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A cDNA encoding vacuolar type β-D-fructofuranosidase (*Osβfruct3*) of rice and its expression in *Pichia pastoris*

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Abstract

A vacuolar type β-D-fructofuranosidase (*Osβfruct3*) was cloned from etiolated rice seedlings cDNA library. It encodes an open reading frame of 688 residues. The deduced amino acid sequence had 58% identity to the vacuolar type β-D-fructofuranosidase of maize (*Ivr1*). *Osβfruct3* exists as a single copy per genome. Northern analyses showed that *Osβfruct3* undergoes organ-specific expression and is involved in the adjustment of plant responses to environmental signals and metabolizable sugars. *Osβfruct3* was also heterologously expressed in *Pichia pastoris*. The recombinant proteins were confirmed to be a vacuolar type β-D-fructofuranosidase.

Introduction

β-D-Fructofuranosidases (EC 3.2.1.26) constitute a family of enzymes that hydrolyze sucrose into glucose and fructose (Tymowska-Lalanne & Kreis 1998, Sturm 1999). Several types of β-D-fructofuranosidase from rice (*Oryza sativa*) have been purified and characterized (Lin & Sung 1993, Sung & Huang 1994, Lin *et al.* 1999, Hsiao *et al.* 2002). However, information about gene expression and regulation of the rice β-D-fructofuranosidase family is limited. In a previous paper, we have reported the cloning of a vacuolar type β-D-fructofuranosidase cDNA (*Osβfruct2*) from rice and its expression in *Pichia pastoris* (Fu *et al.* 2003). In this present study, we describe the cDNA cloning, Southern and Northern analyses of another vacuolar β-D-fructofuranosidase gene (*Osβfruct3*) in etiolated rice seedlings. *Osβfruct3* encodes a functional β-D-fructofuranosidase as demonstrated by the expression of active enzyme in *Pichia pastoris*.

Materials and methods

Preparation and screening of the cDNA library

Total RNA was extracted from shoots of etiolated rice seedlings (germinated for 8 d, *Oryza sativa* L. cv. Tainong no. 67) using the guanidinium · HCl/thiocyanate/phenol/chloroform extraction method. Polyadenylated RNA was isolated using Poly ATtract mRNA Isolation Systems (Promega), and RT-PCR was performed as the cDNA synthesis kit (Gibco BRL) with degenerate primers S1 (5′-AAAAACTGGATGAACGATCCTAATGGT-3′) and AS3 (5′-TCTTCCACCTTGAGCAAAGCTTTCAAC-3′) derived from GenBank according to conserved sequences of vacuolar type β-D-fructofuranosidase of mung bean (Arai *et al.* 1992). The 1.41 kb fragment was used to screen a cDNA library constructed from the same etiolated rice seedlings RNA as used a λZAP-cDNA Synthesis Kit (Stratagene) using a Random Prime Labelling System (Amersham Pharmacia Biotech). A positive clone of *Osβfruct3* was isolated and sequenced. In order to obtain the sequence of the 5′ region of *Osβfruct3* cDNA, an amplification of the

