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Preparation of Magnetic Cross-Linked Amyloglucosidase Aggregates: Solving Some Activity Problems

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Abstract: The preparation of Cross-Linked Enzyme Aggregates (CLEAs) is a simple and cost-effective technique capable of generating insoluble biocatalysts with high volumetric activity and improved stability. The standard CLEA preparation consists of the aggregation of the enzyme and its further crosslinking, usually with glutaraldehyde. However, some enzymes have too low a content of surface lysine groups to permit effective crosslinking with glutaraldehyde, requiring co-aggregation with feeders rich in amino groups to aid the formation of CLEAs. The co-aggregation with magnetic particles makes their handling easier. In this work, CLEAs of a commercial amyloglucosidase (AMG) produced by *Aspergillus niger* were prepared by co-aggregation in the presence of polyethyleneimine (PEI) or starch with aminated magnetic nanoparticles (MNPs) or bovine serum albumin (BSA). First, CLEAs were prepared only with MNPs at different glutaraldehyde concentrations, yielding a recovered activity of around 20%. The addition of starch during the precipitation and crosslinking steps nearly doubled the recovered activity. Similar recovered activity (around 40%) was achieved when changing starch by PEI. Moreover, under the same conditions, AMG co-aggregated with BSA was also synthesized, yielding CLEAs with very similar recovered activity. Both CLEAs (co-aggregated with MNPs or BSA) were four times more stable than the soluble enzyme. These CLEAs were evaluated in the hydrolysis of starch at typical industrial conditions, achieving more than 95% starch-to-glucose conversion, measured as Dextrose Equivalent (DE). Moreover, both CLEAS could be reused for five cycles, maintaining a DE of around 90%. Although both CLEAs had good properties, magnetic CLEAs could be more attractive for industrial purposes because of their easy separation by an external magnetic field, avoiding the formation of clusters during the filtration or centrifugation recovery methods usually used.

Keywords: cross-linked enzyme aggregate; amyloglucosidase; magnetic nanoparticles; bovine serum albumin; polyethyleneimine; starch hydrolysis

1. Introduction

Amyloglucosidase (1,4-α-D-glucan hydrolase, EC 3.2.1.3) is an enzyme that catalyzes the release of glucose from the non-reducing ends of glucose polysaccharides. In addition to selectively hydrolyzing α-1,4-glycosidic bonds, it is also capable of hydrolyzing starch branches (α-1,6-glycosidic bonds),

but in a slower manner [1,2]. Fungal amyloglucosidases, like the ones produced by *Aspergillus niger* (AMG), may present more than one form, with different molecular weights [3–5]. *Aspergillus niger* produces two isoforms, namely G1 isoform, which corresponds to a protein having a catalytic domain (structure on the left in Figure 1), and a starch-binding domain (structure on the right in Figure 1) with a total of 640 amino acid residues and Mw around 68 kDa, and G2 isoform, which contains only the catalytic domain with 470 amino acid residues and Mw around 50 kDa [4,5]. Both isoforms are highly glycosylated by both N-linked and O-linked carbohydrates. These isoforms are derived from the same genetic material, but differ because of a different RNA splicing after transcription [6].
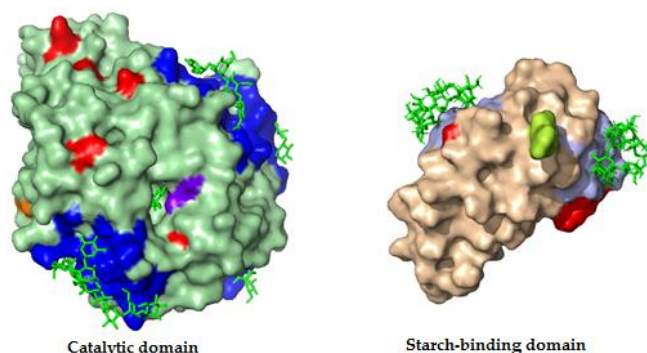

Figure 1. Three-dimensional structures of *A. niger* amyloglucosidase, showing the catalytic domain on the left and the starch-binding domain on the right (3eqa and 1ac0 PDB structures [4,5], respectively). Color patterns: red (lysine residues), purple (active site), orange (N-terminal residue), lemon (C-terminal residue), blue and light blue (glycosylated regions in the catalytic and starch binding domains, respectively). The figures were generated using PyMol (The PyMol Molecular Graphics System; Version 2.1.0; Schrödinger, LLC).

AMG from *A. niger* is extensively used in industrial starch saccharification, almost completely hydrolyzing maltodextrins, amylose, and amylopectins to produce glucose syrup, which serves as a substrate in the production of other syrups for application in the beverage and food industries [2,6,7].

The saccharification of the starch is usually carried out by the enzyme in the soluble form. Because of this, the enzyme is neither recovered nor reused [8]. This drawback can be overcome by the use of enzyme immobilization techniques that allow for enzyme recovery, reuse, and often improvement of the operational stability [9–11]. These advantages could contribute to reduced processing costs and, therefore, a lower final price of the product. Several studies reported the immobilization of amyloglucosidase by different protocols [12–16].

An alternative to the immobilization of enzymes on solid supports is carrier-free immobilization, such as the crosslinked enzyme aggregates (CLEAs) proposed by Sheldon [17–19]. This methodology involves the precipitation of the proteins by the addition of precipitating agents (e.g., salts, organic solvents or nonionic polymers), where the enzymes associate as insoluble aggregates [20]. Thereafter, the formed aggregates are cross-linked using bifunctional (usually glutaraldehyde) or polyfunctional (dextran polyaldehyde, for example) agents [21]. CLEAs of many different enzymes have been reported [18,22–26], including amylolytic enzymes, e.g., β-amylase [27] and amyloglucosidases [28,29].

The preparation of CLEAs may lead to some problems. At first glance, it should be relatively simple to find a precipitant that permits the recovery of high levels of enzyme activity. However, the crosslinking step may be problematic if the enzyme is poor in external free primary amino groups. This problem has been solved using Lys-rich proteins as protein feeders [22,24,27,30], co-immobilizing enzymes and a primary amino rich polymer (e.g., polyethylenimine, PEI) [31] or even enriching the enzyme in primary amino groups via chemical modification [32]. AMG has 13 Lys, but only nine are exposed to the medium [3,5], two of which are located in the starch-binding domain and one close to the active site (see Figure 1). Because of this, the crosslinking of the enzyme with bifunctional

agents may be poor, resulting in a CLEA with low mechanical stability and allowing leaching of the enzyme in the reaction medium [33–36]. In some instances, the crosslinking with glutaraldehyde may lead to enzyme inactivation by altering the active center; in these cases large aldehyde polymers (e.g., aldehyde dextran) or other crosslinkers have been proposed [21,37].

The use of CLEAs also poses some problems. First, CLEAs are mechanically fragile, complicating their recovery. Second, pore sizes may be small and this can lead to high diffusional limitations [38].

The first problem may be solved using magnetic nanoparticles (MNPs) that are co-aggregated with the enzyme to permit magnetic handling of the final CLEAs [39]. The size and functionalization of the MNPs can determine the final properties of the magnetic CLEA [40]. Immobilization of different amylase-related enzymes using CLEA technology associated with MNPs functionalized with 3-aminopropyltriethoxysilane (APTES) has already been reported, showing that its application confers mechanical stability and efficient magnetic separation of CLEAs [28,29,41].

The second problem may be reduced if strategies to enlarge the pores of the CLEA are utilized. The co-aggregation of PEI with the enzyme can significantly improve the crosslinking efficiency, preventing leakage and promoting the generation of a hydrophilic microenvironment that protects the enzyme from organic solvents [20,34–36,42], but that may also result in enlarged pore sizes. Similarly, the use of polymers like starch during the aggregation and crosslinking steps may facilitate the formation of large pores during CLEA production (and perhaps, as it is a substrate of the enzyme, it may protect the active center of the enzyme during the different steps of the CLEA production). As at the final CLEA preparation the starch is degraded by α-amylase and washed away from the CLEA, this strategy may reduce internal mass-transfer limitations and, thus, increase the catalytic efficiency [43,44].

