

Purification and Characterization of a Detergent Compatible Alkaline Protease Produced by *Bacillus raris* Isolated from Vegetable Oil Factory Effluent in Owo, Ondo State, Nigeria

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Abstract

A thermostable alkaline protease was produced by *Bacillus raris* isolated from vegetable oil factory effluent in Owo, Ondo State, Nigeria. Detergents containing enzymes have been reported to possess more effective washing capabilities. The protease was purified and characterized. Biochemical characterization and 16S rRNA sequencing showed the strain was closely related to *Bacillus raris* with accession number NR_042161.1. The protease was purified with a 0.516 purification fold and 19% recovery with a Sephadex G-100 column fraction. The protease was relatively stable at alkaline pH retaining activity at pH 9.0 (100%). The protease was also thermally stable with the highest activity observed at 55 °C. *Bacillus raris* protease activity was stimulated by Ba²⁺, Ca²⁺, Cu²⁺ and Mn²⁺, while enzyme activity was inhibited by Zn²⁺ and Pb²⁺. Protease activity increased with an increase in substrate concentration. The Lineweaver-burk plot revealed Km = 0.14. Protease activity was influenced by the tested surfactants and inhibitors. The protease enzyme showed relative stability to some commercial detergents. Thus, the protease enzyme appears to possess properties desired for detergent formulation and inclusion in other biotechnological applications.

Keywords: Protease, enzyme activity, detergents, alkaline, *Bacillus raris*, purification.

1. Introduction

Enzymes have been used in fermentation processes from ancient times. Earlier reports of their use were reported by the ancient Greeks who used enzymes for baking, brewing, alcohol fermentation and cheese manufacture (Sharma *et al.*, 2017). Literature has also shown that enzymes can perform numerous roles, for example the selective manipulation of protein and lysis of fibroin clusters with advancements in analytical techniques (Sharma *et al.*, 2017).

Proteases are universal enzymes in nature, catalyzing the hydrolysis of protein molecules into peptides and amino acids (Matkawala *et al.*, 2019; Sumantha *et al.*, 2006). Proteases have attracted interests over the years, mainly because of the important roles they play in cellular metabolism and the biotechnology industry (Fatema *et al.*, 2019; Gupta *et al.*, 2002). Proteases are widely used in the food, laundry detergents, leather treatment, bioremediation processes and pharmaceutical industries where they mediate several changes in products taste, texture, appearance, quality and in waste recovery (Mamo & Assefa, 2018; Saha *et al.*, 2011; Sandhya *et al.*, 2005; Vadlamani and Parcha, 2011). In detergency, the combined action of surfactants and enzymes aids in the removal of difficult proteinous stains from fabrics (Olsen and Falholt, 1998). Proteases can be grouped based on

their sites of action as exopeptidases and endopeptidases. Proteases that cleave the peptide bond at the center of the amino or carboxyl termini of the polypeptide chain are referred to as exopeptidases, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (cut at the internal peptide bonds) (Mamo & Assefa, 2018; Rao *et al.*, 1998; Yegin and Dekker, 2013). Proteases are also grouped based on their acid-base behavior viz. acid, neutral and alkaline proteases (Sandhya *et al.*, 2005). Alkaline proteases are optically active in a neutral to basic pH (Sharma *et al.*, 2017). They either possess a serine center or a metallo-type, and are the most researched group of enzymes and are widely used in detergents and allied industries. The high specificity of enzymatic reactions also prevents damage to fabrics and surfaces, which is a matter of concern in chemical detergents (Singh *et al.*, 2016).

Proteases, amylases, lipases and cellulases are used in the starch, textile, detergent and baking industries, representing the second-biggest group, while proteases are the predominant enzyme type, owing to their extensive use in the cleansing and dairy industries (Nguyen *et al.*, 2015; Kirk *et al.*, 2002). Proteases are also used in the modification of proteins to reduce the allergenicity of cow milk for infant formula products in dairy industries (Kirk *et al.*, 2004).

