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Hybrid affinity chromatography of α-galactosidase from *Verbascum thapsus* L.

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Abstract

Purification of α-galactosidase from the roots of *Verbascum thapsus* L. was difficult to achieve using conventional methods due to the presence of coloured contaminants. A newly developed procedure, hybrid affinity chromatography, which was based on a mixed matrix separation procedure, using a substrate analogue and an immobilized metal affinity matrix as ligands, respectively, allowed the purification of this enzyme with good recovery. The method should be applicable to other proteins as well. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Hybrid affinity chromatography; *Verbascum thapsus*; Affinity adsorbents; Galactosidase; Enzymes

1. Introduction

Chromatography is often the method of choice for the isolation and purification of proteins and enzymes from different sources. Unfortunately however, due to the complexity of these natural sources, different chromatographic methods and multiple steps are required to achieve the desired purity. In this work, a new method will be described which we called hybrid affinity chromatography (HAC) for the purification of α-galactosidase. α-Galactosidase (EC 3.2.1.22, α-D-galactoside galactohydrolase) is involved in a variety of natural processes, such as the hydrolysis of the oligosaccharides raffinose and stachyose, and (1-6)-α-D-galactose side chains from storage polysaccharides [1–3]. Oligosaccharides and α-galactosidase are widely distributed in nature, mainly in seeds, roots and underground stems [2,4]. The presence and isolation of α-galactosidase from the roots of *Verbascum thapsus* L. (Great Mullein) has not been reported so far. Methods that were used for the isolation of α-galactosidases from e.g., *Cyamopsis tetragonolobus* [5] were not successful for the isolation of this enzyme from the roots of *Verbascum thapsus* L. as well as other methods described for the isolation of this enzyme [6,7]. The newly developed technique was found to be an essential step to obtain pure α-galactosidase from these roots. The concept of this chromatographic procedure is that the protein of interest is adsorbed onto a matrix which comprises two different ligands; the interaction between ligands and enzyme can be manipulated by changes in pH and competing substances.

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2. Experimental

2.1. Materials

Roots of rosette plants of *Verbascum thapsus* L. were obtained from uncultivated grounds in the neighbourhood of the laboratory. Sepharose G-25, Chelating Sepharose FF and Concanavalin A (Con A)-Sepharose were obtained from Pharmacia Biotech. D-Galactose, *p*-nitrophenyl α-D-galactopyranoside (PNPG), polyvinyl–polypyrrolidone, *N*-ethoxycarbonyl-2-ethoxy-1,2,dihydroquinoline (EEDQ), raffinose and stachyose were from Sigma. All other reagents were reagent grade. The α-D-galactopyranosylamine ammonia complex, *N*-benzoloxycarbonyl-ε-aminocaproic acid, *N*-(*N*-benzoloxycarbonyl-ε-aminocaproyl)-α-D-galactopyranosylamine and *N*-ε-Aminocaproyl-α-D-galactopyranosylamine were prepared essentially as described [8,9].

2.2. Preparation of the hybrid affinity matrix

Chelating Sepharose FF (10 g) was added to 10 ml 50% aqueous ethanol containing 4 mmol EEDQ. The apparent pH was kept constant at 7.0 by the addition of 0.5 *M* HCl using an automatic titration device. After about 1.5 h, when no more reactivity was observed, the activated Chelating Sepharose was washed rapidly with five volumes of 50% aqueous ethanol on a glass filter. A solution of *N*-ε-aminocaproyl-α-D-galactopyranosylamine (4 mmol in 10 ml distilled water) was neutralized with 2 *M* HCl, after which the neutralized solution was mixed with an ethanolic solution of EEDQ (10 mmol in 10 ml). Activated Chelating Sepharose FF (10 g suction mass) was added to the ethanolic mixture and stirred overnight. The hybrid affinity matrix (Fig. 1) was washed on a glass filter subsequently with 10 volumes of 50% aqueous ethanol, 10 volumes of absolute ethanol, 10 volumes of 50% aqueous ethanol and 10 volumes of distilled water. The nitrogen content of the commercially available Chelating Sepharose and the amount of D-galactose derivative, covalently bound to the Chelating Sepharose were measured by the micro-Kjeldahl method. The hybrid affinity matrix was found to contain 40 μmol of covalently bound α-D-galactopyranosylamine per ml.

