Chapter 151
Purification of Alkaline Pectinase in Engineering *Bacillus subtilis*

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Abstract The alkaline pectinase in fermentation liquid of engineering Bacillus subtilis was isolated and purified by using centrifugation, ultrafiltration, ammonium sulfate precipitation, and ion exchange chromatography, and the optimum conditions of ion exchange chromatography were determined. The results showed that, the recovery rate of crude enzymes ultrafiltrated by an ultrafiltration membrane with molecular weight cut-off 10000 Dalton reached 73.3 %, and the specific activity was 259.7 U/mg; after fractional salting out, the recovery rate was up to 62.9 %, and the specific activity was 1084 U/mg. With the DEAE-Sephorose CL-4B anion exchange chromatography under gradient elution with 0–1.0 mol/L NaCl (buffer solution of Gly-NaOH at pH8.6), the specific activity increased to 1230 U/mg. Subsequently, Sephadex-G75 column chromatography was applied and the specific activity increased to 2352 U/mg. The purity was 12.5 times of the crude enzyme, with the recovery rate of 21.6 %. The molecular weight of finally obtained alkaline pectinase was 43 kDa and the SDS polyacrylamide gel electrophoresis displayed that it was electrophoretically pure.

Keywords Alkaline pectinase · Isolation and purification · Ultrafiltration · Chromatography

151.1 Introduction

Alkaline pectinase refers to the pectinase with higher activity under basic condition. Pectate lyase mainly acts in alkaline environment, occupying a large proportion in alkaline pectinase, thus alkaline pectinase generally refers to pectate lyase [1].

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Alkaline pectinase is widely used in leather and silk industry, and shows wide application prospect in food, feed, and particularly in the extraction and washing for plant drugs in the field of medicine.

A strain of *Bacillus subtilis* TCCC11485 with high yield of alkaline pectinase was constructed in our laboratory. The enzyme produced by the engineering bacteria has high expression activity, short fermentation period, fast degumming for pectinase, and small influence on fiber quality, which has potential application in the textile industry.

The purification of alkaline pectinase is the prerequisite and foundation for the effective utilization of alkaline pectinase.

The purification methods for alkaline pectinase produced by bacteria, mold, and yeast reported at home and abroad were different. In the initial separation, salting out or organic solvents were usually used to precipitate it and the commonly used organic solvents are methanol, ethanol, isopropanol, and acetone. Schjter and Marcus precipitated pectin esterase and polygalacturonase from *Botrytis cinereaPers* with 20 % and 66 % acetone respectively, which were purified by 5 times and 6 times respectively [2]. Compared with ammonium sulfate fractionation, the biggest drawback of organic solvent precipitation was the partially inactivation of the enzyme.

DEAE-cellulose chromatography, DEAE-Sephadex, and CM-Sephadex have good application in the purification of pectinase [3–6]. In addition, affinity chromatography also has certain applications in the separation of enzymes. Rombouts and Pilnik crosslinked pectic acid or sodium pectinate (high molecular weight) with epichlorohydrin under alkaline conditions, and methyl esterified the products to separate pectin esterase from the orange. Two peaks of pectin esterase were obtained [7]. Tibensky et al. used crosslinked products without methyl esterification to isolate endo-polygalacturonase from *Aspergillus niger* with the pH 6 elution condition [8].

Sephadex gel filtration was one of the common means used in pectinase purification process. Tagawa and Kaji used Sephadex G-100 to separate polygalacturonase from *Cuoticium rolfsii* after affinity chromatography, having good results [9]. In the separation of pectinases from mutant of *Cladosporium herbarum*, Wu Meihua et al. compared several different Sephadax fixed materials and elution conditions, and found that QAE Sephadex A-25 gradient elution can achieve better results [10]. In fact, Sephadex gel filtration combined with other methods had been widely used in the purification of pectinase.

To sum up, the purification process of pectinase is flexible and diverse, so in the working practice, the methods could be combined according to the specific circumstances, but the primary separation with ammonium sulfate and organic solvent is necessary.

In the fermentation of *B. subtilis* engineering baterium, except alkaline pectinase, there were also other proteins produced by *B. subtilis* and some remaining medium components, which affected the purification of alkaline pectinase. Thus, this article focus on the purification method of alkaline pectinase from *B. subtilis*

TCCC11485, in order to lay the foundation for the further study of structure and properties, such as remixing with other enzymes or auxiliary agents for the application in the textile industry.