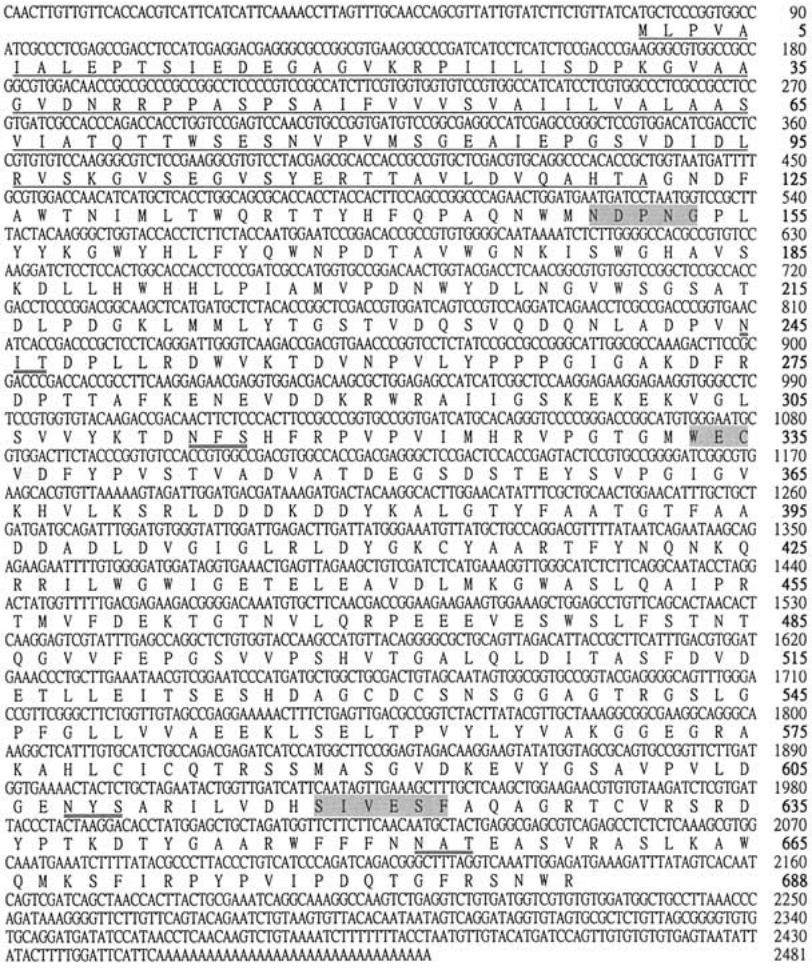

Fig. 1. Entire nucleotide sequence and deduced amino acid sequence of *Osβfruct3*. A putative signal peptide and *N*-terminal extension in the 5′-coding region are underlined. Three conserved regions are marked by shadow boxes. Four potential *N*-glycosylation sites are indicated by double underlines. GenBank accession number of this nucleotide sequence is AY037871.

5′ ends (5′ RACE, Gibco BRL) was performed using a commercial dC-tailed cDNA from etiolated rice seedlings as a template and the following primers: SP1 (5′-GGTGGAGTCGGAGCCCTCGTCGGTGGCCACG TC-3′) and SP2 (5′-GCGCTTGTCGTCCACCTCGT TCTCCTTGAAG-3′). The deduced amino acids were analyzed for the putative targeting signal, isoelectric point and glycosylation sites using the Expert Protein Analysis System of Swiss Institute of Bioinformatics. The BLAST databanks were screened for similarity to other β-D-fructofuranosidase sequences.

Southern and Northern blot analyses

For Southern blot analysis, genomic DNA was prepared from the etiolated rice seedlings using the method of CTAB. Genomic DNA, approx. 10 μg, was separately digested by *Eco*RI, *Nhe*I and *Spe*I, size-fractionated on 0.7% agarose gel and blotted onto nylon membrane (Millipore). The membrane was hybridized with PCR-labelled probes. A 265 bp PCR fragment of the 3′ non-coding region of *Osβfruct3* was prepared using a primer set of IT3-S: 5′-TC AGTCGATCAGCTAACCACTTACTGCGAA-3′ and IT3-AS: 5′-ACTCACACACACAACTGGATC-3′. Hybridization was carried out for 24 h at 42 °C. The blots were washed with 2 × SSPE (3 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.4) plus 0.1% SDS at room temperature for 10 min, twice with 1 × SSPE plus 0.1% SDS at 55 °C for 15 min, twice with 0.1 × SSPE plus 0.1% SDS at 55 °C for 15 min and then exposed to X-ray film. For Northern blot analyses, total RNA was extracted by TRIzol reagent (Gibco

