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Expression of a functional barley sucrose-fructan 6-fructosyltransferase in the methylotrophic yeast *Pichia pastoris*

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Abstract The cDNA encoding sucrose-fructan 6-fructosyltransferase (6-SFT) from barley (*Hordeum vulgare*) has been expressed in the methylotrophic yeast *Pichia pastoris*, using a translational fusion into vector pPICZαC, containing the N-terminal signal sequence of *Saccharomyces cerevisiae* α-factor to allow entry into the secretory pathway. Transformed *Pichia* produced and secreted a functional 6-SFT which had characteristics similar to the barley enzyme, but had a pronounced additional 1-SST activity when incubated with sucrose.

Key words: Fructan; Heterologous expression; Sucrose-fructan 6-fructosyltransferase; *Hordeum vulgare*; *Pichia pastoris*

1. Introduction

Fructans are a class of polysaccharides consisting of linear and branched fructose chains attached to a sucrose moiety. They occur in the prominent plant orders of the Asterales, Liliales and Poales. Among them are important crop species such as wheat, barley, rye and oat. Fructans play a role as a carbohydrate reserve, as an alternative or in addition to starch, and in short-term carbohydrate partitioning. They are important for osmoregulation in flowers and barley growth zones, and might increase drought and freezing tolerance. Fructan is synthesized in the vacuole, as has been shown in barley and in *Helianthus tuberosus* [1–3].

In barley, which contains a branched type of fructan with β(2→1) and β(2→6) linkages, the two key enzymes for fructan synthesis are sucrose-sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99), which produces the trisaccharide 1-kestose, and sucrose-fructan 6-fructosyltransferase (6-SFT), which is responsible for the formation of β(2→6) linkages [2]. We have previously purified, cloned and characterized barley 6-SFT [4]. This enzyme transfers the fructose moiety of sucrose to a multitude of acceptor substrates. Sucrose can be an acceptor, yielding the trisaccharide 6-kestose, but the preferred acceptor is 1-kestose, yielding bifurcose, the simplest branched fructan. The latter reaction can be used to specifically measure 6-SFT activity [5].

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Abbreviations: 6-SFT, sucrose-fructan 6-fructosyltransferase; 1-SST, sucrose-sucrose 1-fructosyltransferase; 6-SST, sucrose-sucrose 6-fructosyltransferase; AOX1, alcohol oxidase 1; BMGY, buffered complex glycerol medium; BMMY buffered complex methanol medium; DP, degree of polymerization; MES, 2-morpholinoethanesulfonic acid; ORF, open reading frame; PCR, polymerase chain reaction; YPDS, rich, complex broth medium with dextrose and sorbitol

Barley 6-SFT cDNA has been expressed in various plant systems before, including tobacco protoplasts and tobacco and chicory plants [4,6].

Here we demonstrate that barley 6-SFT can also be functionally expressed in the methylotrophic yeast *Pichia pastoris*. This yeast, which has been used to produce various heterologous proteins [7–10], is particularly well suited to studying the plant sucrose-metabolizing enzymes formed by way of the secretory pathway, since it does not itself secrete sucrose-metabolizing enzymes such as invertase [11], and since glycosylation of secreted proteins in *Pichia* is expected to be closer to that in plants than that in *Saccharomyces cerevisiae* [12,13].

2. Materials and methods

2.1. Host strains and vectors

Escherichia coli strain DH5α was used for amplification of the recombinant plasmids. *P. pastoris* strains X-33 (wild type), GS-115 (*his4*), KM-71 (Mut^S, *his4*), and the pPICZαC shuttle vector were obtained from Invitrogen BV (Leek, The Netherlands).

