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Heterologous expression and secretion of sweet potato peroxidase isoenzyme A1 in recombinant *Saccharomyces cerevisiae*

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Abstract

A vector system has been developed to express isoenzyme A1 of sweet potato peroxidase (POD) and was introduced into *Saccharomyces cerevisiae*. The system contains the signal sequence of *Aspergillus oryzae* α-amylase to facilitate the extracellular secretion of peroxidase under the control of constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. In a batch culture using YNBDCA medium (yeast nitrogen base without amino acids 6.7 g l^{-1}, Casamino acids 5 g l^{-1} and glucose 20 g l^{-1}), the recombinant strain expressed the *swpa1* gene giving a secretion yield of POD activity of ca. 90% of total expressed peroxidase. Supplementation with PMSF (0.05 mM) and Casamino acids (5 g/50 ml) increased extracellular POD activity to nearly 10 kU ml^{-1}, equivalent to 1.5 kU g^{-1} cell dry wt. This is 9 fold higher than that obtained in medium without PMSF. From SDS-PAGE and native-PAGE analyses POD has an M_r of 53 kDa.

Introduction

Peroxidases (POD, H_2O_2-oxidoreductase, EC 1.11.1.7) catalyse the oxidation of a number of organic molecules with H_2O_2 and are ubiquitously in cells (Van de Velde *et al.* 2001, Welinder 1985). The structural and functional relationships and the catalytic mechanisms of peroxidases have been established in many plants, e.g. horseradish (*Armoracia rusticana*) and in white-rot fungi, e.g. *Phanerochaete chrysosporium*. One method to elucidate above relationships and mechanisms of peroxidases is the extracellular expression of target proteins in recombinant host cells. Furthermore, advances in recombination technology provide a powerful means for clarifying the distinctive properties of peroxidases. Heterologous expression of some peroxidase isoenzymes have been reported in various microorganisms: *Escherichia coli* (Smith *et al.* 1990), *Saccharomyces cerevisiae* (Morawski *et al.* 2000) and fungi (Aifa *et al.* 1999). However, recombinant protein production is often made difficult by the misfolding and aggregation of foreign proteins.

Recently, cDNA (*swpa1*) encoding an isoenzyme of sweet potato peroxidases has been isolated and characterized from a sweet potato cell-line (Huh *et al.* 1997). Sweet potato peroxidases are a family of glycoproteins with molecular sizes of about 40 kDa, and they contain many isoenzymes (Kwak *et al.* 1995). The sweet potato peroxidase isoenzymes have not

been reported as yet to be expressed extracellularly in either prokaryotic or eukaryotic microorganisms. Therefore, in this study, the *swpa1* gene was investigated in a yeast system for secretory expression and its potential productivity.

Saccharomyces cerevisiae was used as a host for recombinant eukaryotic proteins because it is suitable for heterologous expression due to its kinetic stability by glycosylation, high transformation efficiency and high growth rate (Romanos *et al.* 1992). To produce genetically engineered heterologous proteins, a functional promoter, signal sequence and a terminator must be used. In addition, the secretion of the target protein into culture media is desirable because this usually simplifies the seperation and purification of proteins (Romanos *et al.* 1992). Several attempts have been made to improve the secretory expression of heterologous products using the α-amylase signal sequences of *Aspergillus oryzae* (el-Enshasy *et al.* 1999, Hellmuth *et al.* 1995).

In this study, we report the heterologous expression and extracellular production of a sweet potato peroxidase isoenzyme, A1, in *S. cerevisiae*. The vector system was constructed to contain the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and the galactose-1-phosphate uridyltransferase (*GAL7*) terminator, to allow overexpression with efficient regulation. The signal sequence of *A. oryzae* α-amylase was also included to promote the efficient secretion of the active target protein into the culture medium. In addition, the physiological and metabolic parameters of cell growth and the overexpression of the target gene in the recombinant host (Dosoretz *et al.* 1990, Nomura *et al.* 1995, Teichert *et al.* 1989, Vasavada 1995) were correlated with proteolysis and nutritional control in the context of the overproduction of the recombinant protein.

