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Low level of glycosylation of invertase secreted by methylotrophic yeast *H. polymorpha*

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SUMMARY

A purification methodology of external invertase secreted by genetically engineered methylotrophic yeast *H. polymorpha* is described. It was possible to determine that recombinant invertase is less glycosylated than the native external invertase from *S. cerevisiae*. However, it is accumulated almost all in the periplasmic space (about 80%) and only a minor quantity is secreted to the culture media, suggesting a secretion pathway similar to the used in *S. cerevisiae*.

RESUMEN

Se describe una metodología para la purificación de la invertasa externa secretada por una levadura metilotrófica *H. polymorpha*, modificada por ingeniería genética, lo cual permitió su comparación con la invertasa producida por *S. cerevisiae*; sin embargo, alrededor del 80% de ésta es acumulada en el espacio periplasmático, mientras que una mínima cantidad es secretada al medio de cultivo, sugiriendo una vía de secreción similar a la de *S. cerevisiae*.

INTRODUCTION

SUC_2, a single structural gene is well known to encode both secreted glycosylated and cytoplasmic unglycosylated invertase (b-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26) in *S. cerevisiae*. Both invertases differing by a secretion signal sequence, are encoded by separate mRNA (Perlan *et al.*, 1981; Perlan *et al.*, 1984).

The molecular structure of secreted invertase which resides in the periplasmic space consists of a 60 kDa protein subunit to which a variable number of heterogeneous oligosaccharides are N-glycosydically linked (Trimble *et al.*, 1977). The quaternary association of this enzyme has been recognized as a dimer, however in earlier stages the octamer predominates (Chu *et al.*, 1983; Tammi *et al.*, 1987; Esmon *et al.*, 1987); this effect has been associated with its location inside the cell (Esmon *et al.*, 1987).

Concerning the role of the glycosylation for the structure and function of this enzyme, there are different criteria. However, Schulke and Schmed (1988) refer that the mechanisms of renaturation and stability of yeast invertase is not significantly affected by the presence or not of oligosaccharides.

Tschopp *et al.* (1987) transformed the SUC_2 gene from *S. cerevisiae* into the methylotrophic yeast *P. pastoris* under

the control of the alcohol oxidase (aox 1) promoter which resulted in an efficient secretion of biologically active invertase up to 2.5 g/l of growth medium after 250 hours of methanol induction.

This construction has the particularity that the invertase was secreted not only to the periplasmic space (10-20%) but also to the culture medium and with a lower degree of glycosylation than *S. cerevisiae* which was related to the existence of only small oligosaccharide side chains attached to the protein.

A similar result was obtained by Rodríguez *et al.* (1989), that succeed in transforming the methylotrophic yeast *H. polymorpha*, with the SUC_2 gene from *S. cerevisiae* with a secretion level of active invertase higher than 1.0 g/l of culture but remaining almost all the enzyme in the periplasmic space with significant lower molecular weight with a narrower distribution, than the invertase from *S. cerevisiae.*

In this article a scheme of purification of the external invertase from *H. polymorpha* is described, which lead to a highly pure invertase. From this it was possible to establish the principal features that differences both invertase from wild type *S. cerevisiae* and induced invertase secreted by the methylotrophic yeast.

MATERIALS AND METHODS

Analytical procedure

Chromatographic effluents were monitored at 280 nm with an LKB Uvicord S-2138. Protein was quantitated by Lowry *et al.* (1951) procedure with bovine serum albumine as standard. Electrophoretic mobility (SDS-PAGE) of denatured invertase was determined in 12% SDS-polyacrylamide gel (Laemmli, 1970) staining with Coomassie blue.

For invertase assays, samples (100 μl) were incubated with 400 μl 0.1 M sodium phosphate buffer, pH 5 and 500 μl 15% sucrose solution during 10 minutes at 60°C. The invertase activity unit is defined as the amount of the enzyme needed to hydrolyze 1 mmol of sucrose per minute at 60°C in sodium phosphate buffer, pH 5. The reaction was stopped introducing the samples into boiling water during 5 min then to the incubation reaction mixture 1 ml of dinitro salicylic acid (DNSA) reagent was added and boiled 10 min for reducing sugar determination. Carbohydrate content in samples was determined by Anthrone-Sulfuric acid method.

