Lipase-Catalyzed Modification of Konjac Glucomannan

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ABSTRACT: Regioselective acylation of konjac glucomannan (KGM) with vinyl acetate in a solvent-free system was successfully performed using Novozym 435 as a biocatalyst. The degree of substitution (DS) of the modified KGM was used to evaluate the extent of acylation. The influence of various factors, such as water activity (a_w), reaction temperature, shaking rate, enzyme dosage, and the molecular weight of KGM, on the reaction was examined. The water activity of the reaction system played a key role in the acylation of KGM. The optimum water activity, reaction temperature, shaking rate, and enzyme dosage were 0.84, 50°C, 200 rpm and 400 U/mL, respectively. It has also been found that the DS of modified KGM sample decreases with increase in KGM molecular weight. Additionally, the acylation of KGM was shown to be regioselective, with acylation occurring at the C6—OH. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 1335–1340, 2006

Key words: konjac glucomannan; lipase; acylation; water activity

INTRODUCTION

Konjac glucomannan (KGM) is an abundant, naturally occurring polysaccharide isolated from the tubers of *Amorphophallus konjac* plant (Scheme 1). It consists of β-1,4-linked D-glucose and D-mannose units, and the molar ratio of glucose to mannose has been reported to be around 1 to 1.60.[1–3] There may be certain short side branches at the C-3 position of the mannose units and acetyl groups randomly present at the C-6 position of a sugar units.[1,3] The frequency of the acetyl groups ranges from 1/6 sugar units to 1/20 sugar units.[1,4] Furthermore, there are differences in molecular structure of KGM from different species.[2,5] KGM possesses excellent biodegradability, biocompatibility, and many unique pharmacological functions. Various modified forms of KGM have been shown to have promising properties as a novel medicine,[6] an environmentally benign emulsifier,[7] and drug carriers.[8] For example, acetylated KGM is a potential drug candidate for the reduction of blood cholesterol levels and hence used for the prevention of coronary heart disease.[4] KGM can participate in chemical reactions to form their derivatives because of the presence of the hydroxyl groups on the sugar units. However KGM is usually difficult to modify regioselectively via conventional reactions. Typically, such selective reactions require protecting and de-protecting steps that are time-consuming and expensive. Moreover, the catalysts and reagents used for the modification of KGM may cause safety concerns for its eventual use in cosmetic, food, and pharmaceutical industries. An alternative route is a biocatalytic process, which is not only facile and green, but could lead to selective modification of carbohydrates. Indeed, there are a few examples where enzymatic catalysis has been successful in creating valuable modified forms of other polysaccharides.[9–12]

It is well known that enzymatic ester synthesis is thermodynamically unfavorable in conventional aqueous media.[13] Methods have been developed to facilitate the formation of ester bonds over their hydrolysis. One approach is to replace water with an organic solvent to favor synthesis under restricted water conditions.[13] Another is using an activated ester (usually a vinyl ester) as an acyl donor. Vinyl esters are known for their high reactivity in enzyme-catalyzed esterification or transesterification reactions, and the vinyl alcohol formed during the reaction tautomerizes to acetaldehyde, thereby making the reaction irreversible (Scheme 2).

Lipases have been successfully used for a wide range of stereoselective and regioselective acylations of various target molecules in nonaqueous media. Up to now, fewer examples of the use of enzyme technology on modification of polymers have been investi-

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gated relative to its traditional synthetic applications involving small molecules and nothing has been known about enzymatic acylation of KGM. In this paper, we describe the effort to carry out regioselective acylation of KGM via immobilized lipase Novozym 435 in a solvent-free system. As less organic solvent is needed, the process will be more environmental-friendly and cost efficient. Moreover, product contamination is avoided, which could be a problem especially in the food and drug industries.

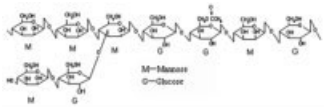
EXPERIMENTAL

Materials

KGM (M_w = 114,000, M_w/M_n = 3.2) was prepared by enzymatic degradation of native KGM powder (M_w = 980,000, M_w/M_n = 1.7; a donation of Multi-Ring Health Products, China).[14] Before use as the substrate for biotransformations, KGM was milled into a fine powder and passed through a 120-mesh sieve. Novozym 435 (Lipase B from *Candida antarctica* immobilized on macroporous acrylic resin; 10,000 U/g) was purchased from Novo Nordisk, Denmark. Vinyl acetate of analytical grade was purchased from Shanghai Chemical, China. All other chemicals used in this work were also from commercial sources and of analytical grade.

