Purification and characterization of trimming glucosidase I from *Saccharomyces cerevisiae*

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Glucosidase I was purified about 1900-fold from yeast microsomal preparations by DEAE-Sephacel chromatography, affinity chromatography on AH-Sepharose 4B-linked N-5-carboxypentyl-1-deoxynojirimycin and Con A-Sepharose chromatography. The enzyme is a glycoprotein with a subunit molecular mass of 95 kDa. Its reaction has a pH optimum close to 6.8 and does not require metal ions. Purified glucosidase I hydrolyses the distal α1,2-linked glucose residue from the Glc_3-Man_9-$GlcNAc_2$ chain of its natural substrate, but is not active against Glc_2-Man_9-$GlcNAc_2$ and aryl-α-glucosides. Like glucosidase I from calf liver, the yeast enzyme is strongly inhibited by 1-deoxynojirimycin (dNM), N-methyl-dNM and N-5-carboxypentyl-dNM with K_i values of 16, 0.3 and 3 μM, respectively.

Trimming enzyme   *Glucosidase I*   *Enzyme purification*   (S. cerevisiae)

1. INTRODUCTION

The biosynthesis of N-linked glycoproteins in *Saccharomyces cerevisiae* resembles in its initial stages the pathway of N-glycoprotein formation in higher eucaryotes involving the transfer of Glc_3-Man_9-$GlcNAc_2$ to selected acceptor sites of the polypeptide [1]. Subsequently, the three glucose and one mannose residue are trimmed off one after the other by specific enzymes: glucosidase I, glucosidase II and a trimming mannosidase, respectively [2,3]. The resulting Man_8-oligosaccharide is then elongated – presumably in the Golgi apparatus – by the addition of a number of mannose residues finally

Dedicated to Professor G. Legler on the occasion of his 60th birthday

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; dNM, 1-deoxynojirimycin; CP-dNM, N-5-carboxypentyl-1-deoxynojirimycin; Nph-, *p*-nitrophenyl-

yielding the outer branches of the yeast mannoproteins [4].

The biological significance of the trimming reactions is largely unknown. To gain some insight into their function, the enzymes concerned have to be separated and characterized. We report here on the purification to homogeneity of glucosidase I from yeast. This enzyme, which is itself a glycoprotein with presumably high-mannose oligosaccharide(s), specifically hydrolyses the terminal α1,2-linked glucose unit from the natural substrate Glc_3-Man_9-$GlcNAc_2$ but is not active on Glc_2-Man_9-$GlcNAc_2$ or on aryl-α-glucosides.

2. MATERIALS AND METHODS

2.1. Materials

UDP-[^{14}C]glucose (spec. act. 260 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. AH-Sepharose 4B and Con A-Sepharose were from Pharmacia, Freiburg; Lubrol PX and Nph-α-glucoside from Sigma, Taufkirchen. Kojibiose was purchased from Koch-Light,

Suffold, England. Baker's yeast was obtained from Uniferm, Monheim, by courtesy of W. Meibaum. dNM was a generous gift from Drs E. Truscheit and D. Schmidt, Bayer, Wuppertal.

2.2. Preparation of crude microsomes from yeast

A suspension of yeast cells (100 g wet wt in 200 ml of 10 mM phosphate buffer, pH 6.8, containing 10 μM PMSF and 1 μg/ml each of pepstatin A and leupeptin) was shaken with 100 g acid-washed glass beads (0.4–0.5 mm diameter) for 30 min with intermittent cooling on ice. Glass beads and cell debris were removed by centrifugation at $8000 \times g$ for 15 min. The supernatant was centrifuged for 2 h at $45000 \times g$ and the resulting pellet washed by resuspending in 5 vols of 200 mM phosphate buffer, pH 6.8, and centrifugation as above. All steps were carried out at 4°C.

2.3. Solubilisation and DEAE-Sephacel chromatography

Washed microsomes were suspended in 10 vols of 200 mM phosphate buffer, pH 6.8, containing 1% Lubrol PX, 10 μM PMSF and 1 μg/ml each of pepstatin A and leupeptin. After 30 min on ice, the suspension was centrifuged at $45000 \times g$ for 2 h. The supernatant was removed, dialysed against 0.2% Lubrol PX in 10 mM phosphate buffer, pH 6.8, and applied to a DEAE-Sephacel column (1.8 × 45 cm) previously equilibrated with the same buffer. Enzyme activity was then eluted by a linear gradient of 0–1 M NaCl.

2.4. Affinity and Con A-Sepharose chromatography

Affinity chromatography on CP-dNM linked to AH-Sepharose 4B was carried out essentially as described in [5] except that elution was done with 50 mM dNM in 0.5% Lubrol PX, 0.5 M NaCl, 40 mM phosphate buffer, pH 7.5. The glucosidase I active fraction obtained after the affinity step was treated with 0.5 ml Con A-Sepharose equilibrated with 0.2% Lubrol PX, 0.5 M NaCl, 40 mM phosphate buffer, pH 6.8. Glucosidase I was displaced by 1 M methyl-α-mannoside in the same buffer.

