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Improved immobilization and stabilization of lipase from *Rhizomucor miehei* on octyl-glyoxyl agarose beads by using $CaCl_2$

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ABSTRACT

A strategy to stabilize octyl (OC)-*Rhizomucor miehei* lipase (RML) and OC-*Candida rugosa* lipase (CRL) at pH 10 is necessary to take full advantage of the immobilization of these enzymes on OC-glyoxyl (OCGLX) supports. $CaCl_2$ and $MnCl_2$ have been reported to stabilize OC-RM and OC-CRL at pH 5.0 and 7.0. After screening different buffers, 5 mM $CaCl_2$ was found to be fully soluble in Gly at pH 10. OC-RML was 15 folds stabilized at this pH value by $CaCl_2$, while OC-CRL was not. This salt was used in the preparation of octyl-glyoxyl (OCGLX)-RML, permitting to maintain almost unaltered the OC-RML activity that was 3 fold higher than that of the free. This preparation was 30% and 3 folds more stable in thermal or acetonitrile inactivations respectively than the standard OCGLX one.

The stabilizing effect of $CaCl_2$ and $MnCl_2$ on the OCGLX preparations was studied. These salts stabilized both OCGLX-RML preparations, although the one prepared using Ca^{2+} during the covalent attachment was more stabilized than the standard one by the presence of Ca^{2+}, even 7-8 folds in the presence of aceonitrile. Thus, this additive permits to recover an OCGLX-RML preparation more stable and active than the standard protocol.

1. Introduction

Lipases are among the most used enzymes in biocatalysis [1–6]. In many instances, it is required their previous immobilization, stabilization and purification before their use as industrial biocatalyst [7–9]. Specific adsorption of lipases on hydrophobic supports via interfacial activation is a methodology successfully used to get this multiple goal [10,11].

Recently, the use of octyl-glyoxyl agarose beads (OCGLX) has been proposed as a methodology that permits the solution of some limitations of octyl-agarose beads (OC), like the undesired release of the enzyme to the medium under certain conditions [12–15]. This heterofunctional support permits to couple the good features of OC (selective immobilization of the open form of the lipase) to the irreversibility of secondary amino enzyme-support bonds provided by the glyoxyl groups, and usually permitted to improve enzyme stability [13,15]. The lipases immobilized on these supports could be submitted even at reactivation strategies via unfolding/refolding protocols without releasing the enzyme [13,16].

However, the immobilization protocol using OCGLX includes the incubation at pH 10 of the enzyme already immobilized on OCGLX to permit the reaction between the amino groups of the lateral chain of Lys of the proteins and the glyoxyl groups of the support [15]. This makes that the immobilization on these supports of some lipases, too unstable at alkaline pH even after stabilization by immobilization on OC, produced a reduction of enzyme activity [13,15]. The lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL) are two examples where the incubation at alkaline pH produced a significant enzyme inactivation [13,15].

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Thus, a method that stabilizes these lipases at pH 10 could permit a significant improvement of the final glyoxyl-octyl lipase biocatalysts features for these two enzymes. Very recently, it has been described that 5 mM of Ca^{2+} and Mn^{2+} are able to stabilize both OC immobilized enzymes. at neutral or acid pH values [17], while alkaline pH was not analyzed due to solubility problems [17]. However, other enzyme formulations from the same enzymes (including the free enzyme and covalently immobilized enzymes) were not stabilized.

As the lipase immobilization on octyl-glyoxyl should produce a very similar enzyme conformation to the immobilizaiton on octyl suppport, in this new paper we intend to use these cations to stabilize OC-CRL and OC-RML at alkaline pH value and that way, improve the activity recovering when immobilizing the enzyme in OCGLX supports without the decrease on activity currently observed [13,15]. Moreover, we will check if these cations may also stabilize the OCGLX preparations as a way of improving their storage or operational stability.

2. Materials and methods

2.1. Materials

Solution of RML (13.7 mg of protein/mL) was a kind gift from Novozymes (Spain). Octyl-agarose beads (OC) were from GE Healthcare and OCGLX was prepared by periodate oxidation of OC support as previously described [13,15]. *p*-Nitrophenyl butyrate (*p*-NPB) and CRL (in powder form, 40% (m/m) of protein content) were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents were of analytical grade.

