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Partitioning of alkaline protease from *Bacillus licheniformis* (ATCC 21424) using PEG–K_2HPO_4 aqueous two-phase system

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ABSTRACT

An aqueous two-phase system (ATPS) containing polyethylene glycol (PEG) and potassium phosphate (K_2HPO_4) was used for partitioning and partial purification of alkaline protease produced by *Bacillus licheniformis* ATCC 21424. The effect of PEG molar mass, pH and concentration of NaCl salt addition on partition coefficient, yield and purification factor (PF) for ATPS were studied. The highest partition coefficient (5.3) was achieved in an ATPS of 25% (w/w) PEG 10,000 (g/mol) and 10% (w/w) NaCl at pH 9. Cation and anion exchange chromatography was employed for purification of alkaline protease from protein mixture and the yield and PF for enzyme fractions were obtained to make a comparison between ATPS and ion exchange chromatography. Although, the results of the study indicated that using ATPS leads to lower PF than ion-exchange chromatography, but considering its simplicity and high yield adding up lower investments make ATPS a promising purification option in industrial scale.

1. Introduction

Proteases, a complex group of enzymes capable of hydrolyzing the peptide bond in a protein molecule [1], can be classified based on the optimum pH on which their maximum activity occurs. [2]. Regarding this classification alkaline protease, a group of proteases with the optimum pH above neutrality [3], can be produced from various natural sources like bacteria, fungi or certain insects [4]. Alkaline protease has wide spectrum of industrial application including food, detergent or tanning processes [5]. From industrial point of view, alkaline protease constitutes 25% of enzyme marketing, advocating to an estimated 1.7 billion dollars trade in 2013 [6].

There are various methods for the separation and purification of alkaline protease from crude biological mixtures. Ultra filtration (UF), precipitation and chromatography are common methods for this purpose [7]. Although, chromatography is considered an appropriate method in purification of enzymes, but in industrial application it is accompanied with several drawbacks like high-cost and time-consuming process [8]. Therefore, demands for alternative industrial methods attract scholars' attention. One of the potential options which successfully fulfill the required criteria for industrial employment is aqueous two-phase systems (ATPSs) [9].

The ATPSs are composed of a mixture of two polymers, or a polymer and a salt which is separated to two phase in an aqueous medium [10]. ATPSs offer many advantages including low-processing time, low-cost material, and low-energy consumption. Literature reports the successful employment of ATPS in enzymes and proteins partitioning [11–16]. However, there is limited number of literature study concerning partitioning and partial purification of alkaline protease in ATPS.In this research, the effects of PEG molar mass (1500–10,000 g/mol), pH and concentration of NaCl salt addition in partitioning and partial purification in ATPS are studied. Then the results were compared with ion-exchange chromatography method regarding the partial purification of alkaline protease from other proteins.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Polyethylene glycol (PEG 1500, 10,000 g/mol), K_2HPO_4, NaCl, Coomassie brilliant blue G250, trichloroacetic acid (TCA) and acetone were purchased from MERK, Germany. Azocasein and bovine serum albumin were purchased from Sigma, Germany. All the chemicals used were of analytical grade.

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2.1.2. Microorganism

Bacillus licheniformis ATCC 21424 was purchased from Persian Type Culture Collection (PTCC) center and was used as microbial source for alkaline protease production.

2.2. Methods

2.2.1. Production of alkaline protease from B. licheniformis ATCC 21424

Media culture contains 6% glucose, 2% soybean meal, 0.04% $CaCl_2$, 0.02% $MgCl_2$ in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer which was prepared at pH 7.6. Incubation was done according to United States Patent Office 3748233. After 48–72 h media culture was centrifuged at 5000 rpm at 4 °C for 20 min and the final enzyme solution was achieved.

2.2.2. Precipitation of enzyme solution

About 7 ml acetone at −20 °C was added to 3 ml enzyme solution in 15 ml tubes and mixed thoroughly. Then, tubes were placed at −20 °C for 2 h; and then centrifuged at 4000 rpm at 4 °C. Finally sediments were collected and stored in −20 °C [17].

2.2.3. Preparation of aqueous two-phase systems

Adding proper amount stoke solutions of PEG, buffer, NaCl and 2 ml protein mixture, ATPS was prepared in 15 ml centrifuge tubes. Protein mixture was obtained from solving protein sedimentation in 4 ml deionized water.