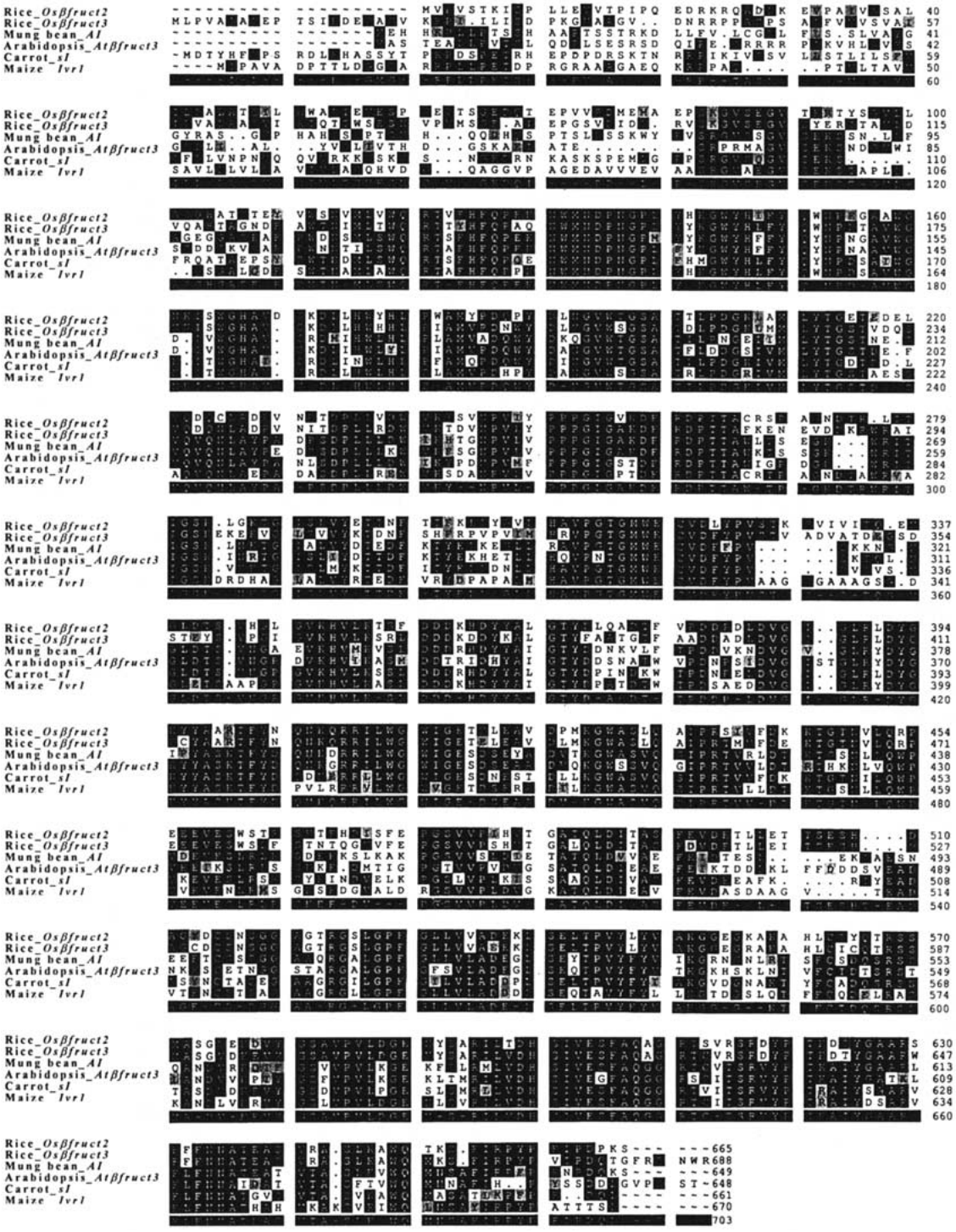

Fig. 2. Alignment of amino acid sequence of *Osβfruct3* with those of homologous vacuolar type β-D-fructofuranosidases. The β-D-fructofuranosidase sequences (abbreviated name and accession number in brackets) are from rice (*Osβfruct2*: AY037870), mung bean (*AI*: D10265), *Arabidopsis* (*Atβfruct3*: X99111), carrot (*sI*: X75352) and maize (*Ivr1*: U16123).