2.2. Determination of enzyme activity

The increase in absorbance at 348 nm produced by the released *p*-nitrophenol (*p*-NP) in the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate at pH 7.0 and 25 °C was determined. The ε of *p*-NP under these conditions is 5150 M^{-1} cm^{-1}.The reaction was initialized by adding 50–100 μL of lipase solution or suspension to 2.5 mL of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-NPB per minute under the conditions described previously. Protein concentration was determined using Bradford's method [18] and bovine serum albumin was used as the reference.

2.3. Determination of $CaCl_2$ and $MnCl_2$ solubility at pH 10 in different buffers

5 mM of each of the studied salts was added to 2.5–50 mM of different aqueous buffers (sodium bicarbonate, sodium borate, glycine/NaOH, sodium phosphate, Tris) at pH 10.0 and 25 °C. After 30 min, the precipitation was visually analyzed.

2.4. Immobilization of enzymes

2.4.1. Immobilization of enzymes on OC

The immobilization was carried employing 10 *p*-NPB units of free lipase activity per g of wet support. This low loading was used to prevent diffusion limitations that could distort the results. The commercial samples of the enzymes were diluted or dissolved in the corresponding volume of 5 mM sodium phosphate at pH 7. Then, the OC support was added (10 g of support in 100 mL of enzyme solution). The activities of both supernatant and suspension were followed using *p*-NPB. After immobilization, the suspension was filtered and the supported enzyme was washed several times with distilled water and stored at 4 °C. Enzyme activity improved versus *p*-NPB as described in many other papers (by a threefold factor using RML) [15].

2.4.2. Immobilization of enzymes on OCGLX

We followed the protocol, described in [13,15], only buffer for the incubation at pH 10 was changed. The immobilization was carried employing 10 *p*-NPB units of lipase per g of wet support. This low loading was used to prevent diffusion limitations that could distort the results. The protocol is described below.

The commercial samples of the enzymes were diluted or dissolved in the corresponding volume of 5 mM sodium phosphate at pH 7.0. Then, the OCGLX support was added (10 g of support in 100 mL of enzyme solution). The activities of both supernatant and suspension were followed using *p*-NPB. After immobilization, the suspension was filtered and the supported enzyme was washed several times with distilled water. RML activity increased after this immobilization by a 3 fold factor [15]. Then, the immobilized enzymes were suspended in 2.5 mM glycine (in some cases plus 5 mM of $CaCl_2$) or 25 mM sodium bicarbonate at pH 10.0 and 25 °C. The activities were measured along time using pNPB. As a reaction end point, 1 mg/ml of solid sodium borohydride was added [13,15]. After 30 min of gently stirring, the suspension was filtered and the supported enzyme was washed several times with 100 mM sodium phosphate pH 7.0 and distilled water a and stored at 4 °C.

2.5. Desorption of the enzyme from the supports

To analyze if the enzymes were really covalently attached to the support, and to keep only the covalently attached enzyme molecules for further studies, the reduced OCGLX derivatives were incubated with a growing concentration of the appropriate detergent, using OC derivatives as reference. This treatment only releases the enzyme molecules adsorbed by interfacial activation [13,15]. Thus, samples of 1 g of different biocatalysts were suspended at 25 °C in 10 mL of 10 mM sodium phosphate at pH 7.0. Then, Triton X-100 was progressively added. Intervals of 30 min were allowed before taking a sample of the supernatant to determine the released enzyme and performing a new detergent addition. A reference suspension, having inert support and the same amount of lipase was submitted exactly to the same treatment, to detect the effects of the detergent on enzyme activity or stability.

2.6. Thermal inactivation of different enzyme preparations

1 g of immobilized lipase was suspended in 10 mL of 5 sodium acetate at pH 5.0 at 60 °C, in some instances containing 5 mM of $CaCl_2$ or $MnCl_2$. Periodically, samples were withdrawn and the enzyme activity was measured using *p*-NPB. Half-lives were calculated from the observed inactivation courses.