In this context, this paper intends the co-aggregation of commercial AMG with MNPs to get a magnetic CLEA. A set of parameters was evaluated to prepare CLEAs of AMG with high activity, reduced leaching, and high thermal and operational stabilities, such as type and amount of precipitants, concentration of glutaraldehyde, time of glutaraldehyde treatment, stirring speed, use of PEI and starch during precipitation, and crosslinking steps to generate larger pores and, perhaps, to protect the active center of the enzyme. The addition of starch or PEI has been studied as a strategy for the formation of CLEAs with larger pores (among other likely effects). The catalytic properties of the most active and stable CLEAs were characterized (optimal pH and temperature for enzyme activity, thermal stability and performance in the hydrolysis of starch at high starch concentration (typical industrial conditions)).

2. Results and Discussion

2.1. Precipitant Selection

Because the biochemical and structural properties differ from one enzyme to another, a screening of precipitants should be performed for the preparation of CLEAs of a particular enzyme [20,45]. Thus, in this work, a screening of five precipitants (acetone, ethanol, iso-propanol, ammonium sulfate and PEG) was carried out aiming at full protein precipitation and high recovered activity of the re-dissolved precipitate. Commercial AMG 300L is relatively pure (Figure 2), exhibiting two main bands (around 70 and 100 kDa), probably corresponding to the G1 and G2 isoforms [3].

Figure 3 shows that acetone, ethanol, and isopropanol were capable of precipitating around 80% of proteins, retaining high activity of the re-dissolved precipitates (around 90%), while ammonium sulfate precipitated only around 50% of the proteins and polyethylene glycol (PEG) did not have precipitation action on AMG (data not shown). The different precipitation yields may be explained by the different mechanisms of aggregation of each precipitant (changes in the hydration state of the molecules, or changes in the dielectric constant of the solution) [45].
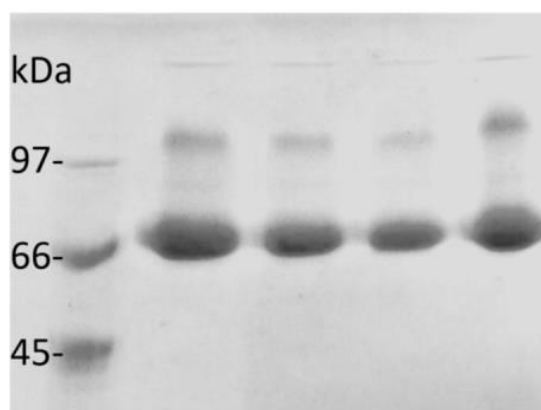
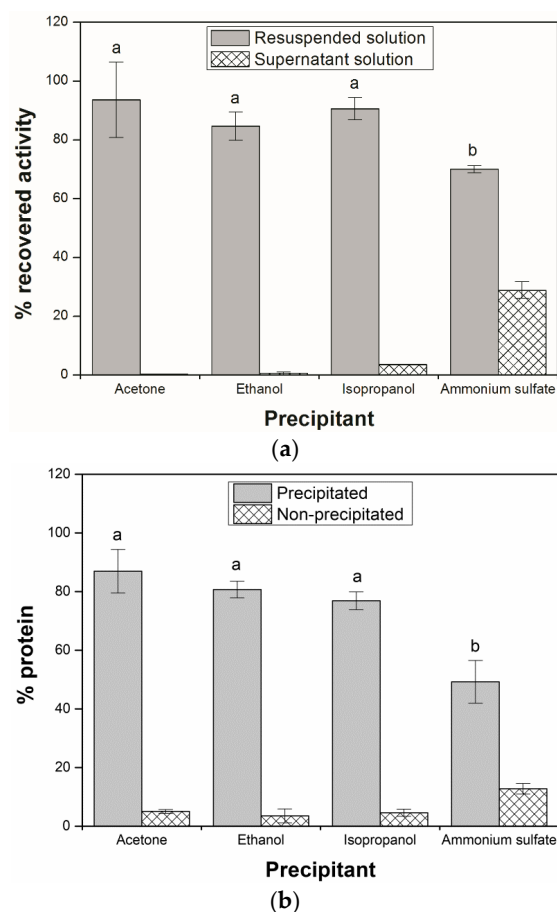**Figure 2.** Electrophoresis gel (10% SDS-PAGE) of commercial amyloglucosidase (AMG, 300L).

Figure 3. Screen of precipitants for amyloglucosidase (AMG 300L), the percentage of recovered activity (**a**) and the percentage of precipitated protein (**b**). Precipitation conditions: precipitant/enzyme solution volume ratio of 9:1, at 4 °C, 60 min precipitation under 150 rpm shaking, and enzyme solution prepared in 50 mM sodium citrate buffer pH 4.5. Note: Values are shown as the mean of triplicate measurements ± s.d. Means followed by the same letter are not statistically different by Tukey's test ($p < 0.05$). Percentage of protein was calculated taken the initial protein as 100%.

Ethanol exhibited good performance at precipitating AMG and is economically and environmentally more friendly (low-cost and -toxicity, and renewable) than the other precipitants evaluated; therefore, it was selected as a precipitant of AMG for the preparation of CLEAs.

Figure 4 shows the influence of the precipitant/enzyme solution volume ratio on the protein precipitation yields and recovered activity of AMG in the re-dissolved precipitate. It can be observed that for ethanol concentrations from 60% to 90% (*v/v*), protein precipitation yields were not statistically different. In terms of recovered activity, 90% (*v/v*) ethanol exhibited a small improvement (around 85% recovered activity). Thus, a volume ratio of 9:1 (precipitant/enzyme solution) was selected for the further assays.
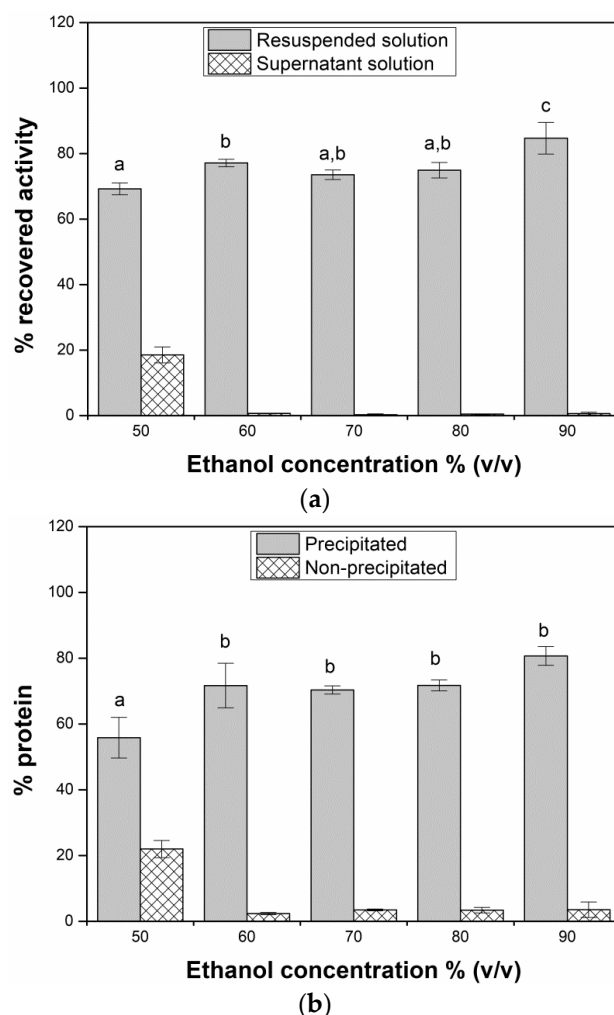

Figure 4. Influence of the ethanol concentration (vol %) on the (**a**) percentage of recovered activity, and (**b**) percentage of precipitated protein of amyloglucosidase (AMG 300L). Assay conditions: 30 min precipitation under static conditions in an ice bath. AMG solution prepared in 50 mM sodium citrate buffer pH 4.5. Note: Values are shown as the mean of triplicate experiments ± s.d. Means followed by the same letter are not statistically different by Tukey's test ($p < 0.05$). Percentage of protein was calculated taken the initial protein as 100%.