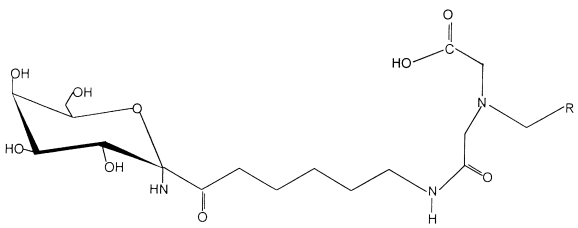

Fig. 1. Partial structure of hybrid affinity matrix.

2.3. Isolation and fractionation of root α-galactosidase

2.3.1. Root extraction

All operations were carried out in the cold (4°C) unless specified otherwise. Fresh roots (500 g) were frozen using liquid nitrogen and powdered in a Waring blender. For each gram of fresh roots, 1 g polyvinyl–polypyrrolidone was suspended in 2 ml buffer containing 0.1 *M* sodium acetate, 0.1 *M* mannitol and 1 *M* NaCl (pH 5.0). The frozen powder was stirred gently into the suspension of polyvinyl–polypyrrolidone until well mixed and the extract was separated from the suspension by squeezing through Miracloth. The cake was extracted and squeezed twice as described above. The combined extracts (4090 ml) were clarified by centrifugation (30 min, 13 700 *g*) and concentrated to 275 ml using an Asahi hollow fibre ultrafiltration unit (PAN-50P). After concentration, the extract was dialysed exhaustively against the pH 5 buffer. The concentrated extract was centrifuged at 30 000 *g* for 30 min. The material was stored at −20°C until further use.

2.3.2. Chromatography

2.3.2.1. Con A chromatography

The concentrated crude extract (295 ml) was dialysed overnight against 2 l buffer consisting of 0.1 *M* sodium acetate, 1 *M* NaCl, pH 6.0. The dialysed crude extract was applied to a column (20×2.5 cm) of Con A-Sepharose, equilibrated with the pH 6 buffer. The column matrix was subsequently washed with six column volumes of the same buffer and eluted with 0.25 *M* methyl α-D-mannopyranoside in the same buffer at a flow-rate of 60 ml/h. α-Galactosidase enzyme activity, as detected in the eluting

fractions, was collected and concentrated using an Amicon concentration device (YM-10 membrane).

2.3.2.2. Hybrid affinity chromatography

The hybrid affinity matrix was packed into a column (10×2.5 cm) and washed thoroughly with distilled water, after which the column was saturated for 75% with a 2 m*M* cupric sulphate solution in distilled water. This HAC column was washed again with distilled water and equilibrated with a 0.1 *M* sodium acetate buffer (pH 6.0), containing 1 *M* NaCl. A Chelating Sepharose FF column (3×2.5 cm) was washed with distilled water. To the column a 2 m*M* cupric sulphate solution was applied until the metal appeared in the eluate. The Cu^{2+} charged immobilized metal affinity chromatography (IMAC) column was washed with distilled water, and equilibrated as described for the hybrid affinity matrix. This IMAC Cu^{2+} column was coupled in front of the HAC column and was used as a safeguard column. The fractions containing α-galactosidase activity obtained from the previous Con A chromatography step was applied to the tandem columns. Fractions of 10 ml were collected at a flow-rate of 60 ml/h. After the unbound material was washed out, a 0.1 *M* sodium acetate buffer (pH 5.0) containing 1 *M* NaCl was applied, resulting in the elution of an inactive protein fraction. A 0.1 *M* sodium acetate buffer (pH 5.0) containing 10 m*M* histidine was applied onto the hybrid affinity column which resulted in elution of a second inactive protein fraction. The histidine containing sodium acetate buffer was completed with 0.1 *M* galactose to displace the adsorbed α-galactosidase (Fig. 2). These pH conditions were selected as they offered the best compromise for binding and elution while maintaining maximal enzyme stability. Histidine was chosen for elution as it was found to give the highest enzyme activity when compared to e.g., imidazole and ammonium chloride. Fractions containing enzyme activity were collected and desalted by G-25 gelfiltration (60×2.5 cm), previously equilibrated with 0.1 *M* sodium acetate (pH 5.0). The α-galactosidase freed from D-galactose and other buffer components was concentrated as described above and stored at −20°C. To demonstrate the effect of the hybrid affinity matrix, the active fractions were once more applied to the HAC column without the capture column and Cu^{2+}, previously