Materials and methods

Strains and plasmids

Escherichia coli XL1-Blue (Stratagene, USA) was used to construct an expression and secretion system pGPD-POD1. *Saccharomyces cerevisiae* 2805 (*Mat α pep4::HIS3 prb1 can1 his3 ura3-52*) (Sohn *et al.* 1995) was used as a host strain for the expression of *swpa1*. A vector pYEGα-HIR525 (a generous gift from Dr E.S. Choi (Korea Research Institute of Bioscience and Biotechnology, Korea)), which contains an *URA3* gene, a yeast 2 μm replication origin, an ampicillin resistance gene, a *GAL10* promoter and a *GAL7* terminator, was used as a template plasmid for genetic manipulation. pYGAP-CT1 was used to introduce a GPD promoter and was kindly provided by Dr S.W. Nam (Dong-eui University, Korea) (Chung *et al.* 1997). The cDNA of *swpa1* from sweet potato cell lines (Huh *et al.* 1997) was used as the source of the peroxidase gene for heterologous expression in *S. cerevisiae*.

Media and culture conditions

E. coli XL1-Blue cells harboring plasmids were grown in Luria–Bertani (LB) medium containing ampicillin (100 μg ml^{-1}) at 37 °C. The *S. cerevisiae* cells were grown in YPD medium (peptone 20 g l^{-1}, yeast extract 10 g l^{-1} and glucose 20 g l^{-1}). YNBDCA medium (yeast nitrogen base without amino acids 6.7 g l^{-1}, Casamino acids 5 g l^{-1} and glucose 20 g l^{-1}) was used to select and maintain the yeast transformants. For bench scale culture, we used a 5 l fermenter with a 6-blade flat impeller. Cells were cultured in 2 l medium with the aeration rate of 1 vvm at 30 °C and at 300 rpm.

Genetic manipulations

General DNA manipulation was performed using typical recombinant procedures (Sambrook *et al.* 1989) and with restriction enzymes (Boehringer Mannheim). DNA fragments were analyzed by electrophoresis on a 0.8% (w/v) agarose gel. Plasmids and DNA fragments were purified using a QIAGEN plasmid kit and a QIAEX II gel extraction kit (QIAGEN), respectively. To introduce the signal sequence of *A. oryzae* α-amylase, a pair of phosphorylated complementary oligonucleotides, namely, 5′-ATTCCCATGGTCGCGTGGTGGTCTCTATTTCTG TACGGCCTTCAGGTCGCGGCACCTGCTTTGGC TCCCGGGG-3′ and 5′-TCGACCCCGGGAGCCAA AGCAGGTGCCGCGACCTGAAGGCCGTACAGA AATAGAGACCACCACGCGACCATGGG-3′, were synthesized and supplied by Takara Shuzo Co., Ltd. (Kyoto, Japan). The oligonucleotides were annealed and cloned between the *Eco*R1 and *Sal*1 site of the shuttle vector, derived from pYEG-αHIR525 (Sohn *et al.* 1995). Both ends of the annealed double stranded oligonucleotide were designed to generate the *Eco*R1 site at 5′ and the *Sal*1 site at 3′. The cDNA of *swpa1* was amplified by polymerase chain reaction (PCR) to modify so that the flanking sequence of the cDNA of