2.2. Preparation of CLEAs

Initially, CLEAs were prepared without co-feeders or any other aid. Figure 5 shows that CLEAs could be only formed using 500 mM glutaraldehyde in the crosslinking step. But the recovered activity was lower than 20%. Using 100 or 300 mM glutaraldehyde, the aggregates were re-dissolved due to inefficient crosslinking.

The co-aggregation with PEI allowed the formation of CLEAs at 100 mM glutaraldehyde (recovered activity around 25%). The increase in the glutaraldehyde concentration led to CLEAs with lower recovered activity, probably due to excessive enzyme modification. The co-aggregation with

starch produced better results (recovered activity around 35%), but required higher glutaraldehyde concentration in the crosslinking step. The combined use of PEI and starch did not improve the recovered activity.
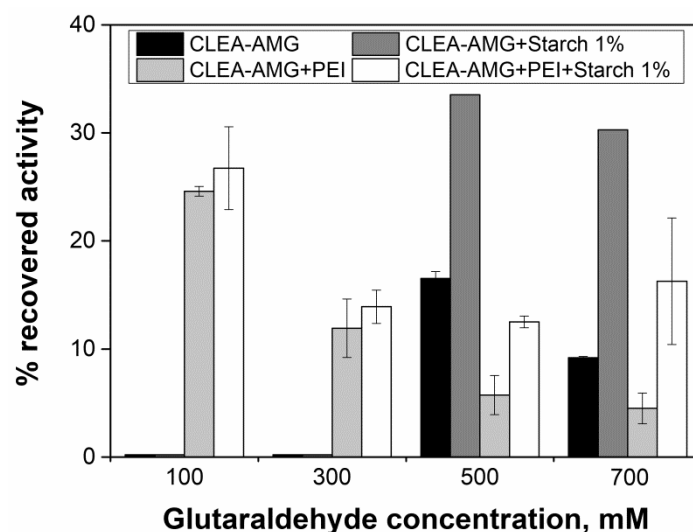

Figure 5. CLEAs of amyloglucosidase (AMG 300L) prepared using only enzyme, co-aggregation with polyethyleneimine (protein/PEI mass ratio of 1:1) and/or starch (1%, *w/v*). Assay conditions: ethanol as precipitant (volume ratio of 1:9, enzyme solution pH 7.0 or 10.0/ethanol), 30 min precipitation in an ice bath, glutaraldehyde as a cross-linker (100–700 mM), 16 h crosslinking under gently stirring at 4 °C. Note: Values are shown as the mean of duplicate experiments ± s.d.

2.3. Preparation of Magnetic AMG CLEAs

In order to prepare easily manageable CLEAs, MNPs were co-aggregated with AMG. A set of commercial MNPs functionalized with amino or amino/hydrophobic groups (Table 1) was evaluated.

Table 1. Content of amino and hydrophobic (octyl or octadecyl) groups in the magnetic nanoparticles supplied by Koop Technologies (São Carlos, SP, Brazil).

Magnetic Nanoparticle	-NH$_2$ Content (µmol/g)		-C8 or -C18 (µmol/g)
NP-N-1	1013.1 [1]		
NP-N-2	265.5 [1]		
DCNP-N	522.1 [1]		Not applicable
N (100%)	275.7 [1]	348 ± 15 [2]	
N (75%) C8 (25%)		136 ± 10 [2]	282 ± 19 [2]
N (75%) C18 (25%)		310 ± 39 [2]	479 ± 21 [2]

[1] Amino content quantified according to TNBS method [46]. [2] Amino content quantified according to CNHS method. Note: Column 1 lists the names of commercial magnetic nanoparticles (MNPs) as provided by the manufacturer. N, C8 and C18 indicate chemicals used for the synthesis of the MNPs, such as (3-aminopropyl)triethoxysilane, triethoxy(octyl)silane and n-octadecyltriethoxysilane, respectively.

Figure 6 shows the recovered activity of CLEAs prepared by co-aggregation with MNPs in a AMG/MNPs mass ratio of 1:1 [39]. As described above, using a glutaraldehyde concentration below 500 mM did not form CLEAs using only AMG, which was fully leached after resuspension in buffer or washing steps. However, using MNPs CLEAs were formed for all glutaraldehyde concentrations evaluated, despite the low recovered activity (less than 20%). The magnetic nanoparticle DCNP-N, containing 522 µmol of amino groups per gram, did give better results even at the lowest glutaraldehyde concentration; therefore, it was selected for further experiments.
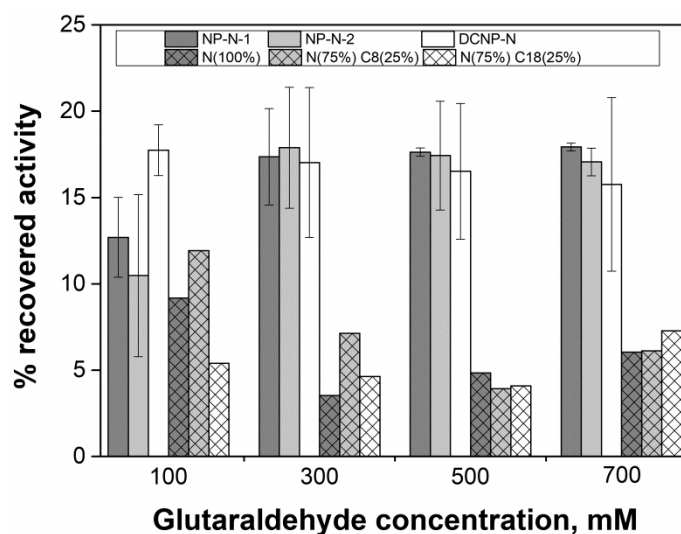
Figure 6. Effect of different commercial aminated magnetic nanoparticles (NP-N-1, NP-N-2, N(100%), N(75%)-C8(25%), N(75%)-C18(25%)) on the recovered activity of CLEAs of amyloglucosidase (AMG 300L). Synthesis conditions: enzyme/magnetic nanoparticle mass ratio of 1:1, ethanol/enzyme solution volume ratio of 9:1 (in 100 mM carbonate buffer pH 10.0), glutaraldehyde concentration ranging from 100 to 700 mM, precipitation and crosslinking under static conditions in an ice bath. Activity of AMG CLEAs was measured with 1% (*w/v*) dextrin in 50 mM sodium citrate buffer pH 4.5–10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of triplicate experiments ± s.d.

2.4. Evaluation of Glutaraldehyde Effect on Enzyme Activity

In order to investigate the probable deleterious effect of glutaraldehyde on the AMG, the activity of free AMG was measured after incubation of the enzyme with glutaraldehyde at different concentrations in the same conditions used in the preparation of CLEAs (16 h at 4 °C), but in the absence of ethanol. Starch was also added to the enzyme/glutaraldehyde solutions to evaluate whether the active site would be protected in the presence of a natural substrate. Table 2 shows that more than 75% of activity was lost when free AMG (without starch) was incubated in the presence of glutaraldehyde. On the other hand, when starch was added to the enzyme solution, slight protection was observed. The interaction of the starch with the amino acid residues at the active site could avoid distorting changes in the tertiary structure of AMG promoted by excessive cross-links with glutaraldehyde and/or could prevent the establishment of a covalent link between glutaraldehyde and the ε-amino group from the lysine residue located close to the active site. The increase of the starch concentration from 1% to 5% (*w/v*) did not cause an improvement in the residual activity, probably due to the high viscosity of the medium at low temperature (i.e., 4 °C), preventing the formation of a homogeneous mixture [43].

Table 2. Effect of glutaraldehyde on the free amyloglucosidase activity in the presence or absence of starch. Residual activity was measured after 16 h incubation at 4 °C and the initial activity was taken to be 100%.