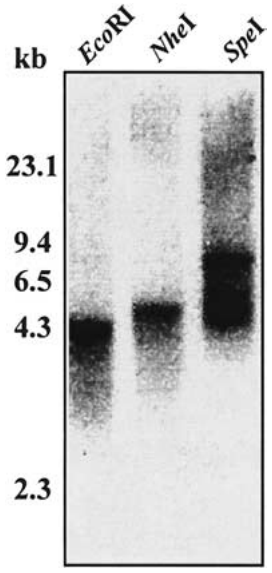

Fig. 3. Genomic DNA Southern blot analysis of etiolated rice seedlings DNA using probes specific to the *Osβfruct3*. The sizes of molecular mass markers are indicated at the margin.

BRL), electrophoresed through agarose gels containing formaldehyde. Transfer, hybridization, detection conditions were as described for genomic Southern blots. The amount of *Osβfruct3* mRNA was quantified using a Bioimaging analyzer (BAS1000, Fuji Co., Japan).

Expression of recombinant protein in yeast

Osβfruct3 was expressed in the methylotrophic yeast *Pichia pastoris* X-33 (EasySelect *Pichia* Expression Kit, Invitrogen), with the secretory expression vector pPICZαA. The DNA sequence, corresponding to putative mature protein regions of *Osβfruct3* between nucleotides 433 and 2148, was amplified by PCR and translationally fused behind the α-factor signal sequence of pPICZαA. The *Pme*I-linearized vector with insert was transformed by EasyComp Kit (Invitrogen) into *P. pastoris*, and transformants were selected on YPDS-Zeocin agar plates (1% yeast extract, 2% peptone, 2% D-glucose, 1 M sorbitol, 2% agar, 100 μg Zeocin ml^{-1}). A BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M potassium phosphate, 1.34% (w/v) yeast nitrogen base without amino acids, 0.00004% (w/v) biotin and 1% (v/v) glycerol] was inoculated with freshly prepared positive colonies and incubated for 48 h at 30 °C. The cells were collected by centrifugation, transferred to a BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M potassium phosphate, 1.34% (w/v) yeast nitrogen base without amino acids, 0.00004% (w/v) biotin and 5% (v/v) methanol] and incubated at 28 °C under aerobic conditions. Every day, 100 μl methanol was added to 100 ml culture medium. Samples, 5 ml, were collected for up to 156 h.

Purification of recombinant protein

A nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) was pre-equilibrated with 50 mM sodium phosphate buffer, pH 8, containing 300 mM NaCl and 5 mM imidazole. The yeast transformants were removed by centrifugation and the concentrated supernatant (Ultrafree15, Millipore) was loaded on to the column and washed with 50 mM sodium phosphate buffer, pH 8, containing 300 mM NaCl and 20 mM imidazole. Finally, the column was eluted with 50 mM sodium phosphate buffer, pH 8, containing 300 mM NaCl and 250 mM imidazole. Fractions containing acidic β-D-fructofuranosidase activity (method to see legend of Figure 5) were pooled and concentrated.

Results and discussion

Molecular characterization of vacuolar type β-D-fructofuranosidase from rice

A new vacuolar type β-D-fructofuranosidase isozyme cDNA (*Osβfruct3*, GeneBank accession number: AY037871) has been cloned here from rice. The cDNA fragment contains 2481 nucleotides with a termination codon TGA and putative polyadenylation signal (AATAAT) (Figure 1). The cDNA encodes 688 amino acids with calculated relative molecular mass of 76 kDa and isoelectric point of 5. The protein contains all the elements characteristic of vacuolar type β-D-fructofuranosidase, including the β-fructosidase motif 'NDPNG' and the putative active site 'WECVD'. The protein also contains four potential *N*-linked glycosylation sites (Figure 1). The *N*-terminus region, like other vacuolar type β-D-fructofuranosidase, contains signal peptide and *N*-terminal extension of 68 and 53 residues, respectively (Figure 1). The mature protein have calculated molecular mass of 63 kDa and calculated *p*I value of 5.1.

