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Enhancing the synthetic utility of aldolase antibody 38C2

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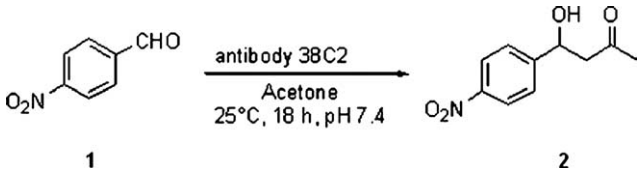
Abstract—Three-phase partitioning (TPP) treated aldolase antibody 38C2 was evaluated for aldol reaction between *p*-nitrobenzaldehyde and acetone to give 4-(4′-nitrophenyl)-4-hydroxy-2-butanone. While TPP-treated 38C2 transformed 65% of *p*-nitrobenzaldehyde, the untreated 38C2 gave only 24% transformation in 18 h at 25 °C. However, since TPP-treated 38C2 also gave an additional (unidentified) product, its synthetic utility was limited. Crosslinked aggregate of 38C2, however, gave the biocatalyst which gave a single product and could be reused at 40 °C five times without loss of activity.

The aldolase antibody 38C2 is among the first antibody preparation which is commercially available and has already proved its usefulness in catalyzing self aldol,[1] crossed aldol,[1] retro aldol,[2] Robinson annulation reactions,[3] and decarboxylation of β-ketoacids.[4] Its conjugate with a water-soluble polymer has been used for prodrug activation for selective chemotherapy for cancer.[5] Earlier, an organoinsulin molecule and etopside derivative have been reported as prodrugs for diabetes and cancer chemotherapy, respectively.[6,7] The antibody 38C2 is reported to have 40 times less catalytic efficiency as compared to the aldolase.[8] It is also an expensive biocatalyst. The present work is aimed at enhancing its catalytic efficiency and reusability.

The aldol reaction used to monitor the catalytic efficiency of 38C2 aldolase antibody was the reaction between *p*-nitrobenzaldehyde (**1**) and acetone (Scheme 1).[1] HPLC was used to estimate the disappearance of *p*-nitrobenzaldehyde.[1,10]

The first approach attempted was that of three-phase partitioning (TPP). It has been observed that when a pure enzyme is subjected to TPP, the treated enzyme shows enhancement of the catalytic efficiency.[11–15] The conditions for subjecting 38C2 aldolase antibody were optimized for obtaining the maximum amount of protein in the interfacial layer. The parameters varied were protein



Scheme 1. Synthesis of 4-(4′-nitrophenyl)-4-hydroxy-2-butanone by aldol reaction between *p*-nitrobenzaldehyde and acetone.

concentration, ammonium sulfate concentration, and the volume of *t*-butanol added to 200 μL protein solution. The optimized conditions are as described.[16] Under these conditions, 82% of the starting protein was found in the interfacial layer in the precipitate form.

The time course of the reaction with TPP-treated 38C2 (Fig. 2, curve B) indicates that it shows a higher catalytic conversion as compared to untreated 38C2 (Fig. 2, curve A). Whereas untreated 38C2 gave 24% conversion in 18 h, the TPP-treated 38C2 gave 65% conversion in the same time.

The nature of the product(s) formed is also different in the case of TPP-treated 38C2 (Fig. 1). Apart from the expected aldol product (**2**, Scheme 1)[17] with a retention time of 6.62 min, the TPP-treated 38C2 also gave an additional product (retention time, 7.74 min) (Fig. 1). However, while the enhancement in catalytic efficiency of 38C2 upon TPP-treatment is in agreement with earlier results with other enzymes such as α-chymotrypsin,[14] proteinase K,[12] and lipase,[16] the formation of an additional product indicated that TPP-treated biocatalysts may function in a different way mechanistically. The