2.5. General procedures

[14C]Glc3-Man9-GlcNAc2 was prepared as in [6,7]. Glucosidase I and aryl-α-glucosidase ac-

tivities were assayed as in [5]. SDS-PAGE was performed according to [8]. The synthesis of N-methyl-dNM, CP-dNM and the preparation of the affinity resin followed the procedures as described in [5]. Inhibition studies were carried out as in [5].

3. RESULTS AND DISCUSSION

3.1. Purification of yeast glucosidase I

The purification of glucosidase I was followed with [14C]Glc3-Man9-GlcNAc2 and Nph-α-glucoside as substrates. The enzyme was isolated from crude microsomes by a four-step procedure involving detergent/salt extraction, DEAE-Sephacel chromatography, affinity chromatography on AH-Sepharose 4B with CP-dNM as the ligand and Con A-Sepharose chromatography as described in section 2. The results of a typical purification experiment are summarized in table 1.

Glucosidase I was extracted quantitatively from the yeast crude microsomes with 1% Lubrol PX in 200 mM phosphate, pH 6.8. Chromatography of the solubilized enzyme on DEAE-Sephacel resulted in an about 25-fold enrichment of Glc3-hydrolysing activity. Glucosidase I was eluted from the ion-exchange column as a sharp peak at 300 mM NaCl of the salt gradient and clearly separated from Nph-α-glucosidase(s), the bulk of which eluted earlier. The key step in the purification procedure was the affinity chromatography by which the specific activity was again increased about 8–10-fold. Binding of glucosidase to the affinity resin and elution was performed in batches. The elution of the enzyme from the affinity resin was found to be a slow process, critically dependent on the salt and pH conditions. The best results were obtained when elution was carried out at pH 7.5 and in high salt. Analysis by SDS-PAGE of the enzyme preparation from the affinity step revealed several bands on the gel, indicating that the preparation was not yet homogenous (fig.1, lane 4). Further purification was achieved by Con A-Sepharose chromatography of the affinity eluate. This treatment removed most of the contaminating proteins which, in contrast to glucosidase I activity, do not bind to the immobilized lectin. Elution with 1 M methyl-α-mannoside yielded a glucosidase I preparation which was virtually homogenous as shown by SDS-PAGE (fig.1, lane

Table 1

Purification of glucosidase I from yeast

Fraction	Volume (ml)	Protein (mg)	Aryl-α-glucosidase		Glucosidase I		Purification (-fold)
			Activity (mU)	Spec.act. (mU/mg)	Activity (U)	Spec.act. (U/mg)	
Crude microsomes	40	2700	12×10^4	44	3200	1.2	1
Detergent extract (1% Lubrol PX in 200 mM phosphate, pH 6.8)	78	1800	8×10^4	42	3900	2.2	1.8
DEAE-Sephacel eluate	63	19	100	5	1200	63	52
Affinity chromatography eluate	33	1.4	n.d.	–	720	510	425
Con A-Sepharose eluate	15	0.25	n.d.	–	570	2300	1900

Glucosidase I activity was assayed with [^{14}Glc$_3$-Man$_9$-GlcNAc$_2$ as substrate, aryl-α-glucosidase activity with Nph-α-glucoside. For definitions of units, see [5]. n.d., not detectable
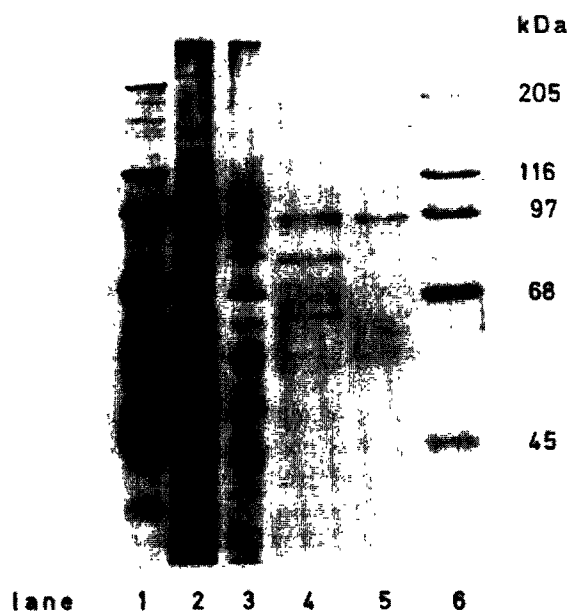

Fig.1. SDS-PAGE of purified glucosidase I. SDS gel electrophoresis was carried out under reducing conditions according to Laemmli [8]. Lanes: 1, molecular mass standards; 2, yeast crude microsomes; 3, glucosidase I fraction eluted from DEAE-Sephacel; 4, material eluted from the affinity resin; 5, purified glucosidase I after Con A-Sepharose chromatography; 6, molecular mass standards. Proteins in lanes 1–3 were silver-stained [11], those in lanes 4–6 stained with Coomassie blue.