151.2 Materials and Methods

151.2.1 Materials

Bacillus subtilis TCCC11485 with the high yield of alkaline pectinase was constructed by Enzyme and Applied Microbiology Laboratory in Tianjin University of Science and Technology. Polygalacturonic acid was purchased from sigma. Molecular weight makers, acrylamide, bis acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), coomassie brilliant blue, DEAE-Sephorose CL-4B, and Sephadex-G75 were purchased from Sangon Biotech (Shanghai) Co., Ltd. (China), and Bradford Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Shanghai) Co., Ltd. (China),and neutral protease was bought from Tianjin Noao Science and Technology Co., Ltd.(China), and other reagents were analytically pure and made in China.

151.2.2 Preparation of Alkaline Pectinase Crude Enzyme

Single colony of engineering *B. subtilis* TCCC11485 was selected and inoculated in LB seed culture medium with the culture at 37 °C and 200 r/min for 12 h, which was then inoculated in a 5 L fermentation tank with the inoculation amount of 4 % for fermentation of 24 h. The dissolved oxygen concentration was controlled to 15–20 % in the fermentation process, and the temperature was controlled at 41 °C in 0–4 h, and at 35 °C in 4–22 h, and the speed was 500 r/min in 0–5 h, and 600 r/min after 5 h. The initial pH of the fermentation medium was 7.0.

After fermentation, the fermentation liquid was centrifuged at 4 °C and 6,000 r/min for 20 min to collect the supernatant, which was alkaline pectinase crude enzyme, and then preserved at 4 °C.

151.2.3 Purification of Alkaline Pectinase

151.2.3.1 Ultrafiltration of Alkaline Pectinase Crude Enzyme

The crude enzyme was concentrated by an ultrafiltration membrane with molecular weight cut-off of 10,000 Dalton to 1/5 of the original volume, and the concentrate was collected.

151.2.3.2 Ammonium Sulfate Fractionation

First according to ammonium sulfate fractionation curve, the suitable saturation was selected for salting out.

500 mL of concentrated crude enzyme was put in ice bath, and 50 % saturation ammonium sulfate salting out was conducted overnight for precipitation of other proteins, then it was centrifuged in high-speed refrigerated centrifuge at 9000 r/min for 15 min to remove the precipitation, and 80 % saturation ammonium sulfate was added to the supernatant for overnight to precipitate target enzyme, subsequently, it was centrifuged in high-speed refrigerated centrifuge at 9000 r/min for 15 min to remove the supernatant, and the precipitation was the fractionation of crude enzyme.

Dialysis was conducted using a dialysis bag immersed into balance buffer with the volume more than 5 times that of the crude enzyme in 4 °C refrigerator. After a few hours, balance buffer was replaced and repeated until the precipitation cannot be seen after dropping 10 % BaCl2 solution.

151.2.3.3 DEAE-Sephorose CL-4B Anion Exchange Chromatography

(1) The choice of pH value of ion exchange eluate

1 mL anion exchanger was aspirated to 10 test tubes, and washed by buffer with different pH values for 10 times. After the ion exchange resin being equilibrated, 1 mL buffer was added, and 100 μL alkaline pectinase solution salted out with ammonium sulfate was added and mixed and then in the marinade for 10 min. The activity of the enzyme in the supernatant was detected to judge whether target protein existed.

(2) Purification of alkaline pectinase

The samples after desaltation were added into DEAE- Sephorose CL-4B anion exchange chromatography column being balanced with pH 8.6 Gly-NaOH buffer, and eluted with pH 8.6 Gly-NaOH buffer at 1 mL/min for 120 min, and then linearly gradient eluted with pH 8.6 Gly-NaOH buffer containing 0–1 mol/L NaCl, with the eluting rate of 1 mL/min. The elution solution was collected automatically with the speed of 1 min/pipe.

