Volume 289, number 1, 64–68 FEBS 10102 September 1991

ADONIS 0014579391008116

Cloning and sequencing of the inulinase gene of *Kluyveromyces marxianus* var. *marxianus* ATCC 12424

Olivier Laloux[1], Jean-Pol Cassart[1], Jean Delcour[1], Jozef Van Beeumen[2] and Jean Vandenhaute[1]

[1]*Lab. Génétique Moléculaire, Facultés Universitaires Notre-Dame de la Paix, rue de Bruxelles 61, B-5000 Namur, Belgium* and [2]*Lab. Microbiologie, Rijksuniversiteit, Ledeganckstraat 35, B-9000 Gent, Belgium*

Received 19 June 1991; revised version received 28 June 1991

Cell wall inulinase (EC 3.2.1.7) was pirified from *Kluyveromyces marxianus* var. *marxianus* (formerly *K. fragilis*) and its N-terminal 33-amino acid sequence was established. PCR amplification of cDNA with 2 sets of degenerate primers yielded a genomic probe which was then used to screen a genomic library established in the YEp351 yeast shuttle vector. One of the selected recombinant plasmids allowed an invertase-negative *Saccharomyces cerevisiae* mutant to grow on inulin. It was shown to contain an inulinase gene (*INU 1*) encoding a 555-amino acid precursor protein with a typical N-terminal signal peptide. The sequence of inulinase displays a high similarity (67%) to *S. cerevisiae* invertase, suggesting a common evolutionary origin for yeast β-fructosidases with different substrate preferences.

Inulinase; Invertase; *Kluyveromyces marxianus* var. *marxianus*; *Kluyveromyces fragilis*

1. INTRODUCTION

Inulinase (EC 3.2.1.7, 2,1-β-D-fructan fructanohydrolase) and invertase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase) are two enzymes able to cleave similar glycosidic linkages, although with different substrate preferences. The preferred substrate of inulinase is inulin, a storage polysaccharide of plant origin in which β-D-fructofuranose residues are linked together by β(2→1) linkages up to a terminal glucose residue, which is linked to fructose by an α(1→2) bond as in sucrose. Inulinases have been isolated from vegetal tissues and from many microorganisms including the yeast *Kluyveromyces marxianus* var. *marxianus* (in short *K. marxianus*), formerly known as *K. fragilis* [1]. Inulinases of different origins display distinct biochemical properties with respect to the mode of hydrolysis (endo or exo) and to their ability to hydrolyze substrates other than inulin. Inulinases from yeasts, in particular, are able to also hydrolyze sucrose very efficiently. Invertase from *Saccharomyces cerevisiae*, on the other hand, preferentially cleaves sucrose, but can also hydrolyze inulin at low efficiency [1]. Thus, invertase and inulinase from yeasts are functionally very similar, since they differ only by their relative rates of sucrose versus inulin hydrolysis (S/I ratio), and thus their status as separate enzymes has been a matter of debate [2]. Recently, Rouwenhorst et al. [3] claimed that they are indeed distinct enzymes. Their conclusion was based upon kinetic evidence as well as on the lack of homology between their reported 20-amino acid N-terminal sequence of *K. marxianus* inulinase and the corresponding region of *S. cerevisiae* invertase. In this paper, we report the complete genomic sequence of inulinase from *K. marxianus* showing, to the contrary, that yeast invertase and inulinase display a high level of evolutionary conservation.

Correspondence address: J. Vandenhaute, Lab. Génétique Moléculaire, Facultés Universitaires Notre-Dame de la Paix, rue de Bruxelles 61, B-5000 Namur, Belgium. Fax: 32 81 230391.

The nucleotide *INU1* sequence presented here has been submitted to the EMBL/GenBank database under the accession no X57202.