2.7. Solvent inactivation of different enzyme preparations

1 g of immobilized lipase was suspended in 10 mL of 25% acetonitrile in 5 mM Tris buffer/NaOH at pH 7.0 at 30 °C, in some instances containing 5 mM of $CaCl_2$ or $MnCl_2$. Periodically, samples were withdrawn and the enzyme activity was measured using *p*-NPB. Half-lives were calculated from the observed inactivation courses. The presence of acetonitrile in the activity assay has no effect on the enzyme activity.

3. Results

3.1. Solubility of $CaCl_2$ and $MnCl_2$ at pH 10.0 in different buffers

5 mM $CaCl_2$ or $MnCl_2$ precipitated at all the concentrations of many of the different used buffers at pH 10 (sodium phosphate,

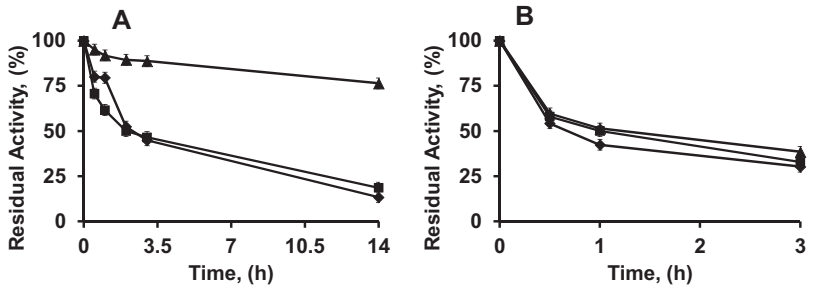

Fig. 1. Effect of $CaCl_2$ on the stability of OC-RML (A) and OC-CRL (B) at pH 10. The temperature was 30 °C and the buffers were 5 mM sodium bicarbonate at pH 10 (rhombus), 5 mM glycine at pH 10 (squares) or 5 mM glycine plus 5 mM $CaCl_2$ (triangles). Experiments have been performed as described in Section 2.

sodium bicarbonate, sodium borate, Tris/NaOH, Gly/NaOH). However, 5 mM $CaCl_2$ was soluble in Gly at pH 10.0, at concentrations of Gly as high as 100 mM. At this pH value and buffer, $MnCl_2$ remained insoluble and was discarded in these studies. The utilization of Gly has a problem for the covalent reaction between the enzyme and the glyoxyl groups of OCGLX support. This is the existence of a primary amino group that can block the glyoxyl groups, making more complex to support-enzyme covalent reaction [19,20]. However, it is possible to use a very low concentration of this buffer, and the near presence of the aldehydes and the side amino of Lys groups may help the enzyme-support covalent reaction, minimizing the impact of the competition of the Gly by the glyoxyl groups [21,22].

Thus, 2.5 mM Gly/NaOH/5 mM $CaCl_2$ was selected to analyze the stabilizing effect of Ca^{2+} at pH 10.0 for OC-CRL and OC-RML.

3.2. Effect of $CaCl_2$ on the stability of OC-CRL and OC-RML at pH 10

Fig. 1 shows that OC-RML became quite stabilized using 5 mM $CaCl_2$ at pH 10. Thus, while OCRML at pH 10.0 and 30 °C in the absence of $CaCl_2$ maintained only around 15% of initial activity, in the presence of this salt preserved more than 75%. The half-live of the octyl preparation increased by around 15 folds. This stabilizing result was similar to that obtained by using $CaCla_2$ at pH 5.0 and 7.0 [17]. In that paper, the stabilizing effect of the cations was explaining by the adsorption of the cation on some pocket, very likely involving some ionic bridge, but the explanation on the stabilizing effect only of the OC-lipase preparation was difficult to give [17]. However, the results were very different using OC-CRL. First, OC-CRL is far less stable at pH 10 than OC-RML (Fig. 1). Second, now $CaCl_2$ did not present any protective effect at pH 10.0 and the activity decrease was very similar in absence or the presence of the salt. This result is in strong opposition previously reported on Ca^{2+} stabilization of OC-CRL at pH 5 and 7 [17]. It seems that at this pH value the enzyme place where Ca^{2+} should be adsorbed on OC-CRL did not recognize it (perhaps because some conformational change), or that the inactivation takes place in different areas of the protein different at this pH value that at pH 5 or 7, or the inactivation at this pH value is not caused by conformation changes (e.g., chemical modification), and that is not prevented by Ca^{2+}. Thus, this salt only seems to be useful for our purpose using OCGLX-RML.

