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Thermal stability and activity regain of horseradish peroxidase in aqueous mixtures of imidazolium-based ionic liquids

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Abstract

Thermal deactivation kinetics of horseradish peroxidase (HRP) were studied from 45 to 90 °C in phosphate buffer and 5–25% (v,w/v) 1-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4] and 1-butyl-3-methylimidazolium chloride [BMIM][Cl]. HRP activity at 25 °C was not affected by the presence of ionic liquids up to 20% (v,w/v). Increasing the ionic liquids concentration up to 25% (v,w/v) changed the biphasic character of deactivation kinetics to an apparent single first-order step. The presence of 5–10% (v/v) [BMIM][BF_4] significantly improved HRP thermal stability with lower activation energies for the deactivation second phase (83–87 kJ mol^{-1}). After deactivation, enhanced activity regain of the enzyme, up to 70–80% of the initial activity, was found in 25% (v/v) [BMIM][BF_4] and 10% (w/v) [BMIM][Cl] and correlated to prevalence of the deactivation first phase.

Introduction

Peroxidases (EC 1.11.1.7; donor:hydrogen-peroxidase oxidoreductase) are ubiquitous heme-containing enzymes of industrial interest for their widespread application in clinical chemistry (Krieg & Halbhuber 2003), environmental biosensor systems (Botchkareva *et al.* 2003), bioconversion of industrial effluents (Song *et al.* 2003) and synthesis of polymeric materials and aromatic chemicals (Shan *et al.* 2003). Such applications are often carried out in systems containing organic solvents which greatly enhance solubilization of reactants and products, and improve yields, however, these systems often suffer from reduced activity, selectivity or stability of the enzyme, as compared to aqueous media (Klibanov 1997).

In recent years, room temperature ionic liquids (low melting point salts) have attracted increasing attention as alternative 'green' solvent media for biocatalysis. This is mainly because of their high chemical and thermal stability, lack of vapour pressure, tunable polarity, along with easier product isolation and catalyst reuse (Park & Kazlauskas 2003, van Rantwijk *et al.* 2003, Wilkes 2004). Since the pioneer study of Erbeldinger *et al.* (2000), biotransformations performed in ionic liquids have shown potential benefits with regard to enzyme activity, stability and (enantio)selectivity (Okrasa *et al.* 2003, Chiape *et al.* 2004, Irimescu & Kato 2004, Hongwey *et al.* 2005). Recently, Pugin *et al.* (2004) reported the potential importance of ionic liquids/water combination ('wet' ionic liquids) in enantioselective catalysis. Such a mixture allowed constituting a new class of solvents with superior catalyst recycling and activity compared to conventional organic solvents and ionic liquids/organic co-solvents media. In this way, because of the large range of biotechnological applications where peroxidases have been used in aqueous

mixtures of organic solvents, it is therefore important to characterize their activity and thermal deactivation behaviour in 'wet' ionic liquids, which have not been reported to date. For this purpose, the present study examines the effect of mixtures of phosphate buffer with water-miscible ionic liquids, [BMIM][BF_4] and [BMIM][Cl], on the isothermal deactivation kinetics of horseradish peroxidase. Spontaneous HRP activity regain following thermal deactivation was also quantified and discussed from the viewpoints of a relationship between deactivation profiles and enzyme reactivation yields.

Materials and methods

Materials

Horseradish peroxidase type I (HRP, EC 1.11.1.7, RZ 1.3) and, [BMIM][BF_4] and [BMIM][Cl] were purchased from Sigma and Fluka, respectively. All the other reagents were of analytical grade. The buffer used was sodium phosphate (0.1 M, pH 7.0).

Measurement of enzyme activity

Peroxidase activity was determined spectrophotometrically according to the Worthington's procedure (Weng *et al.* 1991). The reaction was initiated by the addition of 50 µl enzyme solution (0.032 mg HRP ml^{-1}) to 1.45 ml substrate (9.75 mM H_2O_2, 0.16 M phenol and 2.3 mM 4-aminoantipyrine in 0.1 M phosphate buffer, pH 7.0). The slope of the linear increase in absorbance monitored at 510 nm and 25 °C, every s over 1 min, was used to quantify enzyme activity.

