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Cross-linked Enzyme Crystals of Fructose Diphosphate Aldolase: Development as a Biocatalyst for Synthesis

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Abstract: *Crystals of rabbit muscle aldolase were grown from 45% saturated ammonium sulfate solutions and cross-linked with glutaraldehyde to produce a biocatalyst that has stability for months, can be reisolated and reused, and has excellent solvent stability. This catalyst showed excellent reactivity with non-natural substrates.*

The use of enzymes has emerged as a complementary tool to synthetic strategies using chiral auxillaries. In synthetic studies toward the synthesis of tagetitoxin,[1] we have developed a scheme using fructose diphosphate aldolase (FDP) in the key carbon-carbon bond forming reaction. FDP has proven to be a very effective synthetic tool but it and enzymes in general have several limitations including water insolubility of unnatural substrates, cost and enzyme instability. We report here the development of a superior FDP aldolase biocatalyst consisting of cross-linked enzyme crystals. This aldolase can be stored as a powder at room temperature for months, has excellent solvent and thermal stability, is stable to proteolytic enzymes, and can be reisolated for reuse. In general, this form of rabbit muscle FDP aldolase (RAMA) has greater stability as a chemical reagent, and we think this new biocatalyst will extend the use of RAMA to an even broader range of targets.[2]

Richards and coworkers showed in 1964 that treatment of carboxypeptidase-A with glutaraldehyde had little effect on the diffraction pattern but increased enzyme stability.[3] These cross-linked enzyme crystals (CLC) retained only 30% of their original activity. This was the first study of many to show that glutaraldehyde would insolubilize enzymes without destroying their activity while causing a remarkable increase in their stability under unnatural conditions.

In our studies, the monoclinic crystalline form of aldolase was grown in small test tubes from 45% saturated ammonium sulfate solutions at 22°C.[4] Crystals appeared within a few days and crystallization was complete after 2 weeks. One drawback to immobilization techniques can be loss of enzyme activity; in order to maximize the amount of activity retained upon cross-linking, we examined the effect of varying the concentration of glutaraldehyde (**Figure 1**). In each case 2.5 mg of pulverized RAMA crystals are placed in 1 mL of 0.5 mM triethanolamine buffer with varying amounts of 25% aqueous glutaraldehyde, which had been purified and stored at -20°C to minimize polymerization (as seen by λ_{max} at 235 nm). The cross-linking was carried out by shaking the solutions at 0°C for one hour followed by lyophiliziation. The CLC enzymes were assayed with the natural substrate 1,6-fructose bisphosphate and compared by two methods: the faster coupled assay[5] showed inhibition by any residual ammonium sulfate in the crystals while a hydrazine assay[4] avoided this problem but was much less sensitive. We determined that cross-linking the crystals at a 10 mM glutaraldehyde concentration was optimal. The product crystals are centrifuged and washed successively with

water and lyophilized. These conditions produced a biocatalyst that is microcrystalline, has excellent activity, and does not discolor with time, unlike the amorphous solid that was formed with phosphate buffer.[6] No

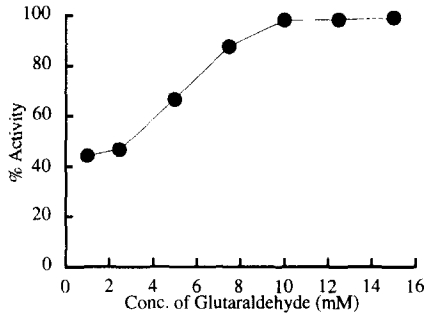


Figure 1. Percent of activity of CLCs of aldolase as a function increasing concentration the glutaraldehyde

reaction was observed when glutaraldehyde was stirred overnight with TEA buffer in the absence of enzyme. We speculate that the triethanolamine is acting as a catalyst for the 1,4-addition of the enzyme amines to the α,β–unsaturated aldehyde of the polymerized glutaraldehyde and/or for reversible formation of aldimines;[7b] use of amine salts has been shown to greatly improve the formation of adducts with α,β–unsaturated aldehydes.[7c]

The cross-linked enzyme crystals (CLC) have remarkable stability. **Figure 2** shows a comparison of dilute (0.2 mg/mL) and concentrated (10 mg/mL) solutions of RAMA and FDP aldolase from *E. coli*, and the cross-linked crystals of RAMA. We included the readily available *E. coli* FDP aldolase from an overexpression

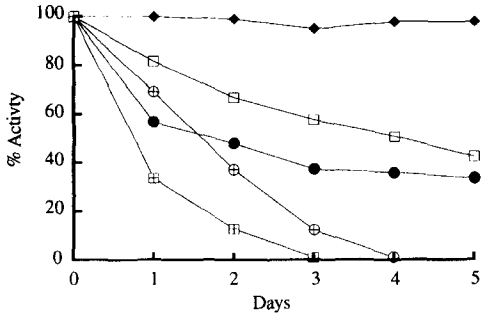


Figure 2. Effect of cross-linking on stability of aldolase

system but found it to be less stable than the commercially available RAMA. The soluble aldolases at high concentration (10 mg/mL) lose the majority of their activity after five days while dilute solutions (0.2 mg/mL) are completely denatured after three days. In contrast, the original CLCs of aldolase showed no change in activity after 6 months of sitting at room temperature (data not shown).

