Production of fungal fructosyl amino acid oxidase useful for diabetic diagnosis in the peroxisome of *Candida boidinii*

Yasuyoshi Sakai[a,*], Hiroyuki Yoshida[a], Hiroya Yurimoto[a], Nobuyuki Yoshida[a], Hiroshi Fukuya[a], Keiji Takabe[b], Nobuo Kato[a]

[a] *Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan*
[b] *Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan*

Received 25 June 1999; received in revised form 7 September 1999

Abstract A high-level production of fructosyl amino acid oxidase (FAOD), whose production was toxic in *Escherichia coli*, was investigated through attempts to utilize the peroxisome of *Candida boidinii* as the place for protein accumulation. The alcohol oxidase-depleted strain (strain *aod1*Δ) produced FAOD at a four to five times higher level than the wild type strain in terms of protein amount and enzyme activity, although the transcriptional level was similar. As a result of this study, we could improve FAOD productivity approximately 47-fold from the original transformant, and FAOD accumulated within membrane-bound peroxisomes up to 18% of the total soluble proteins.

Key words: Glycation; Diabetes; Diagnosis; Peroxisome; Methylotrophic yeast; Gene expression

1. Introduction

Many kinds of oxidases are used for quantitative and qualitative colorimetric assay coupled with a peroxidase reaction. We and others have reported that fructosyl amino acid oxidase (FAOD) from several fungal species can be used for enzymatic determination of the quantity of glycated proteins [1–4]. Non-enzymatic glycation of proteins has been implicated in the pathogenesis of diabetic complications. Since glycation of blood proteins is not affected by transient increases in blood glucose, the level of glycated proteins is a good index for monitoring patients with diabetes mellitus during therapy. Since the original fungi did not produce a sufficient quantity of FAOD and since the cultivation of fungi is costly, a high-level and economical means of producing FAODs needs to be established to supply the large amount of FAOD needed for clinical use. Although the active form of FAOD can be obtained from *Escherichia coli* [1], high-level expression was toxic to the host strain probably because of the fortuitous production of H_2O_2 or the retrieval of the coenzyme, flavin adenine dinucleotide (FAD), via covalent bonding to FAOD [2].

*Corresponding author. Fax: (81) (75) 753-6385.
E-mail: ysakai@kais.kyoto-u.ac.jp

Abbreviations: FAOD, fructosyl amino acid oxidase; FAD, flavin adenine dinucleotide; strain *aod1*Δ, the alcohol oxidase-depleted strain of the methylotrophic yeast *Candida boidinii*; ODC, orotidylate decarboxylase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

To avoid the problems encountered in the *E. coli* expression system, peroxisomes of the alcohol oxidase-depleted strain of the methylotrophic yeast *Candida boidinii* [5] (strain *aod1*Δ) were chosen as the place for enzyme production. The rationale and the merits for our strategy are as follows. (1) Since many kinds of oxidases are compartmentalized together with catalase in peroxisomes [6], the toxicity of heterologous oxidase was expected to be minimized when produced in a membrane-bound compartment. (2) Methanol-grown *C. boidinii* has huge peroxisomes which contain two major matrix proteins, alcohol oxidase and dihydroxyacetone synthase [7]. Heterologous oxidase can be transported by the peroxin molecules (machinery proteins involved in protein transport to peroxisomes) which originally transported alcohol oxidase [8], and can occupy the intraperoxisomal space in strain *aod1*Δ. Therefore, we could expect efficient peroxisomal transport and folding of the heterologous peroxisomal protein. (3) Many oxidases contain FAD as a coenzyme, and overproduction of oxidases could lead to a depletion of FAD. Alcohol oxidase, which is a FAD octameric enzyme [9], makes up 10–20% of the total soluble protein in methylotrophic yeast under batch-culture conditions [10]. Therefore, in strain *aod1*Δ, we could expect that FAD, which was originally used in the production of alcohol oxidase, would be available in sufficient amounts for production of the putative protein. This study was conducted to seek an economical means of producing *Penicillium janthinellum* FAOD [2] with the aim of establishing a general expression method for toxic proteins or oxidases in *C. boidinii* strain *aod1*Δ.

