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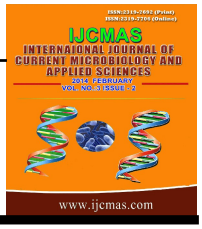

Original Research Article

Purification and characterization of *Staphylococcus aureus* βeta-Lactamase from Lebanese Community

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Keywords

Purification; characterization; *Staphylococcus aureus;* βeta-lactamase.

A B S T R A C T

Staphylococcus aureus produces β-lactamase as a resistant factor for β-lactam antibiotics. The enzyme was produced by the bacterial cells and secreted extracellularly and was partially purified from the cell-free extract by ammonium sulphate precipitation with 75.79% yield and a purification factor of 3.04 fold. Further purification includes ion exchange chromatography in CM-Sephadex C_{50} followed by anion exchange chromatography in DEAE-Sephadex $A_{50.}$ The overall purification technique resulted in 58.74% yield with a purification factor of 102.262 folds. The maximum enzyme activity was recorded at pH 7.0 and 30ºC with a linear relationship concerning the increase in enzyme concentration. The effect of substrate concentration showed a progressive increase in the enzyme activity in a concentration dependent manner till it reaches a plateau where saturation was reached. The kinetic parameters (Km and Vmax) of S. aureus β-lactamase against penicillin-G were 111µmole and 16.66 µmole/mg protein/ml/min respectively.

Introduction

While previously recognized antibiotic-resistant bacteria continue to increase in frequency and numbers globally, new resistance problems have recently emerged which further complicate and impede treatment of critical infectious diseases (Levy, 2005). Since the discovery and subsequent widespread use of antibiotics, a variety of bacterial species of human and animal origin have developed numerous mechanisms that render bacteria resistant to some, and in certain cases to nearly all antibiotics (Dzidic et al., 2008). Bacterial resistance to β-lactam antibiotics can be achieved by any of three strategies: the production of β-lactam-hydrolyzing β-lactamase enzymes, the utilization of β-lactam-insensitive cell wall transpeptidases, and the active expulsion of β-lactam molecules from Gram-negative cells by way of efflux pumps (Wilke et al., 2005). β-lactamases is the most prevalent mechanism of bacterial resistance to the β-lactam family of

antibiotics (Issa et al., 2010). Beta-lactam drugs, whose mechanism of action is inhibition of the last sage of bacterial cell wall synthesis, are the largest family of antimicrobial agents and the most widely used in clinical practice (Suarez & Gudiol, 2009). Penicillin is an antibiotic that belongs to this family of antimicrobial agents. Penicillin resistance in *Staphylococcus aureus* isolates recovered from hospitalized patients was reported shortly after penicillin was introduced in 1941 (Feghaly et al., 2012). Today, more than 90% of human *S. aureus* isolates are resistant to penicillin (Chambers & Deleo, 2009). In view of the antimicrobial resistance observed in *Staphylococcus aureus*, the present study was aimed to purify and characterize the β-lactamase from a local strain of *S.aureus* isolated from Lebanese community.

Materials and Methods

Microorganism

The bacterium used throughout the present study namely *Staphylococcus aureus* which was kindly provided by Al-Bekaa hospital, and identified using phenotypic characterization according to Bergy`s manual of determinative Bacteriology(Buchanan & Gibbson, 1974) and Cloverleaf β-lactamase test (Feghaly et al., 2012).

Media and cultivation

The bacterium under test was maintained on nutrient agar slants and stored at 4□C with subsequent transfers at monthly intervals. Tryptic soy broth (TSB) was the fermentation medium used for cultivation and enzyme production having the following composition (g L^{-1}): enzymatic digest of casein, 17.0; enzymatic digest of soybean meal, 3.0; sodium chloride, 5.0; dipotassium phosphate, 2.5; glucose, 2.5.

The pH of the medium was adjusted to 7.3. Penicillin G (150uM/50 ml broth was added to the culture media) acts as a substrate that induces the production of the enzyme. The flasks were inoculated with 2mL bacterial suspension (O.D $\leq$ 1.0 at 540 nm), and incubated at 30^{o}C on a rotary shaker for 48 hours.

