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Short communication

Single step purification of a series of wheat recombinant proteins with expanded bed absorption chromatography

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Abstract

Expanded bed absorption chromatography (EBA) was used to improve and simplify the purification of several wheat recombinant proteins. Binding and elution conditions were set to allow the purification of the over expressed protein in a single step. In comparison with our previous multi step protocol, same purity was obtained while EBA required less time (one day instead of five) and gave a higher yield (63% instead of 10%). This new procedure was then used for the successful purification of five other wheat ns-LTP. Despite their important polymorphism (identity from 44 to 97 %—pHi from 8 to 10), the EBA protocol allowed their purification in a single step.

Keywords: Recombinant protein purification; Expanded bed absorption chromatography; *Pichia pastoris*; Wheat ns-LTP

1. Introduction

Structural biochemistry requires analysing the structure and function of a lot of proteins. Simplifying the purification procedure of these proteins might be very helpful. We have selected expanded bed absorption chromatography (EBA) for the processing of several wheat recombinant proteins secreted by the yeast *Pichia pastoris*. EBA was used successfully with a variety of non-specific lipid transfer proteins (ns-LTP). ns-LTPs form a multigenic family with two groups of type 1 (9 kDa) and type 2 (7 kDa) LTP. They can mediate the transfer of phospholipids between membranes in vitro and play a role in the plant defence mechanisms under stress [1]. They exhibit surface-active properties useful for food industry or pharmacology [2]. With a high polymorphism (e.g. more than 20 genes in wheat) together with a wide ligand spectrum, ns-LTPs are an interesting model for analysing how variations in primary and tertiary structures can modify activity.

More accurate dissection of the potential role of wheat ns-LTP in food technology as well as structural analysis require high quantities of protein. To reach high expression level as well as to avoid misfolding of these wheat cysteine rich proteins, we used the *P. pastoris* system [3]. Secreted recombinant proteins are usually purified using multiple step protocols, which include cell removal via centrifugation or filtration, protein concentration via insolubilisation or ultra filtration followed by one or more chromatography purification steps. Klein et al. [4] produced large quantity of a wheat 9 kDa LTP but get a low purification yield. We have turned to EBA to optimise the purification of this same LTP [5]. This method allowed the capture of LTP in the fermentor bulk as well as its purification in a single step. This new protocol was then used for the successful purification of five other LTP.

This paper is intended to point out how easy it is to adapt an EBA protocol established with one particular protein to other related proteins. We present the experiments required for setting up the EBA protocol, which is compared with the multi step protocol it replaces. Then we describe the adaptation of this new protocol for the purification of a set of proteins of the same family.

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2. Experimental

2.1. Chemicals and enzymes

Chemicals, restriction endonucleases, DNA-modifying enzymes and synthetic oligonucleotides were from ICN (Irvine, CA, USA), Appligene (Illkirch, France) and Eurogentec (Liège, Belgium).

2.2. Recombinant strain and culture conditions

Recombinant strains and the cultures were described by Klein et al. [4] and de Lamotte et al. [5].

2.3. Samples preparation

For the multi step protocol, cells were removed by centrifugation (10,000 × *g* × 5 min at 4 °C) and the supernatant was concentrated 10 times with a 3 kDa cut-off Ultrasette (Pall, New York, USA). Proteins were unsolubilised by addition of ammonium sulfate at 80% saturation for 15 min on ice with gentle stirring and centrifugation (15,000 × *g* × 30 min at 4 °C). The pellet was re-suspended in a minimal volume of 15 mM MES buffer, pH 5.5. For the single step protocol, cultures were directly loaded on the column.

2.4. Chromatographic equipment and conditions

An Amersham Biosciences Akta Purifier 10 controlled by Unicorn v. 4.10 was used for the chromatographic analysis.

a. For the multi step protocol, the purification of the proteins was achieved using an Amersham Biosciences G-25M (2.5 cm × 14 cm) equilibrated with 15 mM MES buffer pH 5 and eluted with 1.5 column volumes of the same buffer, and an Amersham Biosciences S-Sepharose Fast Flow (2.5 cm × 8 cm). Proteins were loaded on the column equilibrated with 15 mM MES buffer and eluted with a linear gradient (0–1 M NaCl, 15 column volumes).
b. For the single step protocol, the culture medium was directly loaded on an Amersham Biosciences STREAMLINE C50 Column (i.d. 5 cm × 1 m). The matrix (STREAMLINE SP XL) was equilibrated in Buffer A (for composition of Buffer A, see Section 3) and the gel was expanded using upside flow at 300 cm/h until the top of the bed was stable (plunger at the upper position). The culture was applied to the column (upside flow) and the column was washed with buffer A until the absorbance at 280 nm stabilised. The flow was subsequently inverted and the plunger was lowered to 1 cm from the top of the bed. Proteins were eluted with buffer A containing 0.3–1 M NaCl at a 100 cm/h flow rate.