151.2.3.4 Sephadex-G75 Column Chromatography

Glass column packed with Sephadex G-75 dextran gel was balanced with pH 8.6 Gly-NaOH buffer, and sampled, and then eluted with pH 8.6 Gly-NaOH buffer, with the flow rate of 1 mL/min, and the effluent was on-line detected by UV

detector at the wavelength of 214 nm, to record ultraviolet absorption peak curve. The components were collected, used for the determination of enzyme activity and protein content.

151.2.4 Determination of Alkaline Pectinase Activity

(1) The definition of enzyme unit: the amount of the enzyme in 1 mL enzyme solution that catalyzes polygalacturonic acid to degenerate into 1 μmol unsaturated polygalacturonic acid per minute at 45 °C and pH 9.0.
(2) The measurement of enzyme activity [11]: 20 μL crude enzyme dilution and 2 mL glycine-NaOH (0.2 mol/L) buffer solution containing 0.2 % polygalacturonic acid (pH 9.0, containing 0.44 mmol/L CaCl2) were included in measurement system. The solution with no activity was taken as the blank control. Buffer solution containing substrate was added to start enzymatic reaction. The reaction was conducted at 45 °C for 15 min, and 3 mL 0.03 mol/L phosphoric acid was added to terminate reaction, and the absorbance values were measured at 235 nm. Enzyme blank parallel to the samples was used for zeroing.

Enzyme blank: 2 mL of above buffer system was insulated for 2 min, and then 3 mL 0.03 mol/L phosphoric acid was added, subsequently, 20 μL inactivate enzyme solution of the same dilution ratio with the sample was added and mixed. Other operations were same as those for the samples.

$$\text{Enzyme activity(U/ml)} = \frac{10^3 \times \text{t} \times 4,600 \times \text{volume of enzyme preparation}}{\text{OD}_{235} \times 10^6 \times \text{dilution factor} \times \text{volume of mixture}}$$

in which, 4,600 ($L.mol^{-1}$ cm^{-1}) was the molar absorptivity of unsaturated polygalacturonic acid at 235 nm.

t (min) Enzymatic reaction time (within the linear range of enzymatic reaction)
b (cm) The thickness of cuvette

to be simplified as: enzyme activity (U/ml) = 3.6232 × dilution multiple × OD235.

151.2.5 Determination of Protein Content and SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein content was determined by Bradford [12] method, and SDS-PAGE was conducted according to the methods from the literature [13] to identify enzyme purity.

151.3 Results and Discussion

151.3.1 Fermentation Results in 5 L Fermentor

The fermentation curve is shown in Fig. 151.1.

As seen in Fig. 151.1: the whole fermentation process lasted for 26 h. In 0–4 h, due to self-decomposition of nutrients in fermentation medium, pH value declined; after 4 h, with the substantial growth and reproduction of bacteria, pH value of fermentation liquid rose gradually to make the bacteria grow in the optimal environment; at 18 h, nucleic acid reached peak, and bacteria reached stable; at 22 h, enzyme activity reached the maximum value, and alkaline pectinase activity in the final fermentation liquid reached 1536 U/mL. 2133 mL supernatant was obtained by centrifugation to remove bacteria from the fermentation liquid, with the total enzyme activity of 3276288 U.

151.3.2 Ultrafiltration

Fermentation liquid was centrifuged to remove bacteria, and crude enzyme was ultrafiltrated by an ultrafiltration membrane with molecular weight cut-off 10,000 Dalton, and about 500 mL concentrated liquid was collected. After ultrafiltration, pectinase activity was 4,802.6 U/mL, and the total enzyme activity was 240,1307 U. The activity loss was around 26.7 %.

The loss of enzyme activity was the result of some enzymes which were deactivated with the ultrafiltration time. It was probably due to a small part of enzyme molecules adsorbed in hollow fiber membrane.