Glutaraldehyde Concentration	Residual Activity (%)			
	Without Starch	Starch 1%	Starch 2.5%	Starch 5%
100 mM	34.46	48.12	43.18	36.37
300 mM	20.25	30.53	24.25	23.22
500 mM	19.48	29.63	26.80	24.40
700 mM	17.33	24.92	25.73	20.29

Figure 7 shows that the inactivation of AMG is very fast in the presence of glutaraldehyde, mainly at high glutaraldehyde concentrations. For short time periods and low glutaraldehyde concentrations, the residual activity is high, but these conditions do not favor the formation of stable CLEAs [28,47,48]. Thus, 16 h was kept as the glutaraldehyde treatment time in the preparation of CLEAs in the presence of 1% (*w/v*) starch as a pore-making agent. In the CLEA preparation (at 4 °C), the starch hydrolysis rate catalyzed by AMG is very low, thus preserving the starch molecules large enough to serve as pore-making agents.
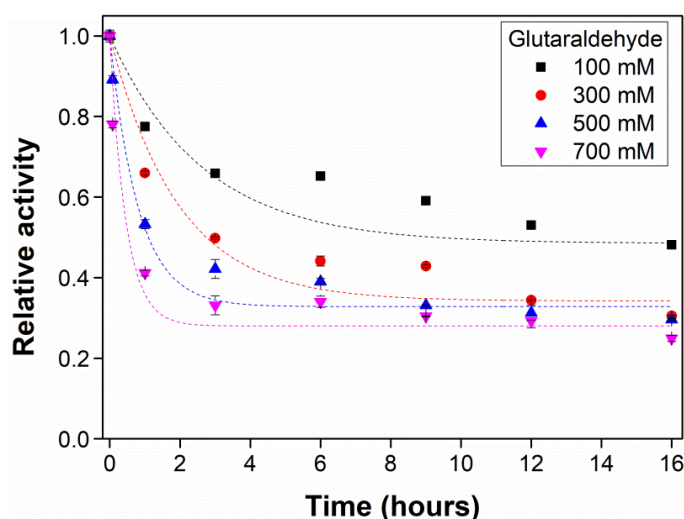

Figure 7. Profile of enzymatic inactivation as a function of glutaraldehyde concentration in the presence of 1% (*w/v*) starch. Assay conditions: water/enzyme solution volume ratio of 9:1 (in 100 mM carbonate buffer pH 10.0), glutaraldehyde concentration ranging from 100 to 700 mM, incubated statically in a refrigerator at 4 °C. Activity of amyloglucosidase was measured with 1% (*w/v*) dextrin in 50 mM sodium citrate buffer pH 4.5, 10 min reaction at 55 °C under 900 rpm stirring. Values correspond to a single assay with activity values measured in duplicate.

Table 3 shows that CLEAs prepared in the presence of 1% (*w/v*) starch (with and without the aminated magnetic nanoparticles DCNP-N) retained 2-3 times more activity, suggesting that the control of the pore size may play an important role in the enhanced activity during CLEA preparation. The combination of starch and DCNP-N yielded CLEAs with around 40% recovered activity using 500 mM glutaraldehyde. Although in the absence of DCNP-N the recovered activity was only a little lower (around 30%), the co-aggregation of AMG with DCNP-N has the advantage of easy separation of CLEAs without the formation of large clusters.

Table 3. Comparison of the effect of glutaraldehyde concentration on the recovered activity of CLEAs (without and with DCNP-N [a]) synthesized in the presence of 1% (*w/v*) starch. Synthesis conditions: 16 h precipitation/crosslinking under static conditions in an ice bath, mass ratio protein/DCNP-N of 1:1, volume ratio enzyme-DCNP-N suspension/ethanol of 1:9.

Glutaraldehyde Concentration	Recovered Activity (%)			
	Without Starch	Starch 1%	DCNP-N	DCNP-N+Starch 1%
100 mM	-	-	17.74 ± 1.47	18.72 ± 0.29
300 mM	-	-	17.03 ± 4.34	18.81 ± 0.31
500 mM	16.53 ± 0.64	33.53	16.52 ± 3.93	39.40 ± 1.44
700 mM	9.20 ± 0.11	30.28	15.76 ± 5.03	29.75 ± 1.15

[a] DCNP-N refer to magnetic nanoparticles functionalized with amino groups.

2.5. Study of the Co-Aggregation of AMG and Polyethyleneimine (PEI)

In order to improve the recovered activity of CLEAs of AMG, PEI was co-aggregated with the enzyme and the MNPs DCNP-N. Figure 8 shows that CLEAs could be formed even at a low glutaraldehyde concentration, yielding higher recovered activity compared to the CLEAs prepared without MNPs (Figure 5).
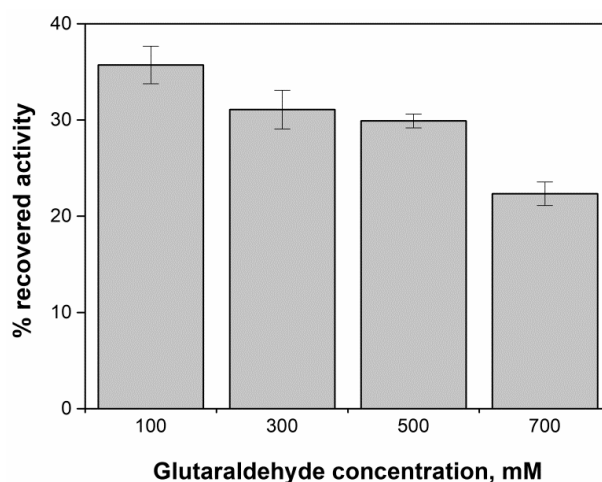

Figure 8. Effect of glutaraldehyde concentration on the recovered activity of CLEAs synthesized in the presence of polyethyleneimine (PEI). Synthesis conditions: PEI/enzyme mass ratio of 1:1 (30 min of gently stirring at 25 °C), addition of the magnetic nanoparticles DCNP-N to a DCNP-N/amyloglucosidase mass ratio of 1:1, precipitation with ethanol (volume ratio enzyme solution in 100 mM phosphate buffer pH 7.00 to ethanol of 1:9), crosslinking with glutaraldehyde concentrations ranging from 100 to 700 mM. Precipitation and crosslinking steps were performed under static conditions in an ice bath. Activity of immobilized amyloglucosidase was measured with 1% (*w/v*) dextrin in 50 mM sodium citrate buffer pH 4.5, 10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of duplicate experiments ± s.d.

Despite the improvement obtained with the addition of PEI, the recovered activity of magnetic CLEAs (maximum around 35% in Figure 8) were lower than that achieved using starch as protective additive (maximum around 40% in Table 3). Therefore, the synthesis of CLEAs of AMG co-aggregated with the magnetic nanoparticles DCNP-N in the presence of both PEI and 1% (*w/v*) starch was also evaluated.

Figure 9 shows that the presence of PEI and starch enabled the formation of CLEAs even at low concentrations of glutaraldehyde (12.5 mM). However, below 100 mM glutaraldehyde the immobilization yields were very low, probably because of insufficient cross-links to form stable structures [33,49]. On the other hand, when the glutaraldehyde concentrations ranged from 300 to 700 mM the immobilization yields were close to 100%, but the recovered activity was very low, probably due to the excessive crosslinking and/or deleterious effect of glutaraldehyde on the tertiary structure of AMG, as previously discussed. Therefore, a minimum concentration of 300 mM glutaraldehyde was selected for future experiments.
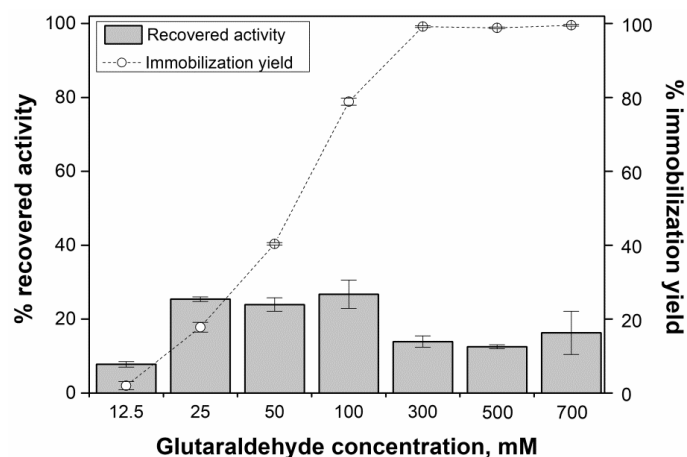
Figure 9. Effect of glutaraldehyde concentration on CLEA of amyloglucosidase (AMG) synthesized in the presence of polyethyleneimine (PEI) and co-aggregated with the magnetic nanoparticles DCNP-N and starch 1% (*w/v*). Synthesis conditions: treatment of AMG with PEI/enzyme mass ratio of 1:1 (30 min under gently stirring at 25 °C), co-aggregation with DCNP-N/enzyme mass ratio of 1:1, ethanol/enzyme solution (in 100 mM phosphate buffer pH 7.0) volume ratio of 9:1, crosslinking with glutaraldehyde concentrations ranging from 100 to 700 mM, precipitation and crosslinking under static conditions in an ice bath. Activity of immobilized AMG was measured with 1% (*w/v*) dextrin in 50 mM sodium citrate buffer pH 4.5, 10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of duplicate experiments ± s.d.