2.5. Electrophoresis analysis

Samples were centrifuged for 10 min at 12,000 × *g* before being analysed on 16% acrylamide SDS-PAGE. LTP was quantified on silver stained gels using Amersham Biosciences Silver staining kit and VDS 1000 digitizer with the corresponding software (Image Master 1D, v. 3.00). The purified LTP was lyophilised and precise amount of this protein was used as standard to quantify the protein in samples from the different steps of the purification process.

2.6. Mass spectrometry and N-terminal sequencing

The N-terminal sequence of the recombinant proteins was determined using a Porton LF 3000 protein sequencer (Beckman Instruments, Fullerton, CA, USA). MALDI-TOF-MS experiments were performed on a BiFlex III mass spectrometer (Bruker, Daltonics, Bremen, Germany), 100 pmol of the protein were solubilised in 10 μl of water containing 0.1% Trifluoroacetic acid (TFA) and 0.5 μl of this solution was mixed with 0.5 μl of matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in water/acetonitrile 50/50 (v/v)) and spotted on the target (dried droplet preparation). Spectra were recorded on the linear positive mode using the pulsed ion extraction.

3. Results

The EBA protocol was first tuned for one recombinant type 1 wheat LTP. This protocol proved to be far superior to the multi step method previously used. It was then adapted to purify type 2 wheat LTPs.

3.1. Type 1 LTP purification

3.1.1. Chromatography optimisation

In order to optimise the binding conditions for wheat LTP, preliminary experiments were performed using clarified fermentor supernatants and a packed STREAMLINE SP XL column (1.25 cm × 17 cm). Taking advantage of the Unicorn 4 buffer prep feature, several pH and buffer compositions were screened by 0.5 pH unit steps (citrate–HCl for pH 2.5–6; acetate–HCl for pH 4–5.7 and Tris–Bis–Tris for pH 6–7.7). Binding and release of the recombinant LTP were analysed by SDS-PAGE and Western blotting. The chromatographic program used is described in Table 1. With this program, 16 buffer compositions and pH were tested in two days. Efficient binding of type 1 LTP as well as low contamination by other secreted proteins was obtained with 30 mM Na–Ac pH 5.5. These conditions were applied to fermentor bulk for large-scale purification.

3.1.2. Large-scale purification

Seven hundred millilitre of cell suspension (99 g l^{-1} dry weigh) were directly applied to the column. The absorbance

Table 1
Chromatographic program used for optimisation of binding and elution of recombinant LTP

Flow rate (ml/min)	10
Equilibration (CV)	3
Sample injection (ml)	50
Wash (CV)	2
Elution step 1 (0.33 M NaCl) (CV)	3
Elution step 2 (0.66 M NaCl) (CV)	3
Elution step 3 (1 M NaCl) (CV)	3
Re equilibrate (CV)	3

at 280 nm increased and then decreased down to baseline after washing with 6 l of starting buffer. The bound type 1 LTP was eluted with 290 ml 30 mM Na–Ac pH 5.5 buffer containing 1 M NaCl. LTP appeared to be the only protein in the eluate as shown in Fig. 1 (lane 5). The fractions containing LTP were pooled and dialysed against distillated water then lyophilised.

3.2. Analysis of the purified protein

The integrity of the recombinant LTP as well as its molecular mass was verified by MS and N-terminal sequencing. No contaminants were detected using these two methods.

