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N-Methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, a new affinity ligand for the purification of trimming glucosidase I

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The paper describes the synthesis of a new type of affinity resin containing *N*-methyl-*N*-(5-carboxypentyl)-1-deoxynojirimycin as the ligand attached to AH-Sepharose 4B, which allows the purification of trimming glucosidase I from a detergent extract of pig liver crude microsomes in one step and with high yield. The structure of the affinity ligand was designed on the basis of the observation that *N*,*N*-dialkylated derivatives of 1-deoxynojirimycin do strongly inhibit trimming glucosidase I, but not nonspecific aryl-α-glucosidases including glucosidase II. The specific binding of glucosidase I eliminates the need of additional purification steps with their associated losses which were required with the previously synthesized *N*-(5-carboxypentyl)-AH-Sepharose 4B resin in order to achieve a homogenous enzyme preparation.

Trimming glucosidase I; Pig liver; Affinity purification; 1-Deoxynojirimycin derivative

1 INTRODUCTION

The complex sequence of *N*-linked oligosaccharide processing is initiated by the action of trimming glucosidase I which removes the distal α1,2-linked glucose residue from the protein-bound Glc^3-Man^9-$GlcNAc^2$ precursor [1]. The enzyme has recently been purified in our laboratory from calf liver, pig liver and yeast and its molecular and enzymatic properties characterized [2–4]. The purification procedure included affinity chromatography on *N*-5-(carboxypentyl)-1-deoxynojirimycin-AH-Sepharose 4B as the key step, together with ion exchange chromatography or polyethylene glycol precipitation. The modest enzyme recovery resulting from the application of the latter techniques, led us to develop a new type of affinity resin containing *N*-methyl-*N*-(5-carboxypentyl)-1-deoxynojirimycin as the ligand. It will be shown that this affinity resin binds glucosidase I activity specifically, thus allowing the isolation of an almost homogenous enzyme preparation from a detergent extract of pig liver crude microsomes in only one step.

2. MATERIALS AND METHODS

2.1 *Materials*

[UDP-^{14}C]glucose (spec. act. 240 Ci/mol) was obtained from the Radiochemical Centre (Amersham), Lubrol PX and *p*-nitrophenyl-α-glucoside from Sigma (Taufkirchen), adipic acid methylester and methyl iodide from Fluka (Neu Ulm) and AH-Sepharose 4B from Pharmacia (Freiburg). 1-Deoxynojirimycin was a generous gift from Dr. D. Schmidt (Bayer AG, Wuppertal).

2.2 *Synthesis of adipic acid monomethyl ester semialdehyde*

Adipic acid methyl ester was converted to the acid chloride with $SOCl_2$ following standard procedures. 12 g (67 mmol) of the distilled acid chloride derivative were dissolved in 150 ml of xylene and reduced under reflux to the semialdehyde with hydrogen gas in the presence of 2.5 g of palladium/charcoal. Completion of the reaction was controlled by measuring the amount of liberated HCl. The catalyst was separated by filtration and the reaction mixture neutralized by the addition of $NaHCO_3$ in water. After separation, the organic phase was dried with $MgSO_4$ and the xylene removed by evaporation. Distillation under reduced pressure of the remaining material gave 8.3 g (58 mmol) of adipic acid methyl ester semialdehyde.

2.3 *Synthesis of N-(5-carboxypentyl)-1-deoxynojirimycin*

100 mg of 1-deoxynojirimycin (0.6 mmol) were dissolved in 3.0 ml methanol/water (1:1, v/v) and reductively *N*-alkylated with hydrogen gas at 1.5 bar under weakly acidic conditions in the presence of 880 mg adipic acid methyl ester semialdehyde and palladium hydroxide on charcoal as catalyst. After 24 h at room temperature, the catalyst was separated by filtration and excess aldehyde extracted from the reaction mixture with petroleum ether. The CP-dNM methyl ester was then hydrolysed with 0.1 N NaOH, followed by acidification of the reaction mixture. For purification the *N*-(5-carboxypentyl)-dNM derivative was bound to Dowex 50 (H^+ form), eluted with aqueous ammonia and crystallised from ethanol (yield 110 mg, 66%). The isolated product was homogenous on TLC using $CHCl_3$/MeOH/NH_3 (5:5:1, v/v) as solvent. NMR data showed that the expected structure had been obtained, m.p. 185–187°C.

