Journal of Chromatography, 469 (1989) 448–452
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 356

Note

Purification of yeast killer toxin KT28 by receptor-mediated affinity chromatography

MANFRED SCHMITT and FERDINAND RADLER*
Institut für Mikrobiologie und Weinforschung, Johannes Gutenberg-Universität, Postfach 3980, D-6500 Mainz (F.R.G.)

(First received September 26th, 1988; revised manuscript received January 27th, 1989)

The ability of certain yeast strains of several genera to secrete protein or glycoprotein "killer toxins", lethal to other, sensitive yeast strains, is a well documented phenomenon[1]. Most *Saccharomyces cerevisiae* killer strains belong to either the K_1, K_2 or K_3 class, in which the killer phenotype is dependent on the presence in the cell of two different double-stranded RNA species[2,3]. Both of these RNA molecules are encapsulated by the same 88-kilodalton protein encoded by the larger L-A-dsRNA. The smaller M-dsRNA carries the information necessary for the production of killer toxin or its precursor protein[4].

Although a considerable amount of information concerning the genetic basis of the killer phenomenon has been published[4–7], comparatively little is known about the killer proteins and their mode of action. This lack of data is due, in part, to the instability of killer toxins (they are rapidly inactivated at pH values above pH 5 and temperatures above 30–35°C) and therefore few killer toxins have been successfully purified and characterized. The K_1-killer toxin of *S. cerevisiae* is a non-glycosylated 18-kilodalton α,β-heterodimer that, after initially binding to a linear β-1,6-D-glucan component of the cell wall, exerts its toxic effect via a specific, but as yet unknown, interaction with the cytoplasmic membrane of sensitive yeast cells[8,9]. The killer toxin KT28 of *S. cerevisiae* differs from the killer groups K_1–K_3 in that it does not bind to glucans but to a 185-kilodalton mannoprotein constituent of yeast cell walls[10,11]. It has already been shown that minor modifications to the structure of this mannoprotein prevent the binding of killer toxin KT28 to the cell walls of sensitive yeast strains and also that these modifications are sufficient to confer resistance to killer toxin KT28[12].

This paper describes how the specific binding of killer toxin KT28 to immobilized mannoprotein was utilized to develop a simple and effective method for the purification of this killer toxin, via a receptor-mediated affinity chromatographic technique.

0021-9673/89/$03.50

EXPERIMENTAL

Microorganisms and culture media

For the production of killer toxin KT28, *Saccharomyces cerevisiae* strain 28 was cultivated in synthetic B-medium at pH 3.5[10]. Sensitive strains of *S. cerevisiae* (381 and X2180-1Aa, obtained from Dr. C. E. Ballou, Berkely, CA, U.S.A.) were grown in YEPD medium as described previously[10]. Methylene blue agar (2% glucose, 2% peptone, 1% yeast extract, 1.5% agar, 1.92% citric acid, 0.003% methylene blue, adjusted to pH 5.8 with K_2HPO_4) was used for the determination of killer toxin activity.

Production of killer toxin KT28 and measurement of killer toxin activity

S. cerevisiae strain 28 was grown in 20 l of B-medium (pH 3.5) for 72 h at 20°C. After centrifugation (5000 *g*, 30 min), the supernatant was concentrated by ultrafiltration (Sartorius type SM 12136, Amicon-PM10) to a volume of 20 ml and then dialysed against citrate–phosphate buffer (10 m*M*, pH 3.5) as described previously[10]. Killer toxin activity in this crude concentrate was assayed by the agar diffusion method[13]. After seeding the sensitive strain *S. cerevisiae* 381 (10^5 cells) on to methylene blue agar plates, killer toxin KT28 (0.1 ml; crude concentrate) was pipetted into wells (10 mm diameter) cut into the agar and the plates were incubated for 72 h at 20°C. The presence of a growth-free zone around the wells is indicative of killer toxin activity. The activity of killer toxin KT28 is expressed in arbitrary units; one unit of KT28 activity corresponds to about 0.1 ng of purified protein[11].