swpa1 may contain *Sal*1 sites at both ends. The PCR with Taq DNA polymerase (Boehringer Mannheim) was performed on a PCR system 9700 (PE Applied Biosystems, USA) by using two primers, primer I (5′-TACGTCGACGATGAAGCGTGTGTTTTCTCGGC CGTT-3′) and primer II (5′-AAAGTCGACATTTTAG AGTGATAGATCGAACTAGT-3′). The PCR conditions were: 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C after denaturing for 5 min at 95 °C. The transformations of *E. coli* and *S. cerevisiae* were performed using a Gene Pulser apparatus (BioRad, USA) with 0.1 cm electrode-gap cuvettes and a modification of the electroporation conditions described. The conditions were as follows: *E. coli* (field strength 2.5 kV cm^{-1}, capacitance 96 μF and a parallel resistance of 200 Ω, with a time constant of 3.8–4 s) (Dower *et al.* 1988), *S. cerevisiae* (field strength 1.5 kV cm^{-1}, capacitance 25 μF and parallel resistance 200 Ω, with a time constant of 5 ms) (Delorme 1989).

PAGE

To determine the molecular size of the expressed POD protein, the culture supernatant with maximum POD activity was partially purified by ultrafiltration (M_r cut-off, 10 kDa, Amicon, USA), ammonium sulfate (70%, w/v) precipitation and an anion exchange chromatography using DEAE-cellulose (Sigma, USA). The column was equilibrated with 10 mM potassium phosphate buffer (pH 6) and POD enzyme was eluted by linear-gradient chromatography with NaCl (0 to 0.5 M; flow rate, 1 ml min^{-1}; column size, 18 × 160 mM). Electrophoreses of SDS-PAGE and native-PAGE were performed using the one-dimensional method with 12% (w/v) polyacrylamide gel. The SDS-gel was then stained with Coomassie blue and the native-gel was detected as reddish-brown band with solutions of pyrogallol and H_2O_2.

Analytical methods

The biomass concentration was determined from the cell dry wt, dried at 100 °C for 10 h to constant wt. Unless otherwise stated, other analyses were performed on the cell-free culture broth. Extracellular POD activity was determined according to the method described (Huh *et al.* 1997) using pyrogallol as a substrate in the presence of H_2O_2. The formation of 1 mg purpurogallin from pyrogallol after 20 s at pH 6 at 20 °C was defined as one unit of POD activity. To determine the total POD activity, 200 μl of the culture sample was placed in a microtube containing glass beads (0.5 mM diameter) and the cells were disrupted by vigorous shaking in a Multi Power Beater (Biogenia Co., Korea) for 2 min at 4 °C. Cell debris was removed by centrifuging for 10 min at 10 000 *g* and the supernatant used to determine total POD activity. Extracellular protease activity was measured with azocasein (Sigma) as a substrate at 340 nm and expressed in relative azocasein digestion units (ACU) (Morita *et al.* 1991). One unit (ACU) of protease activity was defined as the amount of enzyme required to obtain an increase of 0.001 units min^{-1} at 340 nm under the set conditions. Extracellular protein was determined in the culture supernatants using the Bradford method with bovine serum albumin as a standard. The concentration of glucose was analyzed by measuring the amount of reducing sugar using the Nelson–Somogyi method.

Results and discussion

Construction of expression vector

The cloning strategy for the construction of the expression vector is summarized in Figure 1A. For the secretion of *swpa1*, the synthesized signal sequence of *A. oryzae* α-amylase was cloned between the *Eco*R1 and *Sal*1 site of the shuttle vector, derived from pYEG-αHIR525 (Sohn *et al.* 1995). The resulting plasmid pGAL-1 was analyzed by agarose gel electrophoresis (data not shown). In order to introduce the *swpa1* cDNA into the plasmid pGAL-1, the amplified cDNA fragment of about 920 bp, with *Sal*1 site at both ends, was cloned into the plasmid pGAL-1. To complete the expression vector pGPD-POD1 of the *swpa1* gene (Figure 1B), the GPD promoter fragment from pYGAP-CT1 was cloned to replace the *GAL10* promoter, which contained the *Bam*H1 site at 5′ and the *Eco*R1 site at 3′. The plasmid pGPD-POD1 was analyzed by agarose gel electrophoresis (Figure 2).