Many immobilization techniques cause a dramatic reduction of activity but cross-linking of the RAMA crystals gave a catalyst that still had 82% activity compared with the soluble crystals and 65% of the original soluble RAMA (20% activity is lost during crystalization). This is significantly higher than the 10% activity retention in the original carboxypeptidase A studies[3] but compares closely with more recent examples.[8]

Along with increased stability of the catalyst, CLCs of RAMA show great utility due to their stability in organic solvents. Table 1 presents the results of incubation of the CLCs with THF, DMF, acetone, acetonitrile,

Table 1. Solvent Stabilities of CLCs of RAMA Reactions were run on either 2.5 mg of lyophilized RAMA or CLC RAMA, solutions of 1 mL were made from equal volumes of solvent and 5mM phosphate buffer. Enzymes were incubated for 1 hour and then assayed

Solvent	% starting activity lyophylized RAMA	CLC's of RAMA
DMF	25	94
acetonitrile	5	90
acetone	37	92
DMSO	3	89
THF	3	99
dioxane	14	99

and dioxane, none of which caused significant loss of activity (>90%). Soluble RAMA exposed to DMSO was completely inactive after 25 minutes, an interesting example since DMSO has been used as a cosolvent with water insoluble substrates.[2]

We were interested the extent of intra- and intermolecular cross-linking of crystals that occurred as well as the distribution of aldolase activity among the different species. The cross-linked products were fractionated

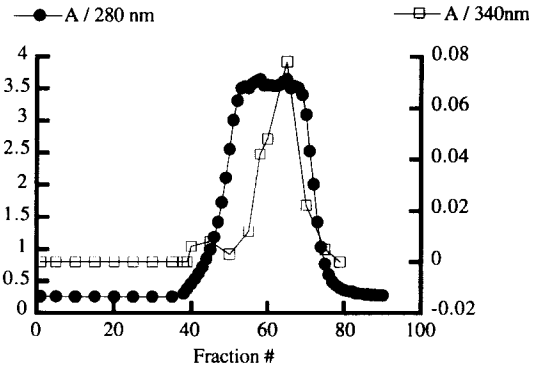


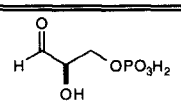
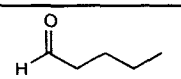
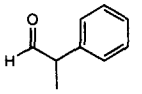
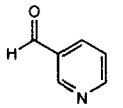
Figure 3. Enzyme activities of cross-linked fractions separated by Sephadex G-200 gel chromatography.

according to molecular weight on a 1.5 x 50 cm Sephadex G-200 column equilibrated with 50 mM potassium phosphate buffer containing 2 mM EDTA and 5 mM 2-mercaptoethanol, pH 7.5, at room temperature. **Figure 3** shows the elution profiles with the activity trace superimposed. From this we calculated the percent of activity in monomeric form to be 10 and that in oligomeric to be 90. These results are quite different from glutaraldehyde cross-linking of soluble RAMA which shows an equal distribution of activity.[9] It is likely that in the ordered form of a crystal, more intermolecular cross-linking occurs. The molecular weights of the fractions obtained from the column were analyzed by SDS polyacrylamide gel-electrophoresis which showed the faster moving species to be monotetrameric with a size of 158 Kd and the oligotetramers were larger than 400 Kd.

In vivo, RAMA catalyzes the condensation of dihydroxyacetone phosphate (DHAP) with D-

glyceraldehyde 3-phosphate (G-3-P) to form D-fructose 1,6-bisphosphate. In the seminal work of Whitesides, he was able to make some generalizations about the substrate specificity and to show RAMA to be a very useful biocatalyst.[2] We chose to re-examine the reactivity of several non-natural substrates from the Whitesides study with our stabilized RAMA. These substrates have low water solubility and showed poor reactivity with soluble

Table 2. Relative Reactivities of Aldehydes with DHAP in CLC aldolase catalyzed reactions.

Substrates	Rates (V_{rel})	Comments
	1.00	These reactions were run in 20% aqueous dioxane. 5 U of cross-linked aldolase were used for each. The CLC aldolase was reisolated and reused for each example. The reactions were followed for 8 hours, and aliquots were removed and analyzed at specific times. Decomposition of DHAP was used as a background correction.
	0.68	
	1.16	
	0.89	

RAMA. With a cosolvent of 20% dioxane, we observed no loss of activity of the cross-linked crystals of aldolase during continuous use. The relative rates we observed show a large increase in the relative rate. Based on our solvent stability studies, we think this increase is due to rapid denaturation of the soluble enzyme with DMSO as a cosolvent in the Whitesides study. Based on our synthetic studies and stability studies, this new CLC form of RAMA can extend the utility of FDP in synthesis for the formation of enantioselective carbon-carbon bonds.

Acknowledgments

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