An improved medium for preparation of filterable *Escherichia coli* hemolysin

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Summary

We found that Tween 80 has potent activity in enhancing passage of *E. coli* hemolysin through a membrane filter and, on the basis of this finding, we devised a medium, heart infusion broth supplemented with 0.05% Tween 80 and 0.1% glucose (HI-TG) for preparation of culture filtrates containing fairly large amounts of hemolysin. When 27 strains of hemolytic *E. coli* were cultivated to late logarithmic growth phase in the HI-TG broth at 37 °C, the culture filtrates of most strains contained 64 to 128 HU50 (50% hemolytic unit) per ml. From these and other results, we established a routine method for partially purifying *E. coli* hemolysin.

Key words: *Escherichia coli*; Hemolysin

Introduction

*E. coli* hemolysin is considered as one of the virulent factors in diseases such as sepsis, urinary tract infections or abscess formation [1–4].

Many investigators have found difficulties in obtaining culture filtrates which contained appreciably large amounts of hemolysin, using ordinary laboratory media [5–7].

We established a routine procedure of partial purification of *E. coli* hemolysin by preparing an ordinary laboratory medium, heart infusion (HI) broth, suitable for preparation of culture filtrates containing large amounts of hemolysin, by supplementation of 0.05% Tween 80 and 0.1% glucose.

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Materials and Methods

Strain

A hemolytic *Escherichia coli* strain, ATCC 25922, was obtained from the American Type Culture Collection. Of the other hemolytic *E. coli* strains used, four were isolated from biopsy samples of rectal mucosae of patients with ulcerative colitis and 22 strains were isolated from feces of healthy adults.

Preparation of crude hemolysin

One drop of fresh *E. coli* culture in HI broth was inoculated into 5 ml of the same broth with or without glucose and/or Tween 80 in 15 x 105 mm glass tubes. After incubation at 37 °C for 4-5 h, most of the cells were removed by centrifugation at 4500 rpm for 10 min at 5 °C. The supernatant was passed through the membrane filter (Millipore Co., Bedford, MA) of 0.45 μm pore and effective area of 4 cm². In some cases, the membrane filter was pretreated with ca. 1 ml of 0.1% Tween 80 in saline or 0.2% bovine serum albumin (Sigma, St. Louis, MO, USA) in distilled water and then washed with ca. 1 ml of supernatant of the centrifuged culture.

Preparation of partially purified hemolysin

Two milliliters of the fresh *E. coli* culture in HI broth was inoculated into 200 ml of the same broth, with or without glucose and Tween 80 in 300 ml flasks. After incubation at 37 °C for 4-5 h with occasional shaking, sodium azide (0.02% w/v) was added and most of the bacterial cells were pelleted by centrifugation at 16000 x g for 30 min at 0-2 °C. The residual bacterial cells in the supernatant were removed by filtration through the membrane filter (Gelman Sciences, Inc., MI, USA, Tuffryn membrane filter, HT-450) of 0.45 μm pore and 12 cm² effective area. Hemolysin was partially purified by polyethylene glycol (PEG) 4000 precipitation [8] with some modification. The hemolysin preparation was stored at −20 °C or below to prevent loss of activity.

Measurement of hemolytic activity

The crude or partially purified hemolysin were serially diluted two-fold in calcium saline [9]. A 0.5 ml of 1% suspension of sheep erythrocytes in the calcium saline was added to 0.5 ml of the diluted hemolysin preparation and to the control (calcium saline). The tubes were incubated at 37 °C for 2 h with occasional shaking. At the end of incubation, unlysed erythrocytes were pelleted by centrifugation at 3000 rpm for 2 min. The supernatant was transferred to another tube containing 2 ml of distilled water. The optical density at 540 nm (OD₅₄₀) was read using the control tube as a blank by spectrophotometer (type 124, Hitachi, Co. Ltd., Tokyo). The inverse of the dilution which caused 50% lysis of the erythrocytes was recorded as 50% hemolytic units (HU₅₀)/ml.