Thermal stability of HRP

Thermal deactivation was started by the addition of 200 µl enzyme solution (0.8 mg ml^{-1} HRP) to 1.8 ml of 0.1 M phosphate buffer (pH 7.0) or buffer/ionic liquid mixtures (5, 10 and 25% v,w/v) previously heated in the range of 45–90 °C. At different times, 100 µl aliquots were removed from the sealed vials and rapidly mixed with 150 µl 0.1 M phosphate buffer (pH 7.0) in Eppendorff tubes placed in a water/ice mixture. Subsequently, the residual enzyme activity was measured within 5 min. Preliminary experiments had shown that upon cooling, the enzyme was able to promptly regain activity, yet not significantly at this short time period.

Deactivation experimental data were described by a double exponential model, regardless of underlying deactivation mechanism(s):

$$\frac{A}{A_0} = (1-\alpha)\exp(-k_1 t) + \alpha\,\exp(-k_2 t) \qquad (1)$$

where A and A_0 are, respectively, the residual activity and the initial activity of the enzyme (µmol of reaction product min^{-1}), α represents the amplitude of the second phase of the deactivation process, in terms of the relative activity of the enzyme at the onset of this phase, k_1 and k_2 are, respectively, the apparent deactivation rate constants of the first and second phases (min^{-1}), and t is the deactivation time (min).

Temperature dependence of the rate parameters was described by an Arrhenius-type relationship according to:

$$k = k_{\mathrm{ref}}\,\exp\left[-\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_{\mathrm{ref}}}\right)\right] \qquad (2)$$

where k is the rate parameter at temperature T, k_{ref} is the rate parameter at a reference temperature T_{ref} (K), E_a is the activation energy (kJ mol^{-1}) and R is the universal gas constant. The T_{ref} selected was the average value of the temperatures tested for each case studied.

Results and discussion

Effect of imidazolium-based ionic liquids on HRP activity

The activity of HRP was firstly investigated in the substrate solution with ionic liquids and buffer to compare the efficiency of these solvents for use as reaction media. The reaction was started by adding enzyme solution to the substrate with 5–25% (v,w/v) [BMIM][BF_4] or [BMIM][Cl] at 25 °C. Figure 1 shows that HRP activity was not affected significantly ($p > 0.05$) by the presence of ionic liquids up to 20% (v,w/v), while a decrease in activity of 19 and 26% was however observed at 25% (v,w/v) [BMIM][Cl] and [BMIM][BF_4], respectively. Therefore, both ionic liquids proved suitable media for enzyme catalytic activity.

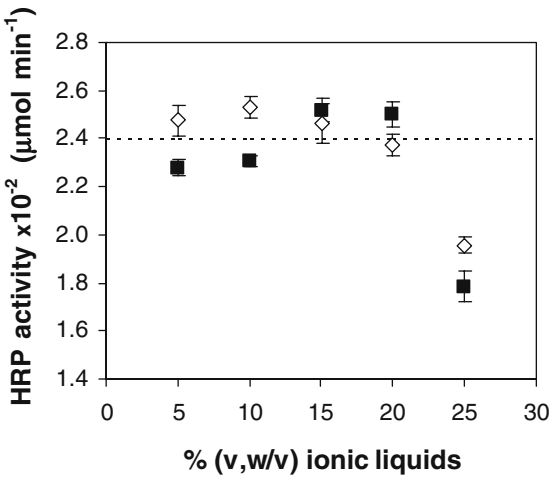

Fig. 1. Horseradish peroxidase (HRP) activity in 0.1 M phosphate buffer, pH 7.0 (dotted line) and different concentrations of [BMIM][BF_4] (◇) and [BMIM][Cl] (■). Error bars represent the standard deviation from the means of three independent measurements. The reaction was initiated by addition of the enzyme (0.032 mg ml^{-1}) to the substrate at 25 °C prepared with buffer or ionic liquids (1.5 ml of total volume reaction in the cuvette). HRP activity is expressed by the amount of enzyme oxidation product, quinol red, in µmol min^{-1} ($\epsilon_{510nm} = 6.58\ M^{-1}\ cm^{-1}$).