3.3. Preparation of OCGLX-RML in presence of 5 mM $CaCl_2$/2.5 mM Gly

After immobilizing RML (immobilization yield over 97%) on OCGLX (having the same hyperactivation than using OC, a 3 fold factor) [15], the immobilized enzyme was incubated at pH 10.0 and 25 °C in the presence or absence of $CaCl_2$ (Fig. 2). As using OC support, the decrease in activity was far higher in the absence of the salt, and after 3 h the activity of the preparation without the salt maintained less than 60% of the initial activity while in the presence of the salt the preparation retained more than 90% of the initial activity. Therefore, this simple additive permitted to reduce 4 folds the activity loses during preparation of OCGLX-RML. The final preparation, starting with 10 U of free enzyme/g of support, exhibited around 26 U of immobilized enzyme/g after the whole protocol.

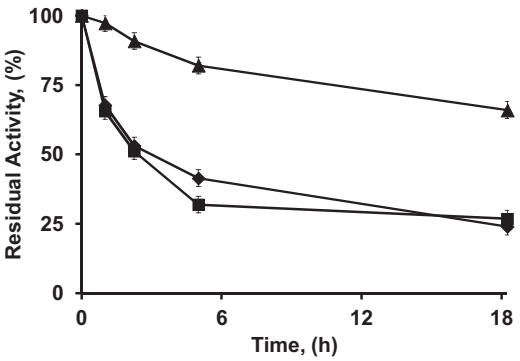

Fig. 2. Effect of $CaCl_2$ on the enzyme inactivation of OCGLX-RML at pH 10. The temperature was 25 °C and the buffers were 5 mM sodium bicarbonate at pH 10 (rhombus), 2.5 mM glycine at pH 10 (squares) and 2.5 mM glycine plus 5 mM $CaCl_2$ (triangles).

After reduction, both preparations were incubated in 0.3% (v/v) Triton to check if the enzyme was really covalently attached to the support. In both cases, less than 15% of the enzyme activity was observed in the supernatant (in agreement with previous reports [17]), suggesting that the presence of 2.5 mM Gly did not avoid the formation of at least one covalent bond between the enzyme and the support. Next, both enzyme preparations have been compared in their stability under different conditions.

3.4. Characterization of OCGLX-RML prepared in the presence or absence of $CaCl_2$ and glycine

3.4.1. Thermal stability at different pH values of different OC-RML preparations

Fig. 3 shows the thermal inactivation of OC-RML, OCGLX-RML and OCGLX-RML prepared in the presence of Ca^{2+} (OCGLX-RML-Ca). The results show that both OCGLX preparations are more stable than the OC one, as it has been previously described [13,15], and that the presence of Gly and Ca^{2+} during the immobilization has negligible effects on enzyme stability, suggesting that the low amount of Gly used in the immobilization has not a negative effect on the number of enzyme-support linkages. In fact, the new preparation is around a 30% more stable than the standard OCGLX preparation and around 12 folds more stable than the octyl preparation. This could be due to a lower distortion of the enzyme molecules during the enzyme immobilization in the presence of

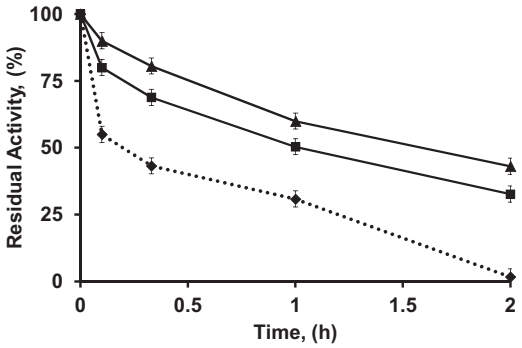

Fig. 3. Thermal inactivations of different RML immobilized preparations. Inactivations were performed at 60 °C in 5 mM sodium acetate at pH 5. Solid line: OCGLX-RML-Ca (triangles), dash line: OCGLX-RML (squares); pointed line: OC (rhombus).