Instrumentations

The molecular weight and molecular weight distributions M_w/M_n of KGM samples were measured at 40°C by GPC using a commercial GPC system (Waters 515, Waters Co.) equipped with a column (Ultrahydrogel 500, Waters Corp., Milford, MA), a pump (Waters 515 HPLC pump) and a Waters 2410 refractive index detector. Dextran was used as standard for calibration. The mobile phase was pure water at a flow rate of 0.6 mL/min. SEM images were obtained with a Quanta 400 scanning electron microscope (FEI Company, The Netherlands) at an accelerating voltage of 20 kV. The ^{13}C-NMR spectra were recorded at room temperature in DMSO-d_6 by using TMS as the internal reference on a DRX-400 NMR spectrometer (Brucker, Germany) at 400 MHz. FTIR spectra were recorded using a Vector 33 spectrometer (Brucker Company, Germany) The potassium bromide (KBr) disks were prepared from powdered samples mixed with dry KBr in the ratio of 1 : 100. The spectra were recorded in a transmittance mode from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}.



Scheme 1 Molecular structure of KGM.

Scheme 2 Enzyme-catalyzed acylation of KGM with vinyl acetate.

Water activity (a_w) control

The vinyl acetate was equilibrated to fixed water activities over saturated salt solutions in a closed container at 25°C. The following salts were used: LiCl (a_w = 0.11), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.54), NaCl (a_w = 0.75), KCl (a_w = 0.84), and K_2SO_4 (a_w = 0.97).[15] The equilibration was monitored by water analysis using Karl Fischer titration until constant water contents were observed. The enzyme and KGM were equilibrated in separate vessels and equilibration was monitored by weighing.

General procedure for enzymatic acylation of KGM

Vinyl acetate is used as the acyl donor to acylate KGM in this work and the vinyl alcohol tautomerizes to acetaldehyde, thereby making the reaction irreversible (Scheme 2). A typical enzymatic acylation was carried out in 6 mL of vinyl acetate (63.9 m*M*) containing 0.5 g of KGM (3.1 m*M* of sugar units) with specified a_w value by the addition of 3000 U Novozym 435 in an air-bath shaker under predetermined reaction conditions. The reaction was terminated by filtering off the enzyme and the vinyl acetate was evaporated under reduced pressure. The remaining solid (namely the modified KGM) was washed thoroughly with ethanol and then dried under vacuum. The nonenzymatic acylation of the KGM was studied by control tests, and no acylation products were detected.

Determination of degree of substitution (*ds*)

The degree of substitution (DS) of modified KGM products was determined by the published titration method,[16] with some modifications. Briefly, the dried sample of powered KGM acetate (0.15 g) was placed in a 100 mL Erlenmeyer flask with a stopper and 75% ethanol (10 mL) were added. The mixture was stirred at 50°C for 30 min, and cooled to room temperature, 0.5*N* KOH (5 mL) was added with swirling. The flask was then kept at 30°C for 72 h with stirring. The excess of alkali was back titrated with 0.1*N* hydrochloric acid using phenolphthalein as an indicator. A blank (to which no KGM acetate sample had been added) was titrated in parallel. Equation (1) can be used to calculate the content of acetyl groups in the sample.

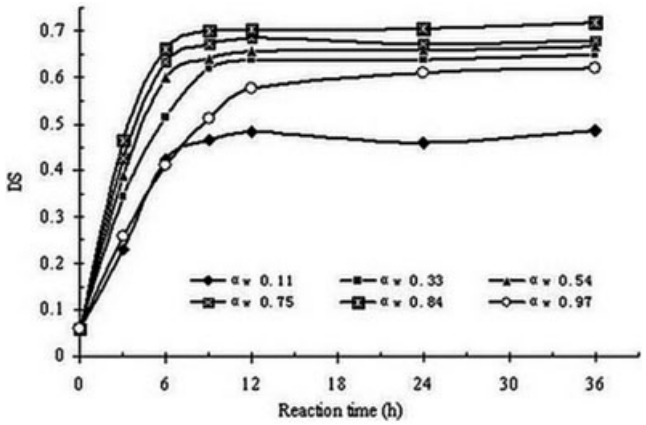


Figure 1 Time course of KGM acylation catalyzed by Novozym 435 at different a_w values. The reactions were carried out at 50°C, 200 rpm and different a_w values by adding 0.5 g KGM (M_w: 114,000) and 3000 U Novozym 435 into 6 mL vinyl acetate.