Expression and secretion of swpa1

The recombinant expression vector (pGPD-POD1) of the *swpa1* gene was introduced into *S. cerevisiae* by electroporation. A superior transformant harboring pGPD-POD was selected and named *S. cerevisiae* GPD-POD1. To characterize the POD proteins produced by *S. cerevisiae* GPD-POD1, the cells were cultured in YNBDCA medium on a bench scale fermenter

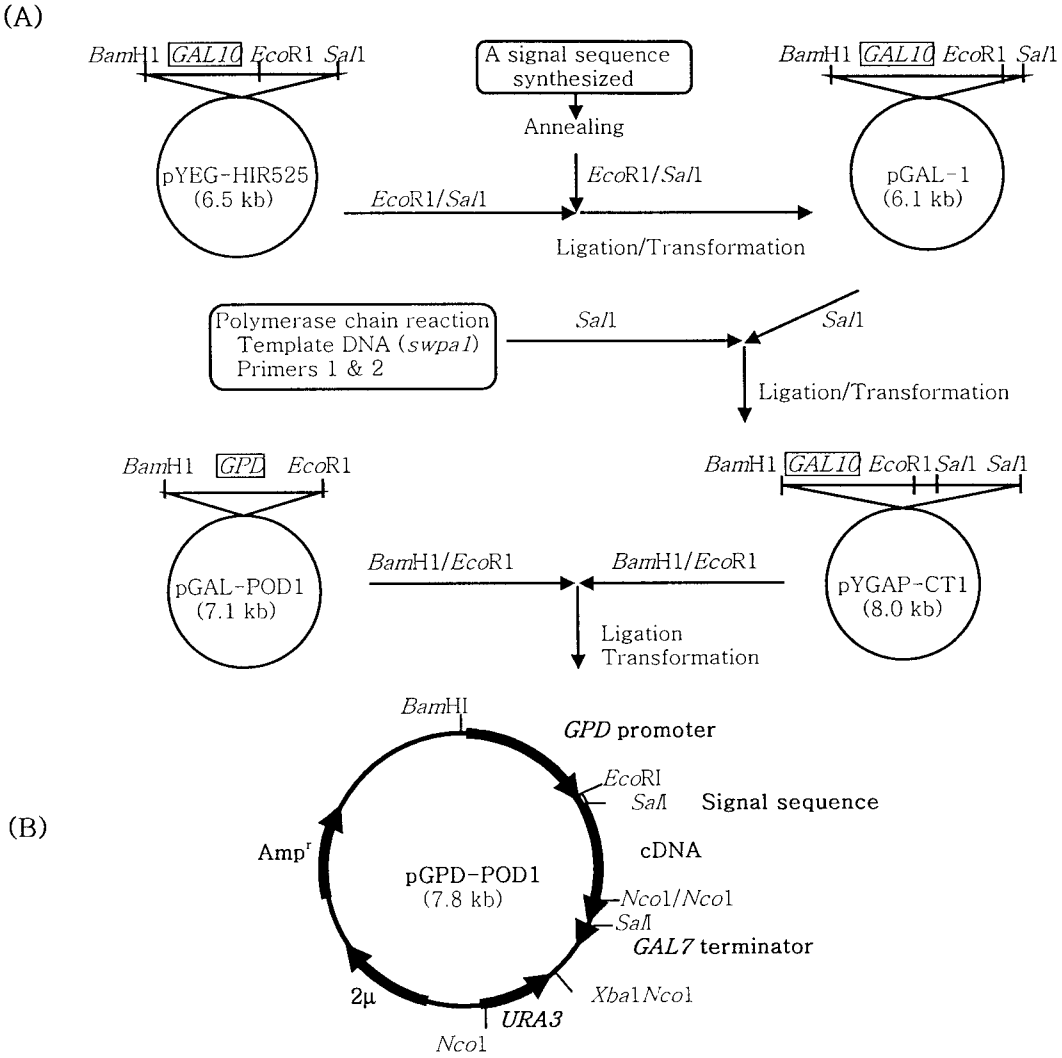

Fig. 1. Schematic diagrams on the construction of the vector pGPD-POD1 for heterologous expression of *swpa1* in *Saccharomyces cerevisiae*. (A) The construction process was set out by using pYEGα-HIR525 and indicated the cleavage sites for restriction enzymes. (B) Map of pGPD-POD1. The vector also contains an *E. coli* ampicillin resistance gene, a yeast 2 μm replication origin and a yeast selectable marker *URA3*.

