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Purification of unstable proteins from *Halobacterium salinarium* crude cell extracts: combined cell disruption and desalting by a hollow-fiber membrane module as an access to perform ion-exchange chromatography

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Abstract

Ion-exchange chromatography, although known as one of the most powerful separation methods, is normally not taken into consideration during the purification of halophilic proteins as those proteins are unstable when exposed to a low ionic strength as necessary for matrix adsorption. This report describes a new, gentle conditioning method that allows the inclusion of ion-exchange chromatography in purification procedures of proteins from halophilic bacteria. A fast, one-step procedure was developed for both on-line cell disruption and on-line lowering of the ionic strength by cross-flow ultradialysis using a hollow-fiber membrane module prior to ion-exchange chromatography.

1. Introduction

Ion-exchange chromatography includes protein binding at low salt concentrations prior to elution by increasing the ionic strength or by shifting the pH of the elution buffer [1–3]. On the other hand, the purification of proteins from halophilic microorganisms is dominated by the need for high salt concentrations in the buffer solutions since a considerably high ionic strength is required for the stability of those proteins [4]. As a consequence, anion-exchange chromatography, although known as an efficient and reliable purification step, is normally not part of the purification of proteins from halophilic bacteria. The method is replaced by other techniques where the ionic strength plays a minor role, e.g. immobilized metal ion affinity chromatography [5], two-phase separation [6] or cell free synthesis combined with functional refolding procedures [7]. Nevertheless, since the deactivation of halophilic proteins under the stress of low ionic strength is a time-dependent process [8], ion-exchange chromatography should, in principle, be possible if the time of exposure to low salt concentrations is very short.

Very recently, the use of a hollow-fiber membrane module (VariPerm M, Bitop, Witten, Germany) was described to continuously exchange the buffer of an eluate during column chromatography [9] or prior to electrophoresis [10] by counter-current ultradialysis. One advantage of using such a module is the possibility to perform dialysis on-line and consequently with-

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out any significant loss of time. Here, we describe a combined cell disruption and desalting procedure making use of on-line dialysis against water via VariPerm M. Without any discontinuous, time-consuming conditioning steps, e.g. centrifugation or batch dialysis at low salt concentrations, the cellular macromolecules derived from *Halobacterium salinarium* were bound to Q-Sepharose fast flow, which was followed by anion-exchange chromatography. It was shown that, in contrast to a conventional conditioning procedure, the enzyme alkaline *p*-nitrophenylphosphate phosphatase remained active even after anion-exchange chromatography when the ionic strength was immediately increased after the run by prefilling the fraction tubes with a concentrated basal salt solution.

2. Experimental

2.1. Chemicals

Bovine serum albumin was purchased from Boehringer (Mannheim, Germany) and *p*-nitrophenylphosphate was from Merck (Darmstadt, Germany); all other chemicals were of analytical-reagent grade appropriate for biochemical use. Water, purified with a Milli-Q system (Millipore, Bedford, MA, USA), was used throughout and all experiments except bacterial growth were performed at room temperature.

2.2. Bacterial growth

Stock cultures of *H. salinarium* were obtained from the Deutsche Sammlung für Mikroorganismen (DSM, Braunschweig, Germany), and 400-ml cultures were grown in illuminated 2-l glass flasks at 39°C until mid-log growth phase was reached as previously described [11].

2.3. Protein measurements

The protein concentrations determined in order to monitor the efficiency of cell disruption were performed according to Lowry et al. [12]. All other protein measurements were carried out as previously described [13] using bovine serum albumin as the protein standard.

2.4. Enzyme measurements

Alkaline *p*-nitrophenylphosphate phosphatase activity was determined spectrophotometrically according to Bonet et al. [14] by monitoring the formation of *p*-nitrophenole at 405 nm.

2.5. Sample preparation and anion-exchange chromatography (including combined on-line cell disruption–desalting)

A 50-ml volume of a *H. salinarium* cell culture was centrifuged at 5000 *g* for 30 min and resuspended in 10 ml of 4.3 *M* NaCl–80 m*M* $MgSO_4$–30 m*M* KCl–10 m*M* sodium citrate–1 m*M* $CaCl_2$ (basal salts). Both cell disruption via osmotic shock and removing of major amounts of salt were carried out by on-line dialysis against water during sample application onto a 10-ml Q-Sepharose fast-flow column (Pharmacia, Uppsala, Sweden) using the hollow-fiber membrane module VariPerm M (Bitop, Witten, Germany) directly connected to the inlet of the column. The extracapillary counter-current flow-rate (water) during sample application was 30 ml/min, and the sample itself was pumped at an intracapillary flow-rate of 0.25 ml/min. Directly prior to elution, the hollow-fiber membrane module was removed and the column was run by a linear gradient of sodium chloride (0–1 *M*) in 30 ml of 20 m*M* Tris–HCl, pH 8.5. Fractions of 2 ml were collected in 10-ml vessels prefilled with 4 ml of 6.1 *M* sodium chloride.

