY production in completely synthetic medium were investigated. During these studies, thiamine was found to stimulate CPY production without affecting cell growth. The stimulatory effect of thiamine was found to occur at the transcriptional stage of CPY expression. Furthermore, it was found that thiamine generally increases the expression of yeast genes other than CPY gene.

Materials and Methods

**Strain and plasmids.** The *S. cerevisiae* strain KSS8-2D[1] is a missorting mutant derived from a cross between KSS1-3C and KSM-169. To examine the effect of thiamine on the expression of the luciferase gene under the control of the GAL10 promoter, we constructed a pGAL-LUC plasmid by replacing the CPY structure gene in the pCY303 plasmid with the luciferase structural gene from the pT3/T7-luciferase expression vector (Clontech Laboratories, Inc. U.S.A.). A DNA fragment (1.9 kbp) corresponding to the luciferase structural gene was excised from the pT3/T7-luc plasmid by *HindIII* digestion and ligated into the large HindIII fragment of pCY303, which lacks the region of the CPY structural gene. The resulting plasmid (pGAL-LUC) with a LEU2 selection marker was used to transform yeast strain KSS8-2D (MATa thr1, leu2, his3, ura3) or DBY746 (MATa his3, leu2, trp1, ura3) and LEU* transformants were selected. KSS8-2D/pGAL-LUC and DBY746/pGAL-LUC, the resulting transformants, were used for testing the effects of thiamine on the expression of the luciferase gene.

**Materials and culture conditions.** Sarcosyl and Antiform A were purchased from Sigma (U.S.A.). Guanidine thiocyanate was obtained from Fluka Chemie AG (Germany); nylon membranes, from Pall (U.S.A.); [γ-32P]ATP, from Amersham (England); CCl₃, from Nakalai Tesque Inc. (Japan); and salmon sperm DNA, from Boehringer Mannheim Co., Ltd. (Germany). Sul1, BgII were from Takara Shuzo (Japan). For the experiments in which CPY and luciferase were assayed, a completely synthetic (CS) medium containing a vitamin mixture (2-2000 μg/liter) with or without thiamine, trace elements (40–500 μg/liter), and salts (0.1–5 g/liter) was prepared in the composition described by Fred Sherman, and was further supplemented with 2% glucose, 2% galactose, 0.2 M potassium phosphate buffer (pH 7.6), 0.5% bovine serum albumin (as stabilizer), and an amino acid mixture (20–3750 μg/liter) without leucine. For the pre-cultures, SD medium containing 0.67% bacto yeast nitrogen base without amino acid (Difco, amount of thiamine–HCl: 400 μg/liter = 1.2 μM) was prepared and supplemented with 2% glucose, 2% galactose, 0.2 M potassium phosphate buffer (pH 7.6), 0.5% bovine serum albumin (as a stabilizer), and an amino acid mixture (20–3750 μg/liter) without leucine. All strains were initially grown in SD medium for 3 days at 26°C. The cultures were then diluted at a 1:99 (v/v) ratio with fresh SD medium in which the cells were then grown for another 3 days at 26°C. One hundred ml of the CS medium was inoculated in 1 ml of seed culture, and the cells were then cultivated for 7 days at 28°C on a rotary shaker (200 rpm). Secretion of CPY was monitored by the measurement of CPY activity by the enzyme assay referenced below. The expression of luciferase genes in the resting yeast cells was measured after the following procedures had been done. KSS8-2D/pGAL-LUC or DBY746/pGAL-LUC cells were grown in SC medium (50 ml) supplemented with thiamine and biotin for 3 days at 26°C. After the cells had been collected by centrifugation at 5K rpm for 5 min, they were washed twice with distilled water and then suspended in fresh SC medium (50 ml) with or without thiamine. The cells were then incubated at 26°C on a rotary shaker (200 rpm) for 4 days.

**Measurement of enzymatic assay.** CPY activity was measured photometrically by a kinetic method as previously described. Luciferase activity was measured spectrophotometrically by a luminometer detection method using a PicaGene kit (Toyo Ink Co., Ltd., Japan).