(5 l) and their expressions monitored (Figure 3). The expression of *swpa1* gene was started after culturing for 20 h. The maximum extracellular POD activity was 1030 U l^{-1} at 32 h, and the corresponding total POD activity was 1140 U l^{-1}, equivalent to 380 U g^{-1} cell dry wt for the whole culture. The secretion yield of POD activity was approx. 90% (U U^{-1}) of total expressed POD. However, a sharp increase in protease activity, followed by an abrupt decrease of POD activity, was observed during the culture (Figure 3B), which suggested that the recombinant proteins were unstable in the presence of the yeast cell proteases (Nomura *et al.* 1995). During the prolonged cultivation, cell growth increased continuously, glucose was not depleted, and the extracellular protein level remained relatively constant (Figure 3A). While an inverse correlation between extracellular protein and protease activity was unclear, the protease activity profile was shown to influence the POD activity during the idiophase of the cell growth.

Identification of POD by electrophoresis

The molecular size of the active POD extracellularly expressed during the growth of the recombinant yeast GPD-POD1 was determined. POD protein,

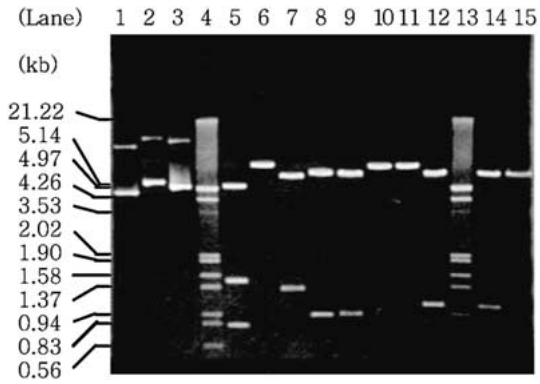

Fig. 2. Restriction analysis of pGPD-POD1. Electrophoresis showing fragments from pGPD-POD1 by restriction enzymes: lane 1, supercoiled pGAL-POD1; lane 2, supercoiled pYGAP-CT1; lane 3, supercoiled pGPD-POD1; lanes 4 and 13, DNA marker (λDNA digested by *Eco*R1 and *Hin*dIII); lane 5, digested by *Nco*1; lane 6, digested by *Xba*1; lane 7, digested by *Eco*R1 and *Xba*1; lane 8, digested by *Sal*1; lane 9, digested by *Eco*R1 and *Sal*1; lane 10, digested by *Eco*R1; lane 11, digested by *Bam*H1; lane 12, digested by *Eco*R1 and *Bam*H1; lane 14, pYGAP-CT1 digested by *Eco*R1 and *Bam*H1; lane 15, pGAL-POD1 digested by *Eco*R1 and *Bam*H1.