The different products in the detergent industry contain proteases as a fundamental component or ingredient and

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are used for cleaning household laundries, false teeth, or contact focal lenses (Razzaq *et al.*, 2019). Successful deployment of enzymes is an integral part of the design and production of economical and environmental (biodegradable) friendly detergents (Dewan, 2017). Microbial alkaline sources have attracted increased demand from industries owing to their cost-adequacy, ready susceptibility to genetic manipulation and relative ease of cultivation (Razzaq *et al.*, 2019). Also, proteases intended for use in detergent formulation need not be in the purest state, unlike those for use in pharmaceutical, medical areas that require a higher degree of purity as a necessity.

The suitability of an enzyme for detergent formulation will depend on its ability to retain its function alongside other detergent constituents such as surfactants and oxidizers under quite harsh operating conditions (Boran, 2018). It should also possess the ability to perform over a range of pH and temperature (Griffin *et al.*, 1992). Certain procedures are also put in place to prevent the denaturation of the detergent enzymes (Boran, 2018). Scientists have also tried to isolate from natural environments, microbial enzymes possessing more tolerance against alkaline pH and chemicals in detergents (Boran, 2018), hence the need for isolation of more detergent-compatible microbial proteases.

This study investigated the production, purification and characterization of a detergent compatible protease using bacteria isolated from vegetable oil effluent soil in Owo, Ondo State, Nigeria.

2. Materials

2.1. Isolation and screening of proteolytic bacteria

Soil samples were collected from vegetable oil factory effluent dump in Owo, Ondo State, Nigeria. Isolation was done by serial dilution on a prepared nutrient agar plate (Oxoid). Single colonies were picked and purified by continuous streaking. Preliminary screening for protease production was done by subjecting the isolates to skimmed milk agar plate (SKMA) containing (% w/v); skimmed milk powder (1.0), peptone (0.1), NaCl (0.5) and agar (2.0) at pH 10, the plates were incubated at 37°C for 48 h. The occurrence of clear halo zones around distinct colonies is indicative of protease production by the bacteria (Bajaj and Jamwal, 2013). Isolate that showed higher protease production potential were selected for further production and assay (Arulmani *et al.*, 2007). Bacterial identification was carried out using morphological, biochemical characterization by 16S rDNA sequencing.

2.2. Protease production

Bacteria selected from SKMA screening were selected for protease production. Protease production media (PPM) consisted of: CaCl₂ (0.01 g/l), K₂HPO₄ (0.05 g/l), peptone (1.00 g/l), MgSO₄ (0.01 g/l), glucose (0.1 g/l), at pH 7.0 and was incubated on a shaker incubator at 37 °C for 48 hours. The cell-free supernatant was then obtained by centrifuging at 10,000 rpm for 10 minutes at 4 °C. The supernatant obtained was used as a crude protease for further studies (Guleria *et al.*, 2016).

2.3. Protease assay

Protease assay was carried out using the casein-pholine method by Cupp-Enyard (2008). Casein (1 % w/v) was dissolved in 0.1 M phosphate buffer at pH 7.0 and was used as the substrate, 1 mL of the enzyme was added to the substrate and incubated at 50 °C in a water bath for one hour. The reaction was then terminated by adding 3 mL of the tricarboxylic acid (TCA). The reaction cocktail was centrifuged at 5000 rpm for 15 minutes. Then 0.5 mL of the supernatant was drawn into a test-tube, 2.5 mL of 0.5 M sodium carbonate was added, vortexed well and incubated for 20 minutes. The reaction was terminated by the addition of 0.5 mL of 2.0 N Folin-phenol reagent and the absorbance was read at 660 nm using a UV-Spectrophotometer (752Pro15041, Spectrum Lab England). Protease produced was estimated and expressed in a microgram of tyrosine liberated by 1 mL of the protease in 30 minutes at 30 °C in tyrosine equivalent (Akhavan and Jabalameli, 2011; Joo *et al.*, 2002). One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine/min under standard assay conditions based on a tyrosine calibration curve (Bhagwan *et al.*, 2015).

2.4. Protein assay

Total protein content was estimated following Lowry *et al.* (1951) using bovine serum albumin (BSA) as reference (Mothe and Sultanpuram, 2016).

2.5. Purification of the protease enzyme

The crude enzyme was subjected to precipitation in a salting-out process (Dixon and Webb, 1971). The precipitated fractions were dialyzed and separated using Sephadex G100 column chromatography (Ding *et al.*, 2012; Wakil and Osesusi, 2017). The fractions obtained were pooled together and their protease activity and protein content determined. These fractions were also used for further characterization.