Cell free extract

After incubation, bacterial cells were harvested by centrifugation at 7000 rpm for 20 minutes at 4^{o}C and washed twice with 0.1 M phosphate buffer (pH 7.0) (Ranade et al., 2013) and the clear supernatant obtained resembles the source of extracellular βeta-lactamase enzyme and was analyzed for its protein content and enzymatic activity.

Estimation of protein content

The protein content was monitored at 280 nm.

Estimation of βeta-lactamase activity

1mL of molybdoarsenic acid-mercuric chloride reagent was added to 100µL of solution containing (250µL penicillin G, 1mL of 0.3M phosphate buffer pH 7.5, and 50µL of the crude enzyme) after 10 minutes incubation period in order to stop the reaction. The resultant blue color was measured by spectrophotometer at 800 nm after 70 minutes of incubation at room temperature (Holm, 1972).

Enzyme fractionation and purification

Ammonium sulphate was added slowly to the cell free extract until 80% saturation.

The mixture was centrifuged under cooling at 7000 rpm for 20 minutes after. The precipitated protein was dissolved in 200mL of 0.005M phosphate buffer (pH 7.2) and dialyzed overnight against the same buffer. Following fractionation, enzyme purification was conducted by the use of two chromatographic steps:

Cation exchange chromatography on CM-Cephadex C_{50}

CM-sephadex C_{50} gel was prepared and stored at 4°C for 2-3 days to ensure complete swelling and then packed in a column (2.5 x 20 Cm) adjusted to vertical position (Blechschmidt et al.,1992). The dialyzed ammonium sulphate fraction was layered carefully on the top of the gel under cooling conditions. 2mL fractions were collected/5min, and the most active fractions showing β-lactamase activity were concentrated against cold sucrose followed by dialyses against 0.005M phosphate buffer (pH 7.2) at 4°C.

Anion exchange chromatography on DEAE-Sephadex A_{50}

The concentrated CM-Sephadex dialyzed sample was applied to (2.5 x 20 Cm) DEAE-Sephadex A_{50} column at 4□C and eluted with 0.1M phosphate buffer containing a linear gradient of 0.01-0.05M sodium chloride. Fractions (2mL) were collected and subjected to protein estimation at 280nm and β-lactamase activity at 800nm.

Enzyme characterization

To determine the optimum pH and temperature for β-lactamase activity, studies were carried out at pH range of 4-9 and 5-50□C respectively. For optimum pH determination according to Issa et al. (2009), the enzyme solution was adjusted using sodium acetate buffer (pH 4 to 6), phosphate buffer (pH 7), and Tris-buffer (pH 8, 9). For optimum temperature determination, the enzyme solution was preheated separately at different temperatures ranging from 5-50□C. The enzyme activity was measured after 70 minutes of incubation at 800 nm after molybdoarsenic acid-mercuric chloride addition.

Similarly, enzyme and substrate concentration were used over a range of 100-1000uM and 50-500uM respectively to study its effects on enzyme activity. For optimum substrate concentration, the assay mixture contained (2.91 ml of 0.05M potassium phosphate buffer (pH 7.0), 40ul of penicillin G (50-500uM), and 50ul of enzyme). The decrease in absorption at 235nm against a reference containing only the enzyme in the buffer was recorded (Ross et al., 1973).

Results and Discussion

Purification

Partial purification of the crude enzyme was achieved by adding ammonium sulphate until 80% saturation at 4□C. The enzyme was concentrated in the precipitate. The specific activity after precipitation increased to 0.719 Abs. at 800 nm recording 75.79 % of the original enzyme activity present in the crude extract. However the total protein decreased from 1873.9 to 465.6 mg with a purification factor of 3.0466 folds (Table 1).

Cation-exchange chromatography of the ammonium sulphate fraction

The enzyme obtained from ammonium sulfate fractionation was further dialyzed

against 0.005M phosphate buffer (pH 7.2) and centrifuged. The supernatant was purified by applying the dialysate to CM-sephadex C_{50} cation exchange column. The protein content and enzyme activity of each fraction were determined (Fig. 1). It was found that the fractions contain only one peak for β-lactamase activity. This result established the purity of the enzyme from contaminants and it agrees with the findings of (Issa et al.,2010) who reported that the result recovered from gel-filtration chromatography revealed one peak for staphylococcal β-lactamase activity. The enzyme was enriched after the first CM-sephadex C_{50} and the specific activity became 2.556 Abs. at 800 nm recording 64.69 % of the original enzyme activity present in the crude extract indicating the preservation of most enzyme activity. The purification factor recorded 10.83 folds and the protein content reduced from 465.6 to 111.91 mg (Table 1) thus cutting off a lot of the associated proteins. Iaconis & Sanders (1990) recorded 108 purification fold in purifying βeta-lactamase from *Aeromanas hydrophila* using CM-Sephadex C_{50} column.