2. MATERIALS AND METHODS

2.1. *Culture conditions*

Yeasts were maintained on YEPD (10 g yeast extract, 20 g peptone, 20 g glucose, each per liter of demineralized water) agar plates. Batch cultures were done at pH 5 and 30°C in shaker flasks containing either YEPD or minimal medium (per liter: 1.75 g yeast nitrogen base without amino acids nor ammonium sulfate, 5 g ammonium sulfate, 25 mg amino acids corresponding to the auxotrophies, 20 g glucose or inulin). For inulinase purification and RNA preparation, an inducing YEPD medium (containing 2% inulin instead of glucose) was used. Bacterial strains were grown on Luria-Bertani medium [4] at 37°C with ampicillin (100 μg/ml) if needed.

2.2. *Inulinase purification*

Cell wall inulinase was purified from *K. marxianus* (ATCC 12424) by a modification of the method of Grootwassing [5]. Cells harvested at the end of the exponential phase were resuspended in 300 mM sodium acetate buffer (pH 7.5) containing 8 mM cystein, at a concentration of 1 g cells (fresh weight) for 10 ml buffer. After 3 h shaking at 30°C, cells were pelleted and the supernatant was recovered, concentrated by lyophilization, resuspended in 30 ml buffer A (50 mM sodium acetate, pH 7.5) and dialyzed extensively at 4°C against the same buffer. The dialyzed extract was applied onto a column (1 × 10 cm) of DEAE-Sepharose CL-6B (Pharmacia) previously equilibrated with buffer A. After washing with buffer A, inulinase was eluted by application of 60 mM NaCl in the same buffer. Active fractions were pooled and stored at −80°C until further use. For cell fractionation

 Published by Elsevier Science Publishers B.V.

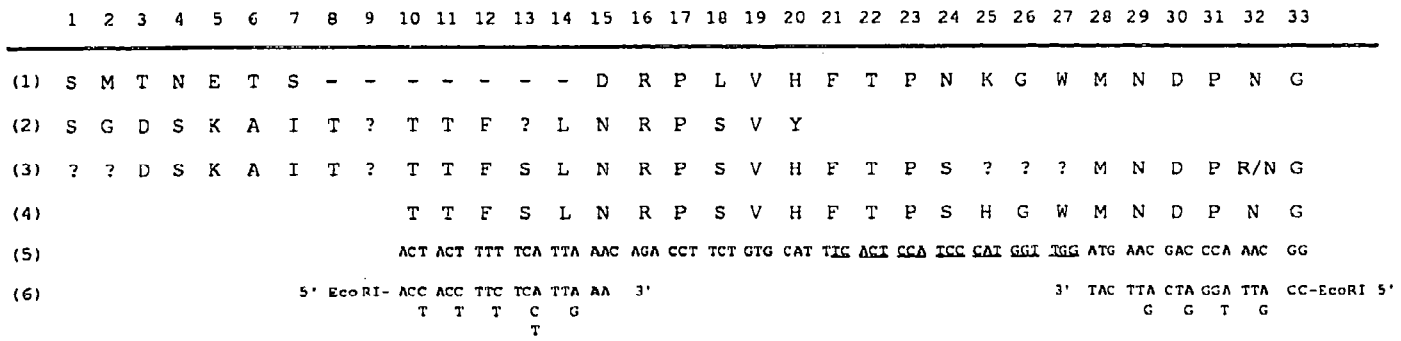

Fig. 1. N-terminal amino acid sequences of *S. cerevisiae* mature invertase (line 1, [18]) and *K. marxianus* mature inulinase (line 2, [3]; line 3, microsequencing, this work; line 4, translation of the PCR-amplified nucleotide sequence) aligned with the nucleotide sequences of the PCR-amplified fragment (line 5, 20-mer oligonucleotide probe used for cloning underlined) and the corresponding degenerate primers extended by *Eco*RI linkers (line 6).

studies, three successive washings were performed and the pooled supernatants were used as the enzyme extract. Supernatant and cell-bound enzyme were prepared according to Rouwenhorst et al. [6]. Protein concentration was measured by the Lowry method [7]. Inulinase or invertase activity was measured by the release of reducing sugars according to Halliwell [8]. One unit of activity was defined as one μmol of reducing sugars produced by min.