2.2. Construction and sequencing of the 6-SFT expression vectors P1 and P2

The 6-SFT cDNA clone B5 [4] was used for the following experiments. For construction of P1, an *Eco*RI restriction site 5′ to the sequence encoding the N-terminus of the mature protein and an *Xba*I site 3′ to the stop codon were introduced by PCR. The resulting insert was designed to be ligated in frame behind the α-factor signal sequence of the *Pichia* expression vector pPICZαC (subsequently called P0). The following synthetic oligonucleotides were used (Fig. 1B): (A) 5′-GCCGTCGACGAGGA**GAATTC**GGCGGGCGGGTTCCCGTCGAGC-3′; (B) 5′-GTTTCAATGCATGCT**TCTAGA**GAGGTTGTGCAGAATGATTCG-3′. For construction of P2, the oligomer (C) 5′-GGAGTTGACGAGCG**GAATTC**AATGGGGTCACACGGCAAGCCACCG-3′ was used instead of (A) to introduce the *Eco*RI site 5′ to the start codon (bold characters in the oligomers indicate the restriction sites). Denaturation, annealing and extension temperatures of 94°C (0.5 min), 58°C (1 min), 74°C (2 min), respectively, were used in the PCR experiments. Deep Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) was used for the reactions. After digestion, the two fragments were ligated at the *Eco*RI-*Xba*I sites of the vector, yielding two constructs named P1 and P2, respectively (Fig. 1C).

The sequences of the inserts created with PCR were determined for both strands by the chain termination method [14]. The Applied Biosystems Dye Terminator Cycle Sequencing kit was used, and sequencing took place on an ABI Prism 310 DNA Genetic Analyzer (PE Applied Biosystems, USA). As a reference the sequence of the B5 cDNA used for cloning was freshly determined using the same method. The sequences obtained were analyzed using the GCG software package, version 9.1 (1997).

2.3. Pichia pastoris transformation and small-scale expression

P. pastoris strains X-33, GS-115, and KM-71 were transformed with 3 μg of *Pme*I-linearized P1 or P2 plasmid respectively, following the EasyComp transformation protocol, and plated on selective YPDS/Zeocin plates (protocol and recipes provided by the manufacturer). As a control the same strains were transformed with the parent plasmid pPICZαC.

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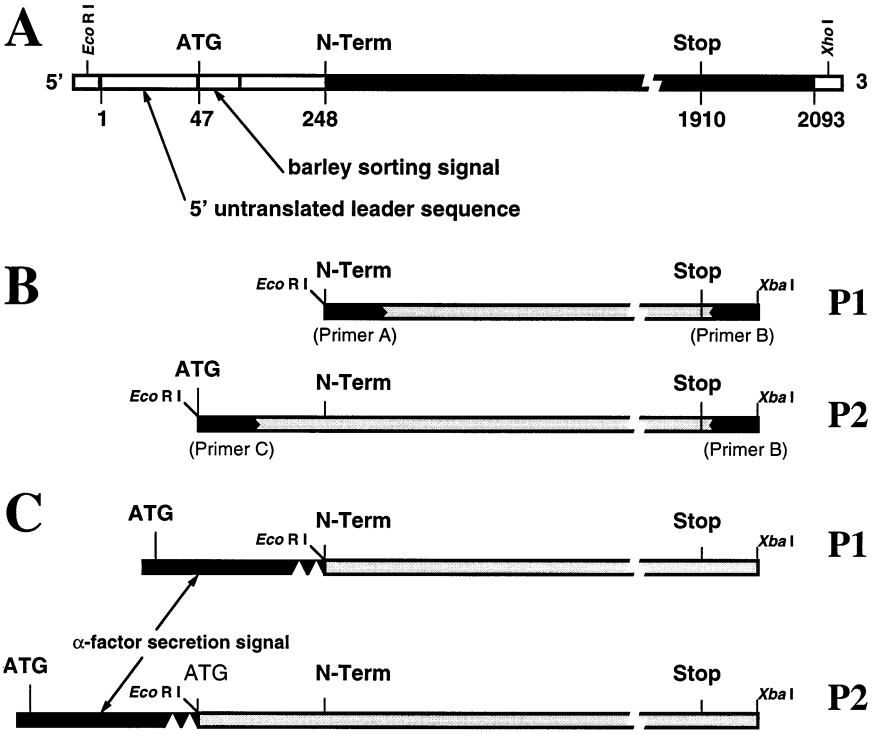

Fig. 1. Constructs created for transformation of *P. pastoris* to express barley 6-SFT. A: 6-SFT cDNA B5. B: Inserts created by PCR lacking (P1) or containing (P2) the barley sorting signal and propeptide sequences. C: Constructs after cloning into the vector pPICZαC.