Anion-exchange chromatography of the cation fraction

The highest active β-lactamase fractions obtained from CM-sephadex C_{50} cation exchange column were loaded on a DEAE –Sephadex A_{50} column pre-equilibrated with 0.1 M phosphate buffer (pH 7.2) at 4^{o}C. The proteins were eluted with a linear gradient of 0.01-0.05M of sodium chloride. The protein content and enzyme activity of each fraction were determined (Fig. 2). The purification step showed one protein band corresponding to β-lactamase. The specific activity increased to 24.134 Abs. at 800 nm with a purification factor equal to 102.262 folds with 58.74% yield. Tigerstrom & Boras (1990) reported that beta lactamase was purified 115 fold with 37% yield. On the otherhand, the protein content reduced to 10.76 mg (Table 1). The affinity of the enzyme to the DEAE-Sephadex A_{50} was found to be not high since it was eluted from the column with lower concentration of the NaCl gradient. At the end of βeta-lactamase purification, the total enzyme activity decreased relative to the crude extract. This could be related to the removal of synergistic enzymes.

Characterization of the purified enzyme

Results in figure 2 indicated the presence of a gradual increase in the enzyme activity as the pH of the reaction mixture increases reaching the climax at pH 7.0. Beyond this pH value, any further increase resulted in activity loss, due to the alteration in the ionization of groups responsible for substrate binding. The high alkalinity and the low acidity of the reaction media would result in the denaturation of the enzyme or decrease in its reaction rates (Blechschmidt et al. , 1992 and Ronade et al. ,2013) .

The optimum temperature for enzyme activity was found to be 30^{o}C. Temperature increase resulted in a decrease in βeta-lactamase activity (fig.4). B-lactamases are thermolabile proteins which inactivate rapidly by heat (Castillo et al., 2001).

The effect of enzyme concentration on the activity of *Staphylococcus aureus* β-lactamase showed that the enzyme activity increased proportionally with the increase in enzyme concentration.

The effect of substrate concentration on the activity was investigated using

Table.1 A summary of the purification of β-lactamase enzyme from *Staphylococcus aureus*

Purification steps	Collected Volume (ml)	Activity Abs at 800nm/ml	Protein mg/ml	Total Activity Abs. at 800nm	Total protein mg	Specific Avtivity Abs. at 800nm/mg protein	Purification factor-fold	yield (%)
Culture filtrate crude extract	350	1.263	5.354	442.05	1873.9	0.236	1	100
Precipitate by ammonium sulfate (85%)	160	2.094	2.91	335.04	465.6	0.719	3.0466	75.79
Filtration on CM-Sephadex C50 column	62	4.613	1.805	286.006	111.91	2.556	10.83	64.69
DEAE-sephadex A50 column	40	6.492	0.269	259.68	10.76	24.134	102.262	58.74

Figure.1 CM-Sephadex C_{50} chromatography of 80% ammonium sulphate fraction from cell free extract of *Staphylococcus aureus*.