Effect of pH on enzyme separation was studied by using buffers at different pHs (7.5, 9 and 10.5). Adding proper amount of KOH and KH_2PO_4 solutions to K_2HPO_4 till K^+ concentration at ATPS reached 0.5 M, the buffers were obtained. PEG concentration was selected 25% (w/w) and NaCl concentrations were chosen 4, 6, 8, 10% (w/w) at ATPS.

In order to make thermodynamic equilibrium between two phases, the ATPS mixture was agitated thoroughly and centrifuged at 3000 rpm and 25 °C for 10 min. Enzyme activity and protein concentration were measured after two final phases were separated.

2.2.4. Enzyme purification by ion-exchange chromatography

Using AKTAprime Plus chromatographer system form GE, cation and anion exchange chromatography were respectively performed on SP Sepharose high performance and Q Sepharose high performance. Column size was a XK16 from Amersham Biosciences with 25 cm height which had been equilibrated with 50 mM sodium phosphate buffer pH 8.0 for cation exchange chromatography and 40 mM Tris–HCl buffer pH 8.5 for anion exchange chromatography. 2 ml of protein mixture was injected to column at the flow rate of 2 ml/min. The column was washed with equilibration buffer until the absorbance of the effluent at 280 nm reached the base line. The bounded proteins were eluted from column using linear gradient of increasing NaCl concentration (0.0–1.0 M) with 50 mM sodium phosphate buffer pH 8.0 and 40 mM Tris–HCl buffer pH 8.5 for cation and anion exchange chromatography, respectively. Fractions at a flow rate of 2 ml/min and a size of 5 ml were collected and analyzed for alkaline protease activity and protein concentration.

2.3. Analytical method

2.3.1. Protein concentration

Bradford method was employed to determine total protein concentration, using Coomassie brilliant blue G250 through absorbance measurements at 595 nm with bovine serum albumin (BSA) as a standard [18]. It was shown that PEG had no effect on Bradford assay method [19].

2.3.2. Protease activity

The alkaline protease activity was determined by modification of azocasein digestion method measuring the absorbance at 440 nm [20]. 0.2 ml of sample was added to 1 ml azocasein 0.5% (w/w) which was prepared in 0.1 M Tris–HCl buffer pH 8.5. After incubation for 30 min at 37 °C the undigested protein was precipitated by adding 1 ml trichloroacetic acid (TCA) 20% (w/w) and was allowed to stand for 30 min at room temperature, followed by centrifugation at 3000 rpm for 20 min then the absorbance of the solution was measured at 440 nm in M501 Single Beam Scanning UV/visible Spectrophotometer.

2.3.3. Polyacrylamide gel electrophoresis

SDS–PAGE (16%) was performed for the determination purity of the enzyme in samples as described by Laemmli [21]. The molecular weight was estimated by using broad range standard molecular weight markers (14.4, 16.4, 25.0, 35.0, 45.0 and 66.2 kDa).

2.4. Calculation

The partition coefficient for a certain protein was defined as the protein concentration in upper phase divided by the correspondent value in the bottom phase as shown in (Eq. (1)). For enzymes partition coefficient was defined as follows (Eq. (2)):

$$K_P = \frac{C_T}{C_B} \tag{1}$$

$$K_E = \frac{A_T}{A_B} \tag{2}$$

where C_T and A_T are, respectively, total protein concentration in mg/ml and enzyme activity of the upper phase, and C_B and A_B are corresponding values for the bottom phase.

The enzyme specific activity (SA, expressed in U/mg of protein), yield recovered in upper phases (Y_T) and purification factor (PF) were calculated according to the given equations:

$$SA = \frac{A}{C} \tag{3}$$

$$PF = \frac{SA}{SA_i} \tag{4}$$

$$Y_T = \frac{100}{1 + 1/(R_V K_E)} \tag{5}$$

$$R_V = \frac{V_T}{V_B} \tag{6}$$

where A and C are activity and total protein concentration (mg/ml) respectively, R_V is the volume ratio, and V_T and V_B are the volumes of upper and bottom phases, respectively. The enzyme specific activity (SA) can be evaluated for both phases, and SA_i represents the specific activity of initial sample (before extraction). Notice, only the concentrations of protein impurities were considered to obtain PF values.