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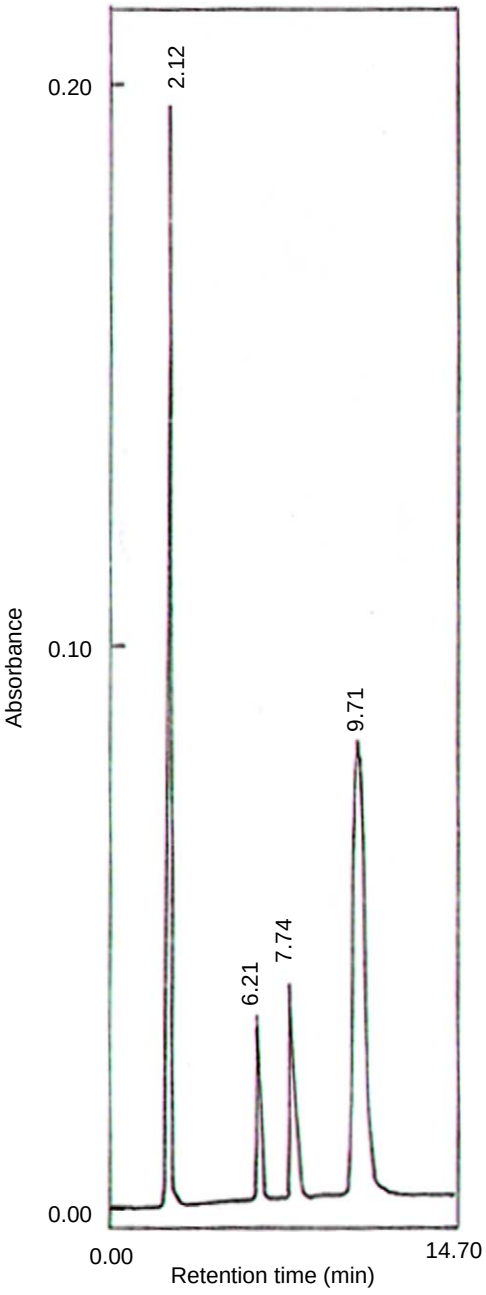

Figure 1. HPLC chromatogram of aldol condensation between *p*-nitro benzaldehyde (t_R = 9.71 min) and acetone (t_R = 2.12 min) with TPP-treated aldolase antibody 38C2 resulting in the formation of the usual aldol product (t_R = 6.21 min) and a new product (t_R = 7.74 min).

above catalytic efficiency was based upon an assay which measures depletion of the substrate. The formation of second product in significant amount implies that product yield of desirable aldol would be considerably lower (see Fig. 2). In that respect, the TPP did not yield the desired biocatalyst with better synthetic utility. A recent biocatalyst design is crosslinked enzyme aggregates (CLEA) which are based upon crosslinking of a protein which is initially precipitated by adding an organic solvent or a salt.[18,19] After screening some precipitating agents (data not shown), precipitation with ammonium sulfate was found to work best. Ammonium

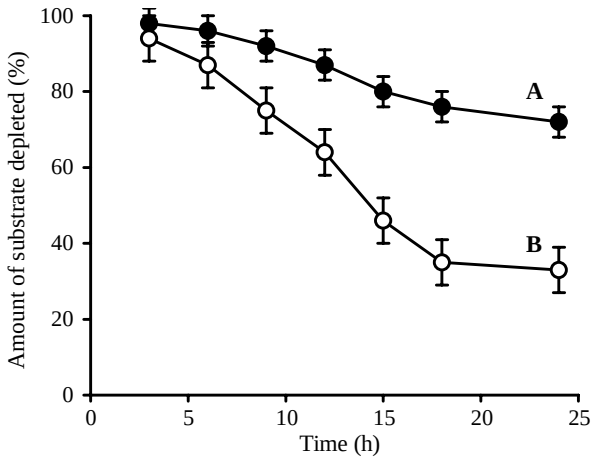

Figure 2. Time course of TPP-treated antibody reaction. The aldol reaction between *p*-nitrobenzaldehyde and acetone was carried out using untreated antibody (●) and TPP-treated antibody (○) as described.[16] Samples were withdrawn at different time intervals and the amount of *p*-nitrobenzaldehyde (substrate) used in the reaction was monitored through HPLC at 254 nm. The experiment was done in duplicate and the error bars show the variation in the readings. The observed standard deviation in each set of readings was less than 0.2%.