**Preparation of cell extract.** Intracellular enzymatic activities were measured in cell extracts that had been prepared as follows. Cells were washed with water and then with 100 mM Tris–HCl (pH 7.5). One gram of cells was resuspended in 4 ml of the Tris–HCl buffer and then disrupted for 3 min with glass beads in a Brown cell homogenizer (Kaiser). The suspension was centrifuged at 15K rpm for 10 min at 4°C, and the
Fig. 1. Courses of Cell Growth and CPY Secretion.
The strain KSS8-2D carrying pCY303 plasmid was grown in a CS medium with (○) or without thiamine (●). The amount of CPY was calculated according to the standard activity curve obtained for authentic CPY.

supernatant fraction was used for assays of the intracellular enzyme.

SDS-gel SDS/PAGE was done by the procedure of Laemmli. 61 Samples of CPY or protein standards were electrophoresed on 8% SDS/PAGE, and stained by silver staining or Coomassie brilliant blue.

Isolation of total RNA and Northern Hybridization of CPYmRNA. After the yeast cells had been cultured for various lengths, cells from a 100-ml culture were pelleted, washed at 4°C with 20 ml of demineralized water, immediately frozen with liquid nitrogen, and stored at −80°C. One g of the frozen cells was ground to a fine powder in a cold mortar with a cold pestle. The total RNA of the yeast cells was purified by the guanidine thiocyanate method of Chirgwin. 62 For Northern analysis the following oligonucleotides were labeled with [32P]ATP and used as probes. These oligonucleotides, [5′-TTGGTTGAAAGGGGGTCCAGGTTGTTCTF-3′] and [5′-TGTCTTTGTCTACCCAGATAGATGGGAA-3′] are part of the structural genes for CPY and actin (ACTI), respectively. CPY or ACTI transcripts were analyzed by autoradiography and a Bioimaging Analyzer (Fuji Film Co., Ltd.).

Results

Thiamine enhanced CPY production

S. cerevisiae strain KSS8-2D/pCY303 is a strain carrying a multicopy plasmid pCY303 carrying the CPY structural gene with the pre-pro CPY region under the control of the S. cerevisiae inducible GAL10 promoter. When KSS8-2D/pCY303 was cultivated in the CS medium with thiamine or without thiamine, no change was observed in the cell growth, as shown in Fig. 1. However, according to the measurement of CPY activity in the medium, the cells grown on the CS medium containing thiamine produced much more CPY than those grown on the medium without it (Fig. 1). Similar results were obtained by SDS–PAGE of CPY protein (data not shown). In addition, the increase of CPY production was observed to depend on the increase in thiamine concentration in the medium (data not shown).

CPYmRNA was stimulated by thiamine

To find whether the increase in CPY production through the addition of thiamine was caused by an increase in CPY transcriptional activity, we examined the effects of thiamine on the level of CPYmRNA. Strain KSS8-2D/pCY303 was used for testing the effect of thiamine on the transcriptional level of CPY. As shown in Fig. 2, the highest level of CPYmRNA was observed when KSS8-2D/pCY303 was cultivated in the CS medium with thiamine for around 24 h.

The level of CPYmRNA after incubation of approximately 24 h was about 6 times higher in the cells grown in the CS medium containing thiamine than in those grown in the medium without it, although the ACTI mRNA level was not affected by thiamine (Table I, Fig. 2). The amount of secreted CPY was proportional to the transcriptional activity (Fig. 2). This phenomenon was also observed with the endogenous CPY mRNA of S. cerevisiae strain KSS8-2D.
Table I. Northern Blot Analysis of CPY Transcriptional Level under the Control of Various Promoters in Various Strains

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<th>(Ratio)</th>
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<tr>
<td></td>
<td>CPY mRNA</td>
<td>ACT1 mRNA</td>
<td>CPY mRNA/ACT1 mRNA</td>
</tr>
<tr>
<td>KS58-2D (CPY promoter)</td>
<td>6.1</td>
<td>0.9</td>
<td>6.7</td>
</tr>
<tr>
<td>KS58-2D/pCY303 (GAL10 promoter)</td>
<td>402</td>
<td>1.1</td>
<td>365</td>
</tr>
<tr>
<td>DBY746 (CPY promoter)</td>
<td>4.3</td>
<td>1.0</td>
<td>4.3</td>
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</table>

KS58-2D, KS58-2D/pCY303, or DBY746 cells were grown for 24 h in a CS medium with 1.2 μg thiamine or without thiamine. The amount of CPY mRNA or ACT1 mRNA was measured using a Bioimaging Analyzer (Fuji Film Co., Ltd.). Each value is given as the relative value normalized to the amount of ACT1 mRNA in the strain KS58-2D grown in a medium without thiamine. The values in parentheses show the ratio of each amount of CPY mRNA/ACT1 mRNA in the cells grown in a medium with thiamine to that in the cells grown in a medium without thiamine in each strain, respectively.