$$\%\ \text{acetyl (w/w)} = \frac{(V_a - V_b) \times N_{\text{HCl}} \times M_{\text{acetyl}}}{m_s} \times 100 \quad (1)$$

where V_a is the volume of hydrochloric acid consumed for the blank in liters, V_b represents the volume of hydrochloric acid consumed for the sample in liters, N_{HCl} stands for the normality of the hydrochloric acid, M_{acetyl} = 43 g/mol, m_s is the mass of the sample in grams. The equation for calculating the DS is as follows:

$$\text{DS} = \frac{M_{\text{anhydrosugar unit}} \times \%\ \text{acetyl}}{(100 \times M_{\text{acetyl}}) - (M_{\text{acetyl}} - 1) \times \%\ \text{acetyl}}$$

$M_{\text{anhydrosugar unit}}$ and M_{acetyl} refer to the molecular weight of anhydrosugar uint and acetyl, namely, 162 and 43, respectively. So, this equation could be changed as follows:

$$\text{DS} = \frac{162 \times \%\ \text{acetyl}}{4300 - 42 \times \%\ \text{acetyl}} \quad (2)$$

RESULTS AND DISCUSSION

Effect of water activity (a_w) on the acylation

Enzyme-catalyzed reactions in nonaqueous media are affected by the nature of the solvent as well as its water content. The water content of the reaction media plays an important role in enzyme catalysis, because it influences the flexibility of the protein, which is responsible for its activity and selectivity.[13] It is therefore particularly important to pay attention to water control in the case of lipase-mediated KGM acylation. The degree of substitution (DS) of the modified KGM was used to evaluate the extent of acylation.

As shown in Figure 1, lipase-catalyzed KGM acetylation showed a clear dependence on a_w. The DS of acylated KGM sample increased with increasing a_w of the reaction system up to 0.84. Further rise in a_w of the reaction system, however, resulted in a sharp drop in DS of products. The presence of water in the reaction system satisfied the requirement of the enzyme for holding essential water layer to perform its catalytic functions properly.[13] The enzyme was uncompletely hydrated and thus showed lower acylation activity when a_w was below the optimum. On the other hand, a_w above the optimum allowed the enzyme completely hydrated, but the competitive hydrolysis of the products took place and hence limited the acylation. The optimal water activity (a_w = 0.84) represented the most appropriate water condition for the balance between the above-mentioned conflicts.

Effect of reaction temperature on the acylation

Reaction temperature has significant influence on the activity, selectivity, and stability of a biocatalyst and the reaction equilibrium as well. As can be seen in Table I, the DS of acylated KGM increased from 0.34 to 0.66 as the reaction temperature increases from 30 to 50°C. However, further increase in temperature resulted in decrease in the extent of acylation. The DS of acylated KGM dropped when the temperature was above 50°C, suggesting the inactivation of the enzyme at elevated temperatures. Thus, the optimal reaction temperature for this reaction was 50°C.

Effect of shaking rate

Shaking rate influences the diffusion and partition of the substrate and the product in the reaction system and thus the rate and yield of a reaction. It is of particular importance to examine shaking rate effect

TABLE I
Effect of Reaction Temperature on Degree of Substitution (DS)[a]

Reaction temperature (°C)	DS
30	0.34
40	0.48
45	0.52
50	0.66
55	0.61
60	0.58
70	0.46

[a]The reactions were conducted at 200 rpm, a_w = 0.84 and different temperatures (30, 40, 45, 50, 55, 60, and 70°C) for 6 h by adding 0.5 g KGM (M_w: 114,000) and 3000 U Novozyme 435 into 6 mL vinyl acetate.