Analysis of invertase oligomers was done by gel filtration chromatography HPLC, was carried out on a TSK-3000 (TOYO-SODA) column (7.5 x 600 mm) with 0.2 M ammonium acetate, 0.2 M sodium chloride pH 6.5 as eluent. Samples were initially dissolved in the carrier buffer, frozen and then thawed and allowed to stand at room temperature, 200 μl (1 mg protein/ml) were injected each time.

Strains and growth conditions

An Ura strain of *H. polymorpha* transformed with the plasmid pAS-24 was used. The plasmid contains the SUC_2 coding region of *S. cerevisiae*, the regulated aox promotor of *P. pastoris*, an URA_3 gene, an autonomous replicating sequence (HARS) and the β-lactamase gene from *E. coli* is present (Rodríguez *et al.*, 1989). A wild type *S. cerevisiae* strain was used as a control, it was grown at 30°C in a culture medium that contains 40 g/l of reducing sugars from final molasses of the sugar industry, 8 g/l $(NH_4)_2SO_4$ and 2 g/l K_2HPO_4. The pH was adjusted to 4.5 and maintained during the fermentation. The modified strain was grown at 37°C in an inorganic salt base medium KH_2PO_4 5 g/l $(NH_4)_2SO_4$ 4 g/l, KCl 0.5 g/l, glycerol 2%, reducing sugar from final molasses 2% and traces elements. The pH was adjusted to 5.5 and maintained during the fermentation. Methanol 0.5% was added during the next 80 hours after the exhaustion of reducing sugars in the culture medium, resulting in an overall production of 35 g (dry weight)/l of biomass and more than 1 000 U/ml.

Deglycosylation of invertase

Pure invertase from *S. cerevisiae* or *H. polymorpha* (1 500 U) was dissolved in 0.1 M sodium citrate buffer pH 5.5 containing 0.02% sodium dodecyl sulfate and heated 7 minutes at 100°C. After cooling to room temperature, 20 mU of Endo β-N acetyl

glucosaminidase H (Endo H) were added, (Endo H concentration 40 mU/ml) and then incubated 16 hours at 37°C after which it is necessary 1 or 2 cycles of treatment with another 10 mU. Samples for SDS-PAGE were heated at 100°C during 10 minutes in presence of 0.5% β-Mercaptoethanol.

Isolation of external invertase

The cell paste from 200 ml culture was suspended in 50 ml of 62.5 mM Tris-HCl, 5 mM EDTA and 2 mM Phenylmethylsulfonyl fluoride pH 8.7 and homogenized either by a Braun homogenizer (5 times for 1 minute each of a 35% cell paste suspension in a 1:1 v/v ratio of liquid to glass beads) or by Biospec Bead Beater (5 times for 1 minute each of 35% cell paste suspension in a 1:2 v/v ratio of liquid to beads).

The homogenate was centrifuged for 30 minutes at 10 000 rpm, the precipitate was washed with 1 volume of the same buffer and the supernatant was mixed; then 1/3 of the total volume of acetone was added and allowed to precipitate at 4°C overnight.

After centrifugation at 10 000 rpm for 20 minutes the precipitate was discarded; then 1 volume of cold ethanol was added to the clear supernatant and allowed to precipitate at least for 4 hours at 4°C, centrifuged at 3 000 rpm and the supernatant discarded. The precipitate was resuspended in 10 mM ammonium acetate pH 6.5, the insoluble material was eliminated and the clear solution was lyophilized or washed several times with acetone and allowed to dry at room temperature giving a white soluble powder (Product I).

Product I was dissolved in 0.1 M sodium chloride (50 mg/ml) and applied to a Sephacryl S-300 (Pharmacia Fine Chemicals) column (2 x 45 cm) previously equilibrated with the same bufffer, the first 58 ml were discarded, the eluent was continuously monitored at 280 nm and fractions of 3 ml were collected, to each one determination of invertase activity and carbohydrate content was made. The fractions with invertase activity were pooled and ultrafiltrated on an Amicon PM-30 filter and washed several times with distilled water. The concentrate was then lyophilized (II).

The lyophilized II once dissolved in 10 mM ammonium acetate buffer pH 6.5 was chromatographed in a Mono Q HR 5/5 FPLC column. Elution was accomplished with a gradient from 20 mM to 1 M of ammonium acetate pH 6.5 in 15 minutes at flow rate of 0.5 ml/min. The peak with invertase activity was rechromatographed, the final product showed to be pure and homogeneous. The pooled fractions were finally lyophilized. The recovery of external invertase was about 30% of the total activity detected in the fresh culture after the fermentation step.

