Purification and Properties of an Extracellular Protease Produced by the Entomopathogenic Fungus *Beauveria bassiana*

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*Beauveria bassiana* GK2016 grown in a medium with gelatin as the sole carbon and nitrogen source produced an extracellular protease. The protease production was highest when the fungus was grown on a semiliquid medium and was purified about 18-fold, with a recovery of 21%. The protease molecular weight was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be about 35,000. It had an optimum activity at pH 8.5 and 37°C and was rapidly inactivated at 50°C. Its enzymatic activity was that of an endopeptidase which hydrolyzed elastin, casein, and gelatin but was much less active on bovine serum albumin and collagen. No trypsin-like activity was detected on N-α-benzoyl-L-arginine-p-nitroanilide. It was, however, inhibited by phenylmethylsulfonyl fluoride, indicating that a serine residue is present in the active site. The protease was unaffected by metal-chelating agents, sulfhydryl reagents, trypsin inhibitor, and chymotrypsin inhibitor.

*Beauveria bassiana* is an entomopathogenic fungus which infects a variety of insects and has potential for insect pest control (5). This fungus generally infects the host insect by passage through the cuticle (19). Germ tubes produced from conidia on the surface of the insect penetrate the host. It is presumed that the fungus orients toward the cuticle and penetrates by mechanical means accompanied by enzymatic action (30). The enzymes involved include extracellular proteases, chitinases, and lipases (4, 6, 13). After crossing the insect integument, the fungus grows within the aqueous hemolymph. There it produces toxins which kill the host. After host death, the mycelium grows throughout the cadaver.

Clarification of the contribution of extracellular enzymes to the fungal infection process is hindered mainly because of unavailability of pure enzyme preparations. Gabriel (6) reported extracellular proteases from *B. bassiana*, and Kucera and Samsinakova (11) partially purified these extracellular proteases. In this case, however, molecular weight, purification, and biochemical characterization of the proteases were not reported.

To elucidate the precise role of the protease in the insect infection process, it was essential first to characterize the enzyme. This article reports the purification and characterization of a protease secreted in vitro by *B. bassiana*.

**MATERIALS AND METHODS**

**Cultures and growth conditions.** For the inoculum, 10⁸ *B. bassiana* GK2016 conidia were inoculated into 100 ml of yeast extract-peptone-glucose broth (YPG) in a 250-ml Erlenmeyer flask. The culture was incubated on a rotary shaker (180 rpm) at 27°C for 3 days. The culture contents were filtered through a 0.45-μm-pore-size filter (HA type; Millipore Corp., Bedford, Mass.). The mycelial mat derived from 16 ml of YPG culture (25 mg [dry weight] of fungal mycelium) was used to inoculate 100 ml of the subsequent growth medium. This medium consisted of 0.5 or 1.0% (wt/vol) gelatin (Difco Laboratories, Detroit, Mich.) in a basal salts solution (pH 7) composed of NaCl, K₂HPO₄, and MgSO₄·7H₂O (0.30 g/liter for each). To compare protease production, *B. bassiana* was grown by two methods. In the first method, submerged culturing, the fungus was grown in an Erlenmeyer flask on a rotary shaker (27°C, 180 rpm). In the second, it was grown in semiliquid culture (3) in a shallow Pyrex (Corning Glass Works, Corning, N.Y.) dish (25 by 44 by 6 cm) containing glass wool (approximately 1% wt/vol) which was impregnated with 1 liter of medium and incubated at 27°C without shaking.

**Isolation of culture filtrate.** After 4 days of growth in 0.5% gelatin medium, the semiliquid culture was filtered by suction through no. 2 filter paper (Whatman Ltd., Kent, England). The filtrate was passed through a 0.45-μm-pore-size filter (Millipore Corp.) and placed on ice.

**Purification of protease.** Cultures filtrates were submitted to (NH₄)₂SO₄ precipitation. The fraction from 60 to 75% saturation, which contained 47% of the total protease activity, was dialyzed (3,000-molecular-weight cutoff) for 24 h at 5°C against three changes of 2 liters of distilled water. The dialysate was lyophilized and stored at −20°C. Twenty milligrams of this material was applied to a Bio-Gel P-60, 60,000-molecular-weight exclusion column (Bio-Rad Laboratories, Richmond, Calif.). The elution buffer was 0.05 M Tris hydrochloride–0.1 M KCl (pH 7). Fractions showing proteolytic activity were dialyzed (12,000-molecular-weight cutoff) against 2 liters of 0.001 M Tris hydrochloride (pH 7) for 24 h at 5°C, lyophilized, and submitted to a second gel filtration with a Bio-Gel P-10, 20,000-molecular-weight exclusion column (Bio-Rad). The elution buffer for the Bio-Gel P-10 column was 0.01 M ammonium acetate (pH 7). Only one peak showing proteolytic activity was observed. All fractions showing proteolytic activity were pooled, and this purified protease was stored lyophilized at −20°C.