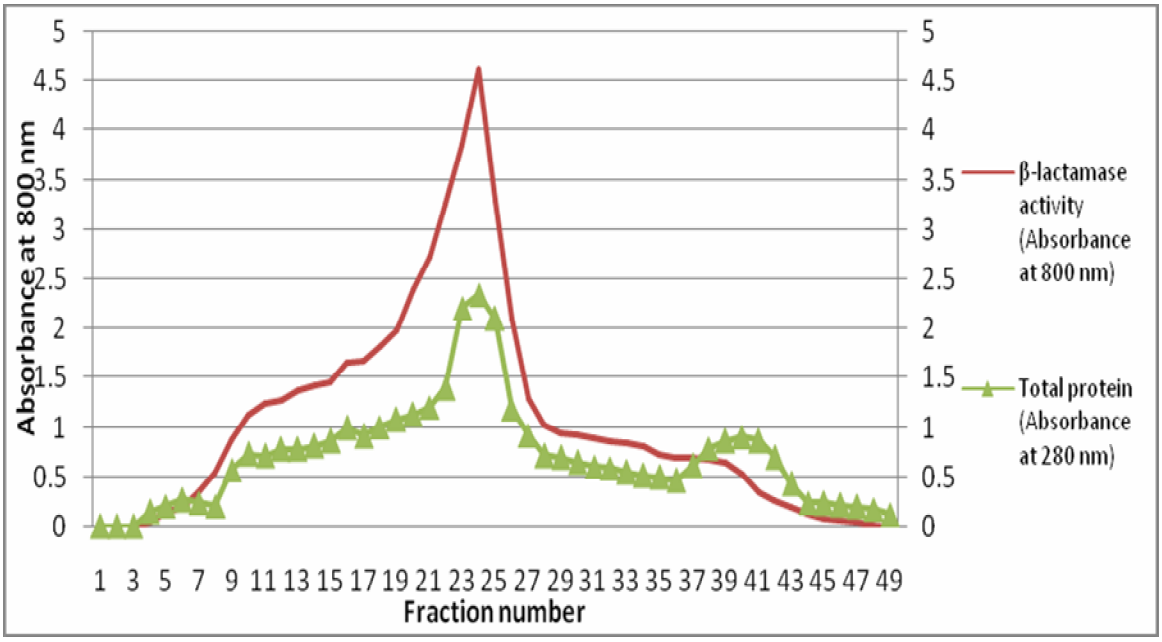


Figure.2DEAE-Sephadex A_{50} of the CM-Sephadex C_{50} gel filtrate fraction of *S.aureus* β-lactamase