3. Characterization of the purified protease enzyme

3.1. Effect of pH

The effect of pH on protease stability was assessed by incubating the protease-substrate mixture in appropriate buffers at intervals of 0.5 pH (Citrate-phosphate for pH 4 - 7 and Tris-HCl for pH 8 - 11) for one hour. Protease activity was determined using the standard assay method. Protease stability was expressed as relative activity (Niyonzima and More, 2014; Priya *et al.*, 2014).

3.2. Effect of temperature

The effect of temperature on the stability of protease was assessed by incubating the protease-substrate mixtures at varying temperatures (20 - 70 °C) at 5 °C intervals in a water bath for one hour. Protease activity was determined using the standard assay method. Protease stability was expressed as relative activity (Niyonzima and More, 2014; Priya *et al.*, 2014).

3.3. Effect of metal ions

The protease stability in the presence of metals was determined by pre-incubating the protease-substrate mixtures with metal (Ca²⁺, Cu²⁺, Mn²⁺, Mg²⁺, Ba²⁺, Zn²⁺, K⁺, Na⁺) for one hour. Metal salt concentrations of 5 mM

were used. Protease activity was determined using the standard assay method. Protease stability was expressed as relative activity (Priya *et al.*, 2014).

3.4. Effect of substrate concentration

The effect of varying substrate concentrations on the protease activity was determined by incubating different substrate concentrations (casein) from 0.2 to 1.2 mg/ml with protease for one hour under standard conditions. Protease activity was determined according to standard methods as previously described. The enzyme kinetics were also determined (Priya *et al.*, 2014). The Michaelis–Menten enzyme kinetic constants K_m and V_{max} were extrapolated from the Lineweaver–Burk plot (Priya *et al.*, 2014).

3.5. Effect of inhibitors and surfactants

Protease stability to surfactants and inhibitors; tween 20, triton X, sodium lauryl sulfate (SLS), sodium dodecyl sulfate (SDS), 2-mercaptoethanol (BME), dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA), were determined by pre-incubating the enzyme substrate mixtures with the surfactants and inhibitors at various concentrations for one hour at a concentration of 1 mM (Gohel and Singh, 2018). Protease activity was carried out according to standard methods as described earlier (Matkawala *et al.*, 2019).

3.6. Effect of detergents

The effect of commercial detergents (Ariel, Klin, Sunlight, Good mama, Canoe and Omo) on protease stability was evaluated by incubating detergents with the protease for one hour at 35 °C. The detergent solutions (0.1 % w/v) were prepared with double-distilled water, the detergent solutions were boiled at 100 °C to inactivate any enzymes already present, then cooled to room temperature. The solutions were then incubated with purified protease at 50 °C for one hour (Lam *et al.*, 2018; Matkawala *et al.*, 2019). Protease activity was determined using the standard assay method.

4. Results and Discussions

4.1. Isolation of proteolytic bacteria

The bacterial isolates that showed appreciable zone of hydrolysis on skimmed milk agar plates were subjected to protease production, and the bacteria with the highest protease production was selected for further study. Quantitative screening for protease production showed that OWO1 had the best protease activity (2.039 U/mL) while AKK1 had the lowest protease activity (0.072 U/mL) as shown in Figure 1. OWO1 was used for further study.

Vegetable oil factory effluents contain an immensely high volume of degradable organic matter. It is rich in nutrients and possesses fertilizing properties even in its raw state (Kamyab *et al.*, 2016). The nutrient-rich nature of the effluents may thus provide a good source of nutrients for proteolytic organisms to flourish.

Microscopic identification showed the isolate as spore-forming, Gram-positive rods, having flat elevation. Further identification showed the bacteria was catalase-positive and utilized citrate, it also utilized a range of hexose sugars, and disaccharide sugars (sucrose, maltose). 16S rDNA showed the isolate to have a 95.75 % homology with *Bacillus raris* NR_042161.1 upon submission of the sequence to the gene data bank of National Center for Biotechnology Information (NCBI). Naidu (2011) reported *Bacillus* species as being excellent protease producers. Sharma *et al.* (2017b) highlighted microbial enzyme activity as a function of several factors including nutritional and cultural variables viz. pH, temperature, carbon and nitrogen sources and duration of incubation of the microorganisms.