Isolation and purification of the primary receptor for killer toxin KT28

The primary receptor for killer toxin KT28, a 185-kilodalton mannoprotein component of the yeast cell wall, was extracted from *S. cerevisiae* X2180-1Aa by first autoclaving (90 min, 121°C) the cells in citrate buffer (30 m*M*, pH 7.0) and then precipitating the mannan complex by addition of four volumes of ice-cold methanol[11]. Following further fractionation of this extract with cetyltrimethylammonium bromide according to the method of Lloyd[14], crude mannoprotein was purified by anion-exchange chromatography on Q-Sepharose followed by gel filtration on Superose 12 HR 10/30 as described previously[11].

Preparation of mannoprotein–CNBr-Sepharose and affinity chromatography of killer toxin KT28

CNBr-Sepharose 4B (1 g, Pharmacia) was soaked for 15 min in 50 ml HCl (1 m*M*), placed on a sintered-glass filter (Schott, size G 4, average pore size 10–16 μm) and rinsed successively with 200 ml of HCl (1 m*M*) and 5 ml of coupling solution (0.1 *M* $NaHCO_3$–0.5 *M* NaCl, pH 8.3). The gel was then mixed with 55 mg of purified mannoprotein (dissolved in 5 ml of coupling solution), gently shaken for 2 h at 20°C and washed with 15 ml of coupling solution on top of the sintered-glass filter. The carbohydrate content of the receptor bound to the gel matrix was determined indirectly by the phenol–sulphuric acid method as described previously[11]. To inactivate remaining free binding sites on the CNBr-Sepharose, the gel was treated with ethanolamine (1 *M*, pH 8.0) for 2 h at 20°C and then washed three times with 15 ml of coupling solution and 15 ml of acetate buffer (0.1 *M* Na-acetate–0.5 *M* NaCl, pH

4.0). At a flow-rate of 1.0 ml min^{-1}, the mannoprotein–CNBr–Sepharose gel was pumped into a glass column (2.6 × 1.6 cm I.D.) and equilibrated with ten column volumes of citrate–phosphate buffer (10 m*M*, pH 3.5). For affinity chromatography, 2 ml of crude, dialysed concentrate of killer toxin KT28 (specific activity $2.0 \cdot 10^4$ U mg^{-1} protein) was applied to the column and chromatographed with sodium citrate buffer (10 m*M*, pH 3.5, 4°C) at a flow-rate of 0.1 ml min^{-}. Bound killer toxin was eluted by applying a linear gradient of 0–1 *M* NaCl (40 ml) in sodium citrate buffer. Eluted fractions (1 ml) were collected and assayed for protein content and killer toxin activity. The protein content of the eluate was determined spectroscopically at a wavelength of 280 nm[15].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Following affinity chromatography, fractions that contained killer toxin KT28 were dialysed against sodium citrate buffer (10 m*M*, pH 3.5), lyophilized and then dissolved in sample buffer containing 2.5% (w/v) SDS and 5% (v/v) mercaptoethanol. The compositions of the sample and electrode buffer were as described previously[10]. Samples were then applied to an SDS gradient gel (4–30% acrylamide) and electrophoresed for 3.5 h at 150 V. After fixation in an aqueous solution of 25% (v/v) 2-propanol and 10% (v/v) acetic acid, the proteins were rendered visible by staining the gels with Coomassie Brilliant Blue [0.02% (w/v) in 7% (v/v) acetic acid].

RESULTS AND DISCUSSION

Mannoprotein is an important component of the yeast cell wall and constitutes the primary receptor for yeast killer toxin KT28[11]. To enable the affinity chromatography of killer toxin to be performed, cell wall mannoprotein was purified and then covalently linked to CNBr-activated Sepharose 4B. Analysis of the carbohydrate content of the ligand, both before and after coupling mannoprotein to the gel matrix, revealed that about 20% of mannoprotein became covalently bound to the Sepharose gel.

Killer toxin KT28 bound specifically to the immobilized mannoprotein and could be eluted at a conductivity of approximately 7 mS in fractions 49–54 (Fig. 1). This affinity chromatographic procedure resulted in a 115-fold increase in killer toxin specific activity (from $2.0 \cdot 10^4$ to $2.3 \cdot 10^6$ U mg^{-1} protein).

