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Full Length Research Paper

Optimization of cellulase and -glucosidase induction by sugarbeet pathogen *Sclerotium rolfsii*

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The ability to produce cellulose degrading enzymes by sugarbeet pathogen *Sclerotium rolfsii* Sacc. in liquid synthetic media with carboxymethy cellulose (CMC) as inducer was studied. Several cultural conditions were examined to assess their effect in optimizing enzymes production. Shaking cultures gave higher yields of cellulases compared with static ones. Aspargine supplement was the best nitrogen source, especially at 3.0 g/l concentration, in promoting enzyme production. Variation of cellulose/xylan ratio in the culture medium showed that cellulose and xylan induced both cellulases synthesis but cellulose being the most effective specific substrate. The influence of different inhibitors on enzymes production by *S. rolfsii* was also studied. Cyclohexmide and ethidium bromide inhibited protein synthesis by *S. rolfsii*. Moreover, glucose repressed cellulase synthesis in *S. rolfsii*.

Key words: Cellulase, -glucosidase, inhibitors, *Sclerotium rolfsii*.

INTRODUCTION

The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1,4)- -D -glucanase (synonyms: endoglucanase, endocellulase, carboxymethyl cellulase [EC 3.2.1.4]), which cleaves -linkages at random, commonly in the amorphous parts of cellulose; exo-(1,4)- -D- glucanase (synonyms: cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91], which releases cellobiose from either the non-reducing or the reducing end, generally from the crystalline parts of cellulose; and -glucosidase (synonym: cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cellooligosaccharides (Bhat and Bhat, 1997).

Sclerotium rolfsii is a plant pathogen that has been isolated from a wide variety of host species, primarily annuals and herbaceous perennials, but saplings of some woody plants may also be attacked. *S. rolfsii* survives on dead plant material in the soil as sclerotia, which later germinate and attack host plants, causing necrosis by attacking cell walls (Ludwig and Haltrich, 2002; Sachsleh-ner et al., 1997).

In a number of fungi, these various endoglycanases can be quite specifically induced. During the growth of *Trichoderma reesei* and *T. harzianum* on xylan-based media, xylanase activities with low levels of endoglucanase are formed. Growth on cellulose or on heterogeneous native substrates containing both xylan and cellulose results in the production of both endo-glucanase and xylanase. This unspecific effect of cellulose could be explained by xylan impurities found in commercially available cellulose preparations (Hrmovà et al., 1986; Senior et al., 1989).

Cellulases have a wide range of applications. Potential applications are in food, animal feed, textile, fuel, chemical industries, paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Beguin and Anbert, 1993; Coughlan, 1985; Mandels, 1985).

The objective of this investigation is to study the production of cellulases by *S. rolfsii* Sac. isolated from diseased sugarbeet roots (El-Abyad et al., 1988) as well as the influence of different cultural conditions on enzyme production by this species in the laboratory. This organism is known as an excellent producer of cellulolytic enzy-

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mes (El-Abyad et al., 1996, 1997; Kurosawa et al., 1989; Lachke and Deshpande, 1988; Moussa, 1994).

MATERIALS AND METHODS

Micro-organism and culture conditions

S. rolfsii Sacc. was isolated from diseased sugarbeet roots (El-Abyad et al., 1988) and maintained on a medium described by Johnson and Curl (1972) and composed of (g/l): dextrose, 30; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; KNO_3, 2; agar, 20; and 1 ml/l of each of stock solutions (1 g/l) of $FeSO_4.7H_2O$, $MnSO_4.7H_2O$, $ZnSO_4.7H_2O$ and thiamine.

Effect of the state of culture on enzymes production

S. rolfsii was cultivated in 250 ml Erlenmeyer flasks with 100 ml medium described by Haltrich et al. 1994 and containing the following (g/l): peptone, 80; NH_4NO_3, 2.5; $MgSO_4.7H_2O$, 1.5; KH_2PO_4, 1.2; KCl, 0.6 and trace element solution at 0.3 ml/l. The concentration of carboxymethyl cellulose (CMC) was 200 mg/l for production of cellulases. The flasks were inoculated with 5 mm plug cut out from the margin of a 5 day-old culture. Incubation was carried out at 25 ± 2˚C under static and shaking at 100 rpm for 11 days. The culture filtrates were dialyzed against distilled water over night at 4°C and freeze-dried. The concentrated filtrates were used as enzyme source.