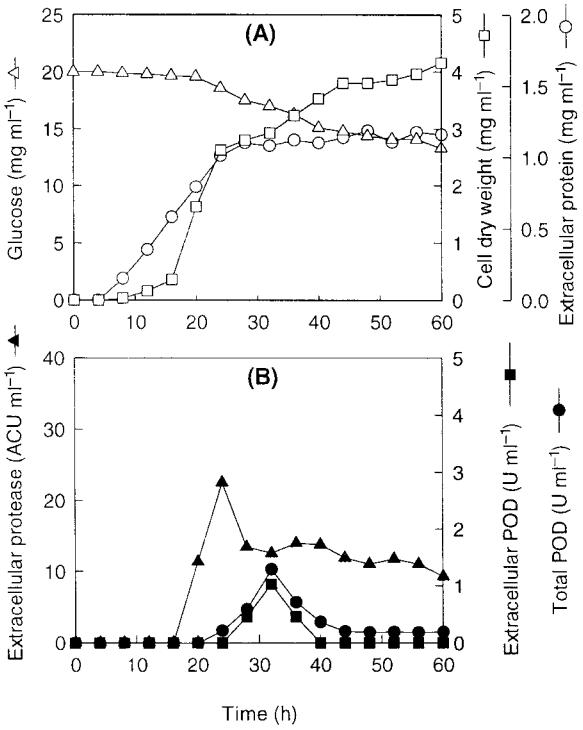

Fig. 3. Time-dependent profiles of expression parameters for the recombinant *Saccharomyces cerevisiae* GPD-POD1. The cells were cultured in the YNBDCas medium in a bench scale (5 l) fermenter with the aeration of 1 vvm at 30 °C and 300 rpm. Glucose consumption (△), cell dry wt (□), extracellular protein (○), extracellular protease activity (▲), extracellular POD activity (■) and total POD activity (●) were analyzed as the parameters.

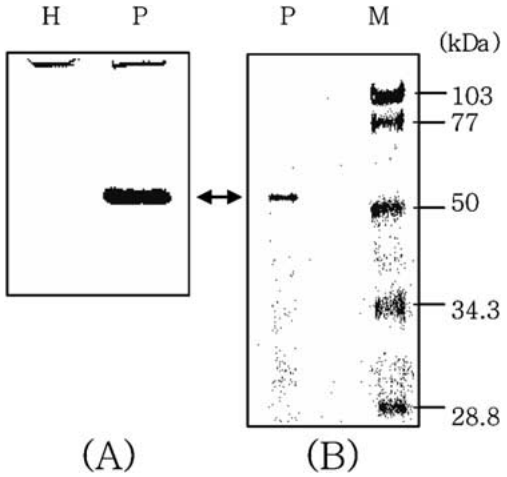

Fig. 4. (A) Native- and (B) SDS-PAGE analyses on extracellular POD protein partially purified by DEAE-cellulose chromatography. The native-gel was stained by pyrogallol and H_2O_2 and the SDS-gel by Coomassie blue R-250. Lane H: the host cell as the control, lane P: purified POD expressed in recombinant yeast GPD-POD1, lane M: molecular size standards (Bio-Rad) (phosphorylase B, 103 kDa; bovine serum albumin, 77 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 34.3 kDa; soybean trypsin inhibitor, 28.8 kDa). All the samples were prepared from extracellular cultures and the recombinant POD of interest is indicated with an arrow.

partially purified by secondary DEAE-cellulose chromatography from the culture supernatant, was analyzed by SDS- and native-PAGE. SDS-gel stained with Coomassie Brilliant Blue showed an intense band of 53 kDa (Figure 4B). The protein produced by recombinant POD, shown in Figure 4B, had a larger molecular size than the native protein (43 kDa) (Kwak *et al.* 1995), and this was attributed to over-glycosylation, which generally occurs in *S. cerevisiae* (Whittington *et al.* 1990). In native-PAGE analysis of the recombinant POD proteins (Figure 4A), a single intense active band was visualized. Although cytochrome *c* peroxidase (cytochrome *c*: H_2O_2 oxidoreductase, EC 1.11.1.5) is known to exist in mitochondria of *S. cerevisiae* with an M_r of ca. 34 kDa (Yonetani 1971), the 19 kDa difference in molecular size indicates that the purified protein visualized in native-PAGE, was the result of over-glycosylation of the recombinant POD protein.