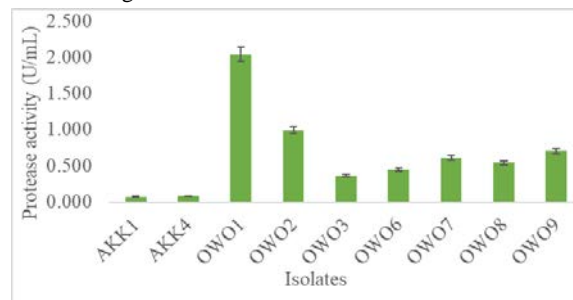


Figure 1. Screening for protease activity

The purification profile (Table I) of *Bacillus raris* protease showed that the total protease activity and total protein content were reduced significantly across the purification steps with the crude fraction having the highest values. The protease purification was found to decrease from 1 to 0.516 purification fold using Sephadex G-100 column chromatography. This is similar to reports by Josephine *et al.* (2012) and Jayashree *et al.* (2014) who reported a rise in the purification fold of *Bacillus* spp protease (Guleria *et al.*, 2016). This disagrees with reports by Naidu (2011) and Bajaj and Jamwal (2013) who reported increased protein content when purification was carried out to obtain the pure protease enzyme.

Bacillus raris protease was relatively stable across varying pH, maintaining relative stability at alkaline conditions up to pH 9.0, after which further rise in pH resulted in reduced protease activity (Figure 2). It was a good candidate for alkaline protease production, which is similar to findings by (Chu, 2007). These findings align with findings by Naidu (2011) who reported high *Bacillus subtilis* protease activity at pH 9.0. Niyonzima and More (2014) also reported that *Bacillus* spp. alkaline protease maintained stability within pH 8.0 – 12.0. Jayashree *et al.* (2014) reported a *Methylobacterium* sp. protease which retained over 50% activity at pH 7.0 - 11.0 but decreased to 30% beyond pH 12.0. Takami *et al.* (1990) attributed the increase in protease activity around the alkaline range to the binding that occurs in the enzyme-substrate complex, because pH greatly influences enzyme-substrate binding (Niyonzima and More, 2014).

Table I. Purification profile of *Bacillus raris* protease

Purification steps	Total protease activity (U/mL)	Total protein (U/mL)	Specific activity (U/mg)	Purification fold	Recovery yield %
Crude extract	1432.27	26.88	53.284	1	100
80% Ammonium sulphate precipitation	935.21	19.25	48.582	0.912	65
Dialysis	657.32	14.13	46.519	0.873	46
Gel chromatography	278.32	10.12	27.502	0.516	19

The results showed that the protease enzyme maintained relative stability after one hour when pre-incubated at varying temperatures between 20 - 70 °C as shown in figure 3, although activity decreased with increasing temperatures. *Bacillus raris* protease had its highest activity at 50 °C (100%), while further temperature rise resulted in reduced protease activity decreasing to 77.042 % at 70 °C. This result agrees with reports by (Adinarayana *et al.*, 2003; Giri *et al.*, 2011; Jayashree *et al.*, 2014; Niyonzima and More, 2014; Ramkumar *et al.*, 2018) who reported protease stability at 50 °C. This contrasts with Naidu (2011) who reported a reduction in protease activity beyond 35 °C. This inherent activity of the protease at alkaline and wide temperature regimes implies its potential for use across a range of washing temperatures (Niyonzima and More, 2014).