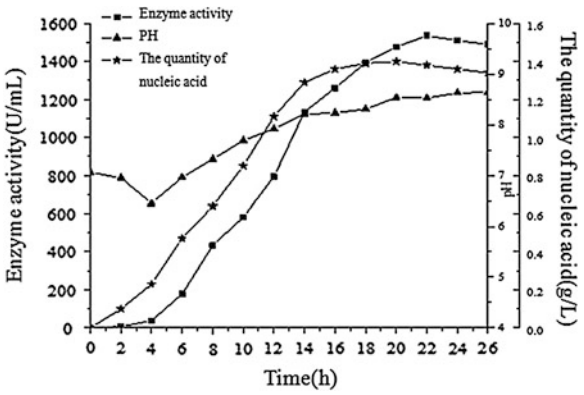

Fig. 151.1 Fermentation curve

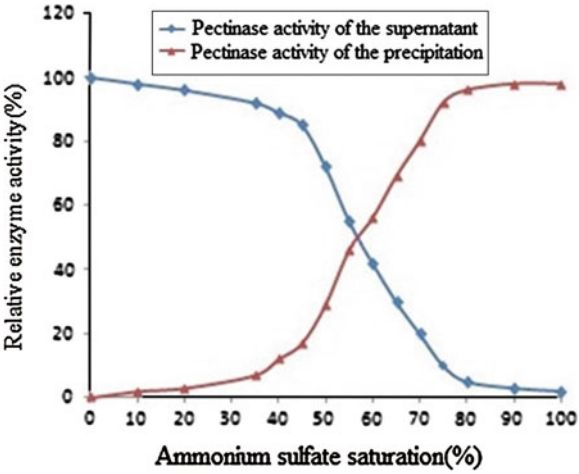

Fig. 151.2 Ammonium sulfate fractionation curve

151.3.3 Ammonium Sulfate Fractionation

The fractionation curve is shown in Fig. 151.2.

Figure 151.2 showed that, when ammonium sulfate was saturated to 50–80 %, enzyme activities of the precipitation increased and that of the supernatant decreased significantly with the saturation. When the saturation rate reached 80–100 %, enzyme activities of the supernatant and precipitation did not change significantly. Thereafter fractional salting out should be adopted. First 50 % saturated ammonium sulfate was added for salting out to remove impure proteins, and then 80 % saturated ammonium sulfate was added to the supernatant to precipitate the target protein.

The target protein after salting out was redissolved with 200 mL buffer. The enzyme activity determined was 10303.2 U/mL, and the total enzyme activity was 2060635 U, with the activity loss of about 37.1 %. The loss of enzyme activity may be due to the protein denaturation under higher salt concentration.

The crude enzyme salted out was desalted and dried in freeze drying conditions to obtain about 1.9 g enzyme powder. The final product was preserved at 4 °C.

151.3.4 Choice of pH Value for DEAE-Sephorose CL-4B Ion Exchange Eluate

The results were shown in Fig. 151.3. When the pH value was 9.0, there was only a very low enzyme activity value of alkaline pectinase determined in the supernatant, which indicated that pectinase was adsorbed to the column at this pH value.

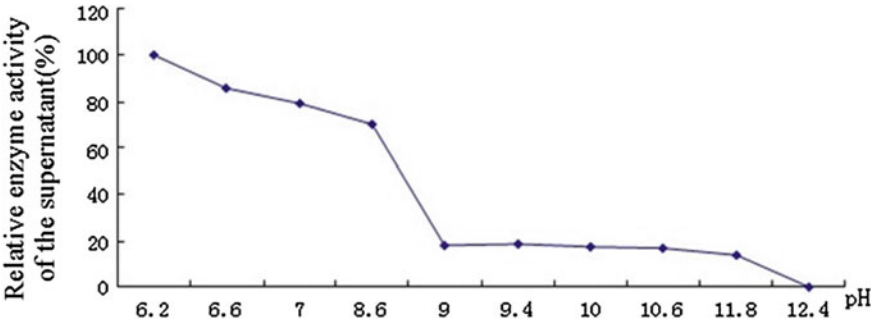

Fig. 151.3 Change of relative enzyme activity of the supernatant (%) with pH value for DEAE-Sephorose CL-4B eluate

When pH was 8.6, alkaline pectinase was not absorbed but impure proteins were adsorbed, thus the crude enzyme can be purified. By comprehensive consideration, the most suitable pH for alkaline pectinase was 8.5, thus pH 8.6 buffer should be chosen.

151.3.5 DEAE-Sephorose CL-4B Anion Exchange Chromatography

0.05 g enzyme powder was dissolved in 10 mL buffer and centrifuged to remove small amounts of insoluble materials. The supernatant was then purified with DEAE- Sephorose CL-4B ion exchange column.