2.6. Influence of Agitation, Glutaraldehyde Treatment Time, Cross-Linker Concentration, and Co-Feeders in the Activity of AMG-CLEAs

It has been reported that at 4 °C, glutaraldehyde treatment time from 6 to 16 h is required to prepare stable CLEAs [28,47,48]. Thus, AMG CLEAs production was evaluated at 4 °C under stirring for 4 and 16 h, and under static conditions for 16 h.

Figure 10 shows that the glutaraldehyde treatment time was a key factor only for CLEAs prepared by co-aggregation with the magnetic nanoparticles DCNP-N and 1% (*w/v*) starch and crosslinking with 500 mM glutaraldehyde. Under these conditions, the immobilization yield increased from 45.3% to 70% and the recovered activity increased around twofold by increasing the time from 4 to 16 h. When PEI was added, no significant difference was observed. For the CLEAs prepared in the presence of PEI, the immobilization yields were higher than 95% for all conditions, but the recovered activity were higher when CLEAs were prepared under gently stirring in 3D Platform Shaker. The gentle stirring promoted the formation of homogeneous CLEAs, which visibly reduced the particle size, thereby probably minimizing mass transfer problems [20].

CLEAs previously prepared with 1% (*w/v*) starch, polyethyleneimine, and combinations thereof were synthesized again under stirring for 16 h at 4 °C; the co-aggregation with the aminated magnetic nanoparticles DCNP-N was compared with the most used co-feeder (bovine serum albumin), and two concentrations of glutaraldehyde (300 and 500 mM) were evaluated. These CLEAs were compared for their recovered activity as well as for their performance in the starch hydrolysis. The hydrolysis conditions were 55 °C under 300 rpm shaking for 6 h, using as a substrate 3% (*w/v*) starch prepared in 50 mM sodium citrate buffer pH 4.5 and pre-hydrolyzed with α-amylase. At the end of the reaction, the Dextrose Equivalent (DE) of the hydrolyzed starch and residual activity of the CLEA were measured.

Figure 11a shows that magnetic CLEAs prepared in the presence of starch (DCNP-N+Starch 1%) yielded the highest DE (83.2), but its residual activity was very low (3.5%). Although the recovered activity was high (around 40%), these CLEAs were not sufficiently stable in starch hydrolysis conditions, even using a co-feeder rich in amino groups and high glutaraldehyde concentration in their preparation. On the other hand, magnetic CLEAs prepared in the presence of polyethyleneimine were also active in the starch hydrolyses (DE above 60) and stable (residual activiy around 40% and 60% for 300 and

500 mM glutaraldehyde, respectively). The addition of 1% (*w/v*) starch in the preparation of these CLEAs was not advantageous from a stability point of view.
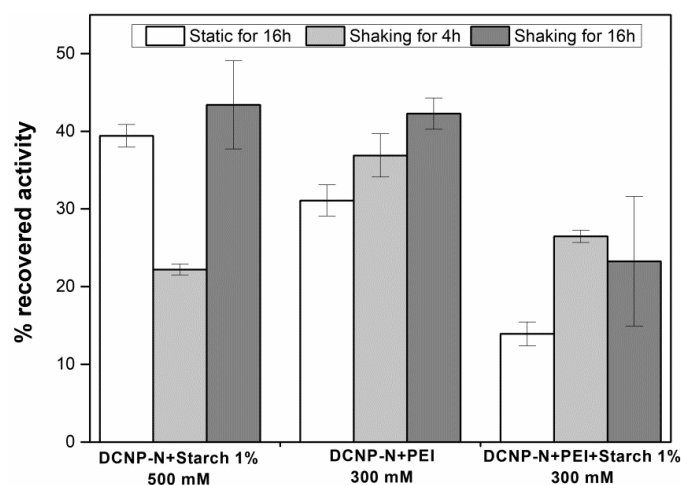

Figure 10. Comparison of the effect of agitation and crosslinking time on the recovered activity of CLEAs of AMG prepared as following: co-aggregation with the magnetic nanoparticles DCNP-N and 1% (*w/v*) starch and crosslinking with 500 mM glutaraldehyde (DCNP-N+Starch 1%-500 mM); co-aggregation with the magnetic nanoparticles DCNP-N and polyethyleneimine (PEI) and crosslinking with 300 mM glutaraldehyde (DCNP-N+PEI-300 mM); co-aggregation with the magnetic nanoparticles DCNP-N, 1% (*w/v*) starch and PEI and crosslinking with 300 mM glutaraldehyde (DCNP-N+PEI+Starch 1%-300 mM). All CLEAs were prepared at 4 °C, mass ratio protein/DCNP-N of 1:1, precipitation with ethanol at volume ratio 1:9 (enzyme solution:ethanol). Values are shown as the mean of duplicate experiments ± s.d.
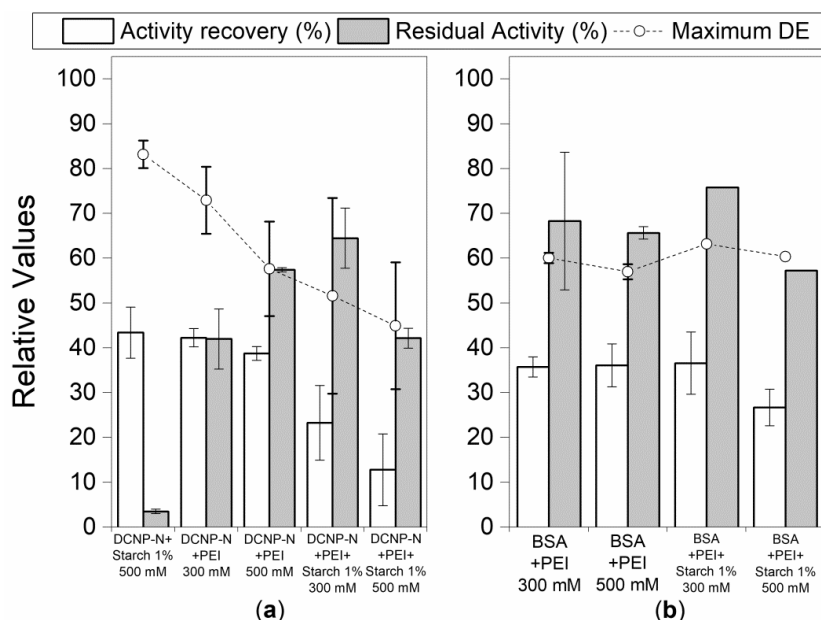

Figure 11. Recovered activity and performance of CLEAs of amyloglucosidase (AMG) in the hydrolysis of 3% (*w/v*) starch (Dextrose Equivalent and residual activity). CLEAs were prepared by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) in the presence of 1% (*w/v*) starch or polyethyleneimine (PEI) or both. A volume of 20 mL of starch (3%, *w/v*) solution was hydrolyzed by 20 U of AMG CLEAs at 55 °C and pH 4.5 for 6 h under 300 rpm stirring. Values are shown as the mean of duplicate experiments ± s.d.