Effect of different nitrogen sources and its concentrations on enzymes induction

Cultivation of *S. rolfsii* was performed on a medium of Haltrich et al. (1994) described above but modified as follows. The NH_4NO_3 was replaced by different nitrogen sources at equimolar nitrogen of KNO_3, $(NH_4)_2SO_4$, $NaNO_3$ or DL-asparagine. The flasks were inoculated with 5 mm plug cut out from the margin of a 5 day -old culture. Incubation was carried out at 25 ± 2˚C under shaking at 100 rpm for 11 days. The culture filtrates were dialyzed, freeze-dried and used as enzyme source.

From the previous experiment the best nitrogen source was DL-asparagine and used in different concentrations based on equimolar nitrogen of NH_4NO_3 (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 g/l) were studied.

Effect of different ratios of cellulose and xylan on enzymes induction

Cultivation of *S. rolfsii* was performed on medium described previously. Carbon sources (carboxymethyl cellulose and Brich wood xylan, Sigma, USA) were added at the ratios as mg/100 ml [C5 (cellulose, 20), C4X1 (cellulose, 16; xylan, 4), C3X2 (cellulose, 12; xylan, 8), CX (cellulose, 10; xylan, 10), C2X3 (cellulose, 8; xylan, 12), C3X4 (cellulose, 12; xylan, 16) and X5 (xylan, 20)]. The flasks were inoculated with 5 mm plug cut out from the margin of a 5 day-old culture. Incubation was carried out at 25 ± 2˚C under shaking at 100 rpm for 11 days. The culture filtrates were desalted, concentrated and used as enzyme source.

Induction and repression of enzymes

For induction and repression of cellulases, *S. rolfsii* was grown on standard medium using glucose (10 g/l) for four days. The mycelium was harvested and rinsed with sterile distilled water, and then transferred to basal medium without nitrogen plus different compounds: CMC, 120 mg/l + 10 g/l glucose; CMC, 120 mg/l + 0.2 mg/l cycloheximide, CMC, 120 mg/l + 0.2 mg/l ethidium bromide; control (glucose 10 g/l). The culture solids (mycelium and undegraded cellulose) were separated from the culture fluids by filtration and then centrifugation at 7000 rpm for 20 min. The culture filtrates were dialyzed, freeze-dried and used as enzyme source.

Enzyme assay

Cellulase activity was determined at 40˚C by using carboxymethyl cellulose (sodium salt, Sigma, USA) as a substrate, in 50 mM acetate buffer, pH 4.5. Reducing sugars released were assayed by the Somogyi method (Somogyi, 1952) modified from Nelson procedure (Nelson, 1944) with glucose as standard. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol/min of reducing sugar expressed as glucose equivalents.

-glucosidase was assayed at 40˚C using *p*-nitrophenyl- -D-glucopyranoside as a substrate, in 50 mM acetate buffer, pH 4.5. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of *p*-nitrophenol /min.

Protein estimation

The protein was measured in the culture supernatant, and estimated by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Statistics

All the results are average of at least three replicates. The data were analyzed by the One-Way ANOVA followed by Tukey-Kramer's multiple comparison tests (p 0.05) (SPSS, 1999).

RESULTS

The results in Figure 1 showed that the influence of culture state on cellulose degrading enzymes production by *S. rolfsii*. Static cultures (Figure 1A) gave lower levels of enzyme as compared with shaken ones. In agitated cultures (Figure 1B), maximal activity for cellulase was at ninth day and tenth day for -glucosidase of growth period. The rate of cellulase production was five times in shaking cultures than in static ones, while, - glucosidase was seven times in shaking cultures than in static ones (Figures 1A and 1B).