2.3. *Carbohydrate analysis*

Sugar content was measured by the orcinol–sulfuric acid method of Dische [9]. Deglycosylation was performed using 0.2 U of endoglycosidase F–glycopeptidase F (Boehringer) per 25 μg of lyophilized enzyme resuspended in 25 μl buffer (50 mM sodium acetate pH 7.0, EDTA 20 mM, SDS 0.1%, Triton X-100 0.5%, β-mercaptoethanol 1%) and incubated for 48 h at 37°C.

2.4. *PAGE analysis*

SDS-PAGE was performed in slab gels according to Laemmli [10]. Gels were stained with Coomassie-blue. Non-denaturing electrophoresis was performed on 5% acrylamide gels in Tris-acetate buffer 100 mM, pH 7.2. In situ detection of enzymatic activity was performed according to Beck and Praznik [11] for inulinase, and to Grossmann and Zimmermann [12] for invertase.

2.5. *Protein microsequencing*

About one nanomole of purified cell wall inulinase was electroblotted from a SDS-PAGE gel onto a polyvinylidene difluoride membrane using 50 mM Tris–50 mM boric acid as transfer buffer. Sequence analysis of the Coomassie blue-stained band was carried out on a 477A pulsed liquid gasphase sequencer with on-line analysis of the phenyl thiohydantoin amino acids on a 120A Analyser (Applied Biosystem, USA).

2.6. *Recombinant DNA techniques*

Routine recombinant DNA techniques were performed following instructions given by Sambrook et al. [4] using the *Escherichia coli* strain *XL 1-blue* (Stratagene). The *K. marxianus* genomic library was made in the *E. coli-S. cerevisiae* shuttle vector YEp351 [13] using *Sau*3A-partially digested total DNA cloned into the vector *Bam*HI site after sucrose gradient purification of 10-kb fragments. Sequencing was performed on denatured plasmid DNA with the T7 sequencing kit (Pharmacia) using synthetic oligonucleotides. Yeast transformation (strain ATCC 20598, invertase negative) was done by the method if Ito et al. [14].

2.7. *Polymerase chain reaction*

The 50-μl reaction contained 5 μg of Poly (A)$^+$ RNA, 50 mM KCl, 6 mM $MgCl_2$, 10 mM Tris-HCl pH 8.4, 4 dNTP (each 1 nM), 1 μl Perfect Match polymerase enhancer (Stratagene) and 10 ng oligonucleotides from the reverse pool (see Fig. 1) to prime the reaction. Incubation parameters were set as follows: 10 min denaturation at 70°C, 30 min annealing at 35°C and, after addition of 3 U of AMV reverse transcriptase (Boehringer Mannheim), 30 min elongation increasing gradually the temperature from 35°C to 42°C. Reverse transcriptase was inactivated by 5 min heating at 100°C. Amplification was performed by adding 1 μg oligonucleotides from each degenerate pool and 5 U of Taq DNA Polymerase (Cetus) and incubating with the following cycle parameters: the first 3 cycles started with a step of template denaturation (92°C, 15 s), primer annealing (37°C, 15 s) and primer extension (72°C, 20 s); the following cycles were identical, except for the primer annealing step performed at 50°C instead of 37°C; a total of 20 cycles were carried out before the final extension step (72°C, 5 min).

Table I

Distribution of inulinase activity in donor and transgenic strains

	Total inulinase activity ($U \cdot mg^{-1}$)[a]	Culture medium	Distribution (%)	
			Cell wall wash	Cell pellet lysate
K. marxianus var. *marxianus* ATCC 12424	1.2	39	60	1
S. cerevisiae ATCC 20598 (pGIOL-01) Exp.A	0.4	64	35	1
S. cerevisiae ATCC 20598 (pGIOL-01) Exp.B	0.36	76	22	2
S. cerevisiae ATCC 20598 (YEp351) Control	0.035	0	100	0

[a]Total inulinase activities are expressed in U (see section 2) per mg of cells (dry weight).