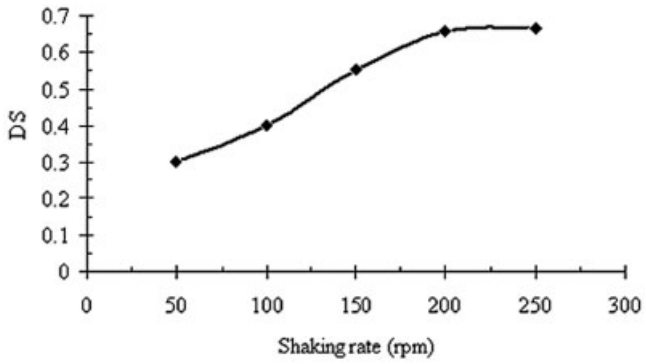


Figure 2 Effect of shaking rate on degree of substitution. The reactions were carried out at 50°C, a_w = 0.84 and various shaking rates from 50 to 250 rpm (in 50 rpm intervals) for 6 h by adding 0.5 g KGM (M_w: 114,000) and 3000 U Novozym 435 into 6 mL vinyl acetate.

on this reaction as the substrate KGM and the product are both of high molecular weight and the reaction medium is quite viscous, which limits the diffusion of substrate and product to and from the active site of the enzyme. Figure 2 shows that the DS of acylated KGM increased rapidly with the increase in shaking rate when it was below 200 rpm, indicating that mass transfer was the rate-limiting step. The optimum shaking rate was thought to be 200 rpm, above which no clear increase in DS of the products was observed with further rise in shaking rate.

Effect of enzyme dosage

As predicted, DS of acylated KGM increased steadily with increasing enzyme dosage up to 400 U/mL and little changes in DS of acylated KGM were observed with further rise in enzyme dosage (Fig. 3). Mass transfer limitation and observable aggregation of enzyme particles may account for this.

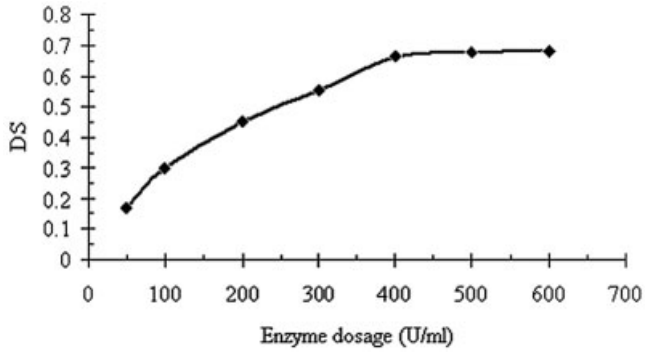


Figure 3 Effect of enzyme dosage on degree of substitution. The reactions were performed at 50°C, 200 rpm, and a_w = 0.84 for 6 h by adding 0.5 g KGM (M_w: 114,000) and various amounts of enzyme into 6 mL vinyl acetate.

TABLE II
Effects of Molecular Weight on DS of Product and Solubility of KGM in Vinyl Acetate

Molecular weight of KGM	DS[a]	Solubility (mg/mL × 10^{-2})[b]
78,000	0.71	42.8
114,000	0.67	31.2
208,000	0.29	12.2
270,000	0.23	7.6
980,000	0.16	1.7

[a]The reactions were carried out at 50 °C, a_w = 0.84, and 200 rpm for 12 h by adding 0.5 g KGM and 2400 U Novozym 435 into 6 mL vinyl acetate.

[b]The solubility of KGM in vinyl acetate was measured at 25°C, using a spectrophotometric assay according to Dubois et al.[17]