3.3. Adaptation of the method to related proteins

The single step protocol was adapted to allow the swift purification of various related proteins. Table 2 lists the similarity matrix and the molecular mass and pHi of these type 2 LTP. They exhibit an important polymorphism and are significantly different from the type 1 LTP used to set up the experiment (Table 2). For each protein, 50 ml of clarified supernatant were processed with the same parameters as the Type 1 LTP. Binding and elution were monitored by SDS-PAGE and western blotting. These conditions appeared to be efficient for LTP 7.1, 7.2, 7.3 and 7.5 but not for 7.6, which exhibited poor binding. For the later one, the same buffer scouting procedure described in Section 3.1.1 was used. All the tests were carried out in 1 day and Tris 30 mM pH 7.5 was selected (Fig. 2). 500 ml of the culture of the five type 1 LTP went through large-scale purification with the selected buffer, using the chromatographic program described in Section 2.4b. The purification efficiency is summarised in Table 3. All the recombinant proteins were successfully purified in one single step.

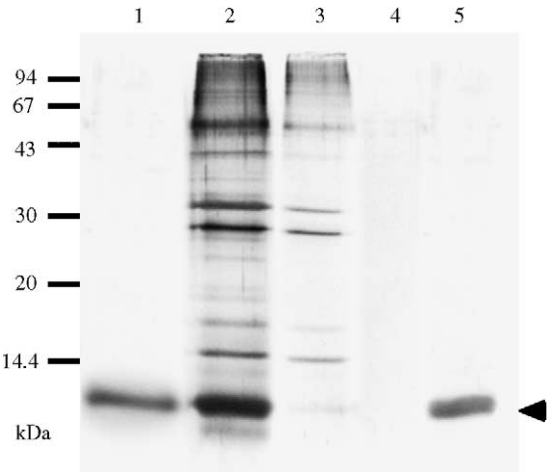

Fig. 1. SDS-PAGE analysis of LTP production and LTP elution from STREAMLINE SP XL. Lanes (1) purified recombinant LTP; (2) fermentor crude medium; (3) column flow through; (4) column wash and (5) Eluate.

4. Discussion

EBA appeared to be a very efficient and time saving method. We also demonstrated that a method could be easily adapted to related proteins.

4.1. Comparison with the multi-step protocol

The former multi-step protocol requires five days while EBA allows the purification of the recombinant LTP in one day. The first three steps of our previous protocol do not lead to any purification of the protein but only prepare the sample to be applied onto the column. The single step EBA protocol replaces clarification of the culture by centrifugation, concentration of the protein by ultra filtration and ammonium sulfate insolubilisation, followed by two chromatographic steps: G-25 and S-Sepharose FF [4]. Quantification of samples from the different purification steps allowed comparing the two methods. The protein purified with the multi step protocol was obtained with a very high purity (>97%) and the yield was 10%. The same protein purified with EBA with a six times higher yield (63%) and the same purity. The output of EBA appears far superior as already reported by de Lamotte et al. [5] or by Shepard et al. 2001 on very large scale (2000 L) [6]. The high purity of the purified protein can be compared with the one obtained by Shepard, also in one step (89% for one protein and 62% for a second one). The fewer steps of the new protocol result in an improvement of the yield by a factor of six. We might assume that the key point is that binding and elution conditions were set up very carefully. This allows LTP to be the only protein that binds to the matrix, and thus, to be purified in one single step.

4.2. Adaptation of the EBA method to related proteins

Modern biochemistry in the high throughput era requires purifications to be done at a high pace. When one has developed an efficient method for the purification of a protein, one would appreciate this method to be as efficient for related proteins. We have challenged the versatility of EBA with the purification of type 2 LTP, which differs significantly form the LTP1 used for setting up the method. They

Table 2
Identity/similarity matrix for the type 2 wheat LTP

	LTP 7.2	LTP 7.3	LTP 7.5	LTP 7.6	MM	pHi
LTP 7.1	74.6/80.6	43.3/56.7	44.8/64.2	59.7/73.1	6979	8.4
LTP 7.2	–	50.7/58.2	43.3/58.2	52.2/68.6	6971	8.5
LTP 7.3		–	63.2/73.5	33.8/52.9	7261	10.0
LTP 7.5			–	41.2/58.8	7093	8.9
LTP 7.6				–	7135	9.4

NB: Type 1 LTP MM = 9607 Da, pHi = 9.8.