**Determination of proteolytic activity.** For a semiquantitative protease assay, gel filtration fractions were assayed with X-ray film (NS2T; Eastman Kodak Co., Rochester, N.Y.). A 100-μl portion of each fraction was diluted twofold 20 consecutive times with 0.2 M phosphate buffer (pH 7). A 15-μl drop of each dilution was placed on the X-ray film and incubated at 27°C in an airtight container with a moist paper towel to prevent evaporation of the samples. After 3 h, the X-ray film was washed under warm tap water. The proteo-
lytic titer from each fraction is defined as the highest dilution which dissolved the film at the location of drop placement under the conditions described above.

For the assay of general proteolytic activity, Azocoll (50/100 mesh; Calbiochem-Behring, La Jolla, Calif.) was used. Azocoll (10 mg) was suspended in 1.0 ml of 1 M glycine-NaOH buffer (pH 8.5)–3.9 ml of distilled water–0.1 ml of enzyme solution in a 25-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (180 rpm) at 37°C for 30 min. The flask contents were filtered through Whatman no. 2 filter paper and measured colorimetrically at 520 nm. One proteolytic unit (PU) is defined as the amount of enzyme which produced an A$_{290}$ of 0.1 under the conditions described above.

Trypsinlike amidase activity was measured with the chromogenic substrate N-$\alpha$-benzoyl-$\delta$-arginine-$p$-nitroanilide (BAPNA; Sigma Chemical Co., St. Louis, Mo.). BAPNA (43.5 mg) was dissolved in 1.0 ml of dimethyl sulfoxide and made to a final volume of 100 ml with 0.2 M glycine-NaOH buffer (pH 8.5). The buffered substrate (4.9 ml) was added to a 25-ml Erlenmeyer flask with 0.1 ml of enzyme solution. The flask was incubated on a rotary shaker (180 rpm) at 37°C for 1 h, and the A$_{290}$ was read. In this case, trypsinlike amidase activity was defined in BAPNA units as that amount of enzyme which produced an A$_{290}$ of 0.1 under the conditions described above.

Proteolytic activity against gelatin, casein, and bovine serum albumin was determined by suspending 20 mg of these proteins in 5 ml of 0.2 M phosphate buffer (pH 7) containing 1.5 $\mu$g of purified B. bassiana protease (PU equivalents, 15). The flask was incubated on a rotary shaker (180 rpm) at 37°C for 1 h. Substrate hydrolysis was measured as micromoles of free amino nitrogen released, as assayed with trinitrobenzene sulfonic acid by the method of Samples et al. (22). Glycine was used as a standard.

Hydrolysis of the insoluble proteins elastin and collagen was assayed by the method of Morihara and Tsuzuki (16). Twenty milligrams of powdered elastin (from bovine neck ligament; Sigma) or collagen (from bovine achilles tendon; Sigma) was placed in 5 ml of 0.05 M Tris buffer (pH 8.5) containing 15 PU of purified B. bassiana protease. The flask was incubated on a rotary shaker (180 rpm) at 37°C for 1 h. Hydrolysis of insoluble protein was measured as the amount of soluble protein found in the solution. Eight replicates were performed for each test with appropriate controls. The hydrolytic activities of B. bassiana protease and Streptomyces griseus protease (Sigma) on different substrates were compared. S. griseus protease was used at concentrations which resulted in the same PU equivalents on Azocoll as with B. bassiana protease.

Protein determination. Proteins were measured by the Bradford method (2), with BSA as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was carried out to assess the number of proteolytic enzymes in the culture filtrate. The gel was polymerized from a mixture of 17.5 ml of 30% acrylamide–0.8% N,N,N',N'-tetramethylethylene diamine–0.70 ml of ammonium persulfate (75 mg/ml). Electrophoresis was performed at 10°C with 0.05 M Tris-glycine buffer (pH 8.3). The protease was specifically located in the gel by application of X-ray film directly on the gel for 2 h at 37°C. Once washed, the film showed the presence of protease activity where the gelatin had been dissolved.