2.6. Sample preparation and anion-exchange chromatography (conventional method)

A 50-ml volume of the same culture as mentioned above was centrifuged at 5000 *g* for 30 min and resuspended in 10 ml of 20 m*M* Tris–HCl–1 *M* NaCl, pH 8.5 in order to disrupt the cells. Debris was removed by centrifugation at 35 000 *g* for 30 min. The supernatant was dialyzed against 20 m*M* Tris–HCl, pH 8.5 for 3 h, applied to a 10-ml column filled with Q-

Sepharose fast-flow (Pharmacia), and anion-exchange chromatography was performed as described above.

3. Results

3.1. *Efficiency of on-line cell disuption and desalting*

The efficiency of counter-current dialysis within a hollow-fiber membrane module depends on the temperature, the pore size, the size of molecules to be exchanged and the extracapillary (EC) and intracapillary (IC) flow-rates. To determine the efficiency of cell disruption and desalting, an unaltered culture of *H. salinarium* in the mid-log growth phase was pumped through VariPerm M membrane module at different IC flow-rates and dialyzed in the counter-current mode against water at an EC flow-rate 30 ml/min. Samples were taken at the IC outlet and centrifuged at 10 000 *g* for 5 min. The dialysis efficiency was judged by measuring the conductivity and the cell disruption efficiency was determined by protein concentration assays of the resulting supernatant. As can be seen, at all IC flow-rates tested the efficiency of cell disruption by osmotic shock was considerably high whereas the efficiency of desalting strictly depended on the IC flow-rate (Fig. 1).

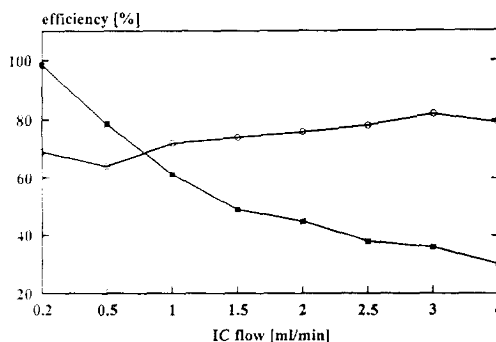

Fig. 1. Efficiency of on-line cell disruption (○, protein concentration measured in the IC supernatant after centrifugation) and on-line desalting (■, conductivity of the IC supernatant after centrifugation) via a VariPerm M membrane module at different IC flow-rates. A 100% efficiency of cell disruption corresponds to the supernatant protein concentration after mechanical cell disruption by glass beads and 100% efficiency of dialysis corresponds to the conductivity of pure Millipore water.

3.2. *Deactivation of alkaline p-nitrophenylphosphate phosphatase at low salt concentrations*

In order to demonstrate the time dependence of halophilic enzyme deactivation at low salt concentrations, the basal salt buffer of a crude cell extract of *H. salinarium* was exchanged against 20 m*M* Tris–HCl, pH 8.5 by molecular sieve chromatography using a PD 10 column (Pharmacia). At different time intervals after desalting, samples were taken and assayed for alkaline *p*-nitropenylphosphate phosphatase activity. As shown in Fig. 2, the activity decreased exponentially with $t_{1/2} = 1$ h.

3.3. *On-line versus conventional sample preparation prior to anion-exchange chromatography*

Two experiments were performed in order to compare combined cell disruption–dialysis with a conventional preparation procedure of a crude cell extract from *H. salinarium*. In contrast to a conventional sample preparation procedure, enzymatic activity of alkaline *p*-nitrophenylphosphate phosphatase was detectable in the case of

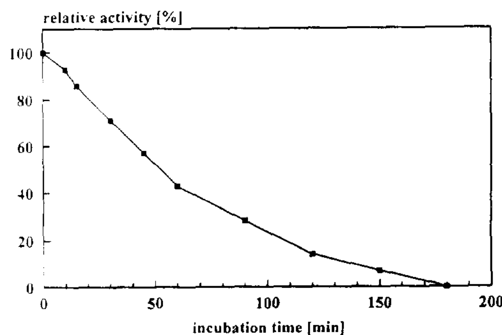

Fig. 2. Time course of alkaline *p*-nitrophenylphosphate phosphatase deactivation after removal of salts by molecular sieve chromatography via PD 10. A 100% activity corresponds to the initial activity observed directly after desalting.