3. RESULTS AND DISCUSSION

3.1. *Partial purification and characterization of cell wall inulinase*

Cell wall inulinase was recovered from *K. marxianus* cells and purified by anion-exchange chromatography as described in section 2. The enzyme eluted as two overlapping peaks following application of 60 mM NaCl. Active fractions from both peaks were pooled separately. SDS-PAGE analysis clearly showed that these two pools contained a highly purified protein visible as a large, diffuse band which was authenticated as inulinase by detection of both invertase and inulinase activities on non-denaturing gel electrophoresis (data not shown). The diffuse aspect of inulinase on PAGE is not surprising since this enzyme is known to be extensively glycosylated [3]. The sugar content measured on four independent enzyme preparations was 36 ± 3% for the first pool and 27 ± 3% for the second, this difference could easily account for the differential elution of the two fractions from the DEAE column. After enzymatic deglycosylation, the apparent molecular weight of both enzyme preparations was reduced by 30% down to a value of about 60 kDa; like yeast invertase [15], deglycosylated inulinase was still active (data not shown).

3.2. *N-terminal protein sequences*

Purified inulinase (pool 1) was used to establish the sequence of a stretch of N-terminal amino acids. Over a total length of 33 amino acids, 27 were identified. It can be seen (Fig. 1) that our sequence is in agreement with the 20-amino acid sequence reported by Rouwenhorst et al. [3] except at residue 20 where we found H instead of Y. As already emphasized by these authors, the 20-amino acid N-terminal segment of the mature inulinase does not resemble that of yeast invertase. However, comparison with our extended 33-amino acid sequence strongly suggested that these two yeast β-fructosidases might be closely related. When residue 15 of inulinase was set in frame with residue 8 of invertase, a box of strong similarity appeared. This point will be discussed further below.