2.4 *N-Methylation of N-(5-carboxypentyl)-1-deoxynojirimycin*

100 mg *N*-(5-carboxypentyl)-dNM were dissolved in 0.5 ml saturated $NaHCO_3$/water and 3 ml of methanol. After the addition of 0.5 ml of methyl iodide the reaction mixture was stirred at room temperature for 12 h. The solvent was removed by evaporation and the residue taken up in methanol/water (1:1, v/v). The *N*-methylated derivative was isolated by binding to and elution from Dowex 50 (H^+

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Abbreviations dNM, 1-deoxynojirimycin; CP-, *N*-(5-carboxypentyl)-; Nph-, nitrophenyl; AH-Sepharose 4B, aminohexyl-Sepharose 4B

form) is described above. The solid material obtained after lyophylization, was homogeneous on TLC (CHCl$_3$/CH$_3$OH/NH$_4$OH 5:4:1 v/v) and its structure confirmed by NMR spectroscopy.

2.5. *General methods*

Pig liver crude microsomes were prepared as in [5]. Details for the synthesis and purification of [^{14}C]Glc$_1$Man$_7$GlcNAc$_2$ are given in [5]. The activity of glucosidase I and II was determined with [^{14}C]Glc$_1$Man$_7$GlcNAc$_2$ and *p*-nitrophenyl-α-glucoside as substrates [2]. One unit of glucosidase I is defined as the amount of enzyme releasing 1% [^{14}C]glucose monitored the radiolabeled oligosaccharide under standard assay conditions (0.5% Lubrol PX in 50 mM phosphate buffer, pH 6.5). One Nph-α-glucosidase (glucosidase II) unit corresponds to the amount of enzyme which hydrolyses 1 μmol of Nph-α-glucoside/min. Inhibition studies were performed as in [2]. Coupling of the affinity ligand to AH-Sepharose 4B and the affinity purification followed the procedures described previously in [2]. Protein was determined by the Lowry procedure [6]. SDS PAGE was done according to Laemmli [7].

3 RESULTS AND DISCUSSION

3.1 *Design and synthesis of* N-*methyl*-N-*(5-carboxypentyl)-1-deoxynojirimycin*

Previous studies demonstrated that 1-deoxynojirimycin (dNM) is a powerful inhibitor for a variety of α- and β-glucosidases including trimming glucosidase I and II [8,9]. Recently we noticed that the inhibitory effect of dNM changed dramatically and in the opposite direction for the two enzymes when the ring nitrogen was alkylated. Thus glucosidase I was significantly more inhibited by the *N*-methyl derivative of dNM than by dNM itself, whereas the inhibition of glucosidase II (nitrophenyl-α-glucosidase) was, in contrast, impaired several-fold by this modification. This is shown for the two enzymes from pig liver in Table I. A strikingly similar inhibition pattern was observed for glucosidase I and II from calf liver and yeast, suggesting that the data may be generally transferable to trimming glucosidases from other cells and tissues [2,4].

The different effects on glucosidase I and II formed the rationale for the design of an affinity ligand, the structure of which is shown in Fig. 1A [2]. Purification of pig liver glucosidase I on a column containing the immobilized *N*-(5-carboxypentyl) dNM derivative yielded a highly enriched, though still heterogeneous enzyme preparation with Nph-α-glucosidase (glucosidase II) activity as the main contaminant [3]. Since the separation of these contaminants by conventional techniques (e.g. ion exchange chromatography, polyethylene glycol precipitation) turned out to affect severely the final yield of glucosidase I, we searched for a new dNM derivative which would allow a greater discrimination between glucosidase I and Nph-α-glucosidase (glucosidase II) activities at the stage of affinity binding. The structure of a compound having these properties is shown in Fig. 1B. The *N*-methyl-*N*-(5-carboxypentyl)-derivative was constructed on the basis of the observation that *N*,*N*-dimethyl-dNM failed to inhibit glucosidase II but retained, in contrast, its high capability for inhibiting glucosidase I (Table I). The quaternary ligand was synthesized by *N*-methylation of *N*-(5-carboxypentyl)-dNM (Fig. 1A) which itself was obtained by reductive *N*-alkylation of dNM with adipic acid semialdehyde (see section 2). The ligand was attached covalently to AH-Sepharose 4B following the protocol described previously for the synthesis of CP-dNM-AH-Sepharose 4B [2]. The concentration of the immobilized ligand was estimated to be 2–3 μmol/ml of swollen gel by an indirect method using [^{14}C]butyric acid [2,3].