The protein profiles of enzyme purification steps were analyzed by sodium dodecyl sulfate (SDS)-PAGE with 12% gels and the buffer system described by Laemmli (12). Low-molecular-weight standards (14,400 to 96,000; Bio-Rad) were run in parallel. The gels were stained with 0.2% Coomassie blue R-250 in methanol-acetic acid-water (3:2:11; vol/vol) at 60°C for 45 min and destained in methanol-acetic acid-water (3:1:1; vol/vol). Gels were scanned at 540 nm with a scanning densitometer (E-C Apparatus Corp., St. Petersburg, Fla.).

Protease inhibitor studies. Purified protease was tested for inhibition by N-tosyl-$\phi$-phenylalanine chloromethyl ketone, phenylmethylsulfonyl fluoride (PMSF), N-$\alpha$-p-tosyl-$\phi$-lysine-chloromethyl ketone, EDTA, cysteine hydrochloride, iodoacetamide, and HgCl$_2$ (Sigma). The reagents were added to the Azocoll assay to yield 10 mM concentrations.

pH and temperature effects. Protease was suspended in Britton-Robinson universal buffer at pH values between 3 and 11, followed by a protease assay by the Azocoll method. Protease activity at temperatures between 5 and 60°C was assessed by the Azocoll method at pH 8.5.

Thermal stability. The reactant vessel was equilibrated to the desired temperature before the protease was added. The temperature stability of the pure protease was determined by holding the enzyme solutions at various temperatures for 0 to 30 min, followed by a protease assay by the Azocoll method. KCl, MgCl$_2$, CaCl$_2$, FeCl$_3$, EDTA, ethylene glycol-bis-$\beta$-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid, mercaptoethanol, dithiothreitol, and glycerol were tested as thermal inactivation protectants of the protease. The compounds were added to yield the concentrations indicated in Results, and the protease solution was kept at 40°C for 30 min. Remaining protease activity was assayed with Azocoll.

RESULTS

Protease production. When B. bassiana was grown in semiliquid culture, proteolytic activity appeared rapidly in the medium. Supernatants of such cultures showed proteolytic activity, with maxima of 7.9 and 5.4 PU at day 4 in 0.5 and 1.0% gelatin media, respectively. The maximum rate of protease production in these cultures was 4.1 and 3.1 PU/day. In contrast, submerged cultures grown in the same medium showed lower amounts of protease activity (Fig. 1). The semiliquid culture with 0.5% gelatin medium was used for production of B. bassiana protease. A zymogram was used to assess the number of extracellular proteases pro-
duced. It showed a single band of dissolved gelatin. *S. griseus* protease (Sigma preparation) showed three bands of dissolved gelatin.

**Protease purification.** Table 1 shows a summary of the purification steps. (NH₄)₂SO₄ precipitation of filtered culture supernatant showed that protease fractionated at 60 to 75% saturation. The (NH₄)₂SO₄ fractions were submitted to P-60 gel filtration, whereupon a single peak with proteolytic activity was observed (Fig. 2A). Here, as with (NH₄)₂SO₄ precipitation, several faint bands appeared on SDS-PAGE from the material under the proteolytic peak (Fig. 3A, lanes a and b). P-10 gel filtration of the protease-containing fractions from the P-60 gel filtration excluded the protease but retained the lower-molecular-weight proteins (Fig. 2B). The material under the proteolytic peak was obtained with a high purity since a single major band appeared after SDS-PAGE, which revealed a monomer with a molecular weight of 35,000 ± 2,000 (Fig. 3A, lane c). The protease was lyophilized and stored at −20°C, and no reduction in activity was observed after 25 days.

**Effects of pH and temperature on protease activity.** With Azocoll as the substrate, the activity of the protease was optimum at pH 8.5 (Fig. 4A); thus, it can be considered an alkaline protease. The optimum temperature of activity ranged between 37 and 42°C (Fig. 4B). The protease was stable at 27°C but was inactivated at higher temperatures (Fig. 5). Approximately 40% activity remained after 30 min at 40°C or after 5 min at 50°C. At 60°C, the protease was rapidly inactivated within 5 min. Several compounds were tested as protectants against thermal inactivation of the protease at 40°C. Dithiothreitol and, to a lesser extent, CaCl₂ showed some protection (Table 2).

**Protease inhibitors.** Various protease inhibitors were used to establish the identity of the protease (Table 3). Inhibition by PMSF indicated the presence of serine-catalyzed protease activity. No inhibition was observed with N-tosyl-L-phenylalanine chloromethyl ketone, thus suggesting that chymotrypsinlike activity was absent. HgCl₂ also decreased proteolytic activity, possibly acting as a denaturant. The alkyllating reagent N-α-p-tosyl-L-lysine-chloromethyl ketone, a specific inhibitor of trypsinlike proteolytic activity, showed no action on *B. bassiana* extracellular protease activity. Iodoacetamide showed no inhibition, suggesting the absence of a thiol protease. No inhibition was observed with cysteine hydrochloride or EDTA, suggesting that the protease is not a metalloprotease.