Single colonies of transformants were inoculated on fresh YPDS/Zeocin plates. The newly grown colonies were inoculated in 25 ml pre-culture medium (BMGY, pH 5.6) for a small-scale expression time course. After 18 h of incubation at 27°C under vigorous shaking (140 oscillations per min), the cells were switched to induction medium (BMMY, pH 5.6) in shake flasks, and incubated at 27°C under the same aerobic conditions. Methanol was replaced every 24 h to a final concentration of 0.5%. 30 ml samples of culture medium were taken after 4, 8, 24, 48 and 72 h of induction and were centrifuged. The supernatant was concentrated down to 0.5 ml, and desalted with 15 ml of 25 mM MES (NaOH) buffer, pH 5.75, by ultrafiltration on VivaSpin concentrators (cut-off at 30 000 Da, VivaScience Ltd, Lincoln, UK). The final volume was 0.5 ml (60-fold concentration). 10 μl of this preparation was incubated overnight at 4°C, with 50 mM sucrose and 50 mM 1-kestose in 100 mM MES (NaOH) buffer, pH 5.75, to a final volume of 20 μl. The carbohydrates produced were analyzed by HPLC (see Section 2.5). The cell pellet from the centrifugation step was weighed to establish a growth curve.

2.4. Characterization of 6-SFT expressed by transformed Pichia

To produce 6-SFT enzyme for characterization experiments, single colonies of transformed *Pichia* X-33 (wild type strain) were inoculated in 25 ml pre-culture medium (BMGY, pH 5.6) and incubated at 27°C as described above. After 18 h the cells were switched to 200 ml BMM (buffered minimal medium, pH 5.6) supplemented with 1% casamino acids as protecting agent against proteases [7]. Cells were then grown under aerobic conditions for 24, 48 or 72 h. Again, methanol was replaced every 24 h to a final concentration of 0.5%. After the time defined by the experiment, the culture medium was centrifuged, and the supernatant was concentrated as described above, and equilibrated in 100 mM citric acid-Na_2HPO_4 buffer, pH 5.75. For characterizations, fresh enzyme preparation was used. The rest of the preparation was mixed 1:1 with 98% glycerol, and stored at −20°C (glycerol stock).

For the second time course experiment samples were taken 24, 48 and 72 h after induction, concentrated 60-fold and assayed for invertase and 6-SFT activity (see Section 2.5).

For enzyme characterization, 200 ml culture medium was harvested 48 h after induction, concentrated 400-fold and assayed in triplicate with sucrose, sucrose and glucose, or with sucrose and 1-kestose as described by Duchateau et al. [5].

2.5. Enzyme assays and carbohydrate analysis

1-Kestose substrate was purified from Neosugar-P (Beghin Say, Toulouse, France) by reverse-phase HPLC [3]. Assays were buffered in 100 mM citric acid-Na_2HPO_4, pH 5.75, contained in the enzyme

Table 1
β-Fructosidase (invertase), 1-SST, 6-SST, and 6-SFT activities of the enzyme from the construct P1, incubated with 0.1 M Suc or 1-kestose alone, or together with 0.1 M of an additional substrate

Substrate	Activities (nkat/ml)				
	β-Fructosidase (invertase)	1-SST	6-SST	6-SFT	Total fructosyl transfer
Suc	4.51	0.23	0.30	0.01	5.05
Glc+suc	3.29	0.22	0.19	0.01	3.71
1-Kestose	0.02[a]	–[b]	0.01[a]	0.05[a]	0.08[a]
Suc+1-kestose	2.21	–[b]	0.12	0.82	3.15

The sum of the four activities was taken as total fructosyl transfer activity. Data are given per ml of the enzyme preparation; 1 ml contained 1.9 mg of protein and corresponded to the amount of activity contained in 405 ml *Pichia* culture medium.
[a]Most probably caused by the presence of a small amount of contaminating Suc in the 1-kestose substrate preparation.
[b]Not determined because 1-kestose was added as substrate.

preparation (see Section 2.4). Invertase activity was measured by incubating enzyme preparations with 100 mM sucrose, and measuring fructose. To measure 6-SFT activity, bifurcose was measured after incubation of enzyme with a combination of sucrose and 1-kestose (100 mM each). Carbohydrates produced were analyzed by HPLC and quantified via external standards. A DX300 chromatography system from Dionex (Sunnyvale, CA, USA) was used, equipped with a CarboPac PA-100 anion-exchange column and pulsed amperometric detection [15].