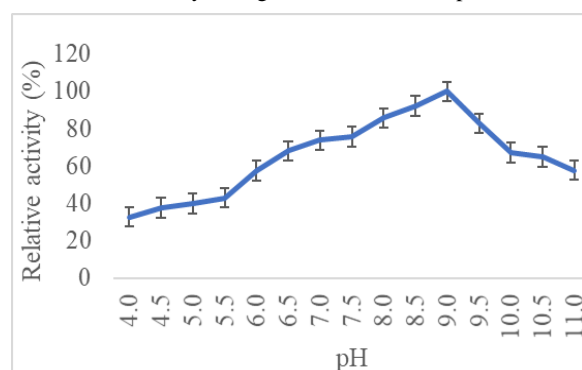
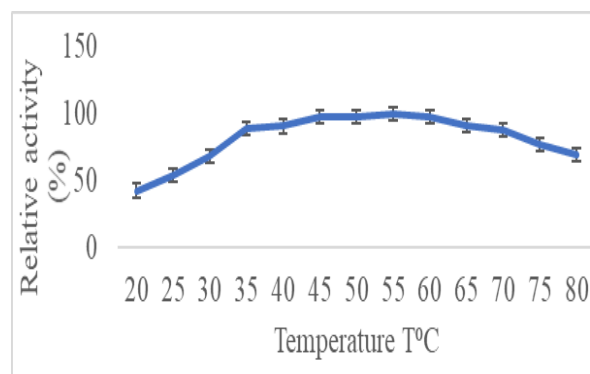
These findings show that *Bacillus raris* protease activity was stimulated by Ba²⁺, Zn²⁺, Cu²⁺, Ca²⁺, Mg²⁺, and Mn²⁺ with K⁺ (Figure 4) supporting the least protease activity, this highlights the protease stability in the presence of metals. This result is similar to reports by (Niyonzima and More, 2015) who reported that Ca²⁺ and Mg²⁺ stimulated protease production. Anandan *et al.* (2007); Niyonzima and More (2015); Sharma *et al.* (2005); Dubey *et al.* (2010) and Kalpana devi *et al.* (2008) positioned the importance of cations in maintaining the enzyme active sites, thus, improving protease thermostability. Also, Lobedanz *et al.* (2016) suggested that the actions of metal ions are dependent on their ability to bind specific sites in the enzyme molecule, ensuring the stability of the active enzyme conformation.

The findings show that increased substrate concentration also resulted in increased protease activity with the highest protease activity obtained at 1.5 mg/ml, while further increase in substrate concentration resulted in reduced protease activity (Figure 5). This corresponds to reports by Devanadera *et al.* (2016); Ramkumar *et al.* (2018); El-Safey and Abdul-Raouf (2004); Sumantha *et al.* (2006) who reported appreciable affinity for an increase in substrate concentration by protease enzyme after which further increase resulted in reduced protease production. The apparent K_m value of protease hydrolysis indicated the higher affinity and efficient catalytic role of *Bacillus* species protease towards their substrates to concentrate the active sites of an enzyme Ramkumar *et al.* (2018), as shown in Figure 6. It is also a measure of the enzyme-substrate (ES) complex. Singh *et al.* (2014) and Singh and Bajaj (2017) highlighted that proteases being designed for biotechnological applications should be robust and possess the structural and kinetic adaptations essential for extremes of industrial microenvironments, such as extreme temperature, pH and presence of inhibitors.

Findings from this study showed that surfactants such as tween 20, triton X, (SLS), (SDS), enhanced the protease activity of *Bacillus raris*, while inhibitors such as 2-

mercaptoethanol (BME), dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA) had varying inhibitory effects on *Bacillus raris* protease as shown in Figure 7. This result is similar to reports obtained by Jayashree *et al.* (2014) who reported improvement of protease activity by tween 20 and triton X and minimal effects of the oxidizing agents on protease enzyme. Further studies on enzyme inhibition could offer more insights regarding the nature of the proteases, its cofactor requirements and its active center (Jayashree *et al.*, 2014). The role of DTT in decreasing protease activity could be due to the excision of the intramolecular disulfide bonds essential for maintaining protease activity and stability (Jayashree *et al.*, 2014; Satyanarayana *et al.*, 2013 and Rai *et al.*, 2010).

The protease maintained relative stability in the presence of some commercial detergents (Figure 8), which is similar to reports by Adinarayana *et al.* (2003) and Ramkumar *et al.* (2018) who reported enzyme addition in detergents. Bajaj and Jamwal (2013) also reported a protease from *Bacillus pumilus* which showed significant stability and compatibility with surfactants and commercial laundry detergents at ambient temperature.