**Substrate hydrolysis.** *B. bassiana* protease hydrolyzed casein, gelatin, elastin, and, to a lesser extent, bovine serum albumin (Table 4). The ratio of *B. bassiana* protease activity to reference *S. griseus* protease activity was approximately 0.5 with casein or gelatin as a substrate. *B. bassiana* protease showed 18-fold higher elastolytic activity than *S. griseus* protease did. Addition of 0.04% SDS to the elastin reaction mixture increased *B. bassiana* protease activity 25-fold over that of the reference protease. Enzymatic activities were absent on collagen. No amide-hydrolyzing activity was observed with *B. bassiana* protease on BAPNA.

**DISCUSSION**

Extracellular protease of *B. bassiana* has been implicated as a component of the insect infection process (24). We have investigated the production, purification, and characterization of one extracellular *B. bassiana* protease.

Polyomorphous growth characteristics for *B. bassiana* in differing culture conditions have been previously observed (14, 23). We have shown the developmental cycle of *B. bassiana* in submerged culture (M. J. Bidochka, T. A. Pfeifer, and G. G. Khachatourians, Mycopathologia, in press). Differentiation in submerged culture is influenced by the choice of medium nutrients (28). In submerged conditions, *B. bassiana* produces blastospores, whereas on solid...
and semiliquid media it produces aerial mycelia and conidia. Our results indicate that extracellular protease production is increased when aerial mycelia arise, as opposed to submerged culture in which blastospores are produced.

Kucera and Samsinakova (11) reported high- and low-molecular-weight proteases produced by a strain of *B. bassiana*. These proteases were toxic when injected into insects. Moreover, it was shown that the ratio of the production of both formed proteases was influenced by the type of nitrogen source (10). More recently, evidence for extracellular protease came from the observation that fluids collected from *B. bassiana* grown on insect cuticle released amino acids from cuticular proteins (27). These descriptions of *B. bassiana* protease are difficult to assess since the purity and characterization of these proteases were not adequately reported.

With different nitrogen sources, several *B. bassiana* proteases may be produced (10). However, *B. bassiana* GK2016 produces a single protease when grown in medium containing gelatin as the sole carbon and nitrogen source. After purification procedures, this protease was attained with high purity as a single major protein band detected by SDS-PAGE (Fig. 3). Based on its inhibition by FMSF, *B. bassiana*

![Image](https://example.com/image1.png)

**FIG. 3.** SDS-PAGE (A) and densitometric scans of SDS-PAGE proteins (B) from each purification step of *B. bassiana* extracellular protease. Lanes: a, 60 to 75% (NH₄)₂SO₄ precipitate; b, P-60 Bio-Gel filtration (fractions 29 to 43); c, P-10 Bio-Gel filtration (fractions 8 to 15); d, low-molecular-weight standards whose sizes (in kilodaltons) are indicated at the left.

![Image](https://example.com/image2.png)

**FIG. 4.** Effect of pH (A) and temperature (B) on *B. bassiana* proteolytic activity. Initial protease concentrations yielded 7 PU by the Azocoll assay.

![Image](https://example.com/image3.png)

**FIG. 5.** Thermal inactivation of the purified *B. bassiana* extracellular protease. Protease solutions were kept at 27, 40, 50, or 60°C for various times and then assayed for remaining proteolytic activity with Azocoll. Initial protease concentrations yielded 7 PU.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>Maximum Activity (%) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>10</td>
<td>40.8 (±0.31)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>41.2 (±0.21)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10</td>
<td>45.1 (±0.61)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>39.9 (±0.52)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10</td>
<td>51.3 (±0.91)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>48.7 (±0.91)</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>10</td>
<td>43.0 (±0.09)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>42.1 (±0.30)</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>44.7 (±0.30)</td>
</tr>
<tr>
<td>EGTA</td>
<td>10</td>
<td>40.4 (±0.21)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>43.4 (±0.0)</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>10</td>
<td>44.7 (±0.30)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>43.0 (±0.09)</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>10</td>
<td>60.1 (±0.30)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>47.8 (±0.60)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10</td>
<td>39.5 (±0.30)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>40.4 (±0.21)</td>
</tr>
</tbody>
</table>

* EGTA, Ethylene glycol-bis(,B-aminoethyl ether)-N,N',N'-tetraacetic acid.
extracellular protease is classified as a serine protease (18). Persson et al. (20) have reported that intracellular protease prepared from mycelial extracts of B. bassiana is inhibited by PMSF (20). The serine proteases are widely distributed among fungi and are almost entirely extracellular, their molecular weights are commonly in the range of 18,500 to 35,000, and they are optimally active at alkaline pHs (18).