3. Results and discussion

3.1. Construction and sequencing of 6-SFT expression vectors

The 6-SFT cDNA clone B5, as described [4], was used for expression (Fig. 1A). The open reading frame (ORF) of B5 starts at nucleotide (nt) 47 of the cDNA with a putative barley vacuolar targeting signal sequence (21 codons long). The sequence encoding the mature protein stretches from the 68th to the 622th codon (nt 247–1912). Since it was not known whether the barley signal sequence interferes with expression, two constructs were created. The 6-SFT cDNA was modified by PCR mutagenesis to add an *Eco*RI site to the 5′ end and an *Xba*I site to the 3′ end of the sequence encoding the mature 6-SFT protein, giving rise to plasmid P1 when ligated into the secretory plasmid pPICZαC. For construct P2, the same restriction sites, but flanking the entire ORF (including the original barley targeting signal sequence), were introduced into the cDNA.

The newly created 2 kb cDNA fragments were sequenced to check for accidental mutations introduced during the PCR experiment. The sequences obtained did not reveal any differences to the freshly sequenced 6-SFT cDNA.

3.2. Expression of 6-SFT in transformed Pichia pastoris strains

The three *Pichia* strains supplied in the expression kit, X-33 (wild type), GS-115 (*his4*), and KM-71 (Mut^S, *his4*), were transformed with the two plasmids P1 and P2. Together with the corresponding controls (*Pichia* strains transformed with the empty parent plasmid P0), nine combinations of *Pichia* strains and plasmids were created. These nine transformants were tested for 6-SFT activity in a small-scale time course. The cells were first grown in a glycerol medium for 18 h. Then they were switched to a medium containing 0.5% methanol to induce the *AOX1* promoter, which controls the expression of heterologous protein in this system. Samples were taken at defined time points, and 6-SFT activity in the medium was measured by quantifying bifurcose production from the combined substrates sucrose and 1-kestose [5]. During this experiment 6-SFT activity was found in the transformants containing the P1 or P2 plasmids, but not in the P0 control transformants (Figs. 2 and 3). The activity rose with increasing induction time. Fructose production also indicated a high β-fructosidase (invertase) activity in all cases where 6-SFT activity was present. All three strains transformed with the P1 plasmid (carrying the sequence for the mature protein only) produced a much higher activity than the P2 plasmid (expressing the entire 6-SFT ORF, including the signal peptide. See Fig. 3, bottom row). The growth curves show that the differences in activity were not due to differences in biomass (Fig. 3, top row). These data show that 6-SFT, a vacuolar enzyme in plants, is functionally expressed in *P. pastoris* and secreted into the medium. The three transformants of any given strain showed similar growth, independent of the vector they contained.