HRP thermal deactivation kinetics

Biphasic deactivation profiles were obtained in buffer and 5–10% (v,w/v) ionic liquids mixtures, though increasing the solvents content up to 25% (v,w/v) yielded a clear change on peroxidase deactivation behaviour (Figure 2). While in the first case the deactivation pattern comprised two distinct first-order phases in a semi-logarithmic plot of the activity vs. time, at the highest content of the ionic liquids the deactivation profiles exhibited different trends. First, stabilization of the enzyme catalytic activity following the first deactivation phase was found in the presence of 25% (v/v) [BMIM][BF_4] (Figure 2b). Secondly, the events underlying the deactivation first phase in 25% (w/v) [BMIM][Cl] occurred faster than could be measured (about 20% of activity was lost within the first 2 min of thermal deactivation). Therefore, in this case, the kinetic analysis was only performed to the deactivation process for the second phase using a first-order decay model (Figure 2c).

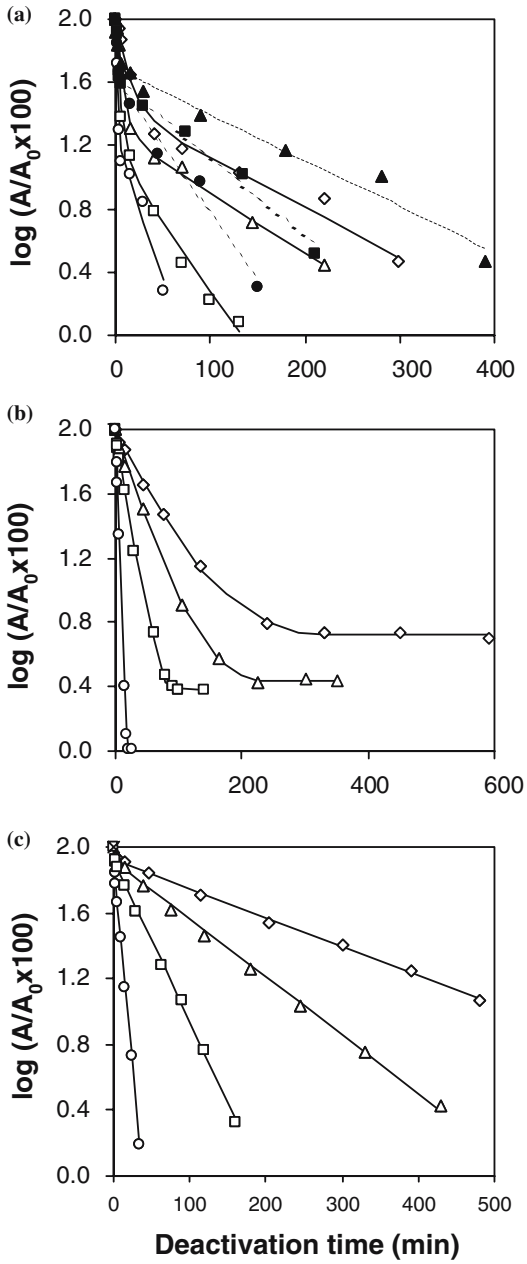

Fig. 2. Deactivation profiles for horseradish peroxidase (0.08 mg ml^{-1}) in (a) phosphate buffer pH 7.0 at 70 °C (◇), 75 °C (Δ), 80 °C (□) and 85 °C (○) and 10% (v/v) [BMIM][BF_4] at 75 °C (▲), 80 °C (■) and 85 °C (●), (b) 25% (v/v) [BMIM][BF_4] at 50 °C (◇), 55 °C (Δ), 60 °C (□) and 65 °C (○), and (c) 25% (w/v) [BMIM][Cl] at 45 °C (◇), 50 °C (Δ), 55 °C (□) and 60 °C (○). Deactivation media were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and all the experiments were duplicated. Solid and dashed lines correspond to the fitted curves with the double exponential equation. Determination coefficients (R^2) of the fits were above 0.99.

Table 1. Rate constants for the deactivation first (k_1) and second (k_2) phases and α values of a double exponential model for horseradish peroxidase (0.08 mg ml^{-1}) thermally deactivated in 0.1 M sodium phosphate buffer (pH 7.0).