CLEAs prepared under the same conditions, replacing magnetic nanoparticles with bovine serum albumin as the co-feeder, showed good performance (Figure 11b) regarding the recovered activity (around 35%), DE in the starch hydrolysis (around 60), and residual activity (above 60%). Although this co-feeder is widely used in the preparation of CLEAs [20,26,27,30,50], the replacement by magnetic nanoparticles is advantageous because of the ease of capture by an external magnetic field [40], avoiding the formation of clusters usually observed in the separation of CLEAs co-aggregated with bovine serum albumin by centrifugation [45]. Moreover, magnetic CLEAs prepared in the presence of polyethyleneimine and crosslinking with 500 mM glutaraldehyde (CLEA DCNP-N+PEI) showed similar performance (recovered activity around 40%, DE in the starch hydrolysis around 60, and residual activity around 60%), so were selected to be kinetically characterized and used in the hydrolysis of starch under industrial conditions. For comparison, CLEAs of AMG prepared by co-aggregation with bovine serum albumin and crosslinking with 500 mM glutaraldehyde (CLEA BSA+PEI) were also selected.

2.7. Characterization of the CLEAs of AMG

The CLEAs AMG+DCNP-N+PEI and AMG+BSA+PEI were characterized regarding the activity as a function of pH and temperature, thermal stability at 55 °C and pH 4.5, and their performance in the hydrolysis of starch under industrial conditions (high starch concentration, i.e., 35%, *w/v*).

Figure 12 shows that the activity profiles as a function of pH for CLEAs of AMG shifted the maximum activity from pH 4.5 to 3.0 compared to the free AMG. This can be associated with a higher stability of immobilized enzyme at this drastic pH.
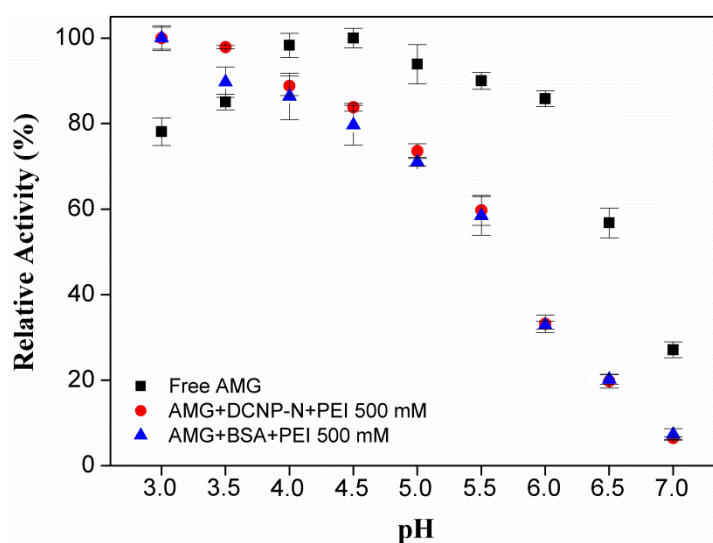
Figure 12. Effect of pH on the activity of free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA). Activity assay conditions: 1% (*w/v*) dextrin solution in 50 mM buffer, 10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of triplicate experiments ± s.d.

Figure 13 shows that the maximum activity of the CLEAs was lowered from 75 °C (for free AMG) to 65 °C. This suggests that the immobilized enzyme was less stable at this high temperature, or that the CLEA structure may change and in that way alter the diffusional problems. However, at 55 °C and pH 4.5 the CLEA activity corresponds to around 80% of the maximum activity, while for soluble AMG the activity at these conditions corresponds to around 50% of the maximum value. Thus, the CLEAs of AMG could be more advantageous from an industrial point of view, because the typical industrial conditions of the saccharification of starch are pH 4.0–4.5 and temperature 55–60 °C [51–53].
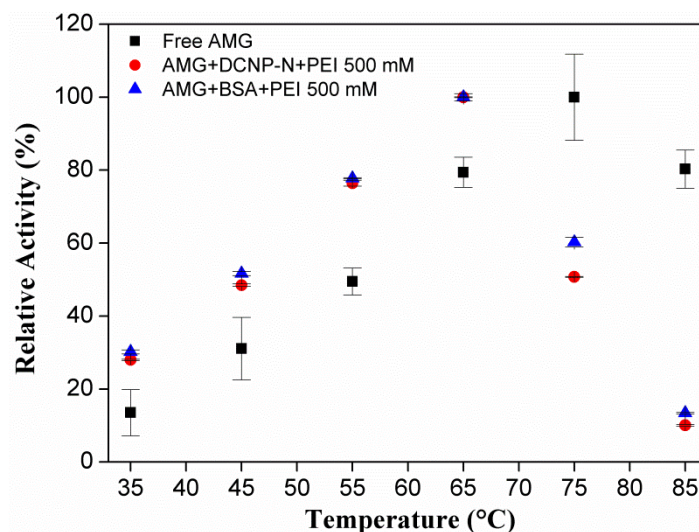
Figure 13. Effect of the temperature on the activity of free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA). Activity assays conditions: 1% (*w/v*) dextrin solution in 50 mM sodium citrate buffer pH 4.5, 10 min reaction under 900 rpm stirring. Values are shown as the mean of triplicate experiments ± s.d.

Figure 14 shows the thermal inactivation profiles of free (45 and 55 °C and pH 4.5) and immobilized AMG at these typical industrial conditions (55 °C and pH 4.5) [51]. Table 4 shows the parameters of the Sadana and Henley model [54] fitted to the experimental data. It can be observed that CLEAs of AMG (with both magnetic nanoparticles and BSA as co-feeders) were around 4 times more stable than the free AMG at 55 °C, having a fraction of immobilized molecules more resistant to the inactivation as indicated by the parameter α (0.36–0.46 for CLEAs and 0.23 for free AMG). This higher stability is indicative of effective crosslinking with glutaraldehyde, aided by polyethyleneimine in the complex and rigid structure of the CLEAs (enzyme and co-feeders). It has been reported that the immobilization tends to restrict the conformational flexibility of the enzyme, which prevents conformational changes [11,38,55].
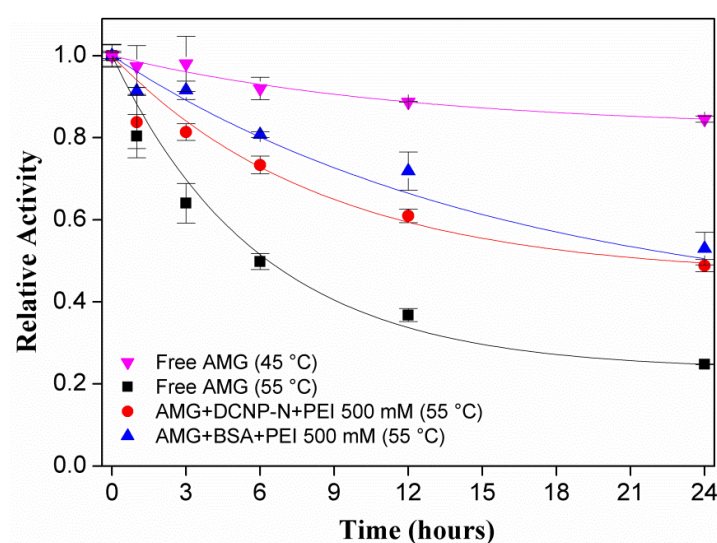

Figure 14. Profile of thermal inactivation of free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) at 45 and 55 °C and pH 4.5 (50 mM sodium citrate buffer). Values are shown as the mean of triplicate experiments ± s.d.

Table 4. Half-life times ($t_{1/2}$) and stability factor (SF) for amyloglucosidase (free and immobilized AMG) at 55 °C and pH 4.5.

