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Short Communication

Purification of bacilli ribonucleases by reversed-phase high-performance liquid chromatography

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Abstract

A two-step purification method is presented that utilizes the specific chromatographic properties of bacilli extracellular cycling ribonucleases. A double gradient system of elution is used. Initial concentration of the sample followed by reversed-phase HPLC gives high yields (90–95%) of homogeneous, active protein on both analytical and preparative scales. The procedure may be applied to the isolation of ribonucleases from different sources without significant modifications

1. Introduction

The extracellular cycling ribonucleases from bacilli provide a convenient model for structure–function studies of enzymes [1–5]. This is primarily due to their small size (M_r 11 000–12 000), the absence of disulphide bonds, easy assay and resistance to denaturation over a wide pH range (3–7.5) [1]. Cloning of the genes coding for ribonucleases from *Bacillus intermedius* (binase, Bi) and *Bacillus amyloliquefaciens* (barnase, Ba) [6,7] has made it possible to apply protein engineering methods to detailed structure–functional analysis of these enzymes [2,4]. To date, a variety of multi-step procedures have been described for the purification of homogeneous binase, barnase, their mutants and other similar extracellular ribonucleases from bacilli, with yields ranging from 20% to 60% [2,8–11]. The present procedure consists of only two steps: the sample is first concentrated, and then subjected to reversed-phase HPLC. This method gives consistently high yields (85–90%) of homogeneous native protein in both analytical and preparative amounts.

2. Experimental

2.1. Materials

Analytical reagent grade salts (Reakhim, Moscow, Russian Federation) were used throughout. isopropyl-6-D-thiogalactopyranoside (IPTG) was obtained from Biopol (Moscow, Russian Federation). Mobile phases were prepared with HPLC-grade acetonitrile (Fluka, Buchs, Switzerland) as the stronger solvent. The aqueous portions of the mobile phase were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, USA) with 0.1% trifluoro-

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acetic acid (TFA), 0.1 *M* ammonium phosphate (pH 2.2), 0.1 *M* ammonium phosphate (pH 3.0), 0.1 *M* ammonium phosphate (pH 3.5), 0.1 *M* ammonium phosphate (pH 4.0), 0.1 *M* ammonium acetate (pH 5.0), 0.1 *M* ammonium phosphate (pH 6.0) and 0.1 *M* ammonium acetate (pH 7.5). All components for growth media purchased from Difco Labs. (Detroit, MI, USA). All buffers and samples for HPLC were filtered through membrane filters (Dia-M, Moscow, Russian Federation) with a pore size of 0.2 μm. The ammonium sufphate fraction of *Bacillus pumiles* culture medium, containing RNAse (Bp), was obtained from Dr. A.A. Dementiev (IMB, Russian Academy of Science). Binase was purified as described by Golubenko *et al.* [10].

2.2. RNase activity assay

RNase activity was assayed at pH 7.5 in 120-μl mixtures containing 0.1 *M* Tris–HCl, 0.1 *M* NaCl and 1.6 mg/ml of ribonucleic acid from yeast (Serva). After incubation at 37°C for 15 min, 300 μl of 2-propanol were added with vigorous mixing. After the mixtures had stood for 20 min at −20°C, they were centrifuged for 10 min at 14 000 *g*. The supernatant was diluted (1:100) in water and the absorbance at 260 nm was measured. The absorbance at 260 nm of the supernatant was a linear function of added enzyme up to an absorbance of at least 1.5.

2.3. Strains and cultivation conditions

The *B. intermedius* 7P strain, a binase producer, was obtained from the Russian National Collection of Industrial Microorganisms (Institute for Genetics of Microorganisms, Moscow, Russian Federation), and cultivation was carried out according to Golubenko *et al.* [10]. The *Eschrichia coli* strain JM105, containing the pMT416 plasmid, also a barnase producer, was provided by courtesy of Professor R.W. Hartley (NIH, Bethesda, MD, USA). Cultivation was according to the scheme of Hartley [7], with the exception that cells were induced immediately upon inoculation. In this step, IPTG was added to a final concentration of 4 μg/ml.