Effect of protease inhibitor (PMSF) addition

Based on Figure 3B, we examined whether protease affected the abrupt decrease of POD activity during the culture at the flask level, using various concentrations of PMSF, which has been reported to be a specific protease inhibitor in extracellular culture broth (Dosoretz

et al. 1990, Tien & Kirk 1984). PMSF at 0.05 mM was the most effective, but above 0.05 mM PMSF, POD activity and cell growth were significantly inhibited (data not shown). This result indicated that the expression of the foreign POD gene in yeast was affected by the presence of protease inhibitor (PMSF) (Dosoretz *et al.* 1990). To investigate the effect of PMSF on a bench scale fermenter (5 l), the recombinant yeast GPD-POD1 was cultured in YNBDCA medium containing 0.05 mM of PMSF (Figure 5). It was noted that PMSF significantly inhibited the protease activity, from 23.8 U mg^{-1} protein to 4 U mg^{-1} protein in the supernatant (24 h) and, accordingly, the POD stability increased and the maximum specific POD activity increased from 0.87 U mg^{-1} protein (32 h) to 3.43 U mg^{-1} protein (40 h) in the supernatant (Figure 5B). Protease activity was observed as primary and secondary activity at the late exponential and stationary growth phases, respectively (Figures 3B and 5B). The addition of PMSF at the initial stage of the culture significantly inhibited the primary protease activity, but did not affect secondary protease activity, suggesting the involvement of the secondary protease in cell growth (Figure 5B). On the other hand, the addition of PMSF made no appreciable difference to the extracellular protein profile, glucose consumption and cell growth by the recombinant GPD-POD1 strain during the culture (Figure 5A).

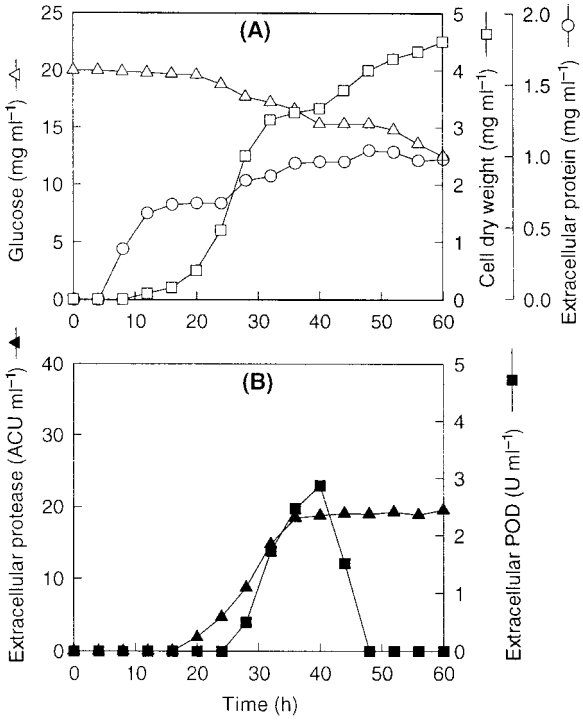

Fig. 5. Effect of PMSF on the activities of extracellular POD and protease during batch culture. The cells were cultured in the YNBDCas medium in a bench scale (5 l) fermenter with the aeration of 1 vvm at 30 °C and 300 rpm, and PMSF added to a final concentration of 0.05 mM at inoculation stage. Glucose consumption (△), cell dry wt (□), extracellular protein (○), extracellular protease activity (▲), and extracellular POD activity (■) were analyzed as the parameters.