The extracellular protease isolated from B. bassiana had optimum activity at pH 8.5 and a molecular weight of approximately 35,000, falling in the range of other fungal serine proteases. Dithiothreitol, a protective agent of sulf-hydryl groups (25), had some stabilizing effect on the extracellular protease against heat denaturation conditions. However, since iodoacetamide did not inhibit protease activity, the sulf-hydryl groups are not located in the active site.

The protease showed no trypsinlike activity, on the basis of absence of inhibition by Nα-p-tosyl-L-lysine-chloromethyl ketone, and no amide-hydrolyzing ability on BAPNA. This is in contrast to proteases produced by the entomopathogenic fungi Lagenidium giganteum (4) and Metarthizium anisopliae (27), which showed trypsinlike activity. In this study, B. bassiana extracellular protease hydrolyzed casein and Azocoll, whereas intracellular proteases of B. bassiana had little or no activity on casein and Azocoll (20). B. bassiana extracellular protease showed elastolytic activity which was enhanced by low concentrations of SDS. Elastolytic activity of most microbial proteases is enhanced by an anionic detergent (8, 16).

Based on studies of substrate hydrolysis, extracellular proteases produced by entomopathogenic fungi may be classified as collagenases or elastases. Proteases produced by the mosquito-parasitizing fungus L. giganteum showed collagenase activity which was inhibited by EDTA (4). An extracellular protease produced by the aphid-parasitizing fungus Entomophthora coronata was strictly defined as a collagenase (9). An elastase isolated from M. anisopliae, an entomopathogen with a wide host range, had a pH optimum of 8 to 9 and a molecular weight of 25,000 (27). More generally, extracellular proteases produced by the fungi B. bassiana, Entomophthora coronata, M. anisopliae, Nomuraea rileyi, and Verticillium lecanii are involved in the partial hydrolysis of insect cuticle proteins (7, 15, 24, 27). Resilin, an elastic tissue unique to invertebrates, and collagen are proteins which occur in insect cuticle (1, 29). The insect cuticle is composed of 55 to 80% protein (17), and its hydrolysis by proteases may be important for fungal penetration. Pretreatment of insect cuticle with protease considerably enhances chitin hydrolysis by chitinase, indicating that cuticular chitin is sheathed by protein (26, 27). However, M. anisopliae mutants having altered protease production had no alteration in virulence against mosquito larvae (21). The degree to which proteolytic activity contributes to B. bassiana growth and insect penetration has yet to be established. Such an investigation is currently in progress.

ACKNOWLEDGMENTS

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LITERATURE CITED


### Table 3. Effects of various inhibitors on B. bassiana extracellular protease activity

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>% (± SD)</th>
<th>Maximum activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>5.7 (±0.24)</td>
<td>3.95</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>67.7 (±0.52)</td>
<td>91.6 (±0.48)</td>
</tr>
<tr>
<td>TLCK</td>
<td>100.0 (±0)</td>
<td>100.0 (±0)</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>99.0 (±0.05)</td>
<td>100.0 (±0)</td>
</tr>
<tr>
<td>EDTA</td>
<td>100.0 (±0)</td>
<td>100.0 (±0)</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>100.0 (±0)</td>
<td>100.0 (±0)</td>
</tr>
</tbody>
</table>

* All were at 10 mM. TLCK, Nα-p-tosyl-L-lysine-chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

### Table 4. Comparison of substrate hydrolysis by B. bassiana extracellular protease and S. griseus protease

<table>
<thead>
<tr>
<th>Substrate</th>
<th>B. bassiana protease</th>
<th>S. griseus protease</th>
<th>B. bassiana/S. griseus protease ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azocoll</td>
<td>15.00</td>
<td>15.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Casein</td>
<td>9.33</td>
<td>21.18</td>
<td>0.44</td>
</tr>
<tr>
<td>Gelatin</td>
<td>7.49</td>
<td>15.26</td>
<td>0.49</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>3.41</td>
<td>3.41</td>
<td>1.00</td>
</tr>
<tr>
<td>Elastin</td>
<td>184.47</td>
<td>10.01</td>
<td>18.43</td>
</tr>
<tr>
<td>Collagen</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BAPNA</td>
<td>0</td>
<td>52.00</td>
<td>0</td>
</tr>
</tbody>
</table>

* Refer to Materials and Methods for measurements of substrate hydrolysis.
* B. bassiana and S. griseus protease activities were standardized on Azocoll.
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