Effect of molecular weight of KGM on the acylation

To gain an insight into the effect of molecular weight of substrate on enzymatic conversions of macromolecules, KGM samples with various molecular weights, which were prepared via enzymatic degradation of native KGM as described in our previous work,[14] were used as substrates for lipase-catalyzed acylation of KGM with vinyl acetate under the same reaction conditions. As can be seen in Table II, the higher the molecular weight of KGM, the lower the DS of acylated KGM. The better solubility of the KGM with low molecular weight in vinyl acetate might be one possible reason for this. Consequently, the solubility of KGM samples with various molecular weights in vinyl acetate was assayed at a_w = 0.84 and 25°C, using a spectrophotometric assay according to Dubois et al.[17] As expected, KGM with high molecular weight is much less soluble than that with low molecular weight (Table II). Moreover, it can be clearly seen from the SEM images of KGM with various molecular weights that the lower the molecular weight of KGM, the less compact and more porous are the particles (Fig. 4). As a result, KGM with the lowest molecular weight was the best substrate for enzymatic acylation owing to its largest accessible surface. Besides, the lower viscosity of the reaction system, higher molecular flexibility, and more effective mass transfer with the case of KGM of lower molecular weight might all be responsible for the higher DS with enzymatic acylation of KGM of lower molecular weight.

Analysis of FTIR spectra

FTIR spectra of KGM (M_w: 114,000) and its acetylated product were shown in Figure 5. It is evident that the hydroxyl stretching band around 3400 cm^{-1} became smaller owing to substitution. At the same time, the carbonyl stretching band at 1752 cm^{-1} resulting from

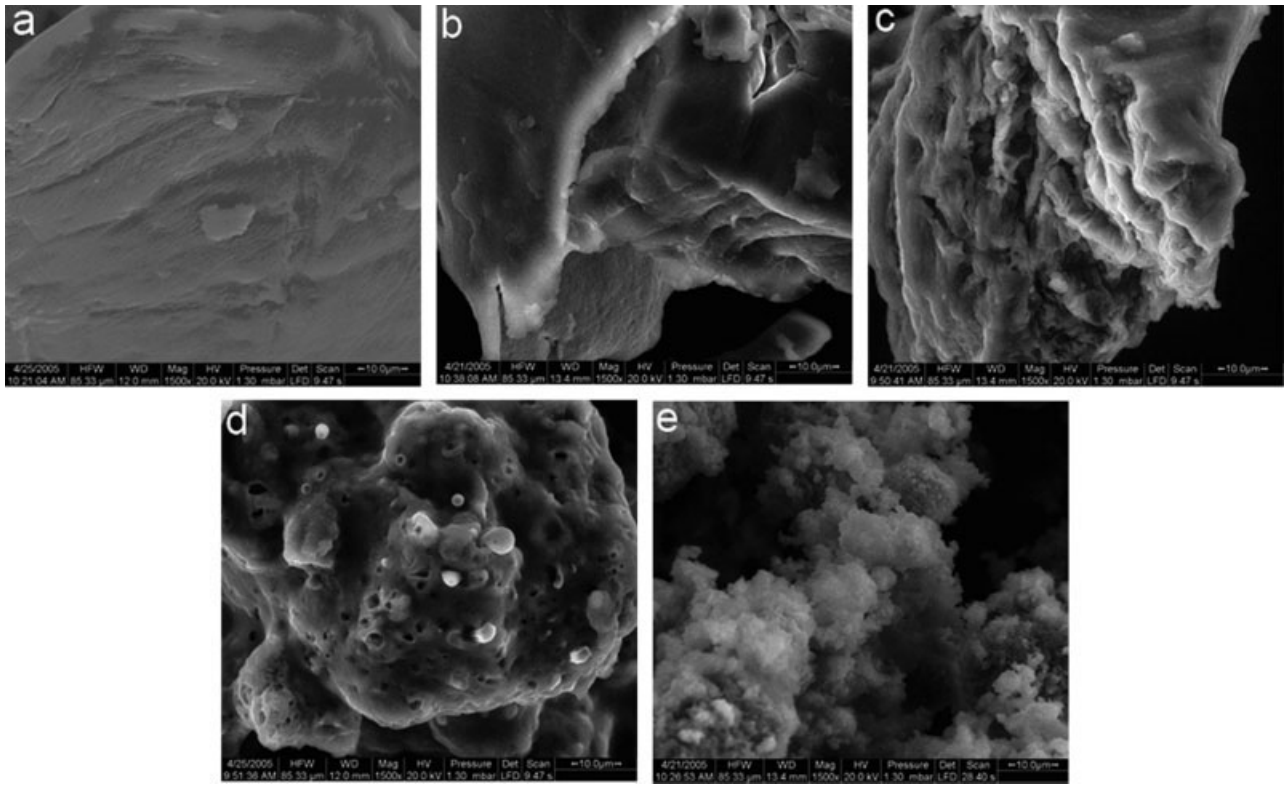


Figure 4 SEM images (20 kV ×1500) of KGM samples with different molecular weights: (a) M_w = 980,000; (b) M_w = 270, 000; (c) M_w = 208, 000; (d) M_w = 114,000; (e) M_w = 78,000.