T (°C)	α	$k_1 \times 10$ (min^{-1})	$k_2 \times 10^2$ (min^{-1})
70	0.21 ± 0.070[b]	0.92 ± 0.54	0.72 ± 0.20
75	0.18 ± 0.023	1.8 ± 0.27	0.89 ± 0.093
80	0.13 ± 0.036	2.9 ± 0.71	2.0 ± 0.32
85	0.17 ± 0.058	8.0 ± 3.2	4.2 ± 1.1
E_a (kJ mol^{-1})[a]		177	146

[a] E_a represents the activation energy of the deactivation process. [b] estimate ± 95% confidence interval.

Tables 1–3 present the kinetic parameters estimated from the analysis of the data sets. The α parameter was not affected by temperature in the presence of buffer or lower contents of the solvents. A tendency to a linear decrease with temperature for the values of this parameter was, however, obtained in 10% (v/v) [BMIM][BF_4]. This same feature became more clear when the content of this solvent was increased up to 25%, where the estimated α values were found to be consistent with the activity degree at which the enzyme onsets activity stabilization (Figure 2b). In general, the amplitude of the deactivation second phase in 5 and 10% (v/v) [BMIM][BF_4] was higher than in buffer only (approximately two to three times higher values of α), indicating that the enzyme was able to retain higher catalytic activity while starting the course of the second phase in these media. Moreover, these results were reflected into less pronounced biphasic profiles for the experimental curves, and contributed to a higher thermal stability, particularly in the presence of 10% (v/v) [BMIM][BF_4] comparatively to the data in buffer (Figure 2a).

Enhanced stabilization of the enzyme against thermal deactivation on addition of 5 and 10% (v/v) [BMIM][BF_4] (at 75–90 °C) compared to buffer (70–85 °C) was reinforced by a joint contribution of the similar to lower values of k_2 deactivation constants in the former media, par-

Table 2. Rate constants for the deactivation first (k_1) and second (k_2) phases and α values of a double exponential model for horseradish peroxidase (0.08 mg ml^{-1}) thermally deactivated in buffer/[BMIM][BF_4] mixtures.

[BMIM][BF_4] % (v/v)	T (°C)	α	$k_1 \times 10$ (min^{-1})	$k_2 \times 10^2$ (min^{-1})
5	75	0.32 ± 0.039[b]	4.6 ± 1.7	0.90 ± 0.071
	80	0.29 ± 0.046	2.7 ± 0.87	1.2 ± 0.10
	85	0.32 ± 0.034	3.1 ± 0.62	2.2 ± 0.14
	90	0.33 ± 0.046	3.6 ± 0.99	2.8 ± 0.20
	E_a (kJ mol^{-1})[a]		ne[c]	83
10	75	0.48 ± 0.065	8.7 ± 6.8	0.76 ± 0.067
	80	0.40 ± 0.082	5.6 ± 3.3	1.1 ± 0.15
	85	0.38 ± 0.082	4.9 ± 2.8	1.9 ± 0.23
	90	0.33 ± 0.052	6.9 ± 2.5	2.7 ± 0.27
	E_a (kJ mol^{-1})		ne	87
25	50	0.050 ± 0.0056	0.18 ± 0.011	0
	55	0.025 ± 0.0049	0.27 ± 0.023	0
	60	0.021 ± 0.0037	0.59 ± 0.036	0
	65	0.0060 ± 0.0014	2.6 ± 0.090	0
	E_a (kJ mol^{-1})		253	–

In the presence of 25% (v/v) [BMIM][BF_4] the enzyme retains some residual activity after the deactivation process ($k_2 = 0$).
[a] and [b] are the same footnotes as in Table 1. [c] Not estimated. The reaction rates did not follow a temperature dependency according to an Arrhenius-type relationship.

Table 3. Rate constants for the deactivation first (k_1) and second (k_2) phases and α values of a double exponential model for horseradish peroxidase (0.08 mg ml^{-1}) thermally deactivated in buffer/[BMIM][Cl] mixtures.

