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Short Communication

Rapid method for preparing a β-glucan-specific sensitive fraction from Limulus (*Tachypleus tridentatus*) amebocyte lysate

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ABSTRACT

By separating Limulus amebocyte lysate by cation-exchange chromatography with an SP-Toyopearl 650C column, a fraction insensitive to endotoxin, yet specifically sensitive to β-glucan, was successfully obtained in the unadsorbed portion. This fraction showed β-glucan dose-dependent clotting enzyme activity, although no sensitivity to endotoxin. This β-glucan-dependent reaction showed no interference in the presence of endotoxin, with the fraction also showing sensitivity towards various kinds of β-glucan, *i.e.* curdlan, pachyman, laminaran and lichenan. The sensitivity towards curdlan was approximately 10^{-10} g/ml.

INTRODUCTION

Limulus amebocytes contain a coagulation system which consists of several protein components and has been reported to be sensitive to bacterial endotoxins [1]. The limulus amebocyte lysate (LAL) test is widely used as a rapid and sensitive method for detecting bacterial endotoxins, reacting not only with endotoxins but also with various types of β-glucan (BG). These two reactions occur through independent pathways, one being triggered by bacterial endotoxin and the other by BG [2,3]. Both of these coagulation-cascade reactions are triggered by the activation of either endotoxin-sensitive serine protease zymogen (Factor C) [4,5] or BG-sensitive serine protease zymogen [3], resulting in the conversion of a pro-clotting enzyme (proCE) to an active clotting enzyme (CE) which transforms coagulogen to coagulin gel [4,6]. The LAL test for either endotoxin or BG detection is mainly performed by determining the gelatination of the LAL, or by measuring the CE activity generated by the endotoxin or BG.

Separating LAL on a dextran sulphate Sepharose column has been reported as a method to produce both endotoxin- and BG-specific LAL [7,8]. However, this

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method requires a later recombination of several fractions for the measurement of the CE activity generated by either endotoxin or BG.

Although Ikemura *et al.* [9] reported a quantification method of BG with LAL, the method requires measurements to be performed twice with two types of LAL, one which is specifically sensitive to endotoxins and the other which is sensitive to both BG and endotoxins.

A new rapid method is reported which purifies a BG-specific fraction from LAL by cation-exchange chromatography, and can additionally provide a method for the specific quantification of BG.

EXPERIMENTAL

Asian Limulus (*Tachypleus tridentatus*) were used for amebocyte collection. Chemicals were obtained as follows: SP-Toyopearl 650C (Tosoh, Tokyo, Japan), curdlan (Wako, Osaka, Japan), lichenan (Sigma, St. Louis, MO, U.S.A.) and laminaran (Tokyo Kasei, Tokyo, Japan). Pachyman was prepared from *Poria cocos* by the method of Whelan [10]. Carboxymethyl (CM) curdlan was prepared from curdlan according to the method of Sasaki *et al.* [11]. CM curdlan was dissolved and diluted with endotoxin-free water, whereas all the other BGs were dissolved in 0.1 *M* sodium hydroxide solution (5 mg/ml) and diluted with endotoxin-free water to a given concentration. Endotoxin from *Escherichia coli* 0111:B4 (Difco Labs., Detroit, MI, U.S.A.) was also dissolved in endotoxin-free water. To avoid contamination of endotoxins and BGs, all glassware was heat-sterilized before use for 2 h at 250°C.

Limulus amebocytes were collected by essentially the method of Nakamura *et al.* [12], with the exception that *n*-propranolol was used instead of caffeine as an anti-agglutination reagent. Limulus haemolymph was bled through the dorsal joint into sterile plastic bags containing 3% sodium chloride solution and 5 m*M* *n*-propranolol. Plasma was separated by centrifugation, and the precipitating amebocytes were washed twice with 20 m*M* Tris–HCl buffer (pH 7.2) containing 3% sodium chloride solution. These amebocytes were then homogenized in fifteen volumes of 20 m*M* Tris–HCl buffer (pH 8.0), and the supernatant was collected by centrifugation at 10 000 *g*.

The lysate (90 ml) was applied to a glass column packed with SP-Toyopearl 650C (200 mm × 26 mm I.D.) equilibrated with 20 m*M* Tris -HCl buffer (pH 8.0) at a flow-rate of 200 ml/h. The unadsorbed portion was washed from the column with the same buffer, and elution was performed in this buffer with a step gradient of 0.05, 0.125, 0.2, 0.5 and 2 *M* sodium chloride solution.