RESULTS AND DISCUSSION

Rodríguez *et al.* (1989) recently demonstrated that the expression of invertase in *H. polymorpha* can be regulated by the aox regulatory sequence from *P. pastoris*, resulting in a protein secretion of more than one order of magnitude respect to the level reported for *S. cerevisiae* (Trimble *et al.*, 1977). This system has the particularity that the secreted enzyme has lower glycosylation degree (Zeigier *et al.*, 1988). Furthermore about 80% of it is accumulated in the periplasmic space.

Enzyme purification

To release the enzyme from the cell it was necessary to homogenize the cell paste by a Bead Beater or Braun homogenizer with more than 90% of initial activity recovery. Once eliminated the cellular debris and acetone clarified, it was possible to obtain a soluble white powder, after precipitation with one volume of ethanol, without enzyme inactivation.

Further purification was achieved with sequential gel filtration chromatography (GFC) on Sephacryl S-300 (Fig. 1) and anion exchange chromatography on Mono Q FPLC (Fig. 2A). By means of the GFC on Sephacryl S-300 it was possible to eliminate not only a high amount of other proteins but also carbohydrates of high molecular weight that were

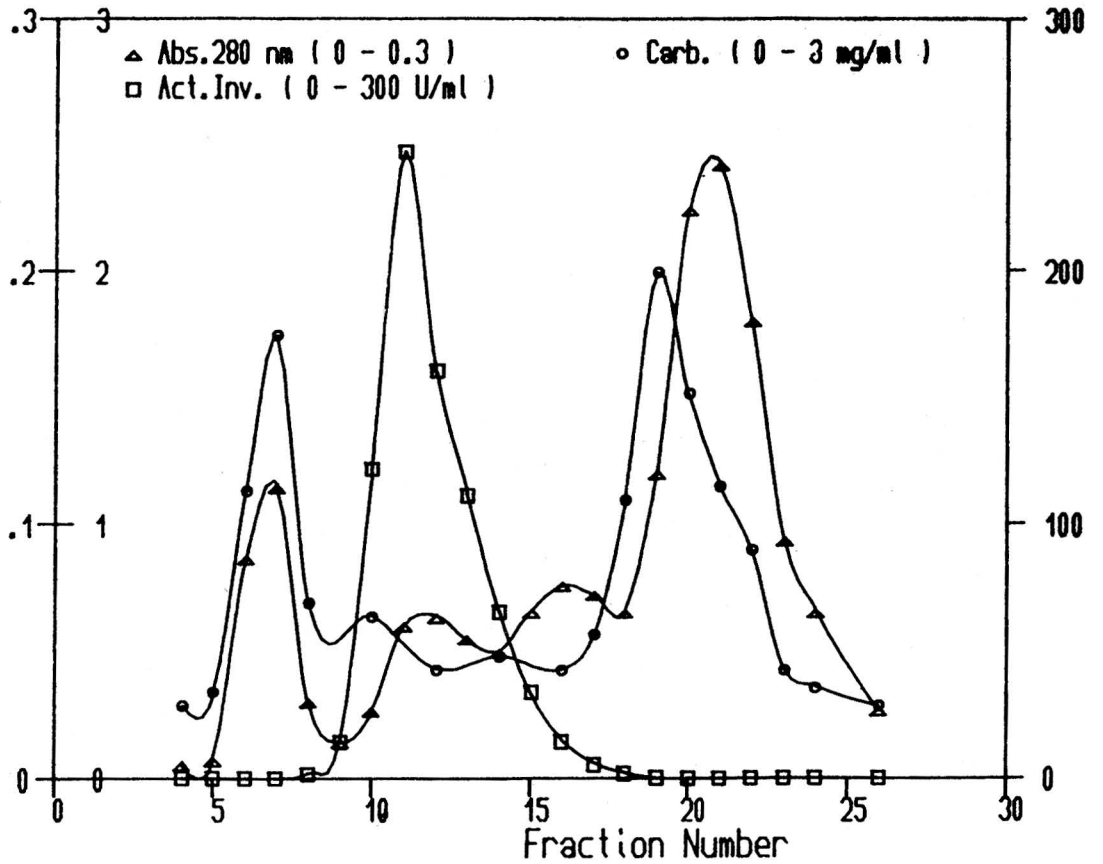

FIG. 1. Gel filtration chromatography of crude external invertase from *H. polymorpha* on analytical Sephacryl S-300 column. Experimental conditions are described in the text. 400 mg of the Product I was dissolved in 2.5 ml of the elution buffer. The fractions (3 ml) were started to collect after the Void volume (50 ml). The eluate was monitored at 280 nm, in each sample were determined the invertase activity and carbohydrate content.

unattached to the protein. A portion of carbohydrate that still remains as impurity was separated in the Mono Q column resulting in a product with only 25-30% of glycosylation and specific activity of 2 800 U/mg of protein.