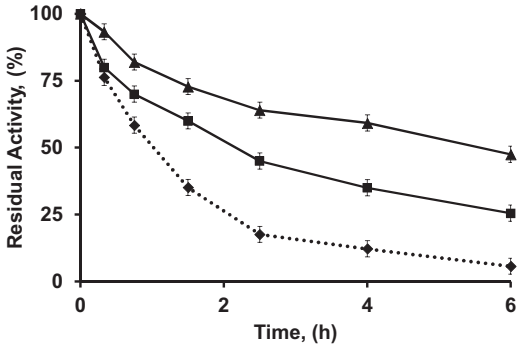

Fig. 5. Solvent inactivation of different RML immobilized derivatives. The biocatalysts were incubated at 30 °C in 25% (v/v) ACN/5 mM Tris HCl at pH 7. Solid line (triangles): OCGLX-RML-Ca; dashed line, (squares): OCGLX-RML and points line, (rhombus): OC-RML.

Ca^{2+}, that do not force the structure of the enzyme like in the absence of the cation.

3.4.2. *Effect of cations on the thermal stability at pH 7.0 of different OC-RML preparations*

Next, we have evaluated the effect of the stabilizing cations (Ca^{2+} and Mn^{2+}) on the three RML preparations, to check if the covalent immobilization has any influence on the stabilization found using just OC preparations [17]. OC-RML has been reported to become stabilized by these cations [17], but the covalent attachment in OCGLX may somehow alter the situation. Fig. 4 shows the results. All preparations are stabilized in the presence of both cations. OCGLX-RML (half-live 2 h) is less stabilized than OC-RML (half-live 1 h) or OCGLX-RML-Ca (half-live 4 h) by Ca^{2+}. Using $MnCl_2$, only OC-RML is more stable (half-live 1.3), while. OCGLX-RML has identical stability than in presence of Ca^{2+}, and OCGLX-RML-Ca is less stable in the presence of $MnCl_2$ than in the presence of $CaCl_2$ (half-live of 2.5 h). Using $MnCl_2$, the differences between both OCGLX preparations are shorter than using $CaCl_2$. This may be due to a change of the pocket where the cations are adsorbed, a kind of mild bioimprinting induced by the Ca^{2+}, that made more effective this action that the Mn^{2+} in stabilizing the enzyme, in opposition to the OC-RML..Thus, OC-RML-Ca is clearly the most stable preparation in presence of Ca^{2+} (by a twofold compared to OCGLX-RML), while OC-RML and OCGLX-RML stabilities become very similar (OCGLX-RML is only 2 folds more stable than OC-RML in the presence of Ca^{2+}). It is remarkable that Mn^{2+} is more stabilizing for OC and OCGLX, while using OCGLX-RML-Ca the best result is obtained using Ca^{2+}, this suggested that the covalent immobilization in the presence of Ca^{2+} reinforces its stabilizing effect, perhaps by making changes in the adsorption pocket where the cation is strongly adsorbed.

3.4.3. *Stability in the presence of organic media of different OC-RML preparations*

Fig. 5 shows the results of the inactivation of the three immobilized RML preparations in acetonitrile. OCGLX is more stable than OC-RML preparation (half live 1.7 h), while OCGLX-RML-Ca is clearly the most stable one (half-live 5.7 h). The differences between both OCGLX preparations are clearer than in the thermal inactivations, the new one is almost 3 folds more stable than the standard OCGLX-RML preparation.

3.4.4. *Effect of $CaCl_2$ and $MnCl_2$ on the stability in organic media of different OCGLX-RML preparations*

The stabilization effect of these cations has not been analyzed previously in the presence of organic solvents. Now, we have decided to assay the stabilization effects of these salts in acetonitrile. Fig. 6 shows that all RMN preparations are stabilized versus the inactivating effect of acetonitrile by these additives. The half-lives using $CaCl_2$ were 5.8, 8.5 and over 60 h for OC, OCGLX and OCGLX-RML-Ca. respectively. Stability using $MnCL_2$ were lower, being 2.5, 4 and 8.5 h respectively. This suggests that these cations are useful to stabilize OC-RML preparations versus any inactivating reagent that cause enzyme distortion. The stabilizing effect is similar for all preparations, and in this case Ca^{2+} is the most stabilizing reagent for all biocatalysts while in thermal inactivations OC and OCGLX-RML were more stabilized by Mn^{2+}. However, the difference between the stabilizing effects of both cations is maximal using while OCGLX-RML-Ca, that in the presence of Ca^{2+} is over 10 fold more stable than OC-RML and 7-8 folds more stable than OCGLX-RML. This may be due to a better adsorption of the Ca^{2+} ion in the enzyme molecules covalently immobilized in the presence of the ion.