3. Result and discussion

3.1. Effect of PEG molar mass, pH and NaCl concentration on partition coefficient of alkaline protease in ATPS

As shown in Fig. 1 and the experimental results in Table 1, K_E values for ATPS contain PEG 10,000 are greater than the similar one with PEG 1500. Increasing NaCl concentration and pH have positive effect on K_E which means highest pH and NaCl concentration with PEG 10,000 provide highest K_E. Maximum K_E (5.3) was obtained with PEG 10,000, pH 10.5 and NaCl concentration 10% (w/w).

The PEG exclusion effect and the PEG–protein binding are two parameters which affect partition coefficient of proteins in ATPS.

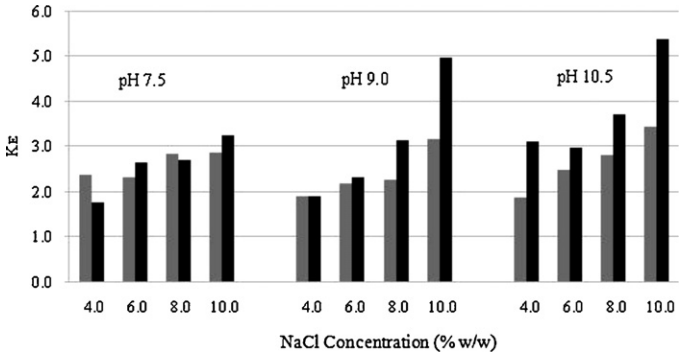

Fig. 1. Effect of PEG molar mass, NaCl concentration and pH on partition coefficient of alkaline protease in ATPS. PEG 1500 (▩) and PEG 10,000 (■).

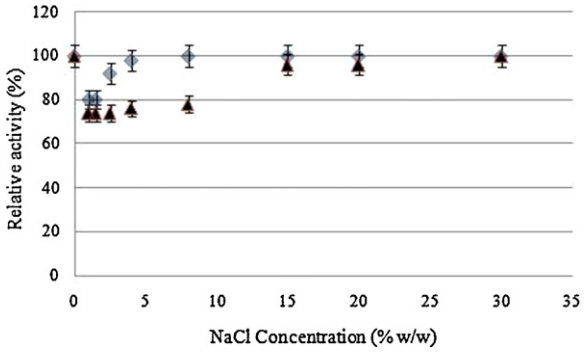

Fig. 2. Effect of presence of PEG on relative activity of alkaline protease. PEG 1500 (◆) and PEG 10,000 (▲). Mixture of PEG and alkaline protease was prepared and activity of alkaline protease was measured. Relative activity in the absence of PEG was referred as 100%.

The PEG exclusion effect alters the free volume available for proteins in the rich PEG phase and changes partition coefficient. The PEG–protein binding is produced through the hydrophobic area of the protein exposed to the solvent. With great hydrophobic surface area exposed to the solvent, proteins desire to transfer PEG rich phase, so partition coefficient increase. Increasing PEG molar mass raises hydrophobic property of it, and if the protein in ATPS has hydrophobic affinity, it transfers to the PEG rich phase, thus K_E increases [22]. Because increasing PEG molar mass from 1500 to 10,000 causes rising K_E, it can be concluded that alkaline protease form this type of *B. licheniformis* has hydrophobic affinity. Another reason for hydrophobic affinity of this enzyme is, taking apart of aggregated alkaline protease molecules in the presence of PEG, because of hydrophobic forces.

Pancera et al. reported that PEG can aggregate enzyme molecules [23]. In Fig. 2 the effect of PEG presence on alkaline protease activity was shown. In the low PEG concentration, alkaline protease molecules aggregate, and results reduction of enzyme molecules surface and its activity. In higher PEG concentration, the hydrophobic forces between alkaline protease molecules and PEG lead to separation of alkaline protease molecules and increasing activity.