BG- and endotoxin-dependent CE activities were calculated from the released amount of 7-aminomethylcoumarin (AMC) by cleavage of N-*tert.*-butoxycarbonyl(Boc)-Leu-Gly-Arg-4-methylcoumaryl-7-amide (MCA). A 0.05-ml aliquot of sample, 0.025 ml of 320 m*M* Tris–HCl buffer (pH 8.0) containing 200 m*M* magnesium chloride solution, 0.1 ml of BG or endotoxin solution and 0.025 ml of 4

m*M* Boc-Leu-Gly-Arg-MCA were mixed and incubated for an appropriate time at 37°C. Acetic acid (2 ml, 10%) was added to terminate the reaction; the amount of released AMC was measured spectrofluorimetrically (Hitachi F-3000) using an excitation wavelength of 342 nm and emission at 410 nm.

The activity of Factor C, an endotoxin-sensitive serine protease zymogen, was assayed by essentially the method of Nakamura *et al.* [5], except that Boc-Val-Pro-Arg-MCA was used as a substrate.

The protein concentration was measured by the method of Lowry *et al.* [13].

RESULTS

The elution profile of the SP-Toyopearl 650C chromatographic separation is shown in Fig. 1. The BG-dependent CE activity appeared only in the unadsorbed fraction. Therefore fractions 7–20 were pooled to obtain a BG-specific sensitive fraction, which showned no Factor C activity and contained no coagulogen during sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The large peak eluted with a 0.125 *M* sodium chloride solution was coagulogen, which appeared almost homogeneous on SDS-PAGE (Fig. 2) and showed no Factor C or proCE activity. Factor C was eluted with sodium chloride concentrations, greater than 0.2 *M*.

This LAL separation method is also effective for the purification of coagulogen and Factor C. The protein content of the lysate applied to the column, the BG-specific fraction and the coagulogen fraction (fractions 93–98) were 288, 153 and 54 mg, respectively.

Fig. 3 shows the endotoxin-dependent CE activities of both the lysate applied to an SP-Toyopearl 650C column and the column's unadsorbed fraction. The lysate showed endotoxin-dependent CE activity, whereas the unadsorbed fraction showed no endotoxin sensitivity.

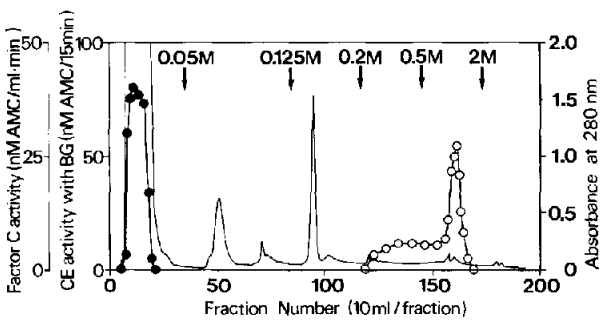

Fig. 1. Elution profile of SP-Toyopearl 650C chromatography of *Tachypleus tridentatus* amebocyte lysate. The BG-dependent CE activity (●) was assayed with a CM curdlan concentration of 100 ng/ml after a 15-min reaction. Solid line indicates absorbance at 280 nm and open circles (○) indicate Factor C activity. Fractions 7–20 were pooled together to form a BG-specific sensitive fraction.

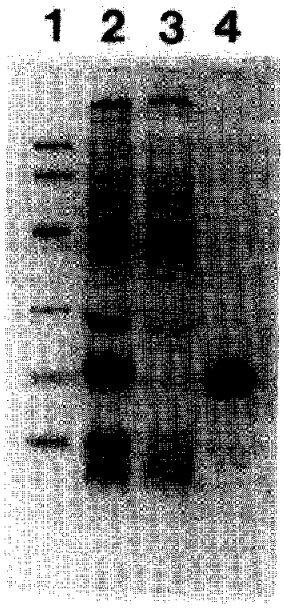

Fig. 2. SDS-PAGE of the chromatographed fractions. (Lane 1) molecular mass marker (phosphorylase b, 94 000 Da; bovine serum albumin, 67 000 Da; ovalbumin, 43 000 Da; carbonic anhydrase, 30 000 Da; soybean trypsin inhibitor, 20 100 Da; α-lactoalbumin, 14 400 Da); (lane 2) lysate applied to the SP-Toyopearl 650C column; (lane 3) BG-specific fraction; (lane 4) coagulogen fraction.

Fig. 4 shows the BG-dependent CE activities of both the lysate applied to an SP-Toyopearl 650C column and the column's unadsorbed fraction, with both fractions showing BG-dependent CE activity. The highest activity was demonstrated at a CM curdlan concentration of 10^{-7} g/ml, indentical to the results of Kakinuma *et al.* [2] which showed that a CM curdlan concentration greater than 10^{-7} g/ml suppressed the activity.