sulfate (80% w/v) precipitated 95% protein which retained 94% aldolase activity. Under optimum conditions, 40 mM glutaraldehyde formed CLEA which showed 120% activity.[20] Marginal increase (in this range) in the activity after CLEA formation has been reported by earlier workers as well.[19] CLEA gave only one product and that was the expected aldol product

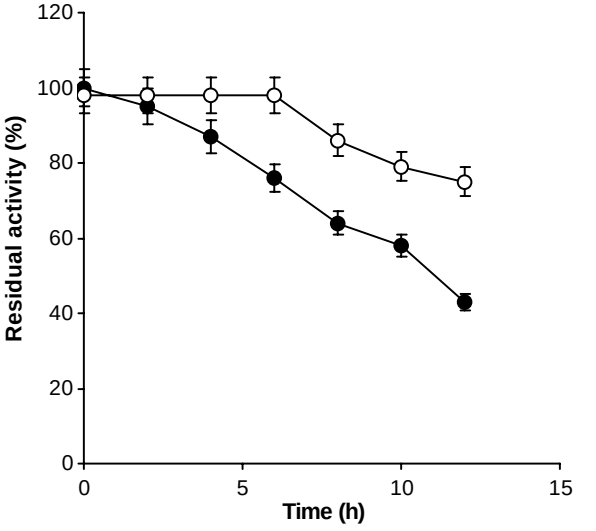

Figure 3. Thermal stability of antibody 38C2 and CLEA of 38C2 at 50 °C. Catalytic antibody 38C2 (100 μg/100 μL) samples (●) were incubated at 50 °C for different periods of time. Thereafter, the sample was cooled to 25 °C and assayed for its activity with *p*-nitrobenzaldehyde (ref). The experiment was repeated with CLEA of 38C2 (○). Residual activity was calculated by taking the activity of 38C2 at 0 time to be 100%. The experiment was done in duplicate and the error bars show the variation in the readings. The observed standard deviation in each set of readings was less than 0.1%.

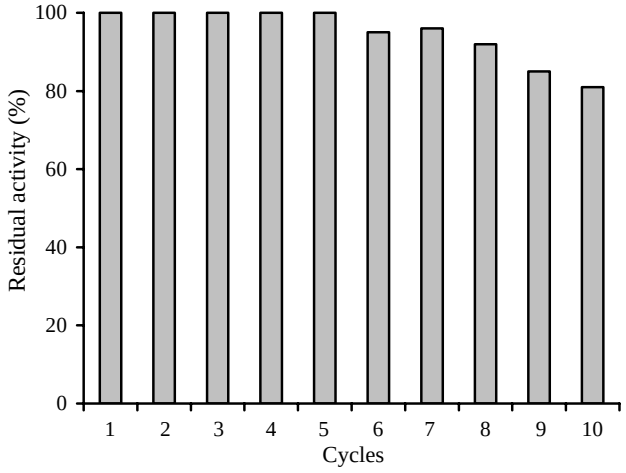

Figure 4. Reusability of CLEA of 38C2. The aldol reaction with CLEA was carried out at 40 °C for 10 h. Thereafter the reaction mixture was removed, CLEA washed with 1 mL buffer and fresh reactants added. After the 5th cycle, loss of protein (5 μg) was detected. After the 10th cycle, the washings were pooled and the amount of protein that leached out was found to be 15 μg (as estimated by the Bradford method[9]). Residual activity of CLEA of 38C2 was calculated by taking the activity of 38C2 in the first cycle as 100%.

(as checked by the HPLC assay). Investigating thermal stability of 38C2 and CLEA of 38C2 showed that CLEA had a higher thermal stability (Fig. 3). At 50 °C, CLEA retained 100% activity, while 38C2 had only 75% activity after 5 h of thermal exposure. However, CLEA also lost 20% activity after 10 h. At 40 °C, both 38C2 and its CLEA retained complete activity even after 12 h. While at 25 °C, free 38C2 gave 10% conversion and CLEA gave 13% conversion in 10 h, at 40 °C the corresponding conversion figures were 33% and 38%, respectively (conversion in the range of 30% has been reported during synthetic application of 38C2).[21] Hence, 40 °C was chosen to evaluate reusability of CLEA. Figure 4 shows the real advantage of working with CLEA. CLEA of 38C2 could be used up to 5 cycles without losing any aldolase activity. Even after 10 cycles, it lost only 20% activity. It may be mentioned that very few immobilized catalytic antibodies have been described in the literature.[22,23] Significant loss in biological activity of catalytic antibody has also been reported during immobilization.[23] Considering the expensive nature of 38C2, CLEA of 38C2 offers an efficient approach for the synthetic application of this versatile biocatalyst.