Effect of pH on K_E depends on the charge of proteins. Charge of a protein itself depends on two parameters: pH of the solvent and isoelectric point of the protein. Isoelectric point of this type of alkaline protease is approximately 9 [24]. According to Fig. 1, changing the pH values has greater effect on K_E at PEG 10,000 than PEG 1500. Rising pH from 7.5 to 10.5, K_E values for PEG 10,000 increase, thus alkaline protease molecules with negative charge prefer PEG rich phase.

Table 1
Result of parameters for ATPS partition coefficient (K_E), yield of separation (Y) and purification factor (PF) for upper phase of ATPS. PEG concentration 25% (w/w) and K^+ concentration 0.5 M at 25 °C.[a]

PEG molar mass	pH	NaCl% (w/w)	K_E	Y%	PF
1500	7.5	4.0	2.4	93.1	2.62
1500	7.5	6.0	2.3	92.1	2.38
1500	7.5	8.0	2.8	93.3	2.45
1500	7.5	10.0	2.9	92.9	2.14
10,000	7.5	4.0	1.8	84.9	1.52
10,000	7.5	6.0	2.7	88.1	1.59
10,000	7.5	8.0	2.7	87.5	1.81
10,000	7.5	10.0	3.3	88.9	1.55
1500	9.0	4.0	1.9	92.2	1.87
1500	9.0	6.0	2.2	91.9	1.72
1500	9.0	8.0	2.3	92.0	1.67
1500	9.0	10.0	3.2	93.8	1.71
10,000	9.0	4.0	1.9	85.5	1.38
10,000	9.0	6.0	2.3	86.7	1.42
10,000	9.0	8.0	3.1	89.4	1.34
10,000	9.0	10.0	5.0	92.6	1.43
1500	10.5	4.0	1.9	92.3	2.03
1500	10.5	6.0	2.5	92.9	2.09
1500	10.5	8.0	2.8	93.6	2.00
1500	10.5	10.0	3.5	93.6	2.21
10,000	10.5	4.0	3.1	91.3	1.44
10,000	10.5	6.0	3.0	89.9	1.37
10,000	10.5	8.0	3.7	91.2	1.39
10,000	10.5	10.0	5.4	93.3	1.42

[a] Standard uncertainties u are $u(T) = 0.1$ °C, pH = 0.1, NaCl% (w/w) = 0.1, $K_E = 0.1$, $u(Y) = 0.1$, and $u(PF) = 0.1$.

3.2. Effect of PEG molar mass, pH and NaCl concentration on yield and PF of alkaline protease in ATPS

From Table 1, yield at PEG 1500 is higher than PEG 10,000. As it can be observed, pH has no effect on PEG 1500, but for PEG 10,000 rising pH causes yield values increase.

Results in Table 1 show PF at PEG 1500 systems are higher than PEG 10,000 for all cases. The results of our study indicate that NaCl has no effect on PF. Also, it can be observed that pH is a significant parameter in PF. The maximum PF in our study was at pH 7.5. This phenomenon can be supported by ion exchange chromatography.

Based on cation exchange chromatography curve (Fig. 4b), two types of protein were detected. The one that passes through the column in washing state, has negative charge and the other which bond to cationic matrix and elutes with NaCl gradient elution, has positive charge at pH 8. If the pH of ATPS adjusts at 7.5, some of proteins have negative and others have positive charge, thus different types of proteins have different tendency to phases in the ATPS and separate each other better than other pHs.

3.3. Statistical study

The experimental data (K_E or PF) were analyzed using MiniTab (version 16). Fig. 3 shows the experimental results plotted in a Pareto chart. The Pareto chart is a bar chart which represents the estimated effects of the variables and the interactions on response in reduce the number of experiments. The line passing through the chart depends on the value of α (it is the value with which the p value of an effect is compared to determine whether an effect exists or not).

It is concluded in Fig. 3a that NaCl concentration is statistically the most important factor for K_E. Also, interaction between PEG MW and NaCl concentration is the shortest bar that passes the vertical line.