Results

Influence of the addition of Tween 80 into culture media on the amount of hemolysins in culture filtrates

The hemolysin titer in the filtrate of HI broth culture of *E. coli* ATCC 25922 was
about 2 units/ml, but in the presence of 0.05−0.1% Tween 80, hemolysin titers in the culture filtrates were 128 units/ml. In the case of 0.025% of Tween 80, the hemolysin titer was slightly lower, i.e., 64 units/ml, than in the cases of 0.05−0.1% of Tween 80.

**Influence of pretreatment of the membrane filters with Tween 80 or bovine serum albumin solution on the amount of hemolysin in culture filtrates**

*E. coli* ATCC 25922 was cultivated in HI broth at 37 °C for 4 h. When the culture was passed through a non-treated membrane filter, the hemolysin titer in the culture filtrate was only 2 units/ml (Fig. 1). When the membrane filter was pretreated with saline containing 0.1% Tween 80, the hemolysin titer in the culture filtrate was as high as 128 units/ml. The same result was obtained when 0.1% Tween 80 was added to HI broth. Pretreatment of the membrane filter with 0.2% bovine serum albumin showed the same effect.

**Influence of addition of glucose into HI broth containing Tween 80 on the amount of hemolysin in culture filtrates**

Two- to four-times higher amounts of hemolysin were usually found in culture filtrates of *E. coli* ATCC 25922 of HI broth supplemented with 0.1% glucose and 0.05% Tween 80 (HI-TG) as compared with those of HI broth supplemented with only Tween 80.

**Influence of incubation times on the amount of filterable hemolysin**

When ca. 6 × 10^6 cells/ml of *E. coli* ATCC 25922 were inoculated into HI-TG broth, the population of *E. coli* increased rapidly and reached the maximum level within 5 h (Fig. 2). Amounts of hemolysin in culture filtrates were also increased concomitantly with growth of the bacteria and reached the maximum level at about 4 h, i.e.,
at the late logarithmic growth phase. The maximum level of hemolysin titer in the culture lasted for at least 24 h of incubation times. On the contrary, when the HI broth lacked supplements, the amount of hemolysin reached the maximum level, 8 units/ml, at 4 h and thereafter declined sharply.

**Amounts of hemolysin in filtrates of 27 strains of various broth cultures**

Figure 3 shows the amount of filterable hemolysin produced by 27 strains of *E. coli* in three kinds of media, HI broth (Nissui), HI broth supplemented with 0.1% glucose and HI broth supplemented with 0.1% glucose and 0.05% Tween 80. Hemolysin titers of filtrates of HI broth cultures of all test strains were as low as 4 units/ml or less. In HI broth supplemented with glucose and Tween 80, hemolysin titers of test strains were as high as 32 to 128 units/ml. In HI broth supplemented with only glucose, some strains produced high amounts of hemolysin but the hemolysin titers of most strains were from 8 to 16 units/ml.

**Optimal concentration of PEG for precipitation of hemolysin**

The hemolysin titer in the filtrate of *E. coli* ATCC 25922 culture in HI-TG broth was 256 units/ml. The hemolysin titers in PEG precipitates obtained by the addition of 15, 20, 25 and 30 g of the PEG into 100 ml of culture filtrates were $1 \times 10^3$, $2 \times 10^3$, $4 \times 10^3$ and $4 \times 10^3$ units/ml, respectively.

**Heat susceptibility of the hemolysin**

When the PEG precipitated hemolysin preparation of 512 units/ml was heated at 56°C for 60 min in a water bath, the hemolysin titer decreased to 64 units/ml.
Comparison of the amounts of hemolysin in culture filtrates of 27 strains of *E. coli* in three media; HI broth, HI glucose (0.1%) broth and HI Tween 80 (0.05%)—glucose (0.1%) broth.