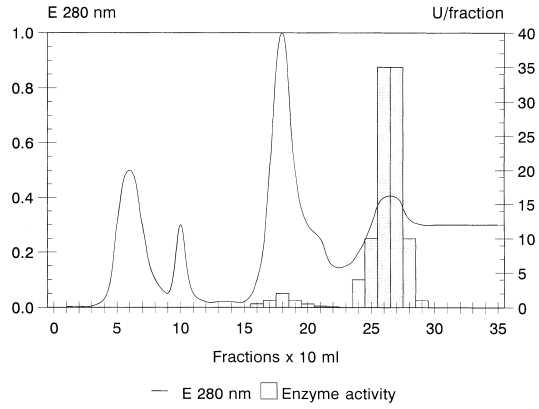

Fig. 2. Hybrid affinity chromatography pattern of α-galactosidase.

equilibrated with 0.1 *M* sodium acetate, pH 5.0. After displacement of the α-galactosidase with the acetate buffer containing 0.1 *M* galactose, the enzyme was concentrated as described before.

2.3.2.3. Chromatofocusing

Hybrid affinity purified α-galactosidase was subjected to G-25 gel filtration for buffer exchange to 25 m*M* histidine–HCl buffer (pH 6.0) and loaded onto a Mono-P column equilibrated with the same buffer. Protein was eluted with polybuffer, which had been adjusted to pH 3.5 with 6 *M* HCl. Fractions were collected and assayed for enzyme activity and pH.

2.4. Assays

2.4.1. Protein assay

Protein concentration was determined by the method of Bradford using bovine γ-globulin (Sigma) as the reference protein.

2.4.2. α-Galactosidase activity assay

Enzyme activity was determined using PNPG as substrate. Hydrolysis of the substrate was followed by determination of the liberated nitrophenolate anion at 405 nm. The reaction mixture consisted of 0.9 ml of PNPG solution (1.1 m*M*) dissolved in 0.1 *M* sodium acetate buffer at pH 5 and 0.1 ml of adequately diluted enzyme solution. Enzyme reactions were allowed to proceed 5 to 10 min at 37°C, and stopped by addition of 2 ml of 10% sodium carbonate solution. Determination of enzyme activity

on isoelectric focusing gels was achieved as follows. The gel was layered with 0.5% agarose solution (45°C) containing 0.1 *M* sodium acetate (pH 5.0) and 1 m*M* 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside. When positive (blue) bands were observed, the gel was rinsed with diluted ammonia and distilled water.

2.4.3. Determination of molecular mass

The molecular mass of the native enzyme was determined using a Superdex 75 column (Pharmacia Biotech) eluted with 0.1 *M* sodium acetate (pH 5.0), containing 0.25 *M* NaCl using the Pharmacia Biotech fast protein liquid chromatography (FPLC) system. The gelfiltration column was calibrated using protein standards (Pharmacia Biotech). The molecular mass was calculated by plotting log M_r versus the V_e/V_o ratio. The molecular mass of the heat denatured enzyme was determined by sodium dodecyl sulphate (SDS) electrophoresis using an ExcelGel (Pharmacia Biotech) following the instructions of the supplier. Protein bands were visualised using silver staining according to the protocol of the supplier.

2.4.4. Isoelectric focusing

Samples (25 μl) were pipetted in duplicate on sample application pieces which were layered on an isoelectric focusing gel (pH range 3.0–10.0, Servalyt Precotes, Serva). Analytical isoelectric focusing was carried out at 10°C in a Multiphor apparatus from Pharmacia Biotech, following the manufacturer's instructions. In separate lanes different isoelectric point (p*I*) markers were used for calibration. One part of the gel was used for protein staining with Coomassie Brilliant Blue R-250. The other part of the gel was used for determination of enzyme activity as described in Section 2.4.2.