In Figure 2A and B shows the effect of the nitrogen source on cellulose degrading enzymes produced by the fungus *S. rolfsii*. *S. rolfsii* cultivated on asparagine promoted the highest yields of cellulose degrading enzymes followed by potassium nitrate, ammonium nitrate, sodium nitrate. Negligible amount of cellulase was observed when cultivated on ammonium sulphate as sole nitrogen source.

The different concentrations of asparagine produced different amounts of cellulose degrading enzymes. The amount of enzymes correlated with asparagine concentration till 3.0 g/l and then decreased with subsequent increase in asparagine concentrations (Figure 2C).

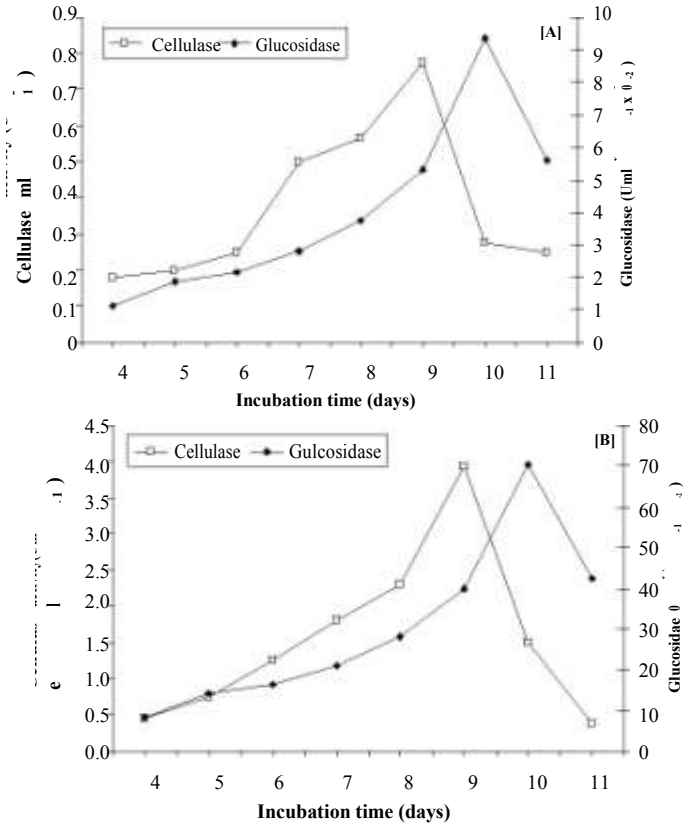

Figure 1. Effect of culture state on cellulose degrading enzymes produced by sugar beet pathogen *S. rolfsii*, [A] static culture and [B] shaking culture.

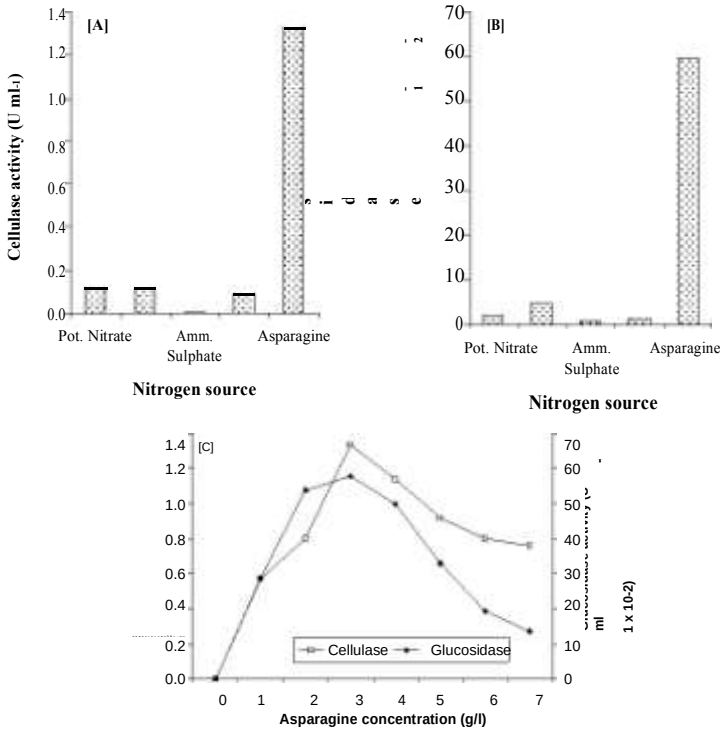

Figure 2. Effect of different nitrogen sources and its concentrations on the production of cellulose degrading enzymes by *S. rolfsii.* [A and B] different nitrogen sources, [C] asparagine concentrations.