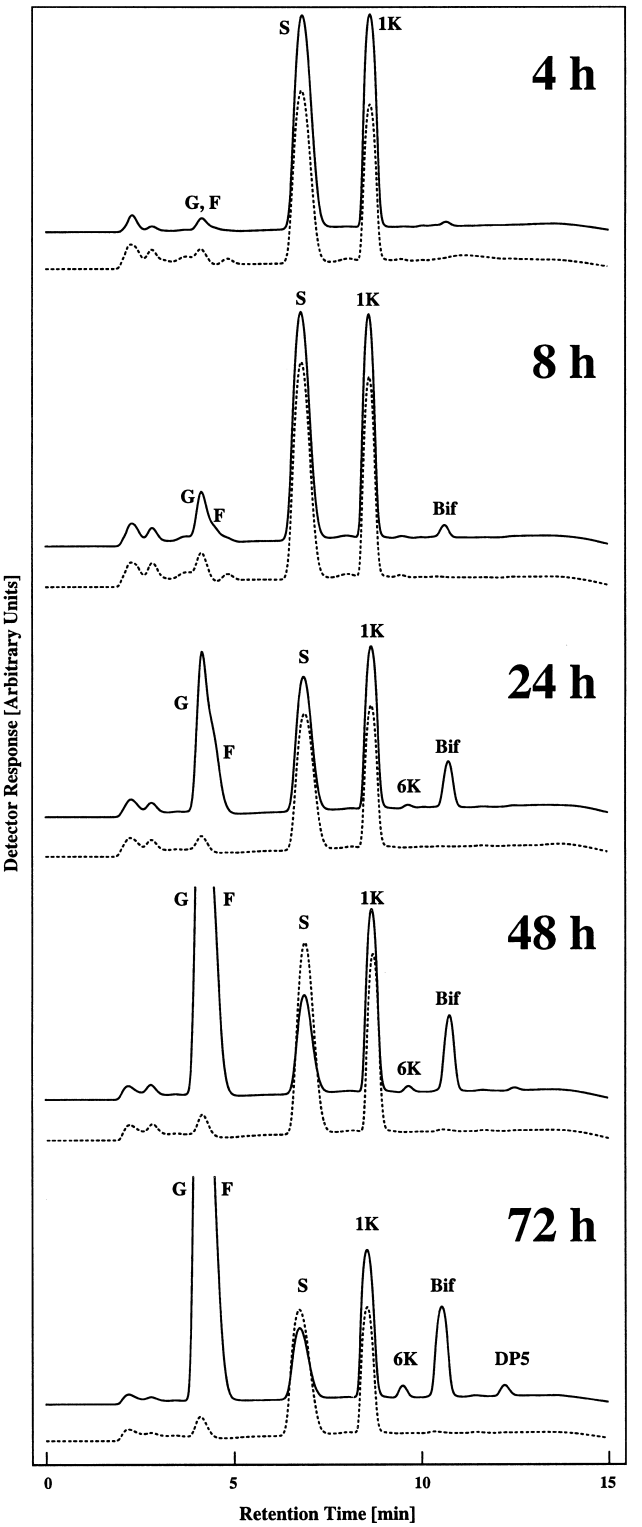

Fig. 2. HPLC analysis of protein activity expressed by the P1 containing *Pichia* strain X-33 over 72 h of incubation. Culture medium was harvested various times after methanol induction. Medium was concentrated, desalted and assayed overnight at 4°C with 50 mM sucrose and 50 mM 1-kestose in 100 mM MES (NaOH) buffer, pH 5.75. Products formed were analyzed by HPLC. The peaks represent glucose and fructose (G and F, peaks not separated), sucrose (S), 1-kestose (1K), 6-kestose (6K), bifurcose (Bif) and DP5 fructan. The dotted line indicates products formed by the control (protein activity from *Pichia* X-33 transformed with the empty parent plasmid P0).