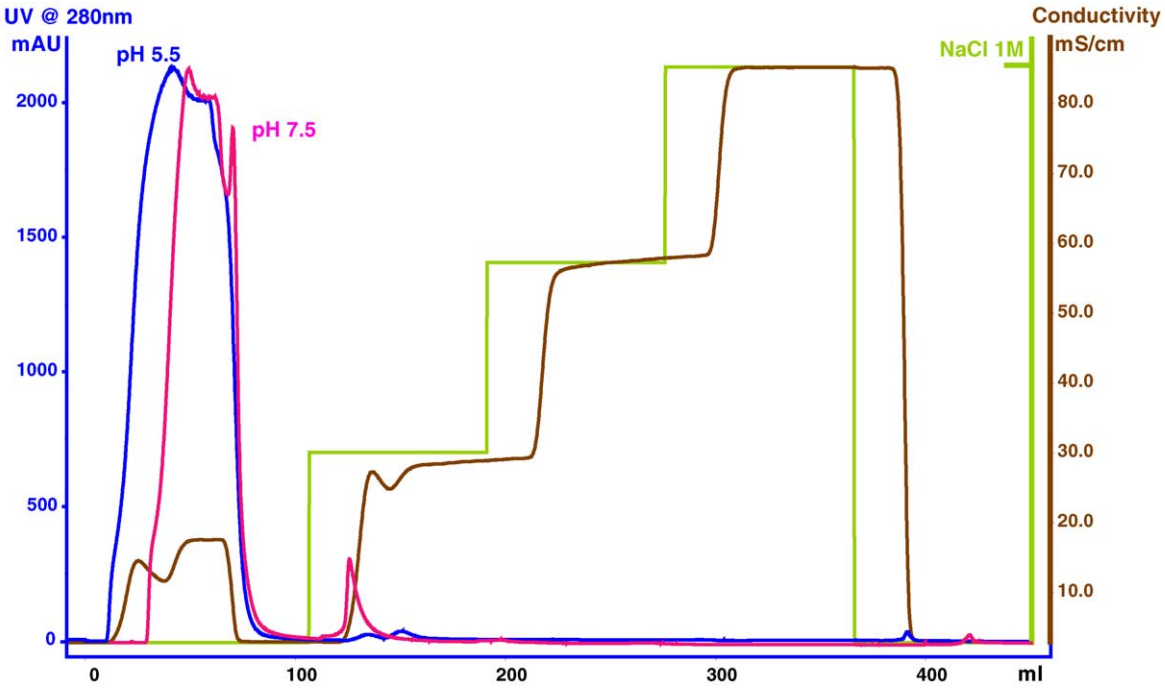

Fig. 2. Elution profiles of recombinant wheat LTP 7.6 at pH 5.5 (blue) and pH 7.5 (red). Elution steps are drawn in green and conductivity in brown.

differ in terms of primary sequence, molecular mass and isoelectric point (Table 2). The method allowed the single step purification of four out of five type 2 LTP tested and the adaptation of the method to the last type 2 LTP was done in one day. Without further modifications, the output of the method appears to be equivalent to the one obtained with the type 1 LTP (75–95%). The lower yield is observed for LTP 7.5. This LTP appears to be the most overproduced, thus the low yield may be explained by the capacity of the column as the amount of purified protein is comparable to the one of the other proteins (114–189 mg). The only modification of the method was made to allow the binding of LTP 7.6. Surprisingly, lowering the pH was not efficient and LTP 7.6 was finally purified using Tris–HCl at pH 7.5. It seems, so far, difficult to give a simple interpretation of this. One might suggest that increasing the pH results in a less important protonation of the protein that could allow unmasking charges at the surface of the protein. The determination of the structure of all these LTP is underway, this may help in giving a final explanation.

4.3. General comments

It appears clearly that, as soon as one protocol has been optimised for one protein, it can be used with related proteins after minor modifications. We noticed that all proteins tested during this project were eluted with 333 mM instead of the usually advised 1M NaCl. It seems interesting to point this out as some enzymes are deactivated by high salt. Furthermore, decreasing the ionic strength of the eluate decreases the time required for the following dialysis step. We might conclude that expanded bed absorption chromatography is a fast,

Table 3
Purification yield

	LTP produced (mg)	LTP purified (mg)	Buffer A	Yield (%)
LTP 7.1	185	150	NaAc pH 5.5	81
LTP 7.2	120	114	NaAc pH 5.5	95
LTP 7.3	248	189	NaAc pH 5.5	76
LTP 7.5	700	100	NaAc pH 5.5	14
LTP 7.6	187	173	Tris pH 7.5	92

efficient and versatile method. Thus we strongly recommend this method to be taken into consideration, not only for industrial processes but also for high throughput biochemistry programs.

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