2.4. Preparation of binase ammonium sulphate fractions

Cells (from 5 l) were pelleted and the supernatant was chilled to 4°C. Ammonium sulphate was then added to the supernatant to a final concentration of 95% saturation while stirring. After overnight stirring (at 4°C), centrifugation (3000 *g*, 30 min) yielded the protein predominantly in the pellet. The pellet was dissolved in 50 ml of 0.1 *M* ammonium acetate (pH 7.5), centrifuged and filtered through a 0.2-μm membrane filter prior to loading on the column. The protein yield (according to the RNase activity) at this stage was 99%.

2.5. Preparation of concentrated barnase

Cell culture was grown overnight at 37°C. Cell growth was halted by chilling followed by the addition of acetic acid to 5%, in order to release that part of the barnase still located in the bacterial periplasm (about 50%). Stirring was continued for a further 15 min. Cells were pelleted by centrifugation for 10 min at 10 000 *g*. SP-Trisacryl cation-exchange resin (5 ml of settled volume per litre of culture medium), previously equilibrated with 50 m*M* sodium acetate (pH 4.5), was added to the supernatant whilst gently stirring. After stirring for 1 h, the resin was allowed to settle and the supernatant decanted. The sorbent was transferred to a glass filter and washed with 2 *M* ammonium acetate (pH 8.0) until the absorbance of the eluate at 280 nm became negligible [12]. The protein yield (according to the activity) at this stage was 91%.

2.6. High-performance liquid chromatography

The HPLC apparatus (Gilson Medical Electronics, Middleton, WI, USA) consisted of Model 305 pumps, a Model 803C manometric module, Model 811B mixer with a 1.5-ml chamber, UV112 detector (set at 280 nm) and Model 7125 injector (Reodyne, Cotati, CA, USA) with 20- and 100-μl and 1- and 2-ml loops. A Model 714 HPLC system controller (Gilson) was used to regulate chromatographic processes

and register results. The following columns were used for analytical separations: Silasorb SPH C_8, $d_p = 9$ μm (150 × 4 mm I.D.) and Silasorb SPH C_{18}, $d_p = 9$ μm (150 × 4 mm I.D.). For preparative purification we used a Silasorb SPH C_{18} column, $d_p = 13$ μm (250 × 10 mm I.D.). All columns were manufactured by Elsico (Moscow, Russian Federation). Elution was performed with a 0–70% linear gradient of buffer B (acetonitrile) in the aqueous buffer at a flow-rate of 1 ml/min for analytical separations and 3 ml/min for preparative separations.

3. Results and discussion

The intention behind this work was to devise a rapid, simple and efficient purification method applicable to the ribonucleases barnase, binase and other similar extracellular RNAses of bacilli. The investigation of the behaviour of barnase on the modified silica gels (C_8 and C_{18}) showed a strong dependence of its recovery on the pH of eluent. At acid pH values (2–2.5) the protein is eluted from the column in 80–98% yield, whereas at neutral pH (6–7.5) its yield falls almost to zero (Table 1). Reiteration of the elution cycle without changing the conditions (pH of the eluent) does not increase the yield of barnase. However, with repeated elution with acidic buffer barnase elutes from the column in high yield. In contrast, the retention time of barnase at different pH values is virtually constant.

The pH of the chromatographic elution buffer was found to influence strongly the protein yield. At an eluent pH of 5 or above, RNase was strongly retained on the column; even repeated cycles with C_{18} phase were unsuccessful in eluting significant amounts of RNAse and only a 20% yield could be reached using a C_8 phase. This also applies to the other bacilli RNases, such as binase, RNAse of *Bacillus thuringiensis* and RNAse of *Bacillus pumiles*, for which retention times were determined to be close to those of barnase itself.