2. Materials and methods

2.1. Strains and media

C. boidinii TK62 (*ura3*) [11] was the original host for transformation. Strain *aod1*Δ was derived by replacing the 1579-bp *Sty*I fragment within the *AOD1*-coding region of *C. boidinii* TK62 with the sequence which encodes *C. boidinii URA3* from pSPR [5]. The *ura3* marker was then regenerated as previously described [12]. The synthetic basal medium (BM) used to grow *C. boidinii* was prepared as previously described [13]. The concentrations of the carbon and nitrogen sources were 1.5% (v/v) methanol, 3.0% (v/v) glycerol, 0.5% (w/v) NH_4Cl, and 0.5% (w/v) methylamine. The cells were aerobically grown at 28°C in a 500-ml or 2-l Sakaguchi flask (Iwaki Glass Co. Ltd., Funahashi, Japan).

2.2. DNA synthesis

The sequence of the synthesized FAOD gene was submitted to GenBank and was assigned accession number AF181866. The synthetic gene was assembled from three separate fragments of the FAOD gene, that is, the 495-bp *Not*I–*Pst*I fragment, the 415-bp *Pst*I–*Hin*dIII fragment, and the 415-bp *Hin*dIII–*Not*I fragment. Each fragment was generated by annealing 9 oligos followed by the

PII: S0014-5793(99)01245-4

conventional PCR reaction using VENT DNA polymerase (New England Biolabs, Beverly, MA, USA).

2.3. Vectors and molecular methods

pNoteI was used as the expression vector [14]. Linearized DNA was transformed to *C. boidinii* strain TK62 and to strain *aod1*Δ. The cells were selected by Ura-plus phenotype. The expected chromosomal integration and the copy number of the integrated plasmid were confirmed by Southern analysis and orotidylate decarboxylase (ODC) activity as previously described [11]. The nucleotide sequence of the synthesized FAOD gene was confirmed using the Shimadzu DSQ-1000L DNA sequencer (Shimadzu, Kyoto, Japan). RNA preparation and Northern analyses were performed as previously described [15].

2.4. Enzyme assay

Cell-free extracts were prepared by disrupting the cells by sonication as described below. FAOD activity in the cell-free extract was determined as previously described [2] using fructosyl valine as the substrate. Quantitation of protein was performed using the method of Bradford [16]. ODC activity was determined by the method of Yoshimoto et al. [17]. Acid phosphatase activity was determined by the method of Toh-e et al. [18], and is expressed as units per unit of optical density at 660 nm.

Western analysis was performed as previously described [19], using an Amersham ECL Detection Kit (Arlington Heights, IL, USA) with rabbit polyclonal antibody raised against purified FAOD [20].

2.5. Purification of FAOD from C. boidinii strain NPESΔa5

Strain NEPSΔa5 constructed in this study was grown in 500 ml of BM containing 1.5% (v/v) methanol, 3.0% (v/v) glycerol, and 0.5% (w/v) methylamine. The cultures were incubated at 28°C with reciprocal shaking for 72 h. Enzyme purification was carried out at 4°C in 50 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM DTT (KPD buffer). Cells were first harvested by centrifugation at $8000\times g$ for 20 min, and washed once with the KPD buffer. The cells in KPD buffer were placed in the Kubota Insonator Model 200M (Kubota Medical Appliance Supply Co., Tokyo, Japan) at 180 W for 60 min. The homogenate was centrifuged at $20\,000\times g$ for 20 min, and the supernatant was used as the cell-free extract (127 mg of total protein/3110 U). Ammonium sulfate was added to the supernatant to 30% saturation, and the precipitates were removed by centrifugation at $20\,000\times g$ for 20 min. The remaining supernatant was applied to a butyl-Toyopearl column (2.2∅×20 cm; Tosoh Co., Tokyo, Japan) equilibrated with KPD buffer containing 30% saturation of ammonium sulfate. The absorbed protein was eluted under a linear gradient of ammonium sulfate (30–0% saturation). The active fractions were collected, and precipitated by adding ammonium sulfate to 80% saturation. The precipitates were dissolved and dialyzed against KPD buffer. They were then loaded on a Sepharose Q column (1.8∅×10 cm; Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with KPD buffer. Enzyme activity was found in the washing fraction. The purified protein (12 mg/2290 U) gave a specific activity of 191 U/mg protein. The purification yield was 75%.