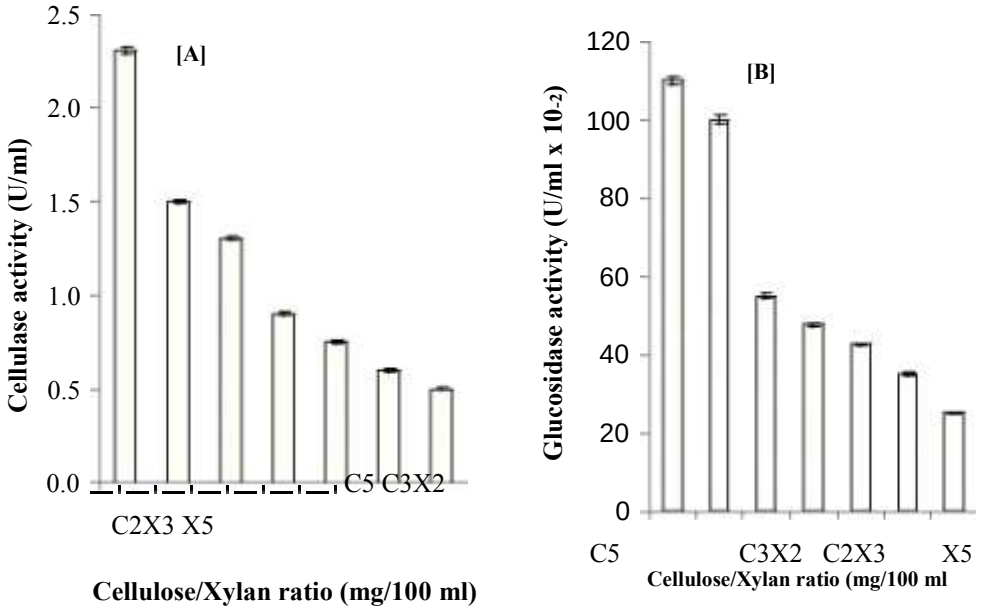

Figure 3. Effect of cellulose/xylan ratio (mg/100 ml) on the induction of cellulose degrading enzymes by *S. rolfsii.* [A] cellulase, [B] -glucosidase.