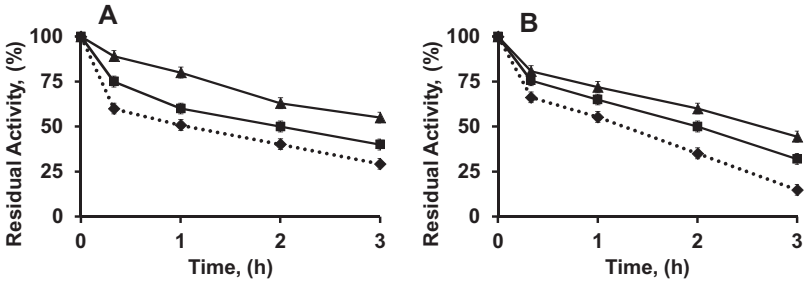

Fig. 4. Effect of cations on the thermal inactivation of different immobilized RML preparations. The inactivation was performed at 60 °C in 5 mM sodium acetate at pH 5 containing 5 mM of $CaCl_2$ (A) or $MnCl_2$ (B). (Triangles): OCGLX-RML-Ca; (squares): OCGLX-GLX; points line, (rhombus) OC-RML.

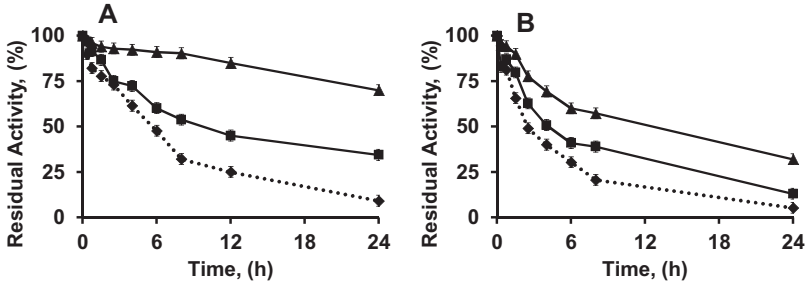

Fig. 6. Effect of $CaCl_2$ (A) or $MnCl_2$. (B) on the solvent inactivation of different immobilized RML preparations. The RML preparations were incubated at 30 °C in 25% ACN/5 mM Tris–HCl at pH 7 containing 5 mM of $CaCl_2$ or $MnCl_2$. OCGLX-RML-Ca: (triangles); OCGLX-RML: (squares) and pointed line (rhombus): OC-RML. Panel A: suspension containing.

4. Conclusions

The use of Ca^{2+} improves the OC-X-RML stability at pH 10.0, while it has no effect on OC-CRL. This has permitted to prepare an OCGLX-RML biocatalyst with an improved activity (90% versus 60%) while also stability is slightly improved. Considering the initial hyperactivation of 300%, the final preparation is 2.7 fold more active than the free enzyme and a 50% more active than the standard OCGLX-RML. The new preparation is more stable than the standard glyoxyl, suggesting that the presence of 2.5 mM Gly in the immobilization buffer is not enough to alter the covalent binding.

Furthermore, the stabilizing effect of Mn^{2+} and Ca^{2+} described in OC-RML but not in other RML formulations [17] is still observed in both OCGLX preparations, although the effect is much clearer using OCGLX-RML-Ca, perhaps because the presence of the cation during the immobilization prevents enzyme distortions and keeps unaltered the stabilization mechanism of this cation.

Moreover, we have found that the stabilization effect of Ca^{2+} and Mn^{2+} previously reported in thermal inactivations may be extrapolated to stabilization in the presence of organic solvents. In fact, the improvement on stability of OCGLX-RML-Ca in the presence of the cations, mainly using Ca^{2+}, is much higher than that of the standard OCGLX-RML.

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