The success of the technique described for purifying killer toxin KT28 was further demonstrated by SDS-PAGE (Fig. 2). In contrast to the crude preparation of killer toxin KT28, which contained several different protein components, purified killer toxin obtained by affinity chromatography was visible as a single band on SDS gels with an R_F value characteristic of the 16-kilodalton glycoprotein KT28.

Not only their instability to higher pH values, temperature and oxygen, but also their tendency to form aggregates causes problems and difficulties in the purification of yeast killer toxins[16–18]. The affinity chromatographic technique described here, based on the biospecific interaction of killer toxin and its receptor, appears to be a simple and effective one-step method for the purification of yeast killer toxin. However, it is apparently insufficient to use preparations of immobilized yeast cell walls as the bound ligand, as only partial purification of killer toxin can then be achieved[19]. Obviously, for the successful purification of killer toxin, isolated and purified recep-

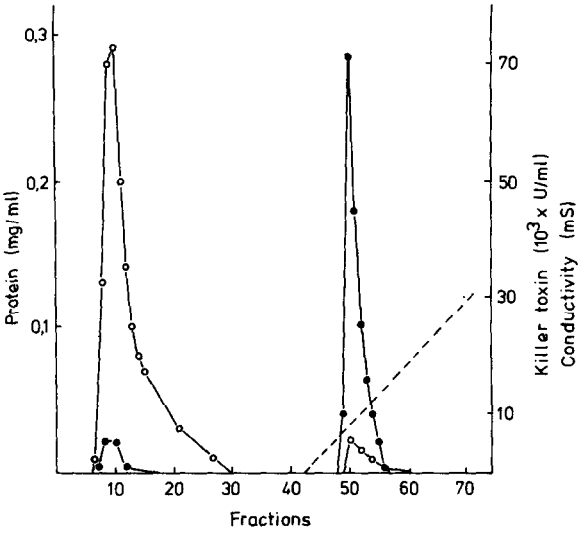

Fig. 1. Receptor-mediated affinity chromatography of killer toxin KT28 with a column of mannoprotein-CNBr-Sepharose 4B. ● = Killer toxin activity; ○ = protein concentration; broken line = conductivity.

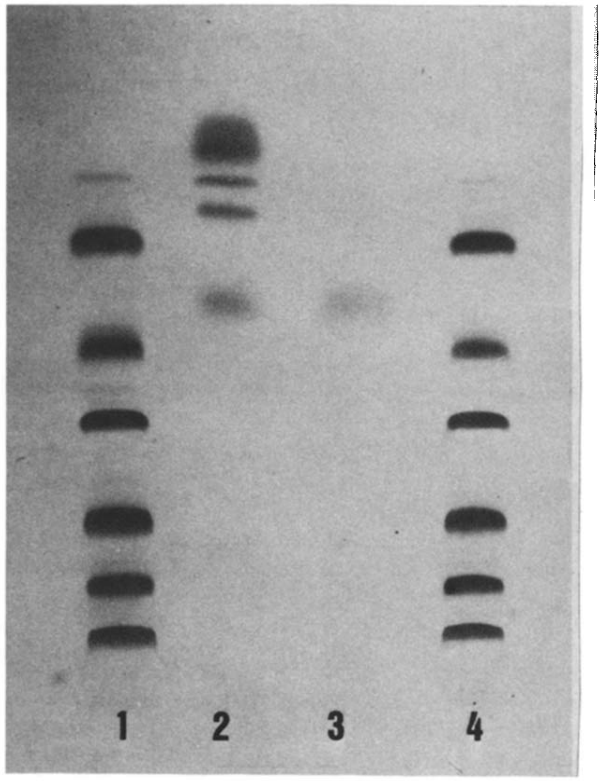

Fig. 2. SDS-PAGE of crude killer toxin KT28 before (lane 2) and after (lane 3) affinity chromatography with mannoprotein–CNBr-Sepharose 4B. Lanes 1 and 4, low-molecular-weight calibration proteins (from top to the bottom: α-lactalbumin, 14.2 kilodaltons; trypsin inhibitor, 20.1 kilodaltons; carbonic anhydrase, 29 kilodaltons; ovalbumin, 45 kilodaltons; albumin, 66 kilodaltons; phosphorylase B, 97.4 kilodaltons).