Biocatalyst	$t_{1/2}$ (h)	SF	k_d	α	Adj. R^2
Free AMG	6.34	1.00	0.167 ± 0.014	0.234 ± 0.005	0.99
AMG+DCNP-N+PEI 500 mM	22.67	3.58	0.117 ± 0.016	0.462 ± 0.027	0.98
AMG+BSA+PEI 500 mM	24.60	3.88	0.062 ± 0.019	0.361 ± 0.119	0.95

Note: SF is the ratio between the half-life of the immobilized enzyme and of the free enzyme; the inactivation parameters (k_d and α) were estimated using the Sadana and Henley model [54]; DCNP-N is magnetic nanoparticles functionalized with amino groups; PEI is polyethyleneimine; BSA is bovine serum albumin. All CLEAs were prepared by crosslinking with 500 mM glutaraldehyde.

Figure 15 shows the profiles of starch hydrolysis catalyzed by free and immobilized AMG at typical industrial conditions (35% starch solution and 2 mL of soluble AMG/kg of starch). When CLEA was used as the catalyst, the amount of CLEA was equivalent to 2 mL of soluble AMG in terms of activity. Because at 45 °C the free AMG is highly stable (more than 80% residual activity after 24 h incubation), starch was also hydrolyzed at 45 °C using free AMG, maintaining the other conditions.
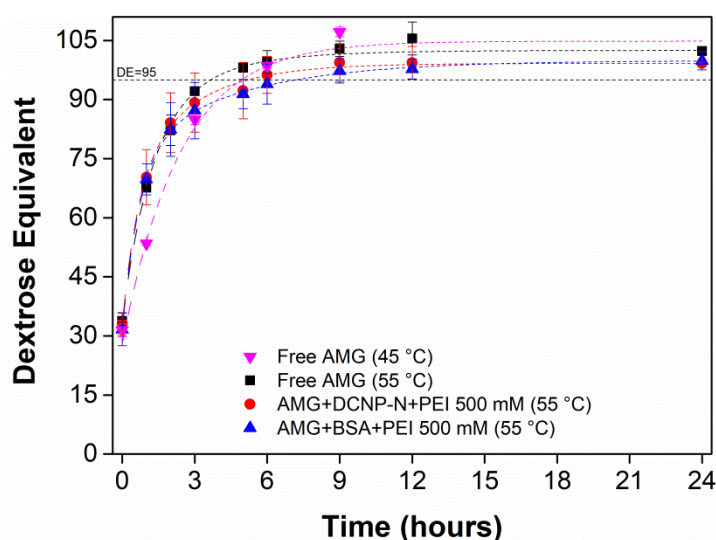

Figure 15. Profile of hydrolysis of 35% (*w/v*) starch (pre-hydrolyzed with α-amylase) catalyzed by free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) at 45 and 55 °C and pH 4.5 under 900 rpm stirring. Amount of AMG: 2 mL of free enzyme/kg of starch (37,820 U/kg of starch) or equivalent units to the CLEAs. The reaction conversion (starch-to-glucose) was monitored by Dextrose Equivalent (DE) measuring reducing sugars by DNS method. Values are shown as the mean of duplicate experiments ± s.d.

It can be observed that the hydrolysis profiles for all biocatalysts (free and immobilized enzymes) were very closed, achieving a Dextrose Equivalent (DE) around 95 after a 6-h reaction, even at 45 °C using free AMG, which shows that the enzyme load is high enough to guarantee high hydrolysis rates. The increase in DE for larger reaction times is very low, not justifying longer hydrolysis because the productivity of the process drops a lot (in $g_{glucose}$ $L^{-1} \cdot h^{-1}$): 6.6 for 6 h, 2.7 for 9 h, and 1.35 for 12 h. Maximum conversions of starch hydrolysis by AMG ranging from 90 to 98% [6] had been previously reported, which shows the excellent performance of CLEAs of AMG prepared in this work.

2.8. Reuse Assays

Because of the similar behavior of hydrolysis at 45 and 55 °C, achieving a DE around 95 after 6 h, the reuse assays were performed at 45 °C because of the high stability of AMG. Figure 16 shows that after five 6-h batches, DE is maintained around 85% using both biocatalysts (CLEAs of AMG prepared

with BSA or MNP in presence of PEI). Handling and recovery of the CLEA were easy when MNP was used applying an external magnetic field (Figure 17).
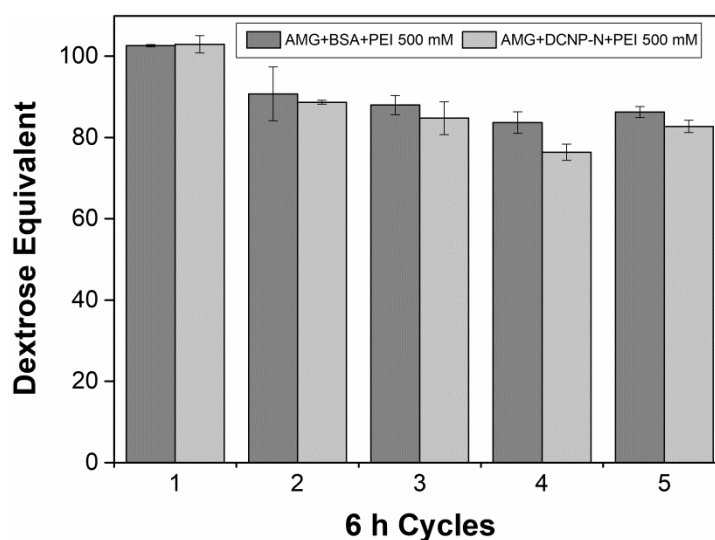

Figure 16. Hydrolysis of pre-hydrolyzed starch with α-amylase (35%, *w/v*) catalyzed by CLEAs of amyloglucosidase (AMG) prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) at 45 °C and pH 4.5 for 6 h reaction. Values are shown as the mean of duplicate experiments ± s.d.
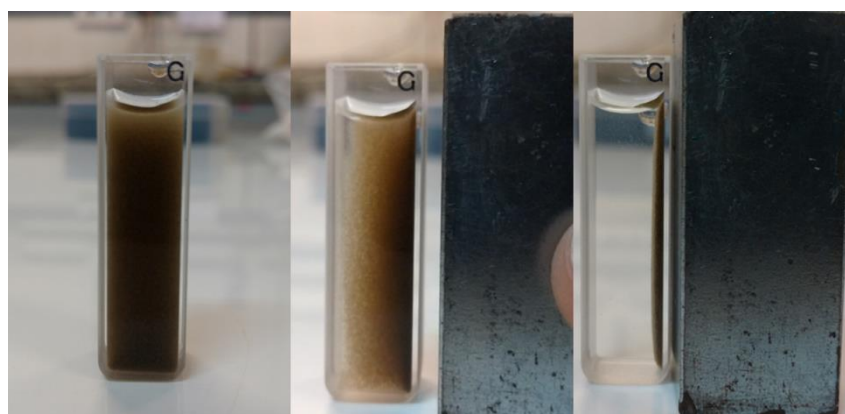

Figure 17. Separation of the magnetic CLEAs of amyloglucosidase by the action of an external magnetic field.

3. Materials and Methods

3.1. Materials

AMG 300L from *Aspergillus niger* (EC 3.2.1.3; glucoamylase; amyloglucosidase) and α-amylase BAN 480L were from Novozymes A/S (Bagsværd, Denmark), dextrin 10, bovine serum albumin (BSA), and polyethylenimine (oligomer mixture, Mn ~423) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Soluble starch and glutaraldehyde 25% (*v/v*) aqueous solution from Vetec (Duque de Caxias, RJ, Brazil). Mono reagent colorimetric enzymatic (GOD-POD) obtained from Gold Analisa (Belo Horizonte, MG, Brazil). The magnetic nanoparticles were purchased from Kopp Technologies (São Carlos, SP, Brazil). All other chemicals (analytical grade) were purchased from Synth (Diadema, SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil).

3.2. Precipitant Screening

The precipitation of amyloglucosidase was carried out by adding the protein precipitant (acetone, ethanol, isopropanol or saturated ammonium sulfate solution) to an enzyme solution (protein concentration of 20 mg·mL^{-1} in 50 mM carbonate buffer pH 4.5) in a volume ratio of 9:1. The mixture was incubated in shaker under 150 rpm at 4 °C for 60 min, the precipitate was recovered by centrifugation (1500× *g* at 4 °C for 5 min) and resuspended in 1 mL of 50 mM sodium citrate buffer pH 4.5. Enzyme activity and protein content were measured in the supernatant and in the re-suspended precipitate as described in Sections 3.7 and 3.8, respectively. For the precipitant chosen based on the highest yields of activity and protein, the volume ratio of precipitant and enzyme solution was also evaluated, but at static conditions in an ice bath for 30 min. Within the volume ratios evaluated, the better one was chosen for further assays.