Table II. The Effects of Thiamine on Expression of Luciferase or CPY Genes in Various Strains

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<tr>
<td>Luciferase activity (RLU)</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>KS58-2D/pGAL-LUC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBY746/pGAL-LUC</td>
<td>74</td>
<td>21</td>
</tr>
<tr>
<td>CPY activity (unit)</td>
<td>108</td>
<td>48</td>
</tr>
</tbody>
</table>

KS58-2D/pGAL-LUC, DBY746/pGAL-LUC, or DBY746 cells were grown for 3 days in a CS medium supplemented with 1.2 μg thiamine or without thiamine. After cell extracts were prepared as described in Materials and Methods, luciferase activity was measured. CPY activity was measured by using the culture medium.

or DBY746 (Table I).

Thiamine also increased the expression of genes other than CPY gene

To examine whether the increase of the transcription of the CPY gene by thiamine is specific to the expression of the CPY gene, we examined the effects of thiamine on the expression of the luciferase gene, which was artifically constructed under the control of the yeast GAL10 promoter. Strain KS58-2D/pGAL-LUC was used for testing the effects of thiamine on the expression level of the luciferase gene. Thiamine also increased the expression of the exogenous luciferase gene under the control of the yeast GAL10 promoter (Table II). Furthermore, thiamine also increased the production of human lysozyme (foreign protein) when the human lysozyme gene under the control of the GAL10 promoter was expressed in strain KS58-2D (data not shown). In addition, the level of invertase in the medium was also increased by thiamine, although this increasing effect of thiamine was observed only at the level giving the greatest CPY production (data not shown). These results suggest that the effect of thiamine may not be specific to CPY production. In addition, as shown in Fig. 3, not all intracellular and/or extracellular proteins were increased by thiamine at the level giving the greatest CPY production. In Fig. 3, the major protein band (arrow in Fig. 3), which was estimated to be the CPY band (M.W. 61,000) and confirmed by western blot analysis using rabbit a anti-CPY antibody in other experiments, in extracellular proteins seemed to be most strongly increased by thiamine. In order to determine whether thiamine enhances protein production in strains other than KS58-2D, we also examined the effect of thiamine on the expression of the exogenous luciferase gene in other yeast strains. Although strain KS58-2D proved to be particularly sensitive, thiamine also increased the expression of the luciferase gene in strain DBY746 (Table II), suggesting that the positive effect of thiamine is not restricted to strain KS58-2D. These results suggested that thiamine generally increases the expression of some yeast genes.

Discussion

During the course of our experiments to improve the
CPY production level, the addition of thiamine to the medium was found to increase the amounts of various proteins (CPY, luciferase, invertase, human lysozyme, etc.) produced by S. cerevisiae. This finding provides the first evidence that thiamine increases the expression level of various genes. As shown in Fig. 3, since not all intracellular and/or extracellular proteins were increased by thiamine, it seems that thiamine does not stimulate the mRNA of all yeast proteins. The expression of actin gene (or histones 2A and 2B genes in the preliminary experiment) was not affected by adding thiamine (Fig. 2). Thiamine may have no effect on the expression of genes encoding many cytoskeletal proteins. Interestingly, it is likely that extracellular proteins rather than intracellular proteins were greatly increased by thiamine (Fig. 3).

And in the extracellular proteins, CPY seems to be the most increased by the addition of thiamine. This finding may have something to do with the fact that the expression unit of CPY gene exists on the plasmid.

Recently, Pekovich et al.\textsuperscript{5)} reported that thiamine has a direct effect on the expression of the transketolase gene, which codes a key pentose phosphate shunt enzyme that is important in the production of reducing equivalents and pentose sugars. It was shown that thiamine pyrophosphate (TPP), a coenzyme form of thiamine, negatively regulates the transcription of thi cluster genes encoding an enzyme of the thiamine biosynthetic pathway.\textsuperscript{6,10)} A subject of great interest is what kind of promoter is regulated by thiamine, as this mode of action of thiamine remains unknown. Further molecular and biological analysis is necessary to discover the mechanism by which thiamine stimulates the transcription of some proteins in S. cerevisiae.

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\textbf{References}

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