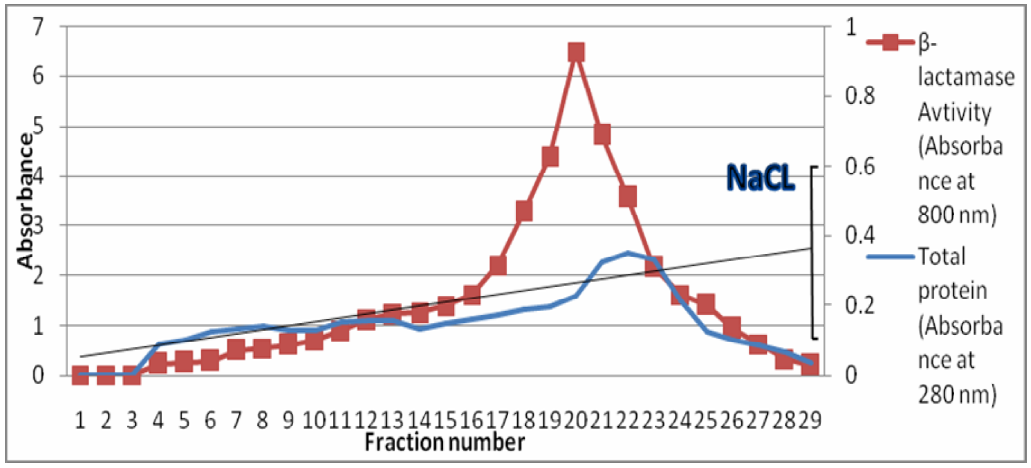


Figure.3 Effect of pH on *Staphylococcus aureus* β-lactamase activity

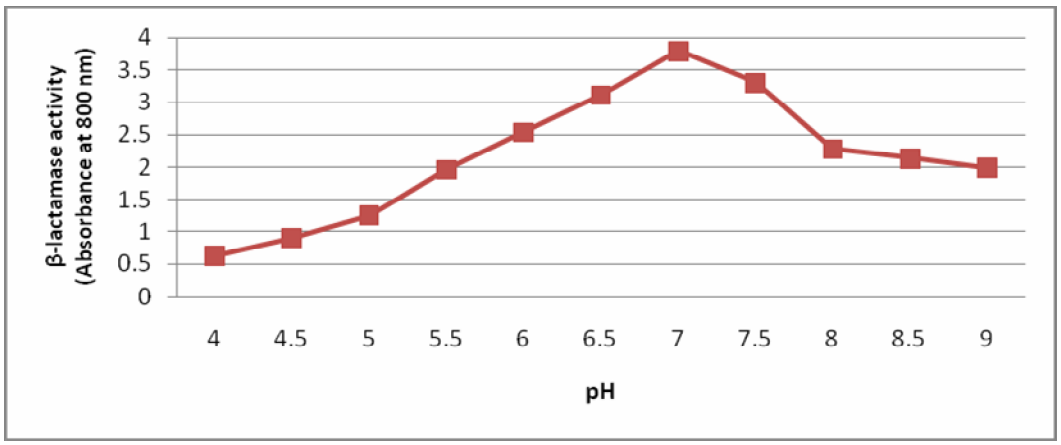


Figure.4 Effect of incubation temperature on the β-lactamase activity of *Staphylococcus aureus*

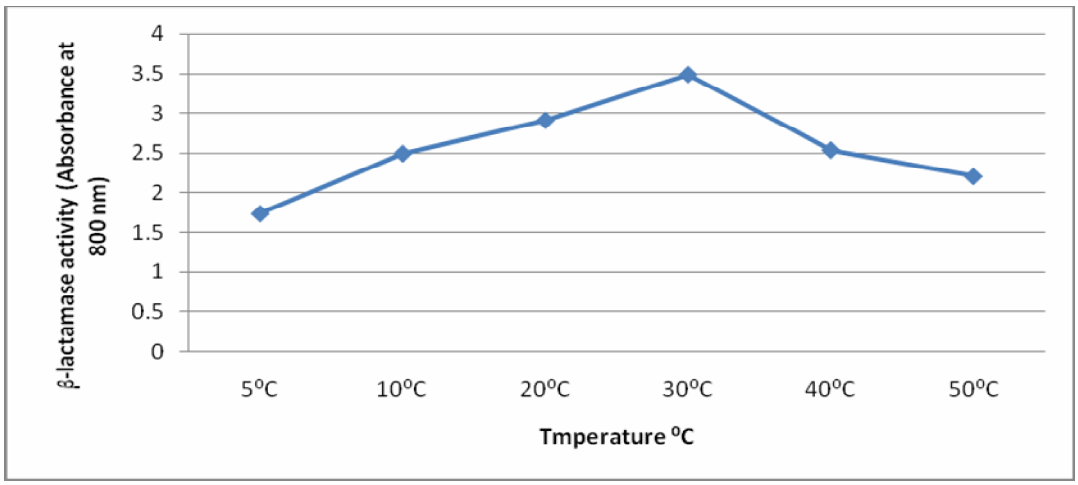


Figure.5 Effect of enzyme concentration on the β-lactamase activity of *Staphylococcus aureus*