2.6. Cytological technique

Subcellular fractionation experiments using *C. boidinii* were performed as previously described [7]. Immunocytochemistry was performed with polyclonal anti-FAOD antibody as previously described [7,20].

3. Results

3.1. Expression of synthetic FAOD gene optimized for C. boidinii codon usage

While the original FAOD cDNA of *P. janthinellum* contains a G or C nucleotide at the third position of most codons [2], highly expressed genes in *C. boidinii* have an A or T at the third position of most codons [21,22]. The synthetic FAOD gene was designed based on the deduced amino acid sequence of FAOD and the preferred codon usage in *C. boidinii*. The original FAOD cDNA and the synthetic FAOD DNA were each ligated to pNoteI [14] under the *AOD1* promoter, yielding pNEP and pNEPS, respectively. After transformation of strain *C. boidinii* TK62 (*ura3*) [11], one-copy transformants of pNEP integrated at the *URA3* locus and one-copy transformants of pNEPS integrated at the *URA3* locus were selected. The level of FAOD expression of the transformants was compared. The copy number of each transformant was estimated from both Southern analysis (data not shown) and the level of ODC activity, which is encoded by the *URA3* gene (Table 1) [11,23]. The pNEPS transformant (strain NEPS1) expressed FAOD at a level approximately 2.3-fold higher than the pNEP transformant (strain NEP1), which reflects a difference in the translation efficiency of the codons. Further studies were performed with pNEPS transformant strains.

3.2. C. boidinii aod1Δ as the expression host

The level of FAOD expressed by the one-copy pNEPS1 transformants of the wild type *C. boidinii* strain (strain NEPS1) and that expressed by one-copy pNEPS1 transformants of *C. boidinii* strain *aod1*Δ (strain NEPSΔa1), were compared. Since strain NEPSΔa1 could not grow on methanol as the sole carbon source, glycerol was added to the media of both transformant strains (methanol+glycerol/NH_4Cl).

Table 1
FAOD production in various transformants of *C. boidinii*[a]

DNA/Strain	ODC activity (fold) (U/mg)	Copy number	FAOD activity (fold) (U/mg)
Control strains			
C. boidinii wild type	0.0258 (1)		n.d.[b]
*C. boidinii aod1*Δ	0.0267 (1)		n.d.
C. boidinii ura3	n.d.		n.d.
*C. boidinii ura3 aod1*Δ	n.d.		n.d.
FAOD cDNA (pNEP)/*C. boidinii ura3*			
Strain NEP1	0.0262 (1.02)	1	0.520 (1.00)
Synthetic FAOD (pNEPS)/*C. boidinii ura3*			
Strain NEPS1	0.0233 (1.03)	1	1.20 (2.30)
Strain NEPS2	0.0553 (2.43)	2	2.75 (5.29)
Strain NEPS3	0.0781 (3.44)	3	4.33 (8.32)
Synthetic FAOD (pNEPS)/*C. boidinii ura3 aod1*Δ			
Strain NEPSΔa1	0.0261 (0.98)	1	4.95 (9.52)
Strain NEPSΔa2	0.0553 (2.07)	2	11.7 (22.5)
Strain NEPSΔa5	0.133 (4.96)	5	24.5 (47.1)

[a]Each transformant was grown on methanol/methylamine medium to the stationary phase (72 h). The enzyme activity was then determined. The copy number of the integrated plasmids was also estimated from Southern analysis.
[b]n.d., not detected.