Previously, we already reported one vacuolar type β-D-fructofuranosidase cDNA (*Osβfruct2*, GeneBank accession number: AY037870) from the same species (Fu *et al.* 2003). Compared to the previous β-D-fructofuranosidase, the newly cloned enzyme has

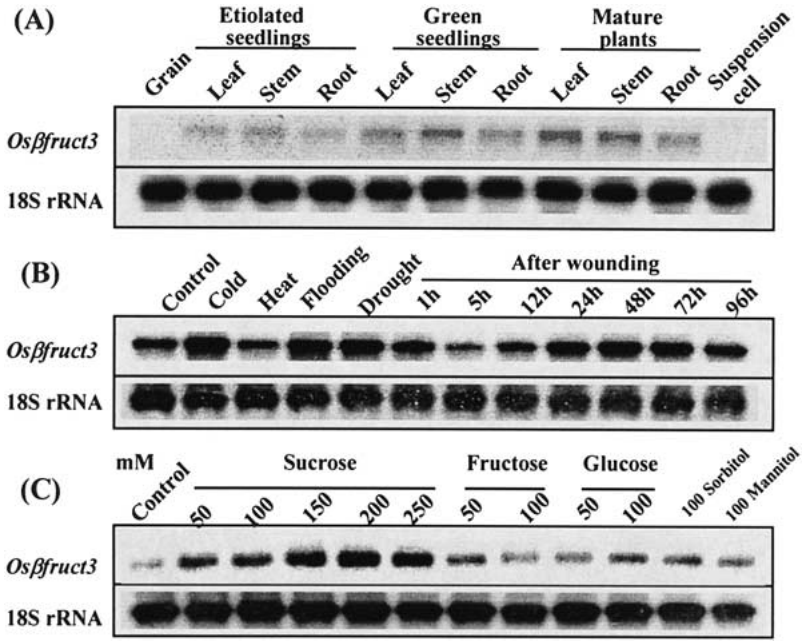

Fig. 4. Northern analyses of etiolated rice seedlings RNA using probes specific to the *Osβfruct3* and 18S rRNA. (A) Tissue and stage specific expression. Total RNA from leaves, stems and roots of 8-d-old etiolated seedlings, 8-d-old green seedlings and mature plant of rice. (B) Stress specific expression. 8-d-old etiolated rice seedlings were exposed separately to stresses for 2 d. Total RNA from shoots of cold (exposed to 10 °C), heat (exposed to 40 °C), flooding (whole plants were submerged in distilled water), drought (dehydrated until losing 30∼40% of the fresh weight) and wounding (excised seedlings tip) treatments. (C) Sugars feeding experiments. Shoots were detached from etiolated rice seedlings (shoots were preadapted to the dark and soaked in distilled water for 5–6 h) and placed into a dish with a feeding solution (sucrose, glucose, fructose, sorbitol or mannitol) for 48 h.

79% identity at nucleotide level and 72% identities at protein level. An alignment of the deduced amino acid sequences with various other vacuolar β-D-fructofuranosidase from plants is shown in Figure 2. *Osβ*fruct3 has a high similarity to the vacuolar β-D-fructofuranosidase of mung bean, *Arabidopsis*, carrot and maize with 60%, 58%, 63% and 58% identities, respectively.

Southern analysis

Total genomic DNA from etiolated rice seedlings digested with *Eco*RI, *Nhe*I and *Spe*I restriction enzymes, respectively, was hybridized with the *Osβfruct3* probes (Figure 3). Only one hybridizing band was observed for *Osβfruct3* showing that the gene is present in one copy per haploid genome.

Northern blot analyses

Osβfruct3 was highly expressed in leaves, stems, and roots of etiolated seedlings, green seedlings and rice plants. This contrasts with no expression in the grains and suspension cells. *Osβfruct3* was also up-regulated by exposure of the plants to light (Figure 4A). For the stress treatments, *Osβfruct3* transcription was increased in cold stress, flooding, drought and wounding (Figure 4B). For the feeding experiments, *Osβfruct3* was strongly up-regulated by sucrose (<200 mM) and glucose (100 mM), but fructose, mannitol and sorbitol had no effect on expression (Figure 4C). Northern analyses showed that *Osβfruct3* undergoes organ-specific expression and is involved in the adjustment of plant responses to environmental signals and metabolizable sugars.