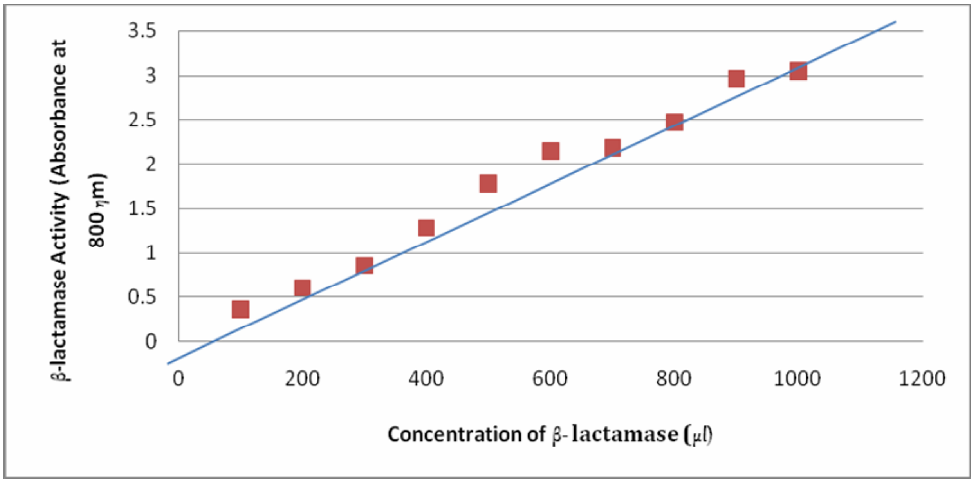


Figure.6 Effect of substrate concentration on β-lactamase activity

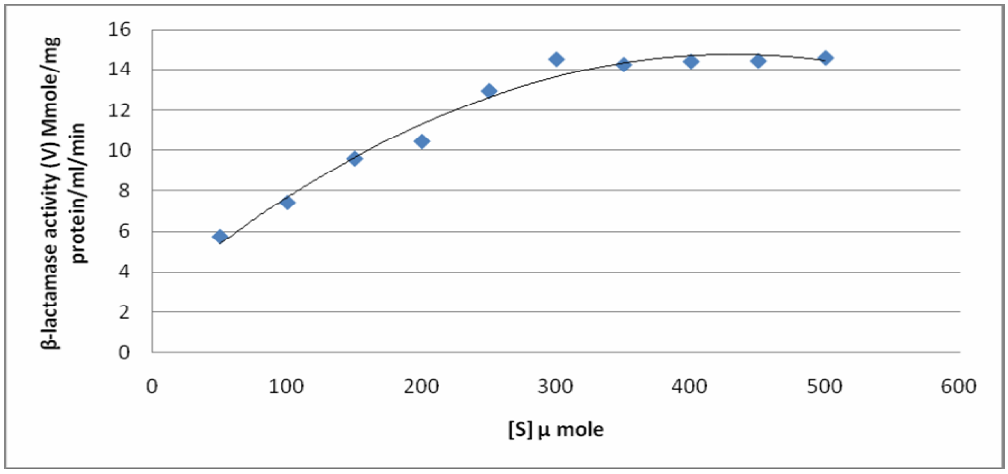
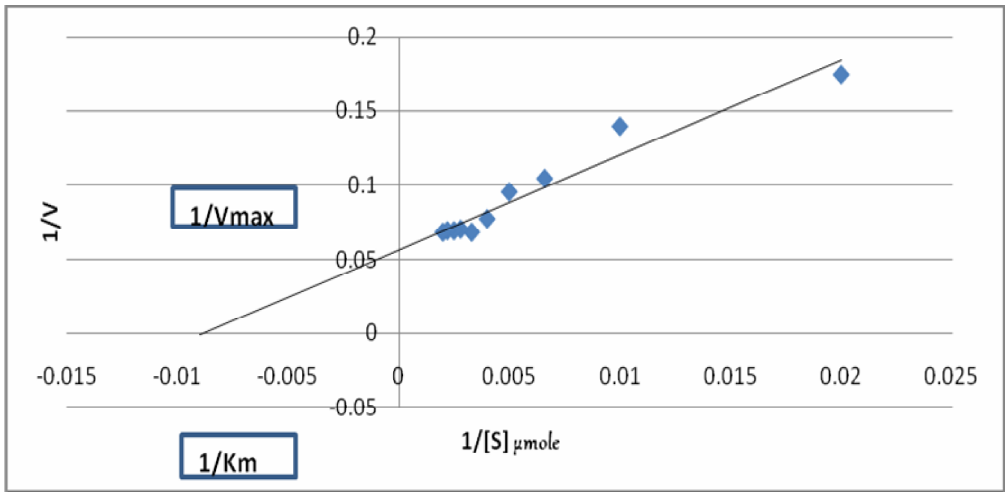


Figure.7 Linweaver-Burk plot of *Staphylococcus aureus* β-lactamase activity



different concentrations ranging from 50-500uM. The relationship between the substrate concentration and the purified *Staphylococcus aureus* β-lactamase was shown in figure 6. The activity of the enzyme increased progressively in a concentration dependent manner till it reached a plateau resembling saturation. As the concentration of the substrate increase, the catalytic sites of the enzyme will start to bind resulting in a steep increase in the activity of the enzyme till it reaches saturation. The rate of formation of product now depends on the activity of the enzyme itself, and adding more substrate will not affect the rate of the reaction to any significant effect.

The initial velocity of β-lactamase reaction was measured as a function of substrate concentration and plotted as double reciprocal in accordance with the Lineweaver-Burk analysis (Figure 7). The plot resulted in a km value of 111 μmole and Vmax of 16.66 μmole/mg.protein /ml/min. This have been studied by Tigerstrom & Boras (1989) who reported that Km for the enzyme was of 116 μmole.

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