However, an unexpected difference was detected when a highly glycosylated external invertase from *S. cerevisiae* was run in the Mono Q FPLC column (Fig. 2B). It was nearly not retained by the matrix suggesting that at pH 6.5 there is not a real interaction between the glycoprotein and the anion exchanger regardless of the fact that it has a very acidic isoelectric point (result not shown). This apparent contradiction might be attributed to the size and conformation of the predominant oligomer (Tammi *et al.*; Esmon *et al.*, 1987).

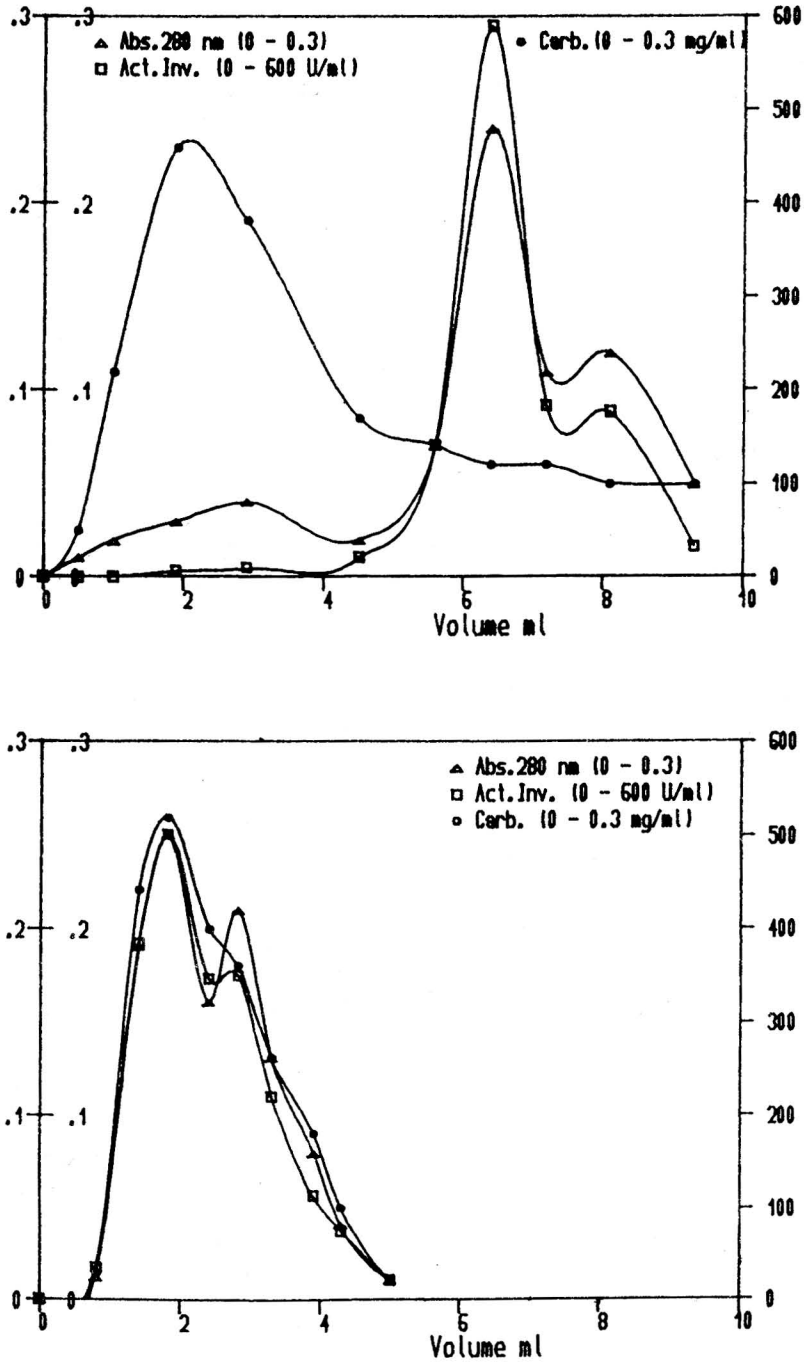

FIG. 2. Ion Exchange chromatography of external invertase from different yeasts. The Mono Q FPLC column was previously equilibrated with 20 mM ammonium acetate, pH 6.5. A linear gradient was established to 1 M of the same buffer in 15 min. A and B, pure invertase from *S. cerevisiae*.