**Figure 2.** Effect of pH on activity of protease enzyme**Figure 3.** Effect of temperature on the activity of protease

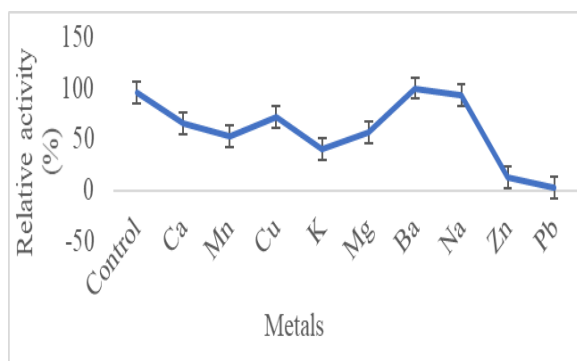


Figure 4. Effect of metal ions on protease activity

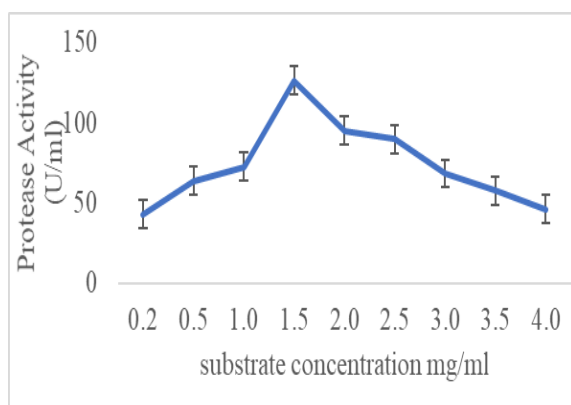


Figure 5. Effect of substrate concentration on protease activity

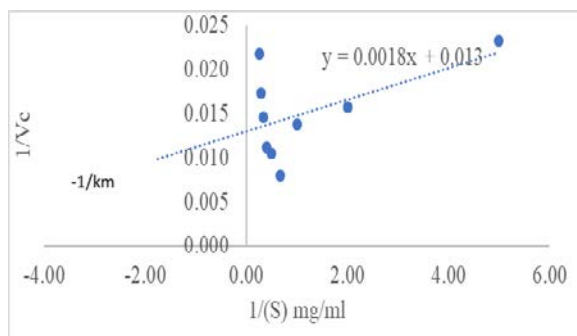


Figure 6. Lineweaver-burk plot for the hydrolysis of casein by the purified protease of *Bacillus raris*

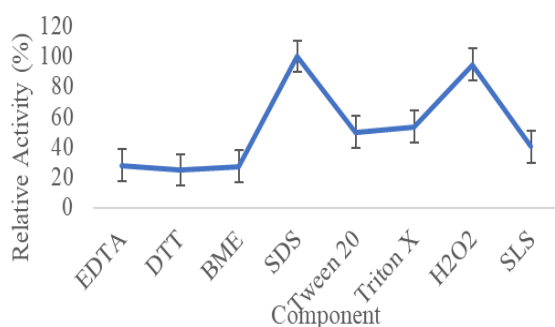


Figure 7. Effects of surfactants, inhibitors and oxidizing agents on protease activity

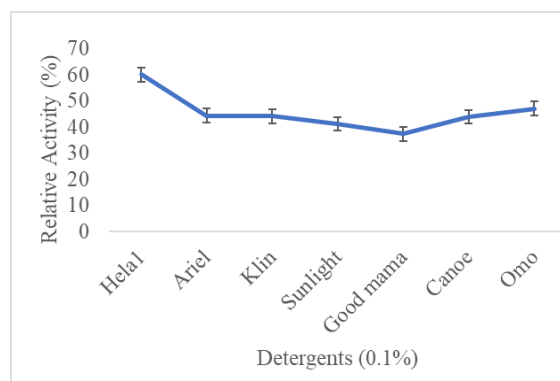


Figure 8. Protease activity in the presence of commercial detergents

5. Conclusion

In this study, an alkaline and thermostable protease was produced using *Bacillus raris* isolated from vegetable oil effluents in submerged fermentation. The protease maintained relative stability to inhibitors and commercial detergents. Protease remains one of the most important groups of industrial enzymes with numerous biotechnological applications. This suggests its potential for inclusion in a detergent formulation as well as other biotechnological uses. Further study on the protein interactions is necessary to tailor the protease appropriately for use.

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