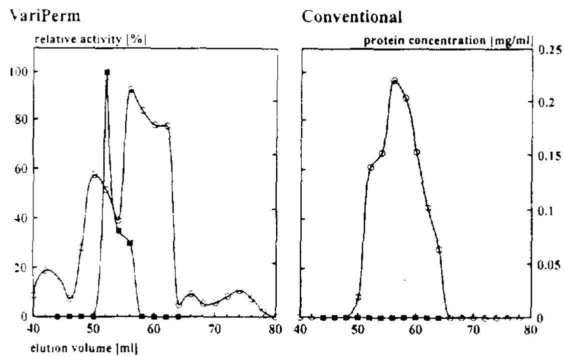

Fig. 3. Comparison of anion-exchange chromatographic runs after conventional sample preparation and after on-line conditioning by a VariPerm M membrane module. Relative alkaline *p*-nitrophenylphosphate phosphatase activities (■) and protein concentrations (○) in the resulting fractions are shown.

fast on-line cell disruption–desalting via the VariPerm M membrane module (Fig. 3). As can be seen, compared to conventional sample preparation the total recovery of protein after anion-exchange chromatography was higher when combined cell disruption–desalting was performed prior to anion-exchange chromatography.

4. Discussion

A new method of on-line dialysis to continuously disrupt the cell walls of halophilic bacteria by osmotic shock and simultaneously remove high amounts of salt by a hollow-fiber membrane module is described. We show that this procedure is necessary when ion-exchange chromatography is planned to (partially) purify halophilic proteins. In contrast to an on-line experiment using the hollow-fiber membrane module, the activity of alkaline *p*-nitrophenylphosphate phosphatase was not detectable after anion-exchange chromatography when the sample was prepared conventionally. We conclude that the main reason for the loss of activity during conventional sample conditioning is the time-dependent deactivation process during exposure to low salt concentrations, as demonstrated in Fig. 2. Since anion-exchange chromatography can be performed within 1–2 h and since the ionic strength is increased again already during chromatography, a halophilic enzyme with $t_{1/2} > 1$ h at low salt concentrations can principally be recovered with a yield of much more than 50%. Further, it may be possible that even the charged groups of the ion-exchange matrix itself act stabilizing on halophilic proteins during chromatography. On the other hand, the use of a conventional sample preparation procedure includes extended time of protein exposure to low salt concentrations, as batch dialysis usually takes more than 3 h to lower the ionic strength necessary for matrix adsorption and additional time is needed for sample adsorption prior to chromatography. This convincingly explains the observed complete deactivation of alkaline *p*-nitrophenylphosphate phosphatase after ion-exchange chromatography.

As a matter of fact, we observed a slightly impaired flow through the column when on-line dialysis was performed prior to chromatography. On the other hand no centrifugation step to remove cell debris was included and, consequently, this may cause a mechanical block. The recently developed Pharmacia Streamline system was especially designed and used to overcome such a problem [15,16]. Since all steps prior to column elution can be performed on-line, combined cell disruption–dialysis by a hollow-fiber membrane module followed by anion-exchange chromatography using the Streamline system should be an ideal advantageous starting procedure in the purification of halophilic proteins. Scaling up of a purification procedure is not limited since hollow-fiber membrane modules are available in any sizes.

The efficiency of cell disruption determined by the protein concentration was 80% compared to a mechanically cell disruption procedure (Fig. 1). It should be noted that due to osmotic pressure within the hollow-fiber membrane module dilution of the crude cell extract occurs. No difference in disruption efficiency was found when the total amounts of protein instead of the protein concentrations were compared.

In this study, the ionic strength of the column eluate after anion-exchange chromatography was

increased immediately by the use of fraction tubes prefilled with high-salt-concentration solutions. We observed that, due to the density difference between the eluate and the high-salt-concentration solution, mixing was not sufficient and additional stirring of each fraction was necessary. A second hollow-fiber membrane module directly connected to the outlet of the column as previously described [9] could solve this problem by gently transferring back the salts to the eluate via counter-current dialysis. The slow increase of the ionic strength should further improve the recovery of enzymatic activity.

Even if no ion-exchange step were considered to purify halophilic proteins, the use of hollow fiber membrane modules as an efficient method of on-line cell disruption can be of great advance. In that case, dialysis with an efficiency yielding a residual intracapillary NaCl concentration of 1 *M* should be sufficient to disrupt the cells (see conventional method) and, if necessary, a second module can be used directly after cell disruption to immediately restore the ionic strength of the resulting crude cell extract. This procedure can be followed either by on-line protein adsorption onto a hydrophobic-interaction Streamline column or by centrifugation using a continuous centrifuge prior to further purification steps.

The data presented here clearly demonstrate that the on-line method described in general allows the inclusion of ion-exchange chromatography in purification schemes of halophilic proteins although the latter are unstable when exposed to low ionic strength. Further, the described method is not limited to sample preparation of halophilic crude extracts prior to anion-exchange chromatography. If the absence of other low-molecular-mass compounds, e.g., coenzymes or substrates, that stabilize a certain target protein is required for protein binding to the matrix, their fast but transient elimination by on-line dialysis would also be useful prior to other chromatographic methods such as affinity chromatography.

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