3.3. *Cloning of the inulinase gene*

Two sets of degenerate PCR primers were derived from the N-terminal protein sequence, spanning residues 10–15 on the left and 28–33 on the right (Fig. 1). PCR amplification of *K. marxianus* first strand cDNA yielded a 71-bp genomic DNA fragment which was subcloned and sequenced. As can be seen in Fig. 1 the amplified sequence was truly that of inulinase. On the basis of the amplified nucleotide sequence, a 20-mer homologous oligonucleotide probe was synthesized and used to screen a *K. marxianus* genomic library by colony hybridization. One of the positive clones was shown to contain a plasmid (named pGIOL-01) with a 10 kb insert, which was used to transform an invertase-nega-

```
   1 TCGAATCCCATAAGTGACACTTTTTTTTTGTTTTATCAATTTAGTTCG
  49 AGATGAAGTTCGCATACTCCCTCTTGCTTCCATTGGCAGGAGTCAGTG
       M  K  F  A  Y  S  L  L  L  P  L  A  G  V  S  A
  97 CTTCAGTGATCAATTACAAGAGAGATGGTGACAGCAAGGCCATCACTA
       S  V  I  N  Y  K  R  D  G  D  S  K  A  I  T  N
 145 ACACCACTTTTAGTTTGAACAGACCTTCTGTGCATTTCACTCCATCCC
       T  T  F  S  L  N  R  P  S  V  H  F  T  P  S  H
 193 ATGGTTGGATGAACGATCCAAATGGTTTGTGGTACGATGCCAAGGAAG
       G  W  M  N  D  P  N  G  L  W  Y  D  A  K  E  E
 241 AAGACTGGCATTTGTACTACCAGTACAACCCAGCAGCCACGATCTGGG
       D  W  H  L  Y  Y  Q  Y  N  P  A  A  T  I  W  G
 289 GTACTCCATTGTACTGGGGTCACGCTGTTTCCAAGGATTTGACTTCCT
       T  P  L  Y  W  G  H  A  V  S  K  D  L  T  S  W
 337 GGACAGATTACGGTGCTTCTTTGGGCCCAGGTTCCGACGACGCTGGTG
       T  D  Y  G  A  S  L  G  P  G  S  D  D  A  G  A
 385 CGTTCAGTGGTAGTATGGTTATCGATTATAACAATACTTCTGGTTTCT
       F  S  G  S  M  V  I  D  Y  N  N  T  S  G  F  F
 433 TCAACAGCTCTGTGGACCCAAGACAAAGAGCAGTTGCAGTCTGGACTT
       N  S  S  V  D  P  R  Q  R  A  V  A  V  W  T  L
 481 TGTCTAAGGGCCCAAGCCAAGCCCAACACATCAGTTACTCATTGGACG
       S  K  G  P  S  Q  A  Q  H  I  S  Y  S  L  D  G
 529 GTGGTTACACCTTCGAGCACTACACCGACAACGCCGTGTTGGACATCA
       G  Y  T  F  E  H  Y  T  D  N  A  V  L  D  I  N
 577 ACAGCTCCAACTTCAGAGACCCTAAGGTGTTCTGGCACGAGGGCGAGA
       S  S  N  F  R  D  P  K  V  F  W  H  E  G  E  N
 625 ACGGCGAAGATGGTCGTIGGATCATGGCCGTTGCTGAATCGCAAGTGT
       G  E  D  G  R  W  I  M  A  V  A  E  S  Q  V  F
 673 TCTCTGTGTTGTTCTACTCTTCTCCAAACTTGAAAAACTGGACCTTGG
       S  V  L  F  Y  S  S  P  N  L  K  N  W  T  L  E
 721 AATCCAACTTCACCCACCACGGCTGGACTGGTACCCAATACGAATGTC
       S  N  F  T  H  H  G  W  T  G  T  Q  Y  E  C  P
 769 CAGGTCTAGTTAAGGTTCCATACGACAGTGTTGTTGACTCTTCGAACT
       G  L  V  K  V  P  Y  D  S  V  V  D  S  S  N  S
 817 CCTCCGACTCCAAGCCAGACTCCGCATGGGTCTTGTTTGTCTCTATCA
       S  D  S  K  P  D  S  A  W  V  L  F  V  S  I  N
 865 ACCCTGGTGGTCCATTGGGTGGTTCCGTTACCCAATACTTTGTTGGTG
       P  G  G  P  L  G  G  S  V  T  Q  Y  F  V  G  D
 913 ACTTCAACGGTACTCACTTCACTCCAATCGACGGCCAAACCAGATTCC
       F  N  G  T  H  F  T  P  I  D  G  Q  T  R  F  L
 961 TAGACATGGGTAAGGACTACTACGCACTACAAACTTTCTTCAACACTC
       D  M  G  K  D  Y  Y  A  L  Q  T  F  F  N  T  P
1009 CAAACGAGAAGGACGTCTACGGTATCGCATGGGCTTCTAACTGGCAAT
       N  E  K  D  V  Y  G  I  A  W  A  S  N  W  Q  Y
1057 ACGCCCAACAAGCCCCAACTGACCCATGGCGTTCATCTATGAGTTTGG
       A  Q  Q  A  P  T  D  P  W  R  S  S  M  S  L  V
1105 TTAGACAATTCACATTGAAAGACTTCAGCACAAACCCTAACTCCGCTG
       R  Q  F  T  L  K  D  F  S  T  N  P  N  S  A  D
1153 ATGTCGTCTTGAACAGTCAACCAGTCTTGAACTATGATGCATTGAGAA
       V  V  L  N  S  Q  P  V  L  N  Y  D  A  L  R  K
1201 AGAACGGTACCACTTACAGTATCACAAACTACACCGTCACCTCCGAAA
       N  G  T  T  Y  S  I  T  N  Y  T  V  T  S  E  N
1249 ACGGCAAGAAGATCAAGCTAGACAACCCATCCGGTTCTCTTGAATTCC
       G  K  K  I  K  L  D  N  P  S  G  S  L  E  F  H
1297 ATCTTGAATACGTGTTTAACGGCTCCCCAGATATCAAGAGCAACGTGT
       L  E  Y  V  F  N  G  S  P  D  I  K  S  N  V  F
1345 TCGCTGATCTTTCCTTGTACTTCAAGGGTAACAACGACGACAACGAAT
       A  D  L  S  L  Y  F  K  G  N  N  D  D  N  E  Y
1393 ACTTGAGATTGGGTTACGAAACCAACGGTGGTGCCTTCTTCTTGGACC
       L  R  L  G  Y  E  T  N  G  G  A  F  F  L  D  R
1441 GTGGCCACACCAAGATTCCTTTCGTGAAGGAGAACTTGTTCTTCACCC
       G  H  T  K  I  P  F  V  K  E  N  L  F  F  T  H
1489 ACCAATTGGCAGTTACCAACCCAGTTTCCAACTACACCACAAACGTCT
       Q  L  A  V  T  N  P  V  S  N  Y  T  T  N  V  F
1537 TCGACGTTTACGGTGTCATTGACAAGAACATCATCGAATTGTACTTCG
       D  V  Y  G  V  I  D  K  N  I  I  E  L  Y  F  D
1585 ATAACGGTAACGTCGTCTCCACCAACACTTTCTTCTCTACCAACA
       N  G  N  V  V  S  T  N  T  F  F  F  S  T  N  N
1633 ACGTTATTGGTGAAATTGACATCAAGTCGCCATACGACAAGGCTTACA
       V  I  G  E  I  D  I  K  S  P  Y  D  K  A  Y  T
1681 CCATTAACTCATTTAACGTTACCCAATTTAACGTTTGATCTGATCTGC
       I  N  S  F  N  V  T  Q  F  N  V  ***
1729 TTACTTTACTTAACGACCAAAGAAAAAACGACAAAA
```