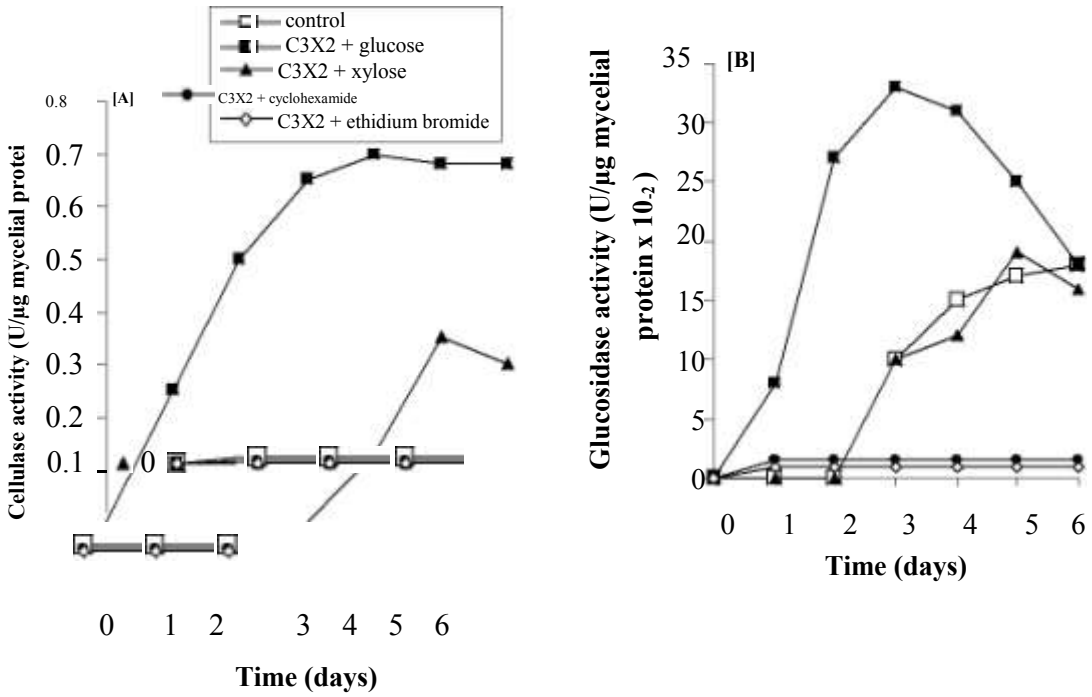

Figure 4. Induction and repression of cellulose degrading enzymes synthesis in *S. rolfsii.* [A] cellulase, [B] -glucosidase.

The data represented in Figure 3 showed that, by varying the relative concentration of cellulose and xylan in the culture medium, both were able to induce and synthesis of cellulose degrading enzymes. The maximum production of cellulose degrading enzymes was when cultivated *S. rolfsii* on pure cellulose and decreased with decreased cellulose concentration (Figures 3A and B).

The inducible synthesis of the cellulase system of *S. rolfsii* was determined by adding cyclohexamide or ethidium bromide to induction media to inhibit protein synthesis, although there was no growth there were very low level of enzymes produced (Figure 4). It may be concluded that in *S. rolfsii* cellulase syntheses are repressed by easily metabolized sugars such as glucose. It is widely accepted for filamentous fungi that cellulase and -glucosidase production are regulated by induction and repression (Figures 4A and B).

DISCUSSION

Since the polymeric substrates are unable to enter the cells by crossing the plasma membrane, the cells receive the signal for an accelerated synthesis of secreted glycanases by means of low-molecular weight fragments, usually disaccharides, derived from the polysaccharides. The fragments are formed by the action of small amounts of the enzymes produced constitutively (Biely, 1993; Bajpai, 1997). Thus, for example, cellobiose is an inducer of cellulose-degrading enzymes (Canevascini et al., 1979; Eberhart et al., 1977; Eriksson and Hamp, 1978; Mandels and Reese, 1957), and xylobiose is an inducer of xylan-degrading enzymes (Biely et al., 1980; Nakanishi et al., 1976).

Purkarthofer et al. (1993) stated that a shaking speed of 120 rpm provided the optimal conditions for enzyme formation. At a decreased shaking speed of 100 rpm, the fungus showed poor growth, and enzyme production was reduced dramatically, at higher shaking speeds of 150 - 250 rpm enzyme production was adversely affected. The lower xylanase activity produced at the slower shaking speed was ascribed to poor oxygen transfer within the medium, whereas the lower xylanase production at higher shaking speeds was thought to be due to greater hyphal branching, mycelial fragmentation and early sporulation (Purkarthofer et al., 1993). Shear stress within the medium, which is directly related to the stirrer speed, has a marked influence on xylanase production by *Thermomyces lanuginosus* SSBP (Reddy et al., 2002; Singh et al., 2000).

The nitrogen source used in the production medium is one of the major factors affecting enzyme production and level. In a study carried out with *Trichoderma harzianum*, $NaNO_3$ and peptone were the best nitrogen sources in production medium (Abdel-Satar and El-Said, 2001), whilst NH_4NO_3 was used in a study with *Schizophyllum commune*, and $(NH_4)_2HPO_4$ was found suitable in another study with *T. lanuginosus* RT9 (Haltrich et al., 1993; Hoq et al., 1994). The effect of various organic nitrogen com-pounds on the production of xylanase by *T. lanuginosus* strains showed that all sources promoted growth of the fungus, but yeast extract had the most pronounced effect (Singh et al., 2003).