3.3. General Crosslinking Procedure Using Glutaraldehyde

Ethanol was added to an enzyme solution prepared in 100 mM carbonate buffer pH 10.0 to a volume ratio of 1:9 (enzyme/ethanol). After 30 min precipitation in an ice bath, volumes of glutaraldehyde 25% (*v/v*) to final concentrations of 100, 300, 500 and 700 mM were added to the precipitated enzyme (crosslinking step). The aggregate suspension was homogenized and incubated statically in a refrigerator at 4 °C for 16 h or under gently stirring in a three-dimensional laboratory agitator (KASVI, K45-4020) at 4 °C for 4 or 16 h. After the incubation period, the suspension was centrifuged (1500× *g* for 5 min at 4 °C) and the precipitate was washed twice with 50 mM sodium citrate buffer pH 4.5, and finally re-suspended in the same buffer. Then, the activity recovery was calculated by Equation (1):

$$Activity\ recovery\ (\%) = \frac{Total\ activity\ in\ the\ CLEA\ suspension}{Total\ activity\ offered\ initially} \times 100. \tag{1}$$

The same procedure described above was used for evaluation of co-aggregation of AMG with magnetic nanoparticles (MNPs) (characteristics described in Table 1) or bovine serum albumin (BSA) as additive or co-feeder, respectively. In this case, the MNPs or BSA was added to the enzyme solution (in 100 mM carbonate buffer pH 10) to a final protein/MNPs (or BSA) mass ratio of 1:1 (20 mg total mL^{-1}). When BSA was used, the CLEAs were separated by centrifugation (1500× *g* for 5 min at 4 °C); on the other hand, when MNPs were used, the CLEAs were separated by applying an external magnetic field.

Starch was also evaluated as a protector additive of the enzyme active site. In this case, a 1% (*w/v*) starch solution was prepared in a 100 mM carbonate buffer pH 10 and was used to prepare the mixture of enzyme and other additives in the CLEA preparation. The other steps remained unaltered.

Polyethyleneimine (PEI) was also evaluated as a crosslinking aid. In this case, an aqueous solution of PEI was prepared and the pH was adjusted to 7.0. This solution was mixed with the enzyme solution (protein concentration of 20 mg mL^{-1} in 100 mM sodium phosphate buffer pH 7.0) to a protein/PEI mass ratio of 1:1, as described by López-Gallego et al. [34]. After 30 min stirring in a tridimensional laboratory agitator at room temperature, the precipitant was added, and the protocol followed the procedure described above.

3.4. Characterization of the Biocatalysts

The activity of soluble and immobilized AMG was measured as a function of the pH (at 55 °C) in the range from 3.0 to 7.0, and as a function of the temperature (at pH 4.5) in the range from 35 to 85 °C. For pH values from 3.0 to 5.5, 50 mM sodium citrate and acetate buffers were used, and for pH values from 5.5 to 7.0 a 50 mM sodium phosphate buffer was used.

Thermal inactivation assays were carried out at 55 °C and pH 4.5 (50 mM sodium citrate buffer). Enzyme activity was measured at regular time intervals until 24 h. The model of Sadana and

Henley [54] was fitted to the experimental data to determine the half-life. The stabilization factor (SF) was calculated as the ratio between the half-life of the immobilized enzyme and that of the free enzyme.

3.5. Hydrolysis of Starch

A solution of starch (35%, *w/v*) was prepared in 50 mM citrate buffer (pH 4.5). The starch was pre-hydrolyzed with α-amylase (BAN 480L, 3 mL/kg starch) at 60 °C for 20 min. The temperature was fitted to 55 °C and soluble AMG (2 mL/kg starch) or CLEAs of AMG (37,820 U/kg starch, equivalent to the amount of soluble enzyme) was added. The reaction was carried out for 24 h in a batch reactor under 900 rpm stirring using a cylindrical impeller without blades to prevent loss of biocatalyst by shearing. Samples of the reaction medium were withdrawn at regular time intervals to measure reducing sugars by the DNS method [56]. Dextrose Equivalent (DE) [57] was calculated by Equation (2) to construct the hydrolysis time profile.

$$Dextrose\ Equivalent = \frac{Amount\ of\ reducing\ sugar\ (expressed\ as\ glucose)}{Starch\ dry\ mass} \times 100 \qquad (2)$$

In the selection of CLEAs, their performance in the hydrolysis of starch was evaluated using 3% (*w/v*) starch. The hydrolysis reactions were performed at 55 °C and pH 4.5 for 6 h under 300 rpm stirring. Reducing sugars and Dextrose Equivalent were determined as described above, only at the final stage of the reaction.

3.6. Reuse Assays

The reusability assays of amyloglucosidase CLEA were performed at 45 °C under 300 rpm shaking with a solution of pre-hydrolyzed starch (35%, *w/v*) prepared in 50 mM citrate buffer pH 4.5. After each cycle of 6 h reaction time, the conversion of starch (DE) was determined and the CLEA was recovered by applying an external magnetic field and/or by centrifugation ($1500\times g$ for 5 min at 4 °C), washed with citrate buffer (50 mM, pH 4.5) and re-suspended in a fresh substrate solution.

3.7. Enzymatic Activity Assay

Enzymatic activity of amyloglucosidase (free and immobilized as CLEAs) was determined by calculating the initial velocity of glucose formation catalyzed by a known amount of enzyme. The standard substrate was dextrin 1% (*w/v*, in 50 mM sodium citrate buffer at pH 4.5). The reaction was carried out at 55 °C for 10 min under 900 rpm stirring. Samples were withdrawn every 2.5 min, the reaction was quenched with 1 M HCl, and glucose was measured as described in Section 3.8. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of glucose per minute under the conditions above.

3.8. Determination of Glucose and Protein Concentration

Glucose was determined spectrophotometrically at 505 nm by glucose oxidase and peroxidase colorimetric enzymatic test (GOD-POD). The GOD-POP solution (1 mL) was added to the sample (10 μL) and incubated at 37 °C for 10 min [58]. The concentration of glucose was quantified using a glucose concentration vs. absorbance curve constructed with glucose as standard.

The protein content was determined spectrophotometrically at 595 nm by the Bradford method, using bovine serum albumin as the standard protein [59].

3.9. SDS-PAGE Electrophoresis

The commercial AMG was characterized by polyacrylamide gel electrophoresis (10% SDS-PAGE) using Coomassie Brilliant Blue for staining [60].

4. Conclusions

This study showed that promising CLEAs of amyloglucosidase could be synthesized by co-aggregation with aminated magnetic nanoparticles (MNPs) or bovine serum albumin (BSA), using polyethyleneimine as an aid in the crosslinking step with glutaraldehyde. Both CLEAs showed around 40% of the offered activity, thermal stability approximately 4 times higher than the soluble enzyme, and small changes in the catalytic properties. Moreover, the starch saccharification at typical industrial conditions, i.e., 35% (*w/v*) pre-hydrolyzed starch with α-amylase, 55 °C and pH 4.5, catalyzed by the CLEAs (co-aggregated with MNPs or BSA) showed similar behavior to the soluble enzyme, yielding a Dextrose Equivalent around 95 after a 6-h reaction. In addition, both CLEAS could be reused in five 6-h cycles at 45 °C and maintain a DE above 85. These findings could be attractive to the amylolytic industry because highly concentrated starch solutions may be processed by immobilized AMG as well as the soluble enzyme (including 10 °C below the conventionally used temperature), having the additional advantage of being easily separated from the reaction medium and reused in the process when MNPs are used instead of the protein co-feeders commonly used in the CLEA methodology, such as BSA, whose cost is too high for the synthesis of CLEAs for large-scale applications.

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