3. Results and discussion

3.1. Isolation and purification

Most of the enzyme activity (90%) present in the roots of *V. thapsus* L., is associated firmly with cell wall fragments, and cannot be extracted. Furthermore the roots of *V. thapsus* L. contain high concentrations of secondary products which interfere seriously in procedures for the isolation of α-galactosidase. Extraction of the roots without special precautions resulted in rapid browning and severe loss of enzyme activity. In our purification strategy, the deep frozen powder is stirred gently into a suspension of polyvinyl–polypyrrolidone in 0.1 *M* acetate buffer pH 5.0, containing 0.1 *M* mannitol and 1 *M* NaCl. This procedure markedly reduced browning. A test for the presence of phenolate, using 10% sodium carbonate was negative. Although the method works reasonably well, the crude concentrated extract was deeply coloured, which could not be cleared by dialysis or other conventional means like precipitation. Furthermore, these contaminants remained bound to the enzyme during further purification. The physical properties of these coloured contaminants were to some extent analyzed. These compounds did not display any characteristic UV spectrum and adhere strongly to proteins, particularly to our protein of interest. A molecular mass distribution for these compounds between 10^4 and $>10^6$ was observed by gel filtration, indicating the presence of polymeric adducts and aggregates. Using common separation techniques such as ion-exchange chromatography, gel filtration, chromatofocusing or a combination of these techniques we were not able to purify the α-galactosidase sufficiently. The application of IMAC, although a very attractive technology [9], did not prove to be of good use in our hands for the isolation and purification of α-galactosidase from the source material. An attractive alternative was affinity purification of the α-galactosidase using a solid matrix with *N*-ϵ-aminocaproyl-*N*-ϵ-aminocaproyl-α-D-galactose as substrate analogue [10]. Testing the substrate analogue column, we observed that the impurities as mentioned above reduced this affinity matrix by nearly 100% of its capacity. To overcome these purification problems a new type of affinity chromatography, called hybrid affinity chromatography, has been developed. The set-up of such a chromatographic system is depicted in Fig. 3.

The hybrid affinity matrix is constructed as a normal substrate analogue derivative consisting of long spacers with a built-in *N*-acetic-amide-glycine structure, capable of chelating divalent metal ions (Fig. 1). The two affinity functions operate independently. Even when the hybrid affinity matrix is fully charged with the transition metal Cu^{2+}, the substrate

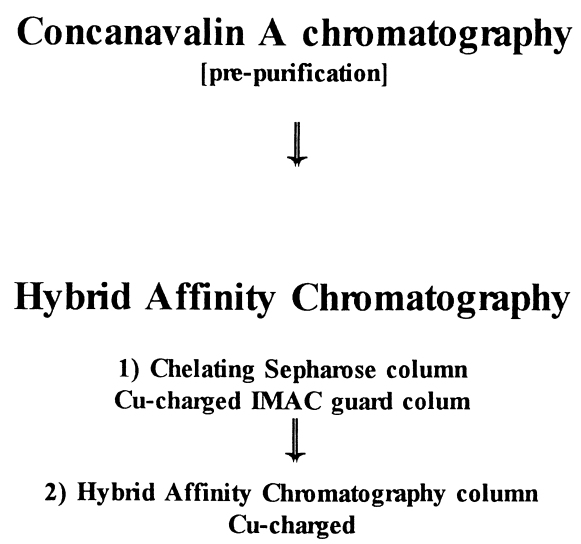

Fig. 3. Hybrid affinity chromatography sequence.