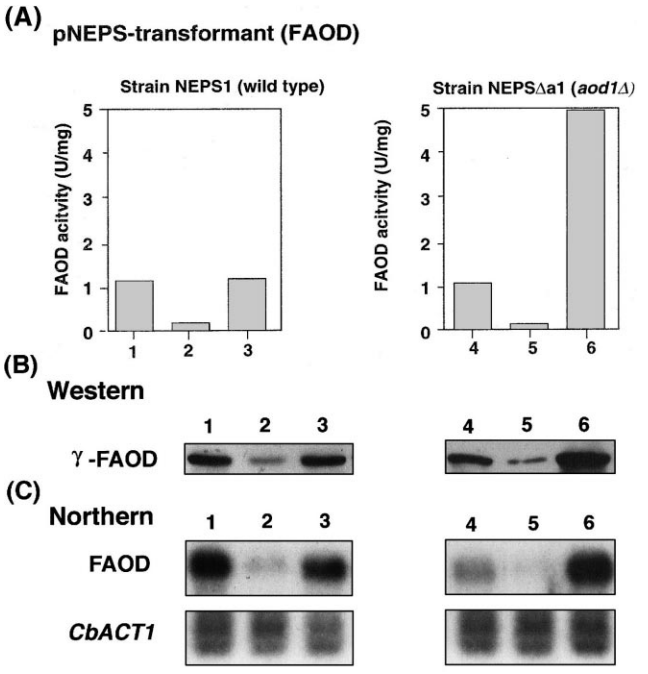

Fig. 1. Expression analysis of the synthesized FAOD gene under the *AOD1* promoter in one-copy integrants on various media. A–C: Lanes 1–3, strain NEPS1 (wild type), and lanes 4–6, strain NEPSΔa1 (*aod1*Δ). Cells were harvested at the early logarithmic phase (36 h). A: FAOD activity. B: Western analysis using anti-FAOD antibody. About 15 μg of protein was loaded on each lane. C: Northern analysis using the ^{32}P-labeled, 1.3-kb synthesized FAOD fragment (upper), or ^{32}P-labeled, 0.9-kb *C. boidinii* actin fragment [7] (lower). About 3 μg of total RNA was loaded on each lane. Each lane number indicates the used carbon and nitrogen sources in the medium: lanes 1 and 4, methanol plus glycerol/NH_4Cl; lanes 2 and 5, glycerol/NH_4Cl; lanes 3 and 6, methanol plus glycerol/methylamine.

Although we expected the enzyme activity in strain NEPSΔa1 to be higher than that in strain NEPS1, the level of FAOD activity and the quantity of FAOD protein detected on immunoblot analysis were the same in both strains (Fig. 1A, lanes 1 and 4 (FAOD activity); Fig. 1B, lanes 1 and 4 (immunoblot)). Unexpectedly, Northern analysis of these transformant strains revealed a smaller amount of FAOD mRNA in strain NEPSΔa1 than in strain NEPS1 (Fig. 1C, lanes 1 and 4), suggesting imperfect activation of the *AOD1* promoter in strain NEPSΔa1. Reduced induction of the *AOD1* promoter in strain *aod1*Δ was also confirmed by using another reporter gene, the *S. cerevisiae PHO5*, which encodes acid phosphatase and which is often used to study transcriptional regulation in *S. cerevisiae* [24]. *AOD1*-promoter-driven induction in *C. boidinii* strain *aod1*Δ is approximately one-third to one-fourth of that in the wild type strain.