The production of both cellulases in media with xylan or cellulose as sole carbon source may be due to substrates contamination or substrate cross-specificity that can ran-ge from absolute for one polymer to about the same affi-nity for both of them (Ferreria-Filho, 1994; Hrmovà et al., 1986; Senior et al., 1989). Nevertheless, concurrent for-mation of cellulase and xylanase has been observed in several fungi using natural and synthetic substrates (Hrmovà et al., 1991; Royer and Nakas, 1990; Sachslehner et al., 1998). There are suggestions of an interaction between xylanase and cellulase induction (Royer and Nakas, 1990), although the xylanolytic and cellulolytic systems in some filamentous fungi are likely to be under separate regulatory control (Bajpai, 1997; Hrmovà et al., 1991; Kulkarni et al., 1999). In *S. rolfsii* there was a high cross induction of cellulolytic and xylanolytic enzymes, in *Aspergillus terreus* it was mainly induced by the respective synthetic dimmers (Hrmovà et al., 1991). This un-specific effect of cellulose could be attributed to xylan impurities found in commercially available cellulose pre-parations (Hrmovà et al., 1986; Senior et al., 1989).

Xylanases are generally produced together with cellula-ses during growth of the fungus on macromolecular subs-trate derived from plant polysaccharides, which inevitably always contain cellulose and xylan. The resulting xylana-se to cellulase ratio has been shown to be directly pro-portional to the xylan/cellulose ratio in the growth subs-trate (Senior et al., 1989). These data seem consistent with results from induction studies, which showed that xylanase and cellulase biosynthesis in *Trichoderma ree-sei* is differentially regulated (Hrmovà et al., 1986). In contrast, the efficient xylanase induction in *T. longibrachiatum* required the simultaneous presence of xylo- as well as cello-oligosaccharides (Royer and Nakas, 1990).

A generally accepted view on the regulation of synthe-sis of enzymes degrading polymeric substrates is that low constitutive levels of polysaccharide hydrolases interact with the polymer and produce small soluble ‘signal’ frag-ments, which enter the cell and induce the synthesis of the corresponding enzyme, thus permitting utilization of polysaccharide.

Studies using inhibitors of protein synthesis have sug-gested that cellulase formation is regulated at the transla-tional level (Nisizawa et al., 1972) . Evidence based on the measurement of mRNA levels documented that the formation of cellulase occurs at the pre-translational level (Kolbe and Kubicek, 1990;

Messner et al., 1991) and the cellulase gene transcription occurs within 20 min, after the addition of inducer (El-Gogary et al., 1989).

The active growth of the fungus is crucial in cellulolysis. When growth was inhibited, cellulolysis remained weak, although cellulase enzymes were present in culture broth. Vaheri, (1983) proposed the participation of an oxidative reaction which is believed to disrupt the hydrogen bonds in crystalline cellulose, rendering it susceptible to attack by endoglucanase. He found that this activity was associated with cell wall in young cells of *T. reesei* in both induced and non-induced conditions. Thus, activities associated with growing cells appear to play a crucial role in the degradation of crystalline cellulose.

Carbon catabolite repression is another regulatory mechanism known to control cellulase production in bacteria and fungi. In this case, the end product of cellulose hydrolysis interacts with a cellular protein and form a complex which interacts with a particular gene at the transcription level and represses cellulase synthesis (Lewin and Genes, 1987). The carbon catabolite repres-sion was reported in *Escherichia coli* (Pastan and Adhya, 1976), *Saccharomyces cerevisiae* (Entian et al., 1985) and *Clostridium thermocellum* (Johnson et al., 1985). However, Canevascini et al. (1979) reported that the cellulase synthesis is regulated by both induction and catabolite repression in *Sporotrichum thermophile.*

The proof for carbon catabolite repression is based on the fact that no cellulase is formed during the growth of a microorganism on glucose, glycerol and other carbon sources related to glycolytic metabolism. Because there is no clear evidence that either glucose or a catabolite in fact controls the transcription of cellulase genes, Kubicek recommended not to use the term "catabolite repression" (Canevascini et al., 1979). The involvement of end product inhibition during crystalline cellulose hydrolysis by a rumen fungus *Neocallimastix frontalis* RK21 was demonstrated (Kubicek, 1992).

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