[BMIM][Cl] % (w/v)	T (°C)	α	$k_1 \times 10$ (min^{-1})	$k_2 \times 10^2$ (min^{-1})
5	65	0.18 ± 0.049[b]	0.59 ± 0.18	0.44 ± 0.093
	70	0.16 ± 0.045	0.87 ± 0.19	0.81 ± 0.19
	75	0.23 ± 0.083	9.9 ± 7.2	2.5 ± 0.37
	80	0.20 ± 0.055	23 ± 14	4.9 ± 0.31
	E_a (kJ mol^{-1})[a]		212	161
10	55	0.21 ± 0.14	0.21 ± 0.073	0.59 ± 0.21
	60	0.21 ± 0.080	0.57 ± 0.15	1.2 ± 0.24
	65	0.24 ± 0.047	1.4 ± 0.18	3.4 ± 0.37
	70	0.13 ± 0.052	5.8 ± 0.98	12 ± 3.1
	E_a (kJ mol^{-1})		256	233
25	45	–	–	0.040 ± 0.011
	50	–	–	0.081 ± 0.025
	55	–	–	0.23 ± 0.10
	60	–	–	1.2 ± 0.3
	E_a (kJ mol^{-1})	–	–	282

In the presence of 25% (w/v) [BMIM][Cl], a first-order decay model was used to estimate the rate constant.
[a] and [b] are the same footnotes as in Table 1.

ticularly for higher temperatures. For example, after 25 min deactivation at 85 °C, the residual enzyme activity was 5% in buffer and 30% in 10% (v/v) [BMIM][BF_4]. Increasing the content of the ionic liquids yielded a considerable opposite effect in the thermal resistance of the enzyme though it was more pronounced in [BMIM][Cl] mixtures. In general terms, the rate constants for the first phase (k_1) were one order of magnitude greater than those of the second, and slower phase (k_2). Since the rapidity at which occur the deactivation events is higher in the first phase of the process, the values of k_1 are generally associated to greater errors and, therefore, poorer statistical significance. The values of the activation energies (E_a) for the deactivation second phase were significantly lower in [BMIM][BF_4] indicating that the presence of this solvent caused a lower sensitivity of deactivation to temperature. The opposite effect was observed for increasing contents of [BMIM][Cl]. Overall, the magnitude of the activation energies for both phases was considerably high thus suggesting that large rearrangements of the enzyme structure might play a major relative role on the deactivation process of HRP (Volkin & Klibanov 1989).

Reversible thermal deactivation of HRP

The analysis of activity regain was performed to the remaining thermally deactivated HRP samples stored at 4 °C. Enzyme activities were assayed for a 48-h period when no significant differences were found between consecutive readings. As Figure 3 shows, both the pattern and the degree of activity regain varied significantly with the deactivation profiles. In the deactivation media where the enzyme markedly displayed biphasic behaviour there was a general trend of activity recovery. Upon an early increase in activity regain, a maximum value is reached (about 30–40% of the initial activity), and thereafter the ability of the enzyme to regain activity becomes progressively less evident with increasing deactivation times (example in Figure 3a). The deactivation times at which occurred both the peak of maximum recovery and the transition to the second phase of the thermal deactivation curves were approximately the same (Figure 3a). On the other hand, a nearly complete recovery of the catalytic activity lost in the thermal treatment was achieved in the presence of 25% (v/v) [BMIM][BF_4] and 10% (w/v) [BMIM][Cl] (respectively, 80 and 70%