The results were shown in Fig. 151.4. When the desalted alkaline pectinase was eluted first with pH 8.6 Gly-NaOH buffer, two kinds of protein components were washed off, and when the column was gradiently eluted with pH 8.6 Gly-NaOH buffer containing 0–1 mol/L NaCl, three proteins were washed off. The elution curve was detected at 214 nm. By the detection of enzyme activity, it was found the first elution peak was active component while other elution peaks were inactive, which illustrated that alkaline pectinase was not adsorbed on ion exchange chromatography column, and can be separated completely from impure proteins.

About 60 mL of the first elution peak was collected with enzyme activity of 565.3 U/mL, and then the protein purity was identified with the SDS-PAGE. The total activity was 1301277 U, and enzyme activity lost around 60.3 %. The activity loss may be resulted from the adsorption of a small amount of enzyme in ion exchange column and was not washed off.

The eluted liquid was dried in freeze drying conditions at −70 °C, and concentrated to be stored at 4 °C.

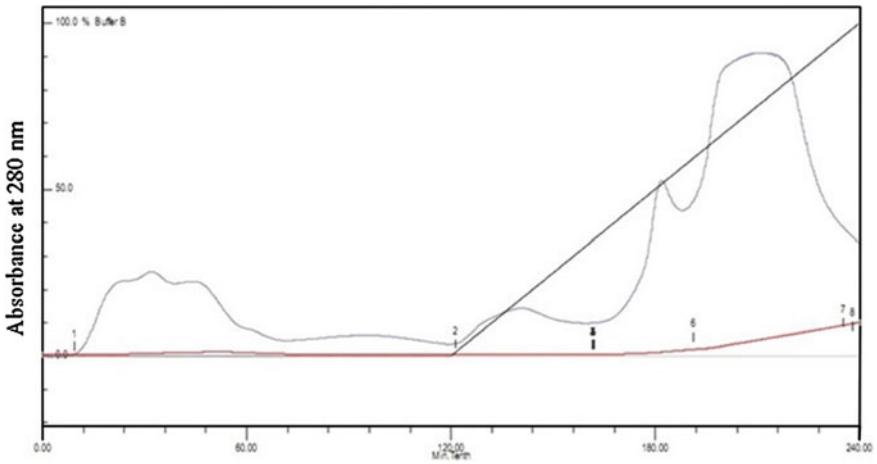

Fig. 151.4 Elution curve of DEAE-Sephorose CL-4B anion exchange chromatography

151.3.6 Sephadex-G75 Column Chromatography

After salting out, dialysis and anion exchange chromatography, crude enzyme solution was subjected to Sephadex-G75 column chromatography to further remove impure proteins. The sample volume was 2 mL. The elution curve was drawn with time as abscissa and absorption of the liquid in each tube as ordinate. As shown in Fig. 151.5, there were two protein peaks in the elution process. By the detection of enzyme activity, the first elution peak was determined as active component, whose shape was nearly symmetric and the baseline was close to zero, which indicated that it could be separated from another protein group.

About 37 mL corresponding to the first elution peak was collected with the enzyme activity of 303.6 U/mL, and total enzyme activity of 707,715 U. After the purification through the steps described previously, the total recovery rate was 21.6 %, and purification multiples reached 12.5 times.

151.3.7 Purity Identification

The molecular weight of the purified alkaline pectinases was detected by Coomassie brilliant blue staining and SDS polyacrylamide gel electrophoresis. Figure 151.6 showed it was 43 kDa, with the electrophoretical purity.

M: molecular weight makers; 1. fermentation liquid; 2. ultrafiltration; 3. concentrated ammonium sulfate salting out solution; 4. DEAE-Sephorose CL-4B ion exchange chromatography; 5, 6. Sephadex-G75 column chromatography.