analogue function is still intact. The adsorption and desorption behaviour of the α-galactosidase to the combined affinity functions is pH dependent. At pH 6.0 the α-galactosidase has little or no affinity for the substrate analogue, while the chelated Cu^{2+} adsorbed the enzyme almost quantitatively. At lower pH (between pH 4–5) the opposite is true, high adsorption capacity for the substrate analogue and a negligible adsorption for the IMAC function. In a number of cases it should be possible to uncouple the combined affinity functions. However, in practice the interaction between the combined functions can easily be manipulated by changes in pH and competing substances. Although it is possible to obtain pure α-galactosidase in a single operation using the hybrid affinity column (with Cu^{2+}) this is not recommended because the contaminants mentioned above influence the column capacity negatively, even after regeneration. To fully exploit and retain the capacity of the hybrid affinity column, we have introduced a safeguard column (IDA-Cu^{2+}) and a Con A chromatographic step. While pure α-galactosidase has negligible affinity for Con A-Sepharose, this purification step can be done successfully, probably due to the presence of a complex of α-galactosidase and carbohydrate-like compounds which have affinity for the Con A-Sepharose. The active fractions obtained from the Con A-Sepharose column can be applied directly to the charged (Cu^{2+}) hybrid affinity column.

To break down the impure α-galactosidase complex and to avoid co-migration, the IMAC function was switched off in a step-by-step mode. First, addition of sodium acetate buffer (pH 5.0) strongly reduced the IMAC function resulting in elution of an inactive protein fraction. Then the IMAC function was switched off completely by addition of histidine to the pH 5.0 buffer, which resulted in elution of all other bound proteins and secondary products except α-galactosidase. Finally α-galactosidase activity was eluted from the hybrid affinity matrix with the histidine containing sodium acetate buffer complemented with D-galactose. Since the purified α-galactosidase showed micro heterogeneity by isoelectric focusing, chromatofocusing was used to purify the enzyme to full homogeneity.

3.2. Purity and physical properties

HAC proved here to be the key step in the purification of α-galactosidase, obtained from the roots of the plant *Verbascum thapsus*. Essential to this is that the enzyme characteristics, with respect to its relative affinities for the immobilized ligands, allow the use of both a specific biological affinity matrix and a less specific (IMAC) affinity matrix. The enzyme purified in this manner (Table 1), appears to be homogeneous as demonstrated by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 4), one single band with M_r=50 000 was detected. In contrast to the single band obtained by SDS electrophoresis, the hybrid purified enzyme could be separated by isoelectric focusing into at least five sharp enzymatically active bands (Fig. 5). The major band of the purified material, visualized by protein and enzyme activity staining corresponded to a p*I* of 4.3. Other bands had p*I* values of 4.13, 4.44, 4.55 and 5.20. To establish the distribution of enzyme activity among the various proteins mentioned above, affinity purified α-galactosidase was fractionated using chromatofocusing as separation technique. Results obtained from these experiments show that about 90% of the applied enzyme activity was located between p*I* values 4.13 and 4.55. The major component of this fraction has a p*I* of 4.3 corresponding to 55% of the total activity, was used

Table 1
Purification of α-galactosidase from *V. thapsus* L.

Step	Volume (ml)	Activity total (U)	Protein total (mg)	Specific activity (U/mg)	Step yield (%)	Purification factor
Crude	4090	231	984	0.24	100	1.0
Concentration	295	222	531	0.42	96	1.75
Con A affinity chromatography	100	153	81	1.90	69	4.5
Hybrid affinity chromatography	50	90	1.8	50.0	59	26.0
Substrate analogue chromatography	50	60	0.96	58.0	67	1.2
Chromatofocusing	3	32	0.53	60.0	53	1.0

for further biochemical investigation. The native root enzyme has a M_r=50 000, as determined by gel filtration (Fig. 2). This agrees well with the molecular mass found by SDS–PAGE (Fig. 4), suggesting that the protein is a monomer.