5). The overall purification factor was about 1900 over crude microsomes.

3.2. *Molecular properties of purified glucosidase I*

The molecular mass of glucosidase I was found to be 95 kDa under reducing and denaturing conditions as calculated from its mobility relative to known molecular mass standards on SDS-PAGE. Whether the enzyme is an oligomer in its native form is unknown. The yeast enzyme is about 12 kDa larger than glucosidase I from calf [5] and pig liver (unpublished). Preliminary data suggest that glucosidase I from yeast does not cross-react with antibodies raised against glucosidase I from pig liver. The strong binding to and successful elution with methyl-α-mannoside from Con A-Sepharose indicate that the yeast enzyme is itself a glycoprotein with high-mannose oligosaccharide(s).

3.3. *Enzymatic properties of yeast glucosidase I*

The pH dependence of [^{14}C]Glc$_3$-Man$_9$-GlcNAc$_2$ reveals a sharp pH optimum of activity at pH 6.7 with half-maximal activity at pH 6.2 and 7.2, respectively. The purified enzyme is not inhibited by either 1 mM EDTA or o-phenanthroline, indicating that it does not require metal ions for activity.

Purified glucosidase I hydrolyses specifically the terminal α1,2-linked glucose residue from the natural Glc$_3$-Man$_9$-GlcNAc$_2$ substrate. Essentially

Table 2

Molecular and enzymatic properties of purified glucosidase I from yeast and calf liver

	Yeast glucosidase I	Calf liver glucosidase I[a]
Molecular mass (SDS-PAGE, reducing conditions)	95 kDa	83 kDa
Con A-Sepharose binding	(+)	(+)
pH optimum	6.8	6.7
Metal ion dependence	(−)	(−)
Substrate specificity		
Glc_3-Man_9-$GlcNAc_2$	removal of the distal α1,2-linked glucose	removal of the distal α1,2-linked glucose
Glc_2-Man_9-$GlcNAc_2$	no hydrolysis	no hydrolysis
Nph-α-glucoside	no hydrolysis	no hydrolysis
Inhibition by[b]		
dNM	K_i 16 μM	K_i 1.0 μM
N-Methyl-dNM	K_i 0.3 μM	K_i 0.07 μM
CP-dNM	K_i 3 μM	K_i 0.5 μM
Kojibiose	K_i 55 μM	not determined

[a] From [10]
[b] Determined at the pH optimum of the enzyme activity

no cleavage of Glc_2-Man_9-$GlcNAc_2$ or of synthetic aryl-α-glucosides occurs under the assay conditions (37°C, 2 h). This observation clearly points to a specific involvement of this enzyme in the N-linked oligosaccharide-processing pathway in yeast.

Having a homogenous enzyme preparation at hand, we have reinvestigated the inhibition of the yeast glucosidase I by various glucosidase inhibitors such as dNM, N-methyl-dNM, CP-dNM and the α1,2-linked disaccharide kojibiose. Under standard assay conditions, the yeast enzyme is inhibited by kojibiose with a K_i of 55 μM, supporting the postulate that the distal glucose residue is attached on α1,2-glucosidically to the Glc_2-Man_9-$GlcNAc_2$ oligosaccharide core. The K_i for dNM determined from linear Dixon plots was about 16 μM, whereas values of 0.3 and 3 μM were found by the same method for the N-alkyl derivatives N-methyl-dNM and CP-dNM, respectively. The stronger inhibition by the N-alkyl derivatives is consistent with earlier observations on glucosidase I from calf [10] and pig liver (unpublished) and may be due to a hydrophobic binding pocket at the active site of these enzymes. The similar enzymatic properties of the yeast and calf liver enzyme (see table 2) suggest an identical, though not yet understood biological function of the glucose trimming in lower and higher eucaryotes.

4. CONCLUSIONS

Our studies demonstrate that affinity chromatography on CP-dNM-Sepharose in combination with conventional methods constitutes a convenient strategy for the rapid isolation of glucosidase I from yeast. A similar procedure has recently been used successfully for the purification of glucosidase I from calf and pig liver [5], suggesting its general applicability for the isolation of these enzymes from other sources as well. The availability of purified glucosidase I from yeast should facilitate further studies on the biosynthesis, subcellular location, topology and biological function of this enzyme.

ACKNOWLEDGEMENTS

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