From these results, we speculated that the *AOD1* promoter is induced not only by methanol but also by formaldehyde, since methanol is not oxidized to formaldehyde in strain *aod1*Δ. However, addition of formaldehyde is not practical for industrial-scale production due to its extreme toxicity and the technical difficulties in the addition itself. To overcome this problem, we changed the nitrogen source from NH_4Cl to methylamine. In *C. boidinii*, methylamine is metabolized to formaldehyde and then oxidized to formate [22]. Therefore, formaldehyde can be supplied continuously in vivo. The level of FAOD expression was 4.1-fold higher and

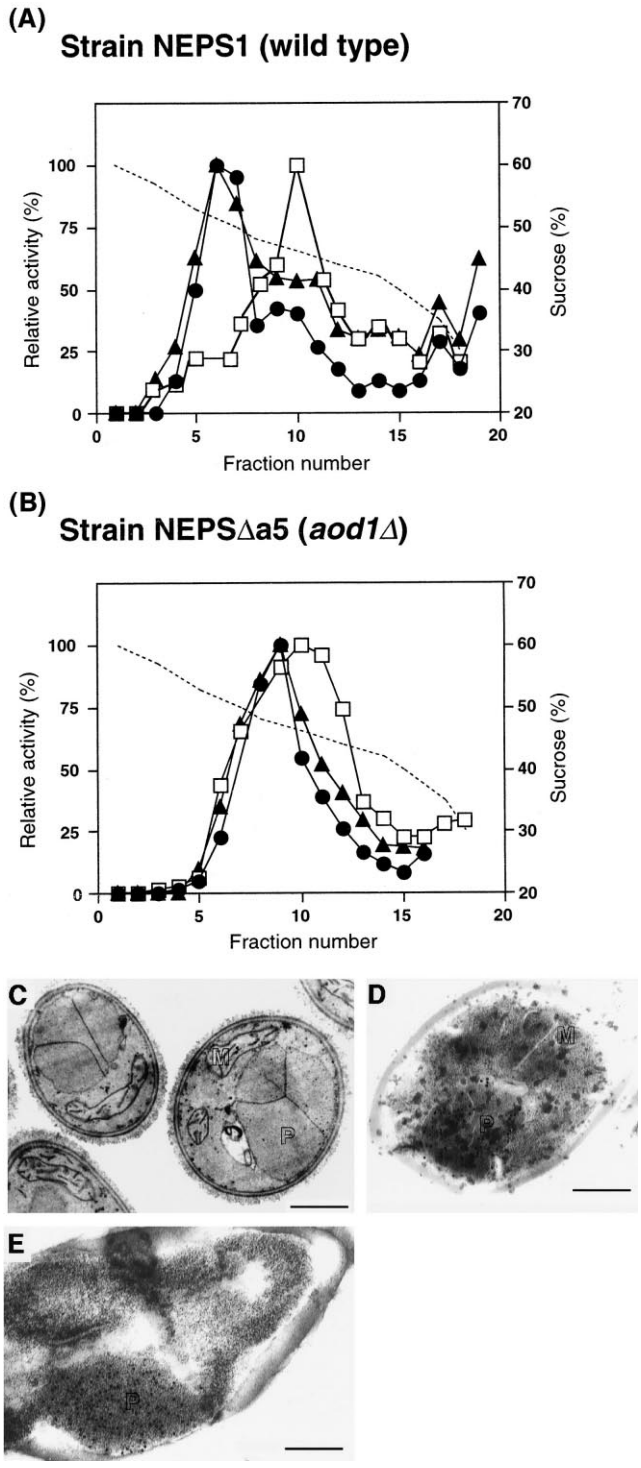

Fig. 2. Localization of the produced FAOD. Results of the subcellular fractionation experiments on (A) strain NEPS1 and (B) strain NEPSΔa5. Catalase and cytochrome *c* oxidase are the peroxisomal and mitochondrial marker enzymes, respectively. ●, FAOD; ▲, catalase; □, cytochrome *c* oxidase. Dotted line, sucrose concentration. C: $KMnO_4$-fixed cells. D,E: Immuno-electron microscopy using anti-FAOD antibody. Methanol-induced cells of (C) the wild type strain, (D) strain NEPS1, and (E) strain NEPSΔa5. Size bars, 1 μm.