Fig. 2. Nucleotide sequence of the *INU1* gene and its encoded protein. Arrows indicate putative cleavage sites for the signal peptide. Arrowhead points to the N-terminus of the cell wall enzyme.

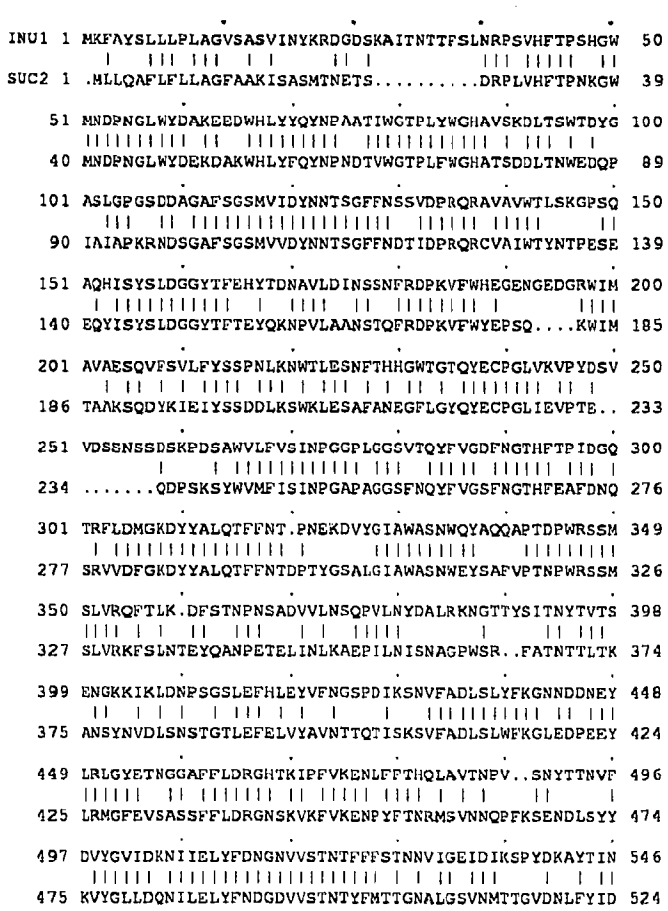

Fig. 3. Amino acid sequence comparison (GAP program, GCG, University of Wisconsin) between the *K. marxianus* INU1 inulinase (this work) and the *S. cerevisiae* SUC2 invertase [18]. Amino acid sequences have been deduced from nucleotide sequences.

tive mutant of *S. cerevisiae*. The transformants obtained were able to rapidly grow on a medium containing inulin as the sole carbon source, indicating that a functional structural gene encoding inulinase was expressed in the transgenic host. Enzymatic assays performed on fractionated cultures clearly demonstrated (Table I) that yeast transformants were producing and secreting inulinase at high level.