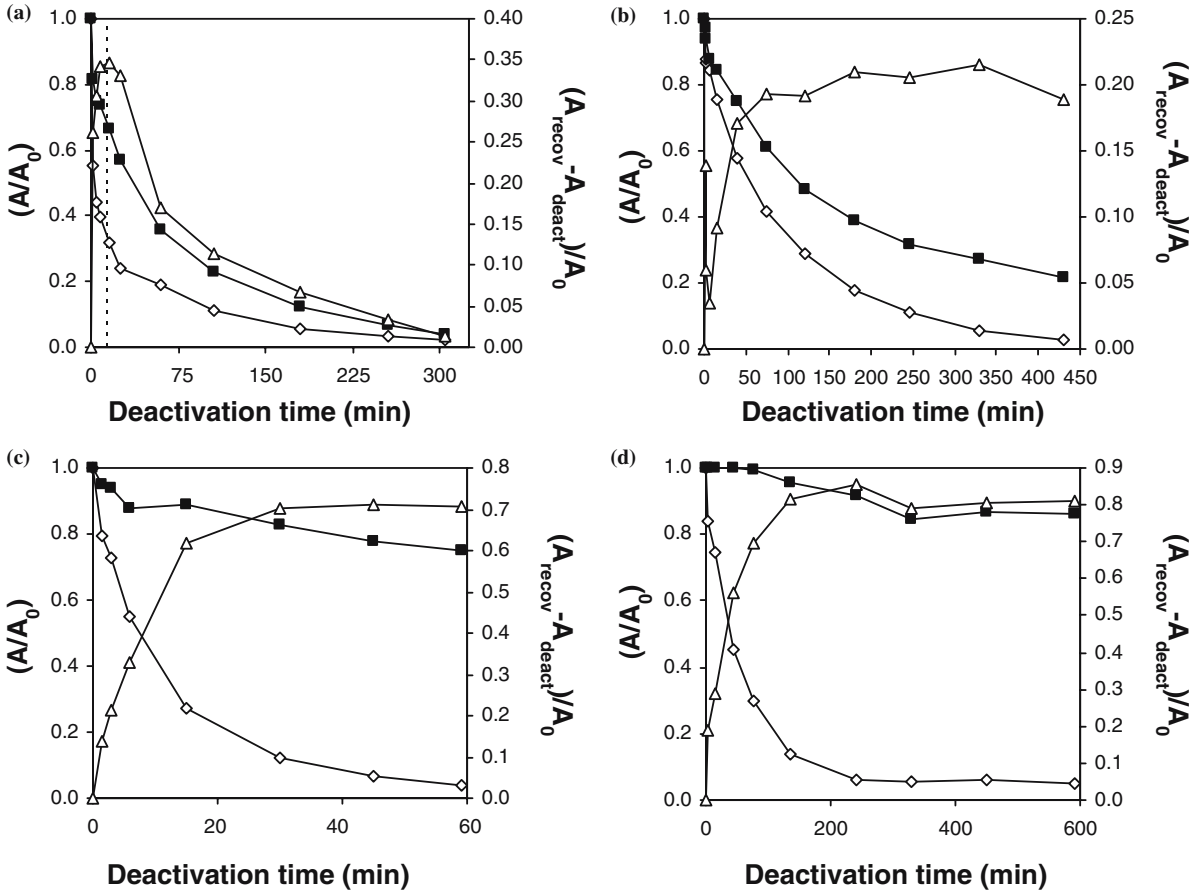

Fig. 3. Activity regain profiles (Δ) of horseradish peroxidase previously thermally deactivated in mixtures of (a) 5% (v/v) [BMIM][BF_4] at 75 °C, (b) 25% (w/v) [BMIM][Cl] at 55 °C, (c) 10% (w/v) [BMIM][Cl] at 65 °C and (d) 25% (v/v) [BMIM][BF_4] at 50 °C. The figure also shows the residual activities used to calculate activity regain: after thermal deactivation (A_{deact}; ◇) and after activity recovery for 48 h from heat removal and storage at 4 °C (A_{recov}; ■). The vertical dashed line in plot A represents the point of transition between the two phases of deactivation before activity regain.

of its initial activity) (Figures 3c, d) where the deactivation first phase dominated. These results suggest that reversible deactivation events prevailed on the first deactivation phase, whereas irreversible ones mainly occurred in the second phase. Such relationship holds significant potential with respect to future advances in studying deactivation pathways for HRP and reducing activity losses.

Conclusions

The catalytic activity of horseradish peroxidase was retained up to 20% of the ionic liquids compared to buffer. Upon increasing the concentration of [BMIM][BF_4] and [BMIM][Cl] up to 25% (v,w/v), the enzyme showed noticeable changes from its typical biphasic deactivation to apparent first-order decay kinetics. HRP thermal stability was improved in the presence of 5 and 10% (v/v) [BMIM][BF_4]. A remarkable recovery of peroxidase activity after thermal deactivation was also observed in 25% (v/v) [BMIM][BF_4] and 10% (w/v) [BMIM][Cl], respectively, 80 and 70% of the initial activity. This, in turn, was associated to thermal profiles where predominated the deactivation first phase. This study showed that water-miscible ionic liquids might thus offer an alternative to aqueous mixtures of conventional solvents, to be used in biocatalysis or biosensors systems with peroxidase.

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