acid phosphatase activity was 4.3-fold higher in the *aod1*Δ strains grown in media which contained methylamine as the nitrogen source instead of NH_4Cl (Fig. 1A, lanes 4 and 6); the

mRNA level and protein level of FAOD was also comparably higher in the *aod1*Δ strains grown in media which contained methylamine (Figs. 1B and 2C, lanes 4 and 6). In contrast, the level of expression of both FAOD and *PHO5* did not change in the *C. boidinii* wild type strain upon changing the nitrogen source from NH_4Cl to methylamine (Fig. 1A–C, lanes 1 and 3). Therefore, FAOD productivity in the *aod1*Δ strain was 4.1–4.9-fold higher than that in the wild type strain under optimized cultivation conditions (Fig. 1A and Table 1).

3.3. Production of FAOD in multi-copy transformants

Multicopy integrants of pNEPS1 were derived from the wild type and from the *aod1*Δ strain. The level of FAOD expressed by these transformants on a glycerol+methanol/methylamine medium was compared (Table 1). The level of FAOD expression was nearly proportional to the copy number of pNEPS1 in both the wild type strain and the *aod1*Δ strain. Transformants with higher copy numbers had larger quantities of FAOD mRNA (data not shown). Therefore, it is suggested that the increase in FAOD expression is due to an increase in the quantity of the mRNA which encodes FAOD. Strain NEPSΔa5, the five-copy integrant of pNEPS in strain *aod1*Δ, was selected as the best producer, which produced FAOD at a level as high as 18% of the total soluble protein. Western analysis did not show any protein aggregates due to improper folding of FAOD (data not shown). As described in Section 2, FAOD was purified from a 500-ml culture through two-step column chromatography (butyl-Toyopearl and Sepharose Q), yielding 75% recovery.

3.4. Characterization of purified FAOD

FAOD expressed by *C. boidinii* strain NEPSΔa5 and FAOD expressed by the original host, *P. janthinellum*, were each purified and subjected to the following tests: (1) the kinetics of the enzyme and reactivity in assay reaction for glycated proteins; (2) stability against temperature and pH; (3) subunit structure; (4) N-terminal amino acid sequence of the purified enzyme, and (5) inhibitor analysis. (6) On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, FAOD protein purified from *C. boidinii* strain NEPSΔa5 co-migrated with FAD fluorescence, which indicates that FAD produced in *C. boidinii* is covalently bound to the enzyme protein (data not shown). FAOD purified from *C. boidinii* strain NEPSΔa5 and FAOD purified from the original host gave the same results on all of these tests.

3.5. Peroxisomal localization of produced FAOD

Although the NEPSΔa5 strain produced a large amount of FAOD, we anticipated that all of the produced FAOD would not be transported into the peroxisomes due to overproduction. To study this issue, we analyzed localization of FAOD in *C. boidinii* strain NEPS1 and strain NEPSΔa5 through subcellular fractionation experiments and immuno-electron microscopy. After osmotic lysis of the FAOD-producing strain [7] and removal of cell debris and nuclei, the sample was centrifuged at $20\,000 \times g$ for 20 min as described previously [7]. The organellar pellet fraction, which contained mainly peroxisomes and mitochondria, was fractionated by sucrose-gradient ultracentrifugation. Since yeast peroxisomes are fragile, only a portion of catalase and FAOD corresponding to approximately 65–75% of catalase and FAOD activity was recovered in the pellet fraction of each strain [7]. Fractionation experiments on each strain indicated that the produced FAOD co-migrated with the catalase fraction but showed a peak distinguishable from that of the mitochondrial marker enzyme, cytochrome *c* oxidase (Fig. 2A,B). The peroxisomal peak fraction was found in lighter fractions of strain NEPSΔa5 than strain NEPS1. This may be due to depletion of the peroxisomal core enzyme, alcohol oxidase.