SDS-PAGE Glycosylation analysis

The enzymatic deglycosylation by means of Endo H (Fig. 3) suggests that both invertases encoded by the SUC_2 gen have the same protein core.

Considering that the protein length and amino acid sequence are the same in *S. cerevisiae* and *H. polymorpha* the possible glycosylation sites would also be the same and thus a lower molecular weight of *H. polymorpha* invertase would imply shorter

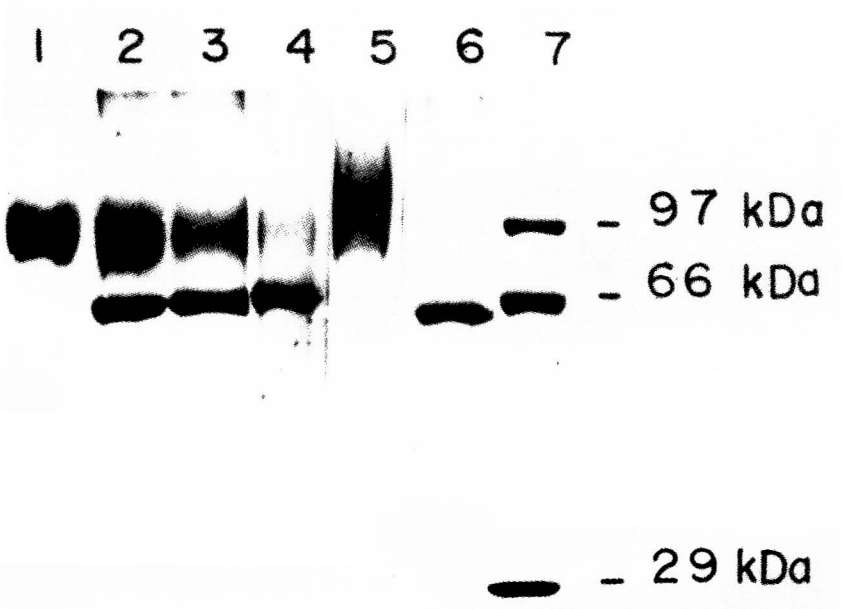

FIG. 3. Deglycosylation treatment of different external invertases with Endo β-N-acetylglucosaminidase *H. polyacrilamide* gel electrophoresis (12%) under denatured conditions was used. The deglycosylation trial is described in *Materials and Methods*. Invertase from *H. polymorpha* (Lanes 1-4) and from *S. cerevisiae* (Lanes 5 and 6). Molecular weight markers (Lane 7). *Lanes 1 and 5)* Invertases without Endo H treatment; *Lanes 2 and 6)* 50 mg invertase, 15 mU Endo H and 16 h reaction time; *Lane 3)* 50 mg invertase, 20 mU Endo H and 32 h reaction time; and Lane 4) 50 mg invertase, 25 mU Endo H and 48 h reaction time.

The SDS-PAGE of denatured invertase (Fig. 4) suggest a lower degree of glycosylation and heterogeneity distribution by its narrower band at 85-90 kDa molecular weight. The fact that there is only a minor coincidence in relative mobility suggest the existence of shorter (if not less) oligosaccharide side chains. This result is in agreement with the above calculated 25-30% glycosylation for invertase from *H. polymorpha*.

oligosaccharide chains (Man_{8-14} $GlcNAc_2$). A minor glycosylation frequency was not considered due to this event occuring soon after the synthesis of the sequon.