the acetate ester became larger. Concomitant with the increase of carbonyl stretching band, C—C(═O)—O stretching band at 1241 cm^{-1} characteristic of acetates was also stronger. In the meantime, on the absorbance mode of FTIR spectra, the ratio of A3400/A2900 for KGM and acetylated KGM was 3.9 and 3.1, respectively, indicating the increase of acetyl content after reaction. These spectroscopic results provided further evidence for the acylation of KGM.

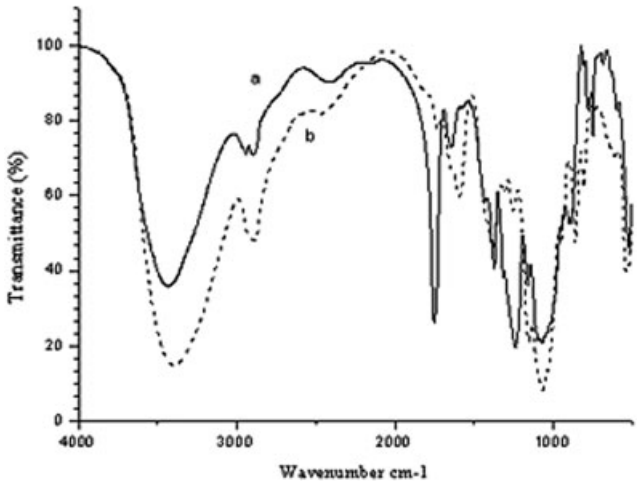


Figure 5 FTIR spectra of (a) acetylated KGM (DS = 0.66) and (b) KGM.

Regioselectivity of enzymatic acylation of KGM

High selectivity represents the most attractive characteristic of enzymatic reactions. ^{13}C-NMR spectra analysis of the KGM and KGM ester were summarized in Table III. It is well known that acylation results in a downfield shift for the chemical shift of acylated car-

TABLE III
Chemical Shifts (ppm) of KGM and Acetylated KGM (DS = 0.66) in DMSO-d_6

Carbon[a]	KGM[b]	Acetylated KGM
G1	103.5	103.1
M1	101.2	101.1
G4	78.6	78.5
M4	77.8	77.8
M5	76.1	71.1 (up)
G5	75.4	70.9 (up)
G3	73.7	73.6
G2	72.8	72.8
M3	72.5	72.4
M2	70.4	70.3
M6	61.2	63.8 (down)
G6	60.9	63.5 (down)
Acetyl		171.1 (C═O); 21.4 (—CH_3)

[a]G: D-glucopyranose residue; M: D-mannopyranose residue.

[b]Signals of ^{13}C-NMR spectrum of KGM were assigned according to Ref. [5].

bon and an upfield shift for its adjacent carbons.[18] Comparison of the chemical shifts of KGM and acetylated KGM shows that acylation of hexose residues caused (a) the signal at 61.2 or 60.9 ppm for C6 of the unmodified substrate to shift downfield to 63.8 or 63.5 ppm and (b) the peak at 76.1 or 75.4 ppm for C5 of the unmodified substrate to shift upfield to 71.1 or 70.9 ppm (Table III). The fact that only the resonances for C5 and C6 showed significant changes demonstrates that the acylation of KGM catalyzed by Novozym 435 is highly selective for C6 of sugar units.

CONCLUSIONS

A novel strategy for the acylation of KGM with biocatalysis in a solvent-free system is reported. The water activity of reaction medium played a key role in the reaction. The optimum water activity, reaction temperature, shaking rate, and enzyme dosage were 0.84, 50°C, 200 rpm and 400 U/mL, respectively. The results also show that the DS of modified KGM sample decreases with an increase in the molecular weight of KGM. Additionally, Novozym 435-catalyzed acylation of KGM was proved to be highly regioselective, which targeted the primary hydroxyl group of KGM.

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