More detailed studies are being carried out to determine the basis for these observations. The purification method now described is based on these chromatographic properties of barnase. A sample containing barnase was applied to a

Table 1
Dependence of binase yield and capacity of the sorbents on the type of buffer used

Buffer	Protein yield (%)		Sorbent capacity (mg protein/ml sorbent)	
	C_8 phase	C_{18} phase	C_8 phase	C_{18} phase
0.1% TFA (pH 2.2)	98.9	97.8	5.3	6.1
0.1 *M* ammonium phosphate (pH 2.2)	98.9	86.8	5.2	6.2
0.1 *M* ammonium phosphate (pH 3.5)	68.7	61.7	5.1	6.0
0.1 *M* ammonium phosphate (pH 4.0)	49.3	19.7	5.1	6.0
0.1 *M* ammonium acetate (pH 5.0)	6.2	5.6	5.2	6.1
0.1 *M* ammonium acetate (pH 6.0)	5.6	2.3	6.3	6.3
0.1 *M* ammonium acetate (pH 7.5)	2.8	1.5	5.5	6.7

Chromatographic conditions: columns, Silasorb C_8 and Silasorb C_{18} (150 × 4 mm I.D.). Mobile phase, linear gradient of acetonitrile, 0–70%; flow-rate, 1 ml/min.

reversed-phase column equilibrated with buffer A1 (pH >7). Contaminants were eluted with an acetonitrile gradient. The column was then washed with buffer A1 and re-equilibrated with buffer A2 (pH 2.2–2.5). A second elution yielded homogeneous RNAse (Fig. 1a).

Although the conditions used failed to achieve a 100% protein yield, the sorbtion of RNase proteins is not irreversible, and it is possible to remove the remainder of the protein from the column by a method described by Henderson *et al.* [13]. The best results were obtained using C_{18} as the stationary phase and buffers A1 (0.1 *M* ammonium acetate, pH 7.5) and A2 (0.1% TFA) as mobile phases. TFA-containing solutions are widely used as RP-HPLC buffers [14,15] owing to their useful properties. Their volatility, in particular, allows desalting of enzyme preparations immediately after lyophilization.

Fig. 1b shows an elution profile with an acetonitrile gradient in 0.1% TFA and Fig. 1a an elution profile with a double gradient of acetonitrile in 0.1 *M* ammonium acetate (pH 7.5) and then 0.1% TFA. Despite having identical separation times, the peaks in Fig. 1b contained contaminants. When a double gradient was used (Fig. 1a), barnase eluted in a homogeneous peak with the second gradient.

A chromatogram of a preparative separation is shown in Fig. 2; the amount of protein in the peak is 100 mg. It demonstrates that even when a large volume of sample is injected, peaks are well resolved.

The method by which the protein is concentrated prior to chromatographic separation was found not to affect significantly the chromatographic efficiency. Therefore, any convenient method may be used for this purpose.

The method described here can provide protein of sufficient purity for amino acid analysis and sequence determination [16]. The method was used to purify RNAses Ba, Bi, Bt and Bp (Table 2) and some other barnase and binase mutant forms.