**Discussion**

We prepared a more satisfactory medium, HI broth supplemented with 0.05% Tween 80 and 0.1% glucose (HI-TG) for preparation of culture filtrates of *E. coli* containing large amounts of hemolysin. When 27 strains of hemolytic *E. coli* were cultivated in HI-TG broth, culture filtrates of most of the strains contained as high as 64—128 units/ml of hemolysin.

Culture filtrates of 27 strains of hemolytic *E. coli* cultivated in HI broth contained only 4 units/ml or less of the hemolysin. When a membrane filter was pretreated with 0.1% Tween 80 or 0.2% bovine serum albumin, the culture filtrate of *E. coli* ATCC 25922 contained as high as 64—128 units/ml of hemolysin. Pretreatment of membrane filters with bovine serum albumin is usually done to prevent the adsorption of proteinous materials. Thus, the low hemolysin contents in the filtrates obtained from HI broth cultures may not be due to a scanty production of the hemolysin but rather to adsorption of the hemolysin to the membrane filters.

Most of the ordinary laboratory media are unsuitable for preparation of filterable *E. coli* hemolysin. Smith [5] devised an alkaline extract broth for preparation of *E. coli* hemolysin. Jorgensen et al. [7] reported that the presence of a putative hemolysin precursor (HP) in the nutrient broth prepared according the the method of Smith [5] and the amounts of hemolysin found in culture filtrates were proportional to the concentration of the HP.

In culture filtrates of the alkaline extract broth, amounts of the hemolysin reached the maximum levels at the initial [7] or late [10] logarithmic growth phase and thereafter decreased sharply. After incubation for 12—16 h, the hemolysin in the culture filtrates was already reduced to the level no longer detectable by the ordinary hemolysis test. In our studies, an identical result was obtained when *E. coli* was cultivated in HI broth as shown in Fig. 2. But, when *E. coli* was cultivated in HI-TG broth, the amounts
of hemolysin in filtrates reached the maximum level at the late logarithmic growth phase and, in contrast to the cases of the alkaline extract broth, the high hemolysin level lasted for at least 24 h of incubation.

In the PEG precipitation of the hemolysin, Bhakdi et al. [8] added 20 g of the PEG to 100 ml of the supernatants of the centrifuged cultures. In our experiments, the maximum hemolysin precipitate was obtained when 25–30 g (ca. 21–24% w/v) of the PEG was added to 100 ml of the culture filtrates.

From these results, we established a routine method of partial purification of *E. coli* hemolysin as follows. Inoculate fresh broth culture of *E. coli* into HI-TG broth (ca. 1–5 × 10⁶ cells/ml) and incubate it at 37°C for 4–6 h, i.e., to the late logarithmic to early stationary growth phase, with occasional shaking. Add NaNO₃ (0.02% w/v) to the culture and centrifuge the preparation at 16000 × g for 30 min at 0–2°C. Pass the supernatant through a membrane filter (0.45 μm pore) and add PEG 4000 (21–24% w/v) to the filtrate. Dissolve the PEG and incubate the preparation for 60 min in an ice bath, with occasional shaking. Centrifuge the preparation at 16000 × g for 30 min at 0–2°C and carefully remove the supernatant. Dissolve the precipitate in the calcium-saline [9] of 1:100 portion of the volume of the original cultures. Check for the absence of viable *E. coli* by spreading 0.1 ml of the hemolysin solution on nutrient agar plates. Store the hemolysin solution at −20°C or below. With this procedure, the hemolysin in the culture filtrates was concentrated 20–40 times and the preparation usually contained 1–8 × 10³ units/ml of hemolysin.

The hemolysin preparation seems to be composed of ca. 90% of thermolabile and 10% of thermostable hemolysin [10].

In SDS-PAGE electrophoresis [11], the hemolysin preparation exhibited a main protein band with a molecular weight of ca. 1.03 × 10⁵ and, in some cases, a faint minor band of molecular weight of ca. 5.8 × 10⁴ (data not shown). The main band is the native hemolysin and the minor one may be a degradation product [8].

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**References**