Next, immuno-electron microscopy was performed on the cells of strain NEPSΔa5 and strain NEPS1. As shown in Fig. 2D,E, immuno-gold labeling was limited to a specific area within the cells. The labeled areas were considered to be peroxisomes due to their similarity to the morphology of peroxisomes in methanol-induced cells of the wild type strain (Fig. 2C). Although the peroxisomes of strain *aod1*Δ were smaller than those of the wild type (data not shown), the peroxisomes of pNEPS1 transformants of strain *aod1*Δ were similar in size to those of the wild type strain (Fig. 2D,E). From these results, we concluded that FAOD produced in *C. boidinii* is transported efficiently to the peroxisomes.

4. Discussion

Many successful examples of heterologous protein production in methylotrophic yeast have been reported [14,25–27]. Other investigators have demonstrated intraperoxisomal production of peroxisomal and non-peroxisomal model proteins in yeast cells to avoid toxicity or to stabilize the produced proteins [28–30]. However, some problems that have been encountered include: (1) the expressed protein was not fully targeted to peroxisomes, (2) the protein was not properly folded, and (3) the quantity of expressed protein was low [28,30]. Using *C. boidinii* strain *aod1*Δ, which contains no alcohol oxidase, we have overcome these problems and have shown the first example of high-level intraperoxisomal production of a commercially important protein. The advantages of using *C. boidinii* are: (1) an efficient gene expression system was established, coupled with robust peroxisome proliferation. Indeed, intraperoxisomal production caused the increase in FAOD productivity. When three amino acids at the carboxy-terminus representing peroxisome targeting signal type 1 of FAOD were deleted, the FAOD productivity was decreased to one fourth (data not shown); (2) alcohol oxidase is encoded by a single gene in *C. boidinii* [5]; (3) glycerol does not repress methanol induction of the *AOD1* promoter and supported the growth of strain *aod1*Δ; (4) the peroxins and FAD originally used for alcohol oxidase would be available for transport and activation of heterologous oxidase to the peroxisomes.

The *aod1*Δ strain could not grow on methanol alone as the single carbon source. Sufficient induction of the *AOD1* promoter in strain *aod1*Δ in the original glycerol plus methanol/NH_4Cl medium was not achieved, probably because methanol was not metabolized further in the *aod1*Δ strain. We used methylamine as the nitrogen source for growth of strain *aod1*Δ, which provides formaldehyde in vivo. This procedure enabled us to activate the *AOD1* promoter to the level of the wild type strain. Although the quantity of mRNA transcripts of FAOD in the *aod1*Δ strain was nearly the same as that in the wild type strain, the level of FAOD expression in the *aod1*Δ strain was 4–5-fold higher than that in the wild type strain. This difference can be ascribed to better efficiency in

post-translational events, e.g. transport of peroxisomes and/or FAD supply.

Another outcome of this study besides the establishment of a new expression system was the achievement of an economical means of producing FAOD. Expression of the active form of FAOD in *C. boidinii* reached as high as 18% of the total soluble protein, which exceeds the expression level in *E. coli* (maximum 0.2%) [2]. Since the purification yield was also high (75%), a 3-day 500-ml culture enabled us to produce and purify 2290 U of FAOD, which was sufficient for assaying approximately 14 000 patient samples for determination of the content of glycated proteins (1 ml of assay volume).

Although many kinds of oxidases and 'assay kits' are currently commercially available, the cost of production has often been the economical limiting factor to large-scale industrial production, as is the case for FAOD. The production system and strategy taken here will not be limited to the production of FAOD from *P. janthinellum*. The same system was also efficient for the production of FAOD from *A. terreus* (data not shown) [2]. Our next interest is to produce non-peroxisomal oxidases in the peroxisomes of *C. boidinii*.

Acknowledgements: We thank Prof. Y. Tani, at the Nara Advanced Institute of Science and Technology for his valuable suggestions, and Prof. S. Harashima, at Osaka University for giving us the *S. cerevisiae PHO5* gene. This study was partly supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan, to Y.S.