Table I

Inhibition of glucosidase I and *p*-nitrophenyl-α-glucosidase (glucosidase II) activity by various 1-deoxynojirimycin derivatives

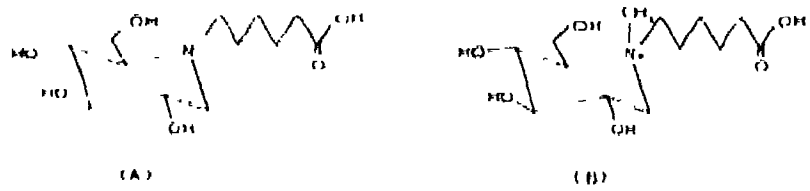
Inhibitor	K_i (μM)	
	Glucosidase I	*p*-Nitrophenyl-α-glucosidase (Glucosidase II)
1-Deoxynojirimycin (dNM)	2.1[a]	8
N-Methyl-dNM	0.1	15
N,*N*-Dimethyl dNM	0.5[a]	<5% inhibition at 1 mM
N-(5-Carboxypentyl)-dNM (CP-dNM)	0.5[a]	45
N-Methyl-CP-dNM	1.4	<5% inhibition at 1 mM

[a] Data taken from [3]

The K_i values were determined using either purified pig liver glucosidase I or pig liver crude microsomes (*p*-nitrophenyl-α-glucosidases) as the enzyme source

3.2 *Purification of pig liver glucosidase I on* N-*(5-carboxypentyl) and* N-*methyl*-N-*(5-carboxypentyl)-dNM-AH-Sepharose*

We have tested the efficiency and specificity of both the CP-dNM- and *N*-methyl-CP-dNM-AH-Sepharose resins by purifying, in a parallel experiment, glucosidase I from a high salt/high detergent extract of pig liver crude microsomes. The work-up procedure followed the protocol described previously in [3]. In order to reduce/prevent unspecific and/or ionic binding, it was essential to extract the crude microsomes with 0.05% Lubrol PX in 20 mM phosphate, pH 6.5, before the final enzyme solubilization and to perform the subsequent operations (binding and elution) in the presence of high concentrations of salt and detergent (1% Lubrol PX, 200 mM phosphate, pH 6.5; solubilization buffer). In general, more than 90% of glucosidase I and between 30% and 50% of Nph-α-glucosidase activity present in the high salt/high detergent extract bound to the CP-dNM-AH-Sepharose 4B resin, from which about 30% of both activities could be eluted with 50 mM dNM. A similar binding and elution of glucosidase I was observed for *N*-methyl-CP-dNM-AH-Sepharose, whereas in this case the Nph-α-glucosidase activity was recovered almost quantitatively in the supernatant. Under standard assay conditions (5 mM Nph-α-glucoside; 1 h incubation at 37°C) nonspecific α-glucosidase activities were not detectable



(A) (B)

Fig. 1. Structure of (A) *N*-(5-carboxypentyl)-1-deoxynojirimycin (CP-dNM) and (B) *N*-methyl-CP-dNM

in the affinity eluate, indicating that the new affinity resin is highly specific for glucosidase I. This specificity is also reflected by the purification factor which was found to be larger than 320-fold (based on the activity in crude microsomes) with the *N*-methyl-CP-dNM affinity resin, whereas it was about 200-fold on using *N*-5-CP-AH-Sepharose 4B.

Fig. 2 summarizes the results of an SDS-PAGE analysis of a typical purification on either affinity resin. As can be seen, chromatography on *N*-methyl-CP-dNM-AH-Sepharose yielded a single major protein band at 85 kDa, representing glucosidase I (lane 5). The affinity eluate from CP-dNM-AH-Sepharose 4B contained, in addition to the 85 kDa protein, an intense protein band at 110 kDa (possibly glucosidase II) and a faint band at 114 kDa (lane 4). We have not characterized these bands further but it is likely that they either constitute nonspecific Nph-α-glucosidases (glucosidase II) and/or catalytically active degradation products of these enzymes. This view is supported by the observation that in particular the glucosidase II protein, unlike glucosidase I, appears to be highly susceptible to proteolytic cleavage after detergent solubilization ([3,11,12], and unpublished observation).