tors are required. Only the primary receptors for killer toxins K_1[9] and KT28[11] have so far been identified, and it is not known whether other types of killer toxins are bound by these receptors[12]. Hutchins and Bussey[9] linked fungal pustulan to Sepharose and were able to purify the K_1 killer toxin of *S. cerevisiae* T158C by affinity chromatography, thereby demonstrating the affinity of K_1 to β-1,6-D-glucan, a component of the yeast cell wall. Similarly, we previously used mannoprotein linked to Sepharose to demonstrate the binding of killer toxin KT28 to cell wall mannoprotein[11]. However, in these experiments, mannoprotein was linked to the Sepharose via the hydroxyl groups of the mannose side-chains essential for the binding activity of the KT28-receptor[12]. Consequently, the affinity of the receptor for killer toxin KT28 was decreased and this made the purification of killer toxin on a preparative scale impossible.

In the experiments described here an affinity gel was used to which the purified receptors for KT28 had been linked covalently via their polypeptide chains, as the protein moiety of mannoprotein is known not to be involved in killer toxin binding[11]. By introducing this modification, killer toxin KT28 could be purified 115-fold and separated from other extra-cellular proteins. The gel matrix of Sepharose itself does not bind the killer toxin; this was demonstrated with a CNBr-Sepharose in which all reactive groups had been blocked with ethanolamine.

Thus, it was shown that the presence of a specific receptor, cell wall mannoprotein, is essential for succesful affinity chromatography of killer toxin KT28. We are optimistic that this procedure will provide an effective means for the selective purification of other yeast killer toxins and, possibly, other proteins with similar biological activities. One immediate candidate for purification by the affinity chromatography method is the killer toxin of *Debaryomyces hansenii*, which, like killer toxin KT28 of *S. cerevisiae*, is bound by the mannan fraction of the yeast cell wall[20].

REFERENCES

1 D. J. Tipper and K. A. Bostian, *Microbiol. Rev.*, 48 (1984) 125.
2 K. A. Bostian, J. A. Surgeon and D. J. Tipper, *J. Bacteriol.*, 143 (1980) 463.
3 R. B. Wickner, *Annu. Rev. Biochem.*, 55 (1986) 373.
4 T. W. Young, in A. H. Rose and J. S. Harrison (Editors), *The Yeasts*, Academic Press, New York, 2nd ed., 1987, p. 131.
5 R. Esteban and R. B. Wickner, *Genetics*, 117 (1987) 399.
5 M. El-Sherbeini and K. A. Bostain, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 4293.
7 T. Fujimura and R. B. Wickner, *J. Biol. Chem.*, 263 (1988) 454.
8 K. A. Bostian, H. Bussey, Q. Elliot, V. Burn, A. Smith and D. J. Tipper, *Cell*, 36 (1984) 741.
9 K. Hutchins and H. Bussey, *J. Bacteriol.*, 154 (1983) 161.
10 P. Pfeiffer and F. Radler, *J. Gen. Microbiol.*, 128 (1982) 2699.
11 M. Schmitt and F. Radler, *J. Gen. Microbiol.*, 133 (1987) 3347.
12 M. Schmitt and F. Radler, *J. Bacteriol.*, 170 (1988) 2192.
13 J. M. Somers and E. A. Bevan, *Genet. Res.*, 13 (1969) 71.
14 K. O. Lloyd, *Biochemistry*, 9 (1970) 3446.
15 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1941) 384.
16 D. R. Woods and E. A. Bevan, *J. Gen. Microbiol.*, 51 (1968) 115.
17 H. Bussey, *Nature New Biol.*, 235 (1972) 73.
18 G. E. Palfree and H. Bussey, *Eur. J. Biochem.*, 93 (1979) 487.
19 V. Jirku, *Biotechnol. Lett.*, 8 (1986) 639.
20 S. Jenkner-Becherer, unpublished results.