Acknowledgments

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10. Murine aldolase antibody 38C2 was purchased from Sigma Chemicals Co. (Cat. No. 479950). *p*-Nitro benzaldehyde was recrystallized from an ethanol–water mixture (1:1, v/v) for further use. Acetone was dried over anhydrous $CaSO_4$, distilled, and further dried over activated molecular sieves before use. Diisopropylamine was distilled over sodium hydride just before use. All other solvents were freshly distilled before use. All antibody catalyzed reactions were carried out in buffered saline (0.01 M phosphate, 0.15 M NaCl, pH 7.4) and monitored by high performance liquid chromatography (HPLC) (Beckmann System Gold HPLC) using a reverse-phase C18 column (150 mm × 4.6 mm). The eluent was 25% acetonitrile in water containing trifluoroacetic acid (0.1%) with a flow rate of 1.0 mL/min. The peaks were detected at 254 nm[1].
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16. Catalytic antibody 38C2 solutions (200 μL, 10 mg/mL in buffered saline) were mixed with 30% saturated ammonium sulfate (w/v). This was followed by the addition of *t*-butanol (200 μL). The solution was gently vortexed and incubated at 25 °C for 30 min. Three phases (i.e., upper layer of *t*-butanol, interfacial precipitate of protein, and lower aqueous layer) were formed. The mixture was then centrifuged at 1800*g* for 5 min. This low-speed centrifugation gave a compact interfacial precipitate and clearly separated the lower aqueous and upper organic layers. The aqueous layer was pipetted out using a Pasteur pipette, after piercing the precipitate layer. The *t*-butanol layer was similarly removed. The precipitate was then dissolved in phosphate-buffered saline so that the total volume was 200 μL. The dissolved precipitates were desalted on a prewashed (with buffered saline) PD10 column (prepacked Sephadex G25) to remove ammonium sulfate

and *t*-butanol. The absorbance of the fractions collected (1 mL each) was read at 280 nm. The fractions containing the protein were pooled and concentrated (against sucrose) to 1 mL. 82% of the starting protein was found to partition to the interfacial layer during TPP as estimated by Bradford's method.[9] To this solution of 38C2, *p*-nitrobenzaldehyde (final concentration 1.04 mM) and acetone (final concentration 0.12 M) were added and kept in an incubator-shaker at 25 °C for different time periods. Thereafter, the reaction was monitored by HPLC at 254 nm. A similar reaction was carried out with untreated-catalytic antibody. The amount of product formed was calculated by taking the substrate concentration at 0 h as 100% and subtracting the amount of substrate that was used up at the given time.

17. Compound **2**. This compound (aldol product) was chemically synthesized as per the method described.[1] HPLC showed the same retention time (6.62 min) as the aldol product obtained in the antibody reaction. Also, upon coinjection of the reaction mixture with an aliquot of the chemically synthesized aldol, the peak for the aldol product was found to be overlapped, thus proving that the chemically synthesized product was the same as in the aldol mixture.
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20. Procedure for preparation of CLEA of 38C2: Catalytic antibody 38C2 solution (100 μg in 100 μL of 0.01 M phosphate buffer, pH 7.4) was added to 2 mg bovine serum albumin (BSA) and the volume made up to 200 μL. Ammonium sulfate (80%, w/v) was then added to precipitate the protein and incubated at 4 °C for 30 min for complete precipitation. Glutaraldehyde (40 mM) solution was then added to the precipitated protein solution and the reaction mixture was incubated at 25 °C for 3 h. The aggregates formed were washed thrice with 1 mL PBS to remove unreacted glutaraldehyde. The CLEA of antibody 38C2 thus formed were used for further reactions.
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