3.4. *DNA sequence analysis*

The recombinant plasmid was used to determine the sequence of the *K. marxianus* inulinase gene. Sequencing was initiated with the 20-mer probe for cloning and was contained by the primer walking strategy. The nucleotide sequence of the gene (named *INU1*) together with the amino acid sequence of the encoded protein are given in Fig. 2. The precursor protein is a 555-amino acid polypeptide displaying a typical signal peptide flanked by 3 consecutive putative cleavages sites as predicted by the '-3-1 rule' [16] (arrows, Fig. 2). The actual N-terminus (arrowhead, Fig. 2) of the mature cell wall enzyme (predicted molecular weight of 59 672 Da) does not coincide with any of these sites, but is located 6, 7 or 8 amino acids further down. This indicates that following secretion inulinase undergoes further proteolysis, as also observed with *Bacillus subtillis* α-amylase [17].

3.5. *Evolutionary comparison*

The *INU1* inulinase displays a high level of evolutionary conservation with the SUC2 invertase of *S. cerevisiae* [18]. Comparison of the amino acid sequences (Fig. 3) revealed 68% of similarity, a value very close to the 63% similarity observed between *S. cerevisiae* and *Schwanniomyces occidentalis* [19] invertases. Therefore, in contrast with recent claims [3] based upon limited protein sequence comparison, it seems reasonable to consider yeast invertase and inulinase as members of the same family of β-fructosidases differing by their degree of preference for sucrose versus inulin.

Acknowledgements: Part of this work was supported by the Belgian National incentive-program on fundamental research in Life Sciences initiated by the Belgian Science Policy Programming Department (Contract BIO 22). Olivier Laloux holds a specialization bursary from IRSIA. We are very grateful to F. Gendre, Gerbaud, R. Kettmann and P. Michels who provided help and encouragement for the realization of this work. The excellent technical assistance of R.-M. Genicot is acknowledged.

REFERENCES

[1] Vandamme, E.J. and Derycke, D.G. (1983) Adv. Appl. Microbiol. 29, 139–176.
[2] Arnold, W.N. (1987) in: Yeast biotechnology, (Berry, D.R., Russel, I. and Stewart, G.G., eds.) pp. 369–391, 2nd ed, Allen and Unwin, London.
[3] Rouwenhorst, R.J., Hensing, M., Verbakel, J., Scheffers, W.A. and Van Dijken, J.P. (1990) Appl. Environ. Microbiol. 56, 3337–3345.
[4] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[5] Lam, K.S. and Grootwassing, J.W.D. (1985) Enzyme Microb. Technol. 7, 239–242.
[6] Rouwenhorst, R.J., Visser, L.E., Van Der Baan, A.A., Scheffers, W.A. and Van Dijken, J.P. (1988) Appl. Environ. Microbiol. 54, 1131–1137.
[7] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[8] Halliwell, G. (1961) Biochem. J. 79, 185–192.
[9] Dische, Z. (1953) J. Biol. Chem. 204, 983–997.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Beck, R.H.F. and Praznik, W. (1986) J. Chromatogr. 369, 240–243.
[12] Grossmann, M.K. and Zimmermann, F.K. (1979) Mol. Gen. Genet. 175, 223–229.
[13] Hill, J.E., Myers, A.M., Koerner, T.J. and Tzagoloff, A. (1986) Yeast 2, 163–167.
[14] Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[15] Gascon, S. and Lampen, J.O. (1968) J. Biol. Chem. 243, 1567–1572.
[16] Von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690.

[17] Takase, K., Mizuno, H. and Yamane, K. (1988) J. Biol. Chem. 263, 11548–11553.
[18] Taussig, R. and Carlson, M. (1983) Nucleic Acids Res. 11, 1943–1954.
[19] Klein, R.D., Poorman, R.A., Favreau, M.A., Shea, M.H., Hatzenbuhler, N.T. and Nulf, S.C. (1989) Curr. Genet. 16, 145–152.