Aqueous-like Activity of α-Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents

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Received February 7, 1994

Unlike aqueous-based enzymology, the vast majority of studies involving enzymes in nonaqueous media have employed biocatalyst suspensions.[1] Efforts to solubilize enzymes in organic solvents have focused on either chemical modification[2] or the use of surfactants either as complexing agents or reversed micelles.[3] The former approach is tedious and often results in substantial enzyme inactivation, while reversed micellar systems often consist of a high local water content in organic solvents (thereby destroying the true nonaqueous character of the organic solvent system) and result in excess surfactant that becomes a burden downstream. In our previous work[4] we observed that enzymes can be extracted from aqueous solutions into organic solvents at very low surfactant concentrations (e.g., <2 mM) via ion-pairing of a surfactant with the protein. The ion-paired enzyme–surfactant complex can exist stably in the organic solvent in the absence of reversed micelles. In the present work, we show that such enzyme–surfactant complexes can be substantially dehydrated in the organic solvent yet remain dissolved, maintain their native structure, and exhibit high catalytic activities which approach that in aqueous solutions.

Extraction of a 1.1 mg/mL aqueous solution of α-chymotrypsin (CT), pH 7.8, into isooctane containing 2 mM Aerosol OT (AOT) results in the solubilization of 1.02 mg/mL (93%) CT.[5] It should be stressed that this approach does not result in the formation of reversed micelles. Calculations of the number of AOT molecules associated per extracted CT molecule[5] indicate that only ca. 30 surfactant molecules interact with the protein, a value too low for reversed micellar incorporation of the protein in isooctane. The water content of the isolated isooctane phase following extraction is 0.3 μL/mL, which can be reduced to 0.035 μL/mL by passing a stream of dry N_2 gas through the organic solvent. This results in an increased concentration of CT dissolved in isooctane of 1.12 mg/mL and a ratio of $[H_2O]/[CT] < 50$, far less than that obtained with CT suspended in isooctane (75–300).[6] Hence, the ion-pairing approach to CT solubilization results in a highly dehydrated, yet organic-soluble enzyme preparation.

Table 1. Activity of Soluble Chymotrypsin in Organic Solvents

solvent[a]	k_{cat}/K_m[b] (M^{-1} s^{-1})	k_{cat}[b] (s^{-1})	K_m[b] (mM)
isooctane	3020	16.6	5.5
toluene	320	3.8	12
tert-amyl alcohol	75	2.4	32
tetrahydrofuran[c]	4.6	0.36	79
tetrahydrofuran + 5 μL/mL H_2O	45	4.4	98
aqueous buffer, pH 7.8[d]	21 479	58.1	2.7
isooctane (suspended)[e]	1.26		

[a] Unless otherwise stated, CT was in the soluble state and prepared by extraction from an aqueous buffer solution, pH 7.8, into isooctane, dried via N_2 gas, and diluted into the specific organic solvent. Transesterification of Ac-L-Phe-OEt with 1-propanol employed an enzyme concentration of 1.25 μg/mL (hydrolysis employed 4.0 μg/mL). The solvents contained no more than 0.03% (v/v) water as measured by Karl–Fischer titration. [b] These values were determined by nonlinear fit of the kinetic data and represent averages of triplicate measurements with standard deviations in each case of less than 5%. Initial rates of formation of Ac-L-Phe-OPr were determined by gas chromatography (530-μm fused silica gum (Hewlett-Packard)). No reaction was observed with CT preinactivated by phenylmethanesulfonyl fuloride (PMSF, 0.3 mM) (Gold, A. M.; Farney, D. *Biochemistry* **1964**, *3*, 783). Saturation kinetics could not be determined for reactions with CT suspended in isooctane. [c] The solubility of CT in THF (up to 10 mg/mL) provides strong evidence that reversed micelles are not involved in solubilization. [d] Hydrolysis of Ac-L-Phe-OEt was performed at pH 7.8 with a pH-Stat. [e] CT was lyophilized from the aqueous buffer (pH 7.8) containing 2.5 mg/mL Ac-L-Phe as described by Zaks and Klibanov.[8]

CT retains nearly all its catalytic sites when dissolved in organic solvents. Active site titration[7] using *N-trans*-cinnamoylimidazole in anhydrous isooctane resulted in the titration of 95% of the number of catalytic sites active in water within 90 min. Transesterification of Ac-L-Phe-OEt with 1-propanol in anhydrous isooctane (Table 1) gave a catalytic efficiency (k_{cat}/K_m) = 3020 M^{-1} s^{-1} (which is within an order of magnitude of the k_{cat}/K_m in aqueous buffer (Table 1)) and a k_{cat} value nearly one-third as high as in aqueous buffer. CT activity strongly depends on the pH of the aqueous solution used in the protein extractions (Figure 1). Thus, the phenomenon of "pH memory" [8] holds for enzymes dissolved in organic media. CT is also soluble and active in several other anhydrous solvents (Table 1). In all cases, the activity of the soluble enzyme is at least 3 orders of magnitude higher than similar activity for suspended CT in that given solvent. The increased catalytic activity of CT in solution as compared to suspension is unlikely to be due to a relaxation in diffusional limitations inherent in heterogeneous suspensions as such limitations would not be expected to contribute more than an order of magnitude in increased activity.[9] Instead, the soluble CT preparations in organic solvents may retain more native structure than their heterogeneous counterparts. Spectroscopic analyses[10] indicate that CT retains nearly all of its native secondary and tertiary structure while dissolved in organic solvents, perhaps as a result of the potential stabilizing effects of ion-pairing.