The existence of Endo H resistant sequons (not related to the oligosaccharide length) in *S. cerevisiae* external invertase was reported before (Zeigier *et al.*, 1988). However, it was observed that invertase from *H. polymorpha* is more resistant towards Endo H treatment than *S. cerevisiae*,

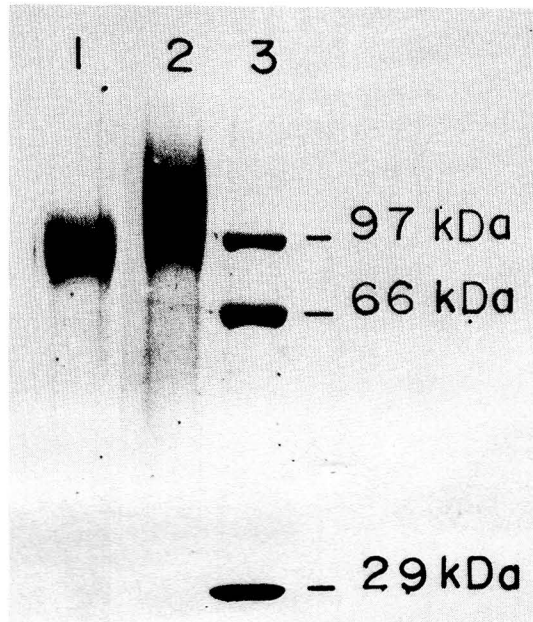

FIG. 4. Electrophoretic mobility (SDS-PAGE) of denatured yeast external invertase, 10 mg of samples were subjected to denaturation in presence of 0.5% β-mercaptoethanol and 4% SDS and heated 10 min at 100°C and then applied in a 12% polyacrilamide gel. The staining was carried out with Coomassie blue. Lanes 1 and 2 represent invertase from *H. polymorpha* and *S. cerevisiae* respectively.

meaning that almost twice the amount of Endoglycosidase and more than 40 hours of reaction are needed.

Aggregation properties

The stability of the multimeric forms of secreted invertase could be a factor to consider in Endo-H deglycosylation resistance due to the establishment of strong hydrophobic interaction between monomeric subunits which if facilitated by protein glycosylation (Tammi *et al.*, 1987; Esmon *et al.*, 1987). A comparison of Kav values on a gel filtration chromatography HPLC column (TSK-3000) is shown in Table 1.

The data showed confirm that external *S. cerevisiae* invertase in solution (1 mg prot/ml) just after thawing exists mainly in its octameric form (M, W 1.0 x 10^6) and after 9 hours at 25°C pH 6.5 more than 80% of invertase molecule is equilibrated to its tetrameric form (M, W 5.2 x 10^5). However external *H. polymorpha* invertase in the same conditions exists in an equilibrium of octameric (M, W 7.2 x 10^5)-hexameric (M, W 5.4 x 10^5) forms which can also be deduced from the elution pattern in Sephacryl S-300 column (Fig. 1) where invertase activity was detected immediately after the vold volume.

Taking into acount the above mentioned results, it is possible to consider that those oligomers that are formed in the E.R. and pass through the secretion pathway in its

Table 1
OLIGOMERIC FORM VARIATIONS OF EXTERNAL INVERTASE FROM *S. cerevisiae* AND *H. polymorpha*

External invertase *S. cerevisiae*	t[1]-0.5 h	t-3.0 h	t-9.5 h
	Kav[2]-%[3]	Kav-%	Kav-%
	1.0-70%	1.0-45%	1.0-12%
	0.22-30%	0.22-55%	0.22-88%
External invertase *H. polymorpha*	t-0.5 h	t-3.0 h	t-50.0 h
	Kav-%	Kav-%	Kav-%
	1.0-95%	1.0-85%	1.0-60%
	0.16-5%	0.16-15%	0.16-30%

[1] Equilibration time after thawing samples of *S. cerevisiae* and *H. polymorpha* invertase at a concentration 1 mg/ml 0.2 M ammonium acetate pH 6.5 buffer.
[2] Kav values from gel filtration chromatography on TSK 3 000 HPLC column. Thyroglobulin (Kav-0.11 MW-669 x 10^3), Catalase (Kav-0.50-MW-232 x 10^3), and BSA (Kav -0.60 MW-67 x 10^3) were used as standard. Blue Dextran and Ribonuclease A (V_o and V_f respectively).
[3] Relative % of the different fractions.

highly aggregate form may be hiding some of the oligosaccharide chains thus increasing the resistance against endoglycosaminidase action. Whether or not the addition of outer chains in Golgi apparatus is blocked in engineered *H. polymorpha*, and the influence of oligosaccharide length in the stability of high oligomeric forms is now under study.

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