At higher salt concentrations, the ions decrease protein solubility by increasing hydrophobic interaction, and the hydration effect of the salt molecule surrounding the protein. So, most proteins

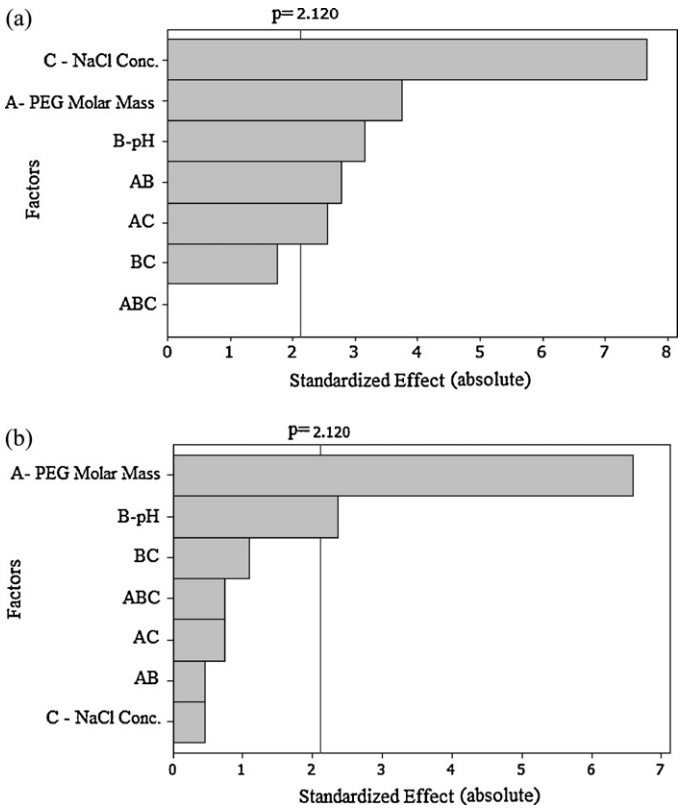

Fig. 3. Pareto chart for the standardized effects of the variables for PEG–K_2PO_4 system with $\alpha = 0.05$, for the partitioning coefficient of alkaline protease (a), and purification factor of alkaline protease (b). PEG MW (A), pH (B), NaCl concentration (C), two-letter and three-letter symbols represent the interaction factors.

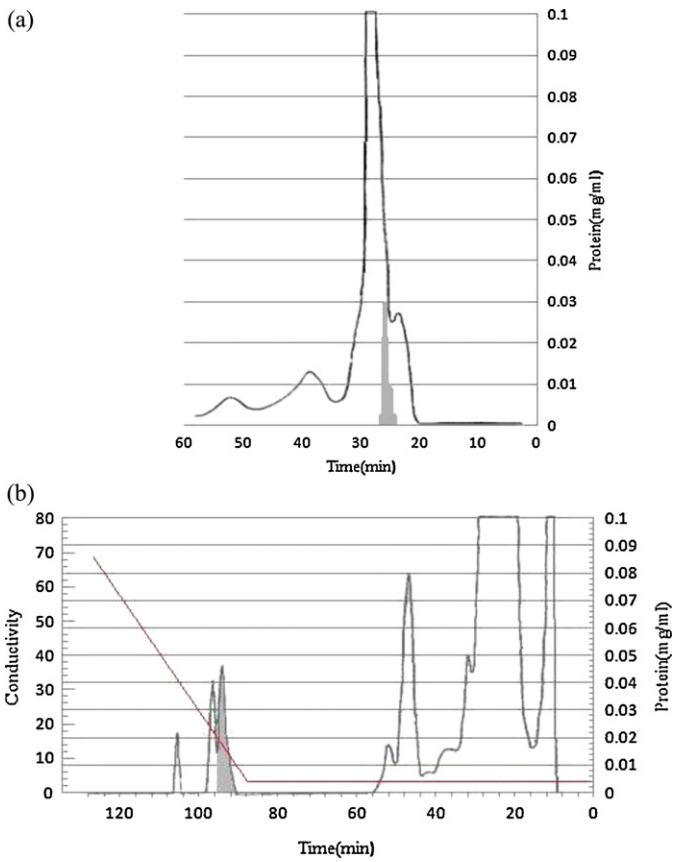

Fig. 4. Ion exchange chromatography curves. Flow rate is 2 ml/min and fraction size is 5 ml. (a) Anion exchange chromatography, alkaline protease pass through column by 40 mM Tris–HCl buffer pH 8.5 washing. (b) Cation exchange chromatography, column was washed with 50 mM phosphate buffer pH 8 for 50 min and then eluted with equilibration buffer and NaCl (0–1 M) gradient elution.

partition to the phase with lower salt concentration, thereby increasing the interaction between the protein and the PEG molecules and ultimately improving extraction of proteins to the PEG-rich phase [25].