Expression and properties of the recombinant Os*βFRUCT3*

To confirm that *Osβfruct3* encodes a vacuolar type β-D-fructofuranosidase, the *Osβfruct3* cDNA was expressed in the *Pichia pastoris*. A clear sucrose hydro-

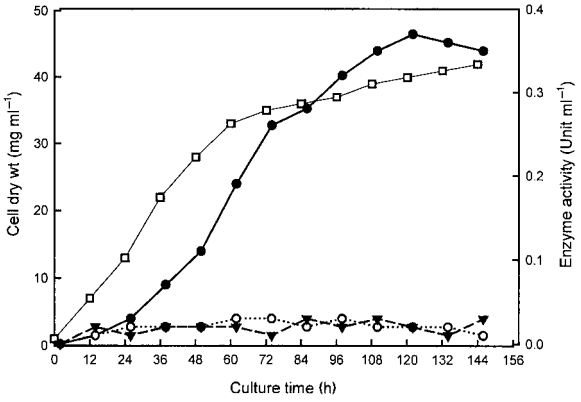

Fig. 5. Time-course of *Osβ*FRUCT3 expression in *P. pastoris* transformed with pPICZαA vector system. (□), Cell dry weight; (●), vacuolar β-D-fructofuranosidase activity in medium (*P. pastoris* was transformed with *Osβfruct3*); (○), vacuolar β-D-fructofuranosidase activity in medium (*P. pastoris* was only transformed with pPICZαA); (▼), intracellular vacuolar β-D-fructofuranosidase activity (*P. pastoris* was transformed with *Osβfruct3*). Protein concentration was determined by the protein-dye binding method. The enzyme activity was assayed in a 0.36 ml mixture of 0.2 M sucrose in 100 mM sodium acetate (pH 5) for vacuolar β-D-fructofuranosidase, at 37 °C for 10 min and the amount of reducing sugar was measured by the Somogyi–Nelson method. One enzyme unit was defined as the amount of enzyme that catalyzed the production of 1 μmol reducing sugar per min at 37 °C and at the optimum pH.

lysis activity was only detected around the transformant with the *Osβfruct3* expression vector (Figure 5). This result suggests that the active recombinant enzyme was expressed in the yeast and secreted from cells. To characterize *Osβ*FRUCT3, the recombinant enzymes were purified and subjected to SDS-PAGE and showed a single protein band of about 67 kDa that matched the calculated molecular mass of *Osβ*FRUCT3 (Figure 6). Recombinant enzymes were incubated for 10 min at various temperatures or in different pH. Activity of the recombinant enzymes was optimal at 30 °C and in pH 4.5 (data not shown). The biochemical properties detailed above are similar to those of vacuolar β-D-fructofuranosidase purified from rice (Lin & Sung 1993) and various other plants (Fu *et al.* 2002).

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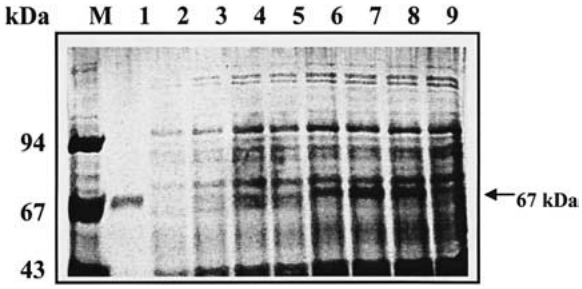

Fig. 6. Production of *Osβ*FRUCT3 in *P. pastoris*. The figure shows the results of 10% SDS-PAGE analysis of the production of *Osβ*FRUCT3 throughout the time course of the fermentation. Silver staining was used to visualize the protein. Lane M, low-molecular-mass markers; lane 1, purification of nickel-nitrilotriacetic acid (Ni-NTA) agarose column; lanes 2–9, cell-free medium (10 μl) 0–144 h after 0.5% methanol induction, respectively. The arrow indicates the position of the 67 kDa of *Osβ*FRUCT3.

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