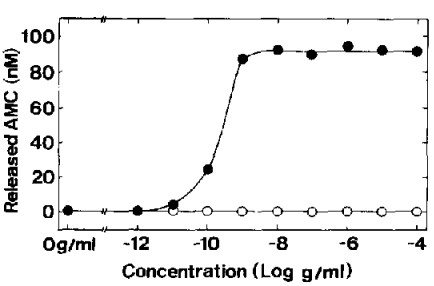

Fig. 3. Endotoxin-dependent CE activity of the lysate applied to the SP-Toyopearl column and the column's unadsorbed fraction. The lysate (●) and the unadsorbed fractions (○) activities were assayed after 15- and 30-min reactions, respectively.

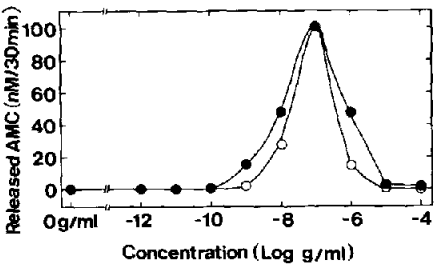

Fig. 4. CM curdlan-dependent CE activity of both the lysate applied to the SP-Toyopearl column (●) and the column's unadsorbed fraction (○). The activity of both samples was assayed after a 30-min reaction.

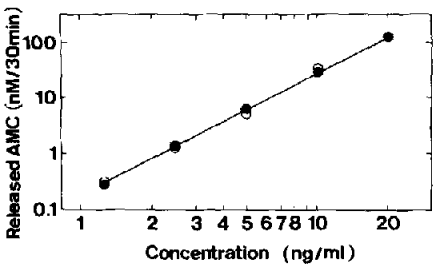

Fig. 5. CM curdlan-dependent CE activity of the unadsorbed fraction of SP-Toyopearl 650C chromatography in the presence and absence of endotoxin. A 0.05-ml sample, 0.025 ml of 320 m*M* Tris–HCl buffer (pH 8.0) containing 200 m*M* magnesium chloride, 0.025 ml of 4 m*M* Boc-Leu-Gly-Arg-MCA, 0.05 ml of CM curdlan solution and 0.05 ml of $2 \cdot 10^{-6}$ g/ml endotoxin solution (○) or endotoxin-free water (●) were mixed and incubated for 30 min.

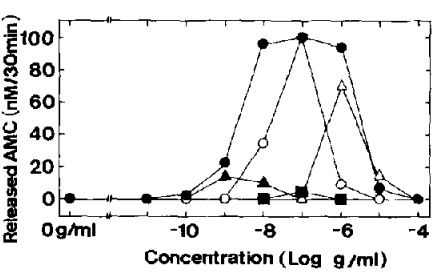

Fig. 6. Sensitivity of the unadsorbed fraction of SP-Toyopearl 650C chromatography towards various kinds of BG. Sensitivity of the BG-specific sensitive fraction to (●) curdlan, (○) CM curdlan, (▲) pachyman, (△) lichenan and (■) laminaran was assayed after a 30-min reaction.

The relationship between the CM curdlan concentration and the amount of released AMC by the unadsorbed fraction is shown in Fig. 5. Logarithmic values of both fractions showed a good mutual relationship in a CM curdlan concentration range between 10^{-9} and $2 \cdot 10^{-8}$ g/ml, with no interference observed when endotoxin was added to the reaction mixture.

Fig. 6 shows the sensitivity of the unadsorbed fraction of SP-Toyopearl chromatography to various kinds of BG. This fraction can be seen to be sensitive to various kinds of BG containing β-(1–3) bonds.

DISCUSSION

A method for the quantification of BG using LAL was reported by Ikemura *et al.* [9], employing an endotoxin-specific LAL reagent and an LAL reagent that is sensitive to both endotoxin and BG. Using this method, however, it is impossible to quantify small amounts of BG at high endotoxin concentrations. Additionally, it is fairly difficult to use because two types of LAL are required for the activity measurements, and all processes have to be performed under endotoxin-free conditions.

A method to obtain BG-specific sensitive LAL by separating LAL with dextran sulphate Sepharose column chromatography has been reported [7,8], but this method requires the later recombination of fractions and cannot separate coagulogen from BG-specific sensitive serine protease zymogen(s).

A rapid method for obtaining the BG-specific fraction from LAL, which cleaves the synthetic substrate of the CE, was achieved by SP-Toyopearl 650C cation-exchange chromatography. This separation technique requires only one-step column chromatography and does not require recombining of the eluted fractions. The column's unadsorbed fraction was effectively separated from Factor C and coagulogen.

This fraction showed BG dose-dependent cleavage of Boc-Leu-Gly-Arg-MCA and no sensitivity to endotoxin, with the presence of endotoxin showing no reaction interference; therefore BG which co-exists with endotoxin is detectable.

Early diagnosis of fungal infections are difficult, and many reports have been published concerning the early diagnosis of these infections by measuring the plasma level of BG with LAL [9,14–19]. Most importantly, the quantification of BG can be easily performed using this BG-specific sensitive fraction and is therefore useful as a diagnostic reagent for fungaemia.

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