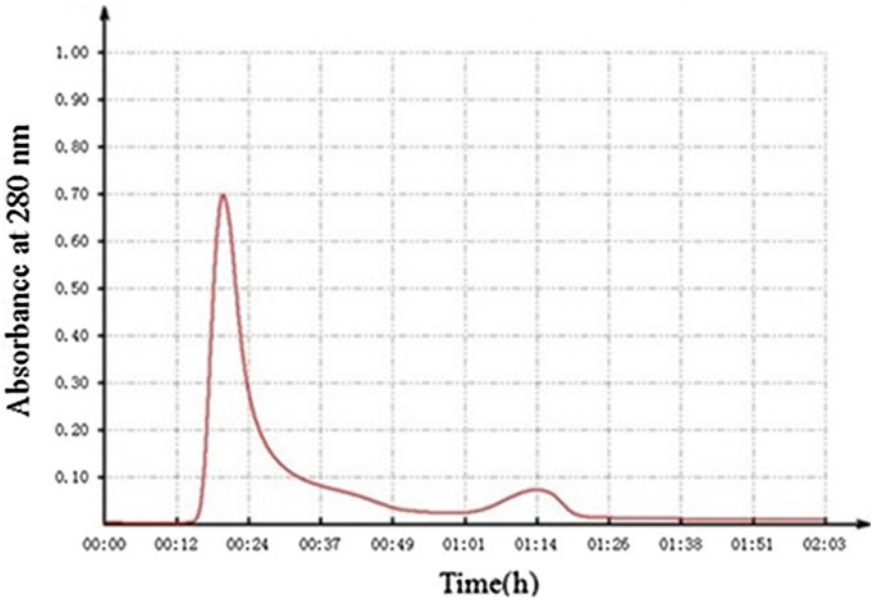

Fig. 151.5 Sephadex-G75column chromatography elution curve

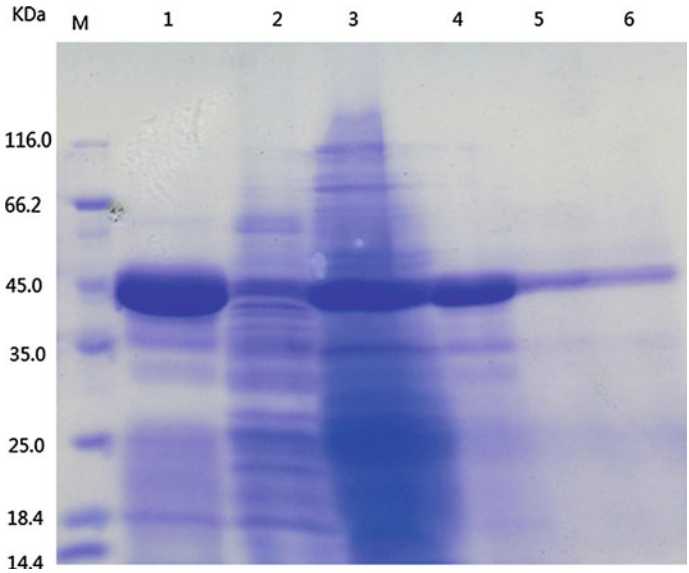

Fig. 151.6 Polyacrylamide gel electrophoretogram

Table 151.1 The purification results of alkaline pectinase

	Total activity (U)	Protein content (mg)	Specify activity (U/mg)	Purification multiple	Recovery rate (%)
Fermentation liquid	3276288	17400	188.2	1	100
Ultrafiltration	2401307	9246	259.7	1.4	73.3
Ammonium sulfate	2060635	1900	1084	5.8	62.9
DEAE-Sephorose CL-4B	1301277	1057	1230	6.5	39.7
Sephadex-G75	707715	301	2352	12.5	21.6

151.4 Conclusions

By the processes of centrifugation, ultrafiltration, ammonium sulfate precipitation, DEAE-Sephorose CL-4B anion exchange chromatography and Sephadex-G75 column chromatography for the fermentation liquid, the specific activity of electrophoretically pure alkaline pectinase finally obtained, which was 12.5 times higher than that of the original enzyme with the recovery rate up to 21.6 %. It can be seen that the purification route described above can improve the purity of the alkaline pectinase, with high recovery rate. It was an effective purification method (Table 151.1).

This experiment uses the previous purification methods of microbial pectinase for reference, and combines ammonium sulfate salting out, ion exchange chromatography, gel filtration, and other conventional separation methods, to establish a kind of separation method for alkaline pectinase with low cost and good purification effect, which will be helpful to further research on enzyme properties and advanced structure of alkaline pectinase, laying the foundation for remixing with other enzymes or auxiliary agents to be applied in degumming in the textile industry.

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