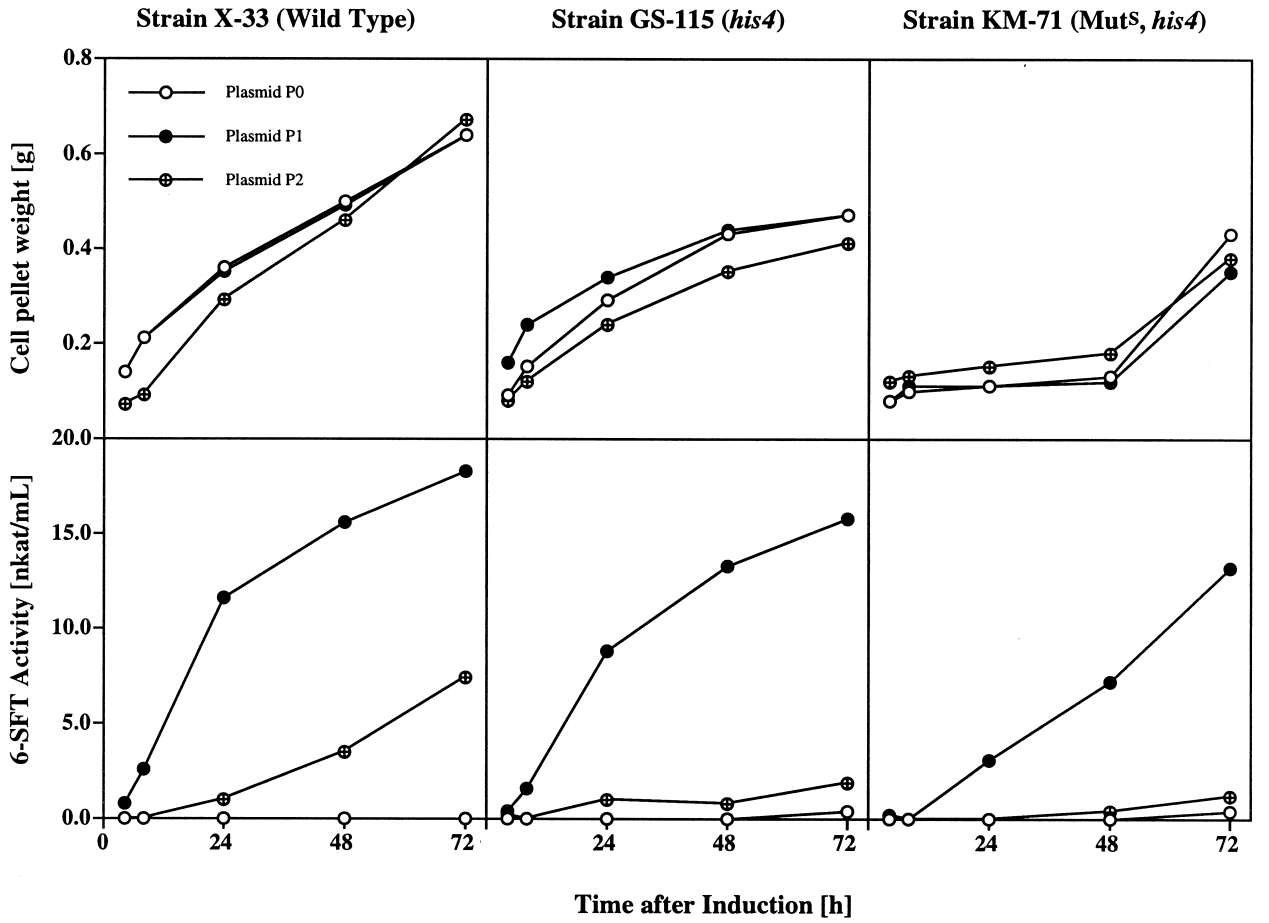

Fig. 3. Growth curves and 6-SFT activities of the *Pichia* strains X-33, GS115 and KM-71 transformed with the plasmids P0 (empty parent plasmid), P1 (construct without barley signal sequence) and P2 (construct containing barley signal sequence) over a time of 72 h. Methanol was replaced at 24 h intervals to a final concentration of 0.5%. At various times after methanol induction 30 ml of culture medium was harvested, concentrated 60× by ultrafiltration, and used for enzyme assays, and the weight of the cell pellet was taken as a measure of cell biomass.

A second time course experiment was carried out under similar conditions. However, only three time points (24, 48 and 72 h after methanol induction) were measured, and only transformants deriving from strain X-33 were used for enzyme production. Further activities were measured in addition to 6-SFT activity by incubating the secreted enzyme with sucrose as a sole substrate. This time the assays were carried out at 27°C for 4 h. Results are shown in Fig. 4. In P1 transformants invertase activity increased in parallel with 6-SFT. The P0 (control) transformants showed neither invertase nor 6-SFT activity, consistent with the results of Sreekrishna et al. [11], who had previously observed that *P. pastoris* cannot use sucrose as the sole carbon source. This indicates that the invertase activity was not secreted by *Pichia*, but rather was an inherent activity of the heterologously expressed 6-SFT, as also described for the purified barley 6-SFT. Both activities of the enzyme produced by P2-transformed *Pichia*, invertase and 6-SFT, were much lower than activities expressed by P1 transformants (data not shown).

The differences in activity of the secreted enzyme between plasmids P1 and P2, observed in both time courses, could be due to improper folding, or the putative barley targeting signal might prevent secretion by interfering with the *Pichia* protein sorting machinery.

3.3. Characterization of enzyme activities produced by P1 transformants

In addition to invertase activity, barley 6-SFT also exhibits 6-SST activity and 6-SFT activity with longer fructan substrates [5].

We characterized the enzyme secreted by *Pichia* strain X-33, expressing the P1 plasmid, and compared it to the known properties [5] of the barley 6-SFT (Table 1). When sucrose was given as a sole substrate, the main products were glucose, fructose and 6-kestose, indicating invertase and 6-SST activity, as in the plant enzyme. However, an additional 1-SST activity, producing 1-kestose, was found in amounts comparable to 6-SST activity. There was also a noticeable shift in activity distribution between invertase and transferases; in barley, 6-SST activity was about 20% of invertase activity, whereas 1-SST and 6-SST combined amounted only to 11% in the recombinant *Pichia* enzyme, when incubated with sucrose.