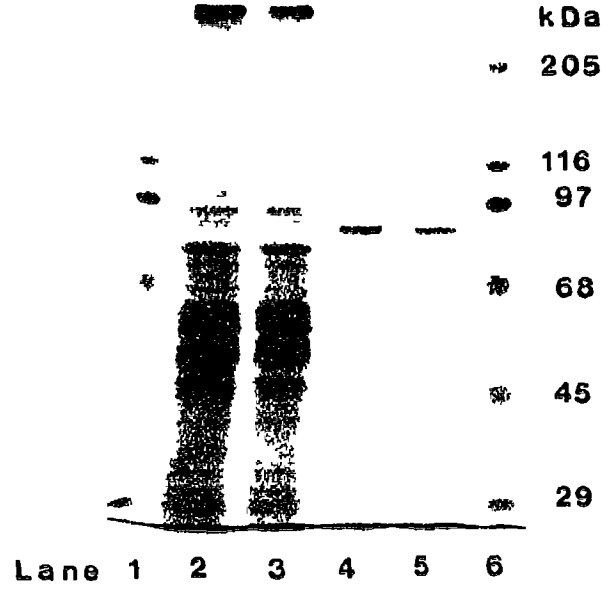


Fig. 2. SDS-PAGE of a typical purification of pig liver glucosidase I on CP-dNM- and *N*-methyl-CP-dNM-AH-Sepharose 4B. Glucosidase I (and *p*-nitrophenyl-α-glucosidase) activity was solubilized from the microsomal membrane with 1% Lubrol PX, 200 mM phosphate, pH 6.5, and equal amounts of the detergent extract (700 ml) treated batchwise with 2 ml of the respective affinity resin (12 h at 4°C). The affinity resins were separated by decantation and washed exhaustively with the solubilization buffer, followed by elution of glucosidase I with 10 ml of a solution of 50 mM dNM in 200 mM phosphate, pH 7.5, containing 1% Lubrol PX. After separation of the inhibitor on a Sephadex G-25 column equilibrated in 0.5% Lubrol PX, 50 mM phosphate, pH 6.5, aliquots of the affinity eluates were subjected to SDS-PAGE [3,7]. Proteins were stained with Coomassie blue. (Lane 1 and 6) Molecular mass standards, (lane 2) crude microsomes; (lane 3) detergent extract, (lanes 4 and 5) affinity eluate from CP-dNM- and *N*-methyl-CP-dNM-AH-Sepharose 4B, respectively.

3.3. *Conclusions*

The paper describes, based on the results of kinetic measurements with various dNM derivatives, the design and synthesis of a new affinity matrix with *N*-methyl-*N*-5-carboxypentyl-dNM as the ligand, the application of which allows the rapid and efficient purification of trimming glucosidase I from a detergent extract of pig liver crude microsomes. The crucial advantage of the new *N*-methyl-CP-AH-Sepharose 4B resin lies in its specificity, namely in its exclusive binding of glucosidase I, thereby making further recovery-affecting purification steps unnecessary. We are currently investigating whether the procedure is transferable, with similar efficiency, to the isolation of glucosidase I from other cells and tissues.

The significantly lower K_i values of the mono- and di-*N*-alkylated derivatives of dNM point to some kind of hydrophobic binding pocket at the active site of glucosidase I (see Table I). Steric factors, on the other hand, could be responsible for the reduced ability of *N*-methyl-dNM and CP-dNM or the failure of *N*,*N*-dimethyl-dNM and *N*-methyl-CP-dNM to bind to glucosidase II. It could also be, however, that effects on the catalytic turnover may account for the different susceptibility of two trimming enzymes, since studies on the pH dependence of inhibition indicated that the protonated form of dNM is the inhibitory species for glucosidase I [10], whereas glucosidase II seems to require the non-protonated form of dNM and *N*-methyl-dNM for inhibition (unpublished results).

It should be of interest to see whether our approach may also be applicable for distinguishing trimming α-mannosidases using 1-deoxymannojirimycin derivatives as probes. Purification of these enzymes by affinity chromatography, as recently shown for trimming Man^9-mannosidase from calf and pig liver [13,14], and

in vivo experiments to investigate the functional/biological role of oligosaccharide intermediates during *N*-glycoprotein processing, using such derivatives would provide further useful information.

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