References

[1] Yoshida, N., Sakai, Y., Serata, M., Tani, Y. and Kato, N. (1995) Appl. Environ. Microbiol. 61, 4487–4489.
[2] Yoshida, N., Sakai, Y., Isogai, A., Fukuya, H., Yagi, M., Tani, Y. and Kato, N. (1996) Eur. J. Biochem. 242, 499–505.
[3] Takahashi, M., Pischetsrieder, M. and Monnier, V.M. (1997) J. Biol. Chem. 272, 3437–3443.
[4] Takahashi, M., Pischetsrieder, M. and Monnier, V.M. (1997) J. Biol. Chem. 272, 12505–12507.
[5] Nakagawa, T., Mukaiyama, H., Yurimoto, H., Sakai, Y. and Kato, N. (1999) Yeast (in press).
[6] Van den Bosch, H., Schutgens, R.B.H., Wanders, R.J.A. and Tager, J.M. (1992) Annu. Rev. Biochem. 61, 157–197.
[7] Sakai, Y., Saiganji, A., Yurimoto, H., Takabe, K., Saiki, H. and Kato, N. (1996) J. Cell Biol. 134, 37–51.
[8] Subramani, S. (1998) Physiol. Rev. 78, 1–18.
[9] Kato, N., Omori, Y., Tani, Y. and Ogata, K. (1976) Eur. J. Biochem. 64, 341–350.
[10] Tani, Y., Sakai, Y. and Yamada, H. (1985) Agric. Biol. Chem. 49, 2699–2706.
[11] Sakai, Y., Kazarimoto, T. and Tani, Y. (1991) J. Bacteriol. 173, 7458–7463.
[12] Sakai, Y. and Tani, Y. (1992) J. Bacteriol. 174, 5988–5993.
[13] Sakai, Y., Rogi, T., Takeuchi, R., Kato, N. and Tani, Y. (1995) Appl. Microbiol. Biotechnol. 42, 860–864.
[14] Sakai, Y., Akiyama, M., Kondoh, H., Shibano, Y. and Kato, N. (1996) Biochim. Biophys. Acta 1308, 81–87.
[15] Sakai, Y., Yurimoto, H., Matsuo, H. and Kato, N. (1998) Yeast 14, 1175–1187.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Yoshimoto, A., Umezu, K., Kobayashi, K. and Tomita, K. (1978) Methods Enzymol. 51, 74–79.
[18] Toh-e, A., Ueda, Y., Kakimoto, S. and Oshima, Y. (1973) J. Bacteriol. 113, 460–479.
[19] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA. 77, 5201–5205.
[20] Sakai, Y., Yoshida, H., Yurimoto, H., Takabe, K. and Kato, N. (1999) J. Biosci. Bioeng. 87, 108–111.
[21] Sakai, Y. and Tani, Y. (1992) Gene 114, 67–73.
[22] Sakai, Y., Murdanoto, A.P., Konishi, T., Iwamatsu, A. and Kato, N. (1997) J. Bacteriol. 179, 4480–4485.
[23] Sakai, Y., Goh, T.K. and Tani, Y. (1993) J. Bacteriol. 175, 3556–3562.
[24] Ohshima, Y., Ogawa, N. and Harashima, S. (1996) Gene 179, 171–177.
[25] Cregg, J.M. (1993) BioTechnology 11, 905–910.
[26] Sakai, Y., Rogi, T., Yonehara, T., Kato, N. and Tani, Y. (1994) BioTechnology 12, 291–293.
[27] Sakai, Y., Tani, Y. and Kato, N. (1999) J. Mol. Catal. B Enzym. 6, 161–173.
[28] Nicaud, J.M., Raynal, A., Beyou, A., Merkamm, M., Ito, H. and Labat, N. (1994) Curr. Genet. 26, 390–397.
[29] Godecke, A., Veenhuis, M., Roggenkamp, R., Janowicz, Z.A. and Hollenberg, C.P. (1989) Curr. Genet. 16, 13–20.
[30] Faber, K.N., Westra, S., Waterham, H.R., Keizer-Gunnink, I., Harder, W., Ab, G. and Veenhuis, M. (1996) Appl. Microbiol. Biotechnol. 45, 72–79.