With barley 6-SFT, invertase activity was reduced by 60% when sucrose was given in combination with glucose. The same phenomenon could be observed with the *Pichia* enzyme, although the decrease in invertase activity was only half as high (27%). 6-SST activity was also reduced (37%, compared to 60% in barley). Interestingly, 6-SST activity also decreased

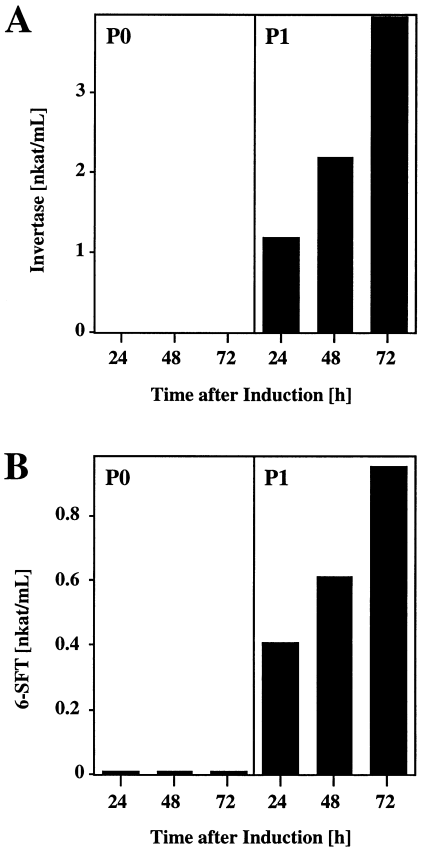

Fig. 4. Enzyme activities of various protein preparations from transgenic *Pichia*: (P0) proteins secreted by the control clone (expressing the empty parent plasmid) into the culture medium; (P1) proteins secreted by the P1 expressing clone (construct without barley N-terminal signal sequence. The medium was harvested 24, 48, and 72 h after methanol induction, and concentrated 60× by ultrafiltration. A: Invertase activity of the above protein preparations. Enzyme was incubated with 100 mM sucrose for 4 h at 27°C, pH 5.75. B: 6-SFT activity of the same protein preparations, incubated with 100 mM sucrose+100 mM 1-kestose under the same conditions.

in both enzymes, but 1-SST in the recombinant enzyme did not. Total fructosyl transfer, the sum of invertase, 1-SST, 6-SST, and 6-SFT activity, was reduced by 27% (barley: 60%).

When the *Pichia* enzyme was incubated with a combination of sucrose and 1-kestose, less invertase activity was found than in assays with sucrose as sole substrate. This reduction in invertase activity had been found for the barley enzyme to be as high as 80%. It was about 50% for the *Pichia* enzyme. 6-SFT activity in the *Pichia* enzyme, however, was still only 37% of the invertase activity, whereas in barley it was 450% of the invertase. Reduction of 6-SST activity was 60% (barley: 84%), and total fructosyl transfer was reduced by 38% (in barley only 11%).

Invertases are known to produce small amounts of 1-kestose when incubated with sucrose [16]. Since the controls did not produce any activity at all, the 1-kestose found in the sucrose assay could not be attributed to an invertase produced by *Pichia*. Thus, unlike the barley 6-SFT, the recombinant enzyme seems to be able to form β(2→1) as well as β(2→6) linkages. A similar change of enzyme specificity has been observed by Tibbot et al. when expressing barley α-glucosidase in *Pichia* [10].

The decrease of invertase or transferase activity in the presence of glucose indicates that a fructosyl transfer to glucose might occur, as has been shown for barley 6-SFT. The sequencing of the PCR fragments used for cloning indicated no difference to the original 6-SFT cDNA. Therefore, the differences in enzyme characteristics described above cannot be caused by a point mutation during PCR. It might be due rather to differences in folding or glycosylation between the *Pichia* and the plant system.

3.4. Conclusions

A functional 6-SFT has been expressed in a heterologous system, *Pichia*. The enzyme had similar properties as the barley enzyme, but there were some substantial differences in detail, most notably the additional 1-SST activity. Although the heterologous system described here is well suited to functionally testing plant cDNAs related to fructosyltransferases, invertases, or fructan hydrolases, care should be taken in analyzing mechanistic details of enzyme activity in the heterologous system.

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