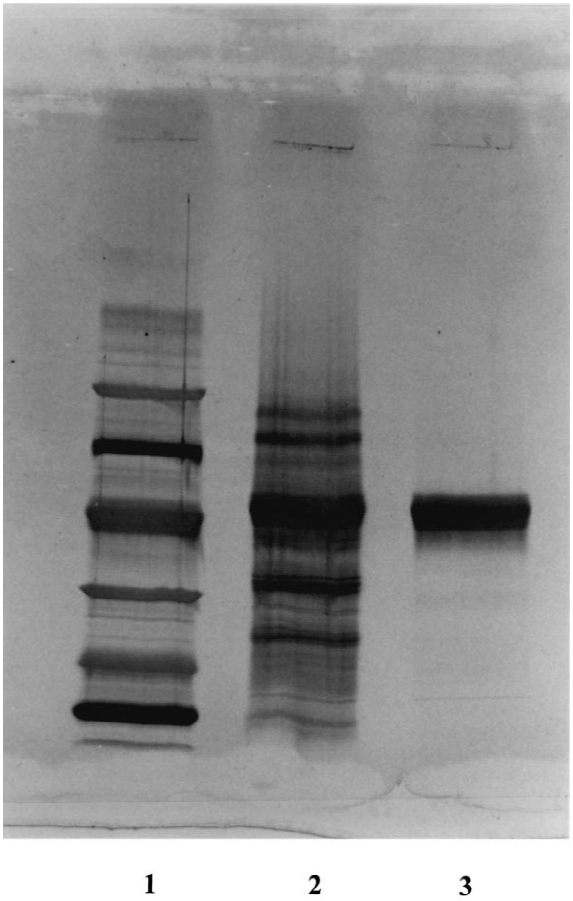

Fig. 4. SDS–PAGE of α-galactosidase. Lanes: 1=marker proteins, 2=concanavalin-purified α-galactosidase, 3=hybrid affinity chromatography-purified α-galactosidase.

4. Conclusion

Application of a HAC technique allowed the isolation and purification of α-galactosidase from the roots of *Verbascum thapsus* that was otherwise very difficult to achieve. More generally, other enzymes that are difficult to separate and purify due to the presence of many interfering substances could pos-

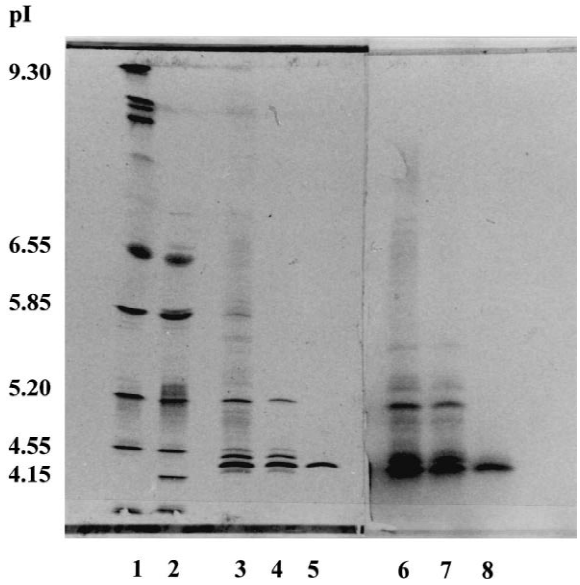

Fig. 5. Isoelectric focusing of α-galactosidase. Lanes: 1=marker proteins broad p*I* range, 2=marker proteins narrow p*I* range, 3–4=hybrid affinity chromatography-purified α-galactosidase, 5=chromatography=purified α-galactosidase, 6–8 lanes 3–4 stained for enzyme activity.

sibly be purified using this approach. α-Galactosidase obtained from hybrid affinity chromatography shows several active bands in isoelectric focusing. The α-galactosidase preparation was purified further using chromatofocusing to homogeneity as shown in SDS–PAGE and isoelectric focusing. The existence of multimolecular forms of α-galactosidase has been reported in enzyme preparations from various origins [11–13]. The physiological significance of multimolecular forms of α-galactosidase in our preparation is questionable, because the roots of *V. thapsus* L. contain a very high content of phenolic compounds. In spite of special precautions, possible occurrence of quinone addition cannot be excluded. Nevertheless, the main fraction purified finally by chromatofocusing shows normal physical characteristics. This fraction was further used to examine the physical and biochemical characteristics of the α-galactosidase. The described HAC technique although not fully developed yet, could be another chromatographic procedure for the isolation and purification of biomolecules.

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