Effect of Casamino acids supplement during batch culture

Proteolysis in yeast is affected by the culture conditions, especially nitrogen control (Nomura *et al.* 1995, Teichert *et al.* 1989) and to be minimized by Casamino acid addition to the medium (Werten *et al.* 1999). Accordingly, we examined the effect of a suitable nutritional supplement by adding 50 ml of 10% (w/v) Casamino acids, under the same culture conditions, after culturing for 36 h (Figure 6). Cell growth and the glucose consumption by the recombinant yeast GPD-POD1 were found to increase slightly after the supplementation. However, the production of extracellular protein was not appreciably affected (Figure 6A). Although the protease activity was increased upon supplementation with Casamino acids, POD activity also increased dramatically, to more than three times the POD activity observed during idiophasic growth (Figure 6B). This was approx 6 times higher than that observed in the YNBDCA medium without the addition of protease inhibitor (PMSF). These results indicate that: (1) the effect of environmental conditions during the growth of yeast cells can affect the production of extracellular enzymes, (2) proteolysis may play an important role under stress conditions, such as protein degradation, cell growth and nutritional starvation (Teichert *et al.* 1989). Therefore, our results suggest that *N*-source supplementation, using Casamino acid, facilitates the production of heterologous POD protein in batch culture.

Conclusions

A peroxidase isoenzyme from a sweet potato cell line (*Ipomoea batatas*) (Huh *et al.* 1997) was overexpressed in *S. cerevisiae* using an expression vector pGPD-POD1. The expression vector consisted of a strong glycolytic GPD promoter (Chung *et al.* 1997), a signal sequence of *A. oryzae* α-amylase (Hellmuth *et al.* 1995), cDNA of *swpa1* gene and a *GAL7* terminator.

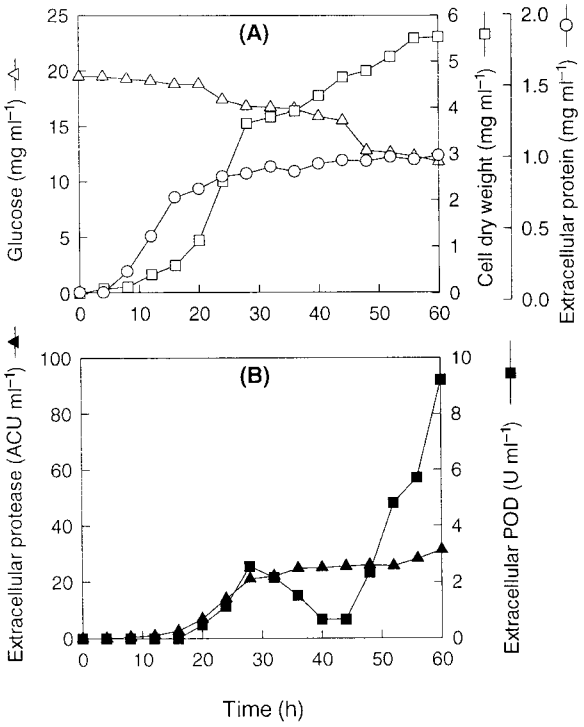

Fig. 6. The effect of Casamino acids on overproduction of extracellular POD by the recombinant *S. cerevisiae* GPD-POD1. The cells were cultured in YNBDCA medium containing 0.05 mM of PMSF as protease inhibitor and Casamino acids (10 g) dissolved in 50 ml was supplemented after culturing for 36 h. The parameters of glucose consumption (△), cell dry wt (□), extracellular protein (○), extracellular protease activity (▲) and extracellular POD activity (■) were analyzed.

When the recombinant strain of GPD-POD1 was grown in batch culture in YNBDCA medium, the *swpa1* gene product was secreted at a yield of approximately 90% (U U^{-1}) of total POD expression, and this was identified to be an active protein of 53 kDa. The recombinant POD secreted had a higher molecular weight than the native protein (43 kDa), possibly due to its higher glycosylation level, since *S. cerevisiae* is generally known to hyperglycosylate heterologous proteins (Romanos *et al.* 1992).

Our finding that the addition of a protease inhibitor (PMSF) and supplementation with a nitrogen source (Casamino acids) affects POD activity in batch culture suggests the involvement of protease activity and the possibility of eliminating or minimizing the abrupt decrease of POD activity observed in nitrogen-limited medium. Further characterization of the POD proteins obtained should lead to further advances in productivity and the biotechnological applications of POD.

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