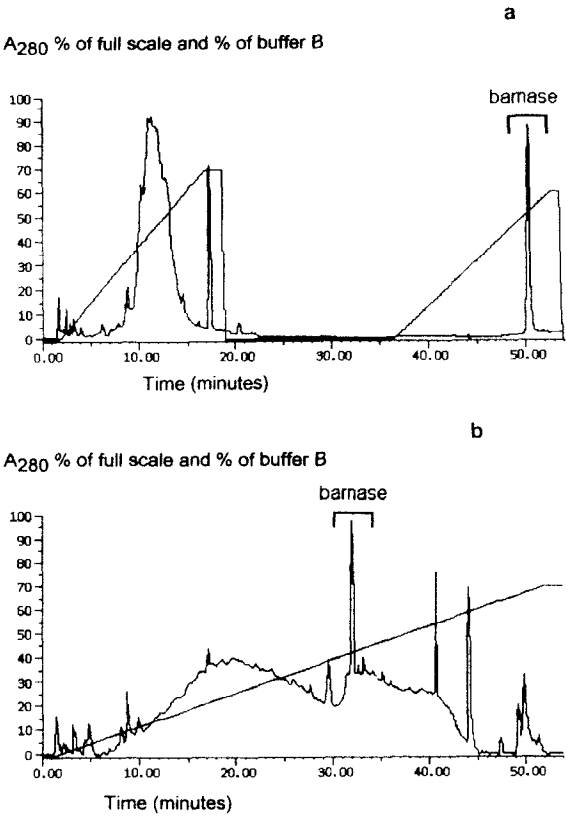

Fig. 1. Analytical separation of 20 μl of ammonium sulphate fraction from cultural liquid of JM105 (pMT416) strain. Column, Silasorb C_{18} SPH (150 × 4 mm I.D.). Mobile phase, (a) 0.1 *M* ammonium acetate (pH 7.5)–acetonitrile (first gradient) and 0.1% TFA–acetonitrile (second gradient). (b) 0.1% TFA–acetonitrile. Flow-rate, 1 ml/min. $A_{280} = 0.1$ AUFS.

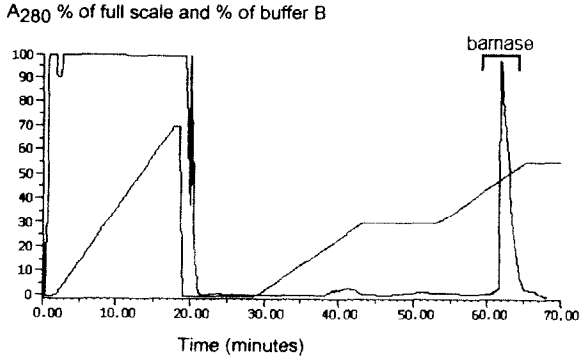

Fig. 2. Preparative separation of ammonium sulphate fraction from JM105 (pMT416) straincultural liquid, 100 mg Ba in peak. Column, Silasorb C_{18} SPH (250 × 10 mm I.D.). Mobile phase, 0.1 *M* ammonium acetate (pH 7.5)–acetonitrile (first gradient) and 0.1% TFA–acetonitrile (second gradient). Flow-rate, 3 ml/min. $A_{280} = 3$ AUFS.

Table 2
Purification scheme for RNAses Ba, Bi and Bp

Stage of purification	Volume (ml)	Total protein (A_{280})	Total activity[a] (units per ml of 1 A_{280})		Yield (%)
Culture liquid, JM105 (pMT416)	2000	16 000	55 000	6875	100
After SP-Trisacryl chromatography	40	7500	$2.5 \cdot 10^6$	$1.3 \cdot 10^4$	91
After RP-HPLC	8	102	$1.2 \cdot 10^6$	$9.5 \cdot 10^5$	89
Culture liquid, *B. intermedius*	2000	18 000	$4 \cdot 10^5$	$4.4 \cdot 10^4$	100
After ammonium sulphate precipitation	15	1375	$5.3 \cdot 10^7$	$5.8 \cdot 10^5$	99
After RP-HPLC	10	648	$7.2 \cdot 10^7$	$1.1 \cdot 10^6$	90
Ammonium sulphate fraction RNAse Bp	15	210	$6.3 \cdot 10^5$	$4.5 \cdot 10^4$	100
After RP-HPLC	10	9.1	$8.5 \cdot 10^5$	$9.4 \cdot 10^5$	95

[a] One unit will produce alcohol-soluble oligonucleotides equivalent to a ΔA_{260} of 1.0 in 30 min at pH 7.5, 37°C, in a 120-μl reaction volume.

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