Interaction between PEG MW and pH is another significant factor for K_E, because negatively charged proteins generally prefer the upper phase in PEG–salt systems [26].

In Fig. 3b PEG MW and pH have important significant effect on PF, but as mentioned, NaCl concentration has not a remarkable effect on it. Similar results for partitioning xylose reductase from *Candida mogii* were reported by Mayerhoff et al. [27].

3.4. Cation and anion exchange chromatography

Color gray in Fig. 4 determines alkaline protease peaks in anion and cation exchange chromatography curves. Results of anion and cation exchange chromatography are shown in Table 2. For ion exchange chromatography method, PF is higher than ATPS and cation exchange chromatography has maximum PF (22.14).

According to cation exchange chromatography, it has better PF than ATPS, but the process is time consuming and lasts 2 h. So, if intermediate purification is needed, ATPS is better than ion exchange chromatography.

The purity of the separated alkaline protease, using ion exchange chromatographies and ATPS methods are shown in Fig. 5. Lane 2 has only one band that indicates high enzyme purification in cation exchange chromatography.Conclusion

The experiment results indicated that pH and PEG molar mass have significant effect on purification factor of alkaline protease at ATPS. The highest partition coefficient (5.3) was achieved in an ATPS of PEG 10,000 and 10% (w/w) NaCl at pH 9, while the highest PF (2.5) obtained in PEG 1500 and 4% (w/w) NaCl at pH 7.5. According to yield and purification factor from ATPS and ion-exchange chromatography, ATPS leads to lower PF than ion-exchange chromatography, but its simplicity and high yield in addition to lower investments makes ATPS as a practical method in industrial scale for alkaline protease partial purification which is used in detergent and tanning processes. Thus for medicine and food applications which demand high purification, cation exchange chromatography is inevitable.

Table 2
Yield of separation (Y) and purification factor (PF) for cation and anion exchange chromatography fractions. $T = 25\,^{\circ}C$ and buffer flow rate = 2 ml/min.[a]

Anion exchange chromatography				Cation exchange chromatography			
Fraction	Minutes	Y%	PF	Fraction	Minutes	Y%	PF
9	20–22.5	0.18	1.92	37	90–92.5	0.06	12.82
10	22.5–25	0.74	3.03	38	92.5–95	0.81	22.14
11	25–27.5	0.08	0.2	39	95–97.5	0.13	3.08

[a] Standard uncertainties u are $u(T) = 0.1\,^{\circ}C$, $u(Y) = 0.1$, and $u(PF) = 0.1$.

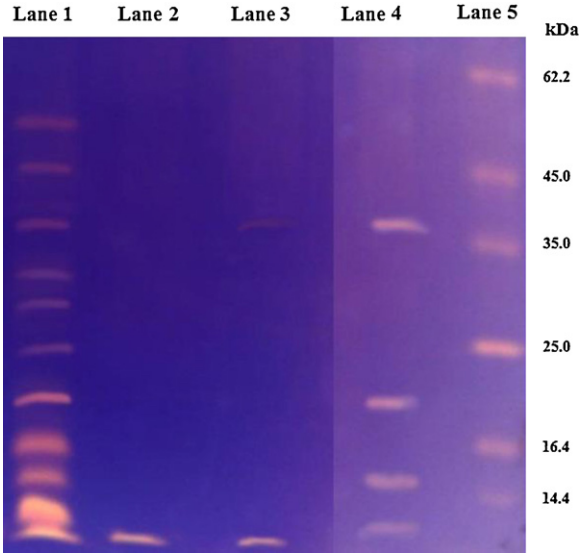

Fig. 5. SDS–PAGE of alkaline protease from *Bacillus licheniformis*. Lane 1: Crude extracellular alkaline protease. Lane 2: Cation exchange chromatography, purified alkaline protease. Lane 3: Anion exchange chromatography. Lane 4: ATPS from top phase of system contain PEG 1500, 4% (w/w) NaCl at pH 7.5. Lane 5: Standard protein markers of different molecular weights.

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