(1) See the following reviews and references therein: (a) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141. (b) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194. (c) Dordick, J. S. *Biotechnol. Prog.* **1992**, *8*, 259. (d) Zaks, A.; Russell, A. J. *J. Biotechnol.* **1988**, *8*, 259.

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(4) Paradkar, V. M.; Dordick, J. S. *Biotechnol. Bioeng.* **1994**, *43*, 529.

(5) The aqueous phase consisted of 10 mM bis-tris propane buffer, pH 7.8, containing 6 mM $CaCl_2$ and 1.10 mg/mL CT. This phase was extracted with an equal volume of isooctane containing 2 mM AOT (sodium bis(2-ethylhexyl) sulfosuccinate) by stirring at 250 rpm at 25 °C for 2 min. The phases were allowed to settle and then centrifuged to effect clean phase separation. The concentration of CT in the organic phase was determined spectrophotometrically. Analysis of the dry enzyme preparation in isooctane by dynamic light scattering (Dawn Model B device from Wyatt Technology Co. (Santa Barbara, CA) using a 5-mW linear polarized He–Ne incident laser beam (632.8 nm)) showed a mean molecular size of 6.8 nm, consistent with a spherical complex of CT ion-pairing with AOT molecules.

(6) Zaks, A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1986**, *108*, 2767.

(7) Isooctane-soluble CT was titrated as follows: A 2-fold molar excess (to enzyme) of *N-trans*-cinnamoylimidazole (NTC) (Schonbaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930) was dissolved in the solvent, and the enzyme solution was incubated at 25 °C for 90 min with mild shaking at 50 rpm. The CT was precipitated from the reaction mixture by addition of 1% (v/v) methanol, and the solids were centrifuged. The supernatant, containing unreacted NTC, was analyzed by gas chromatography.

(8) The ionogenic state of an enzyme in organic media remains the same as in the last aqueous solution from which the enzyme is recovered (Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194).

(9) Khmelnitsky, Yu. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 2647.

(10) Virtually no differences were observed for CT dissolved in isooctane or in aqueous buffer for the UV spectrum (230–310 nm) and the circular dichroism spectrum (220–300) nm). No significant differences were observed in the fluorescence spectra of CT (λ_{ex} = 295 nm; λ_{em} = 328 nm as a maximum) in all solvents (including water) listed in Table 1. Thus, little secondary or tertiary structural difference in CT was evident between aqueous buffer and organic solvents.

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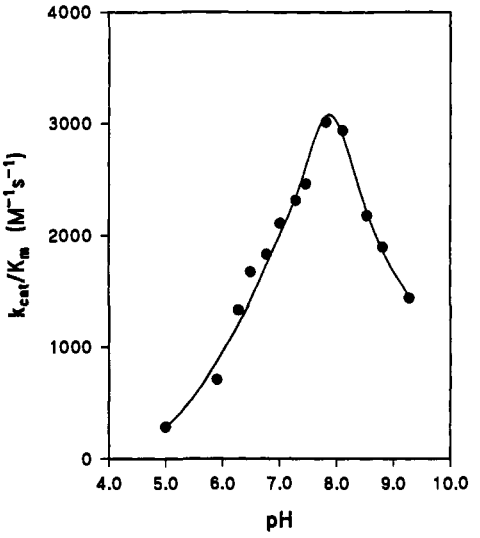

Figure 1. Effect of aqueous-phase pH used in CT extraction on the resulting catalytic efficiency for the transesterification of Ac-L-Phe-OEt with 1-propanol. Values of k_{cat}/K_m were determined as described in a footnote below Table 1.

The lower activity in hydrophilic solvents (Table 1) is consistent with observations of suspended enzymes in similar solvents[1] and may be due to the stripping of water off the soluble enzyme.[11] Lower values of k_{cat}/K_m may also result from the increased ground-state stabilization of the substrate in more hydrophilic solvents, thereby increasing the apparent K_m of the enzymatic reaction.[12] It is important to note that the K_m (apparent) for Ac-L-Phe-OEt increases from 5.1 to 79 mM in going from isooctane to THF, thus partially explaining the drop in k_{cat}/K_m in THF as compared to isooctane.

The high catalytic activity of solubilized CT was applied to the facile synthesis of dipeptides (Table 2). Soluble CT retains its preference for bulky, hydrophobic P_1 substrates,[13] yet even relatively high conversions were obtained for the synthesis of Bz-Ala-Pro-NH_2.[14] Peptide synthesis reactions are marked by several salient features including (i) high conversions using nearly equimolar concentrations of both the acyl donor and the nucleophile; (ii) nearly quantitative yields; and (iii) high reaction rates (and therefore relatively low enzyme concentrations required). CT-catalyzed peptide synthesis was extended to gram-scale synthesis: Incubation of 5 mmol of Bz-L-Tyr-OEt with 7.5 mmol of L-Phe-NH_2 in 0.1 L of isooctane (containing 30%, v/v, THF) resulted in the synthesis of 1.76 g of Bz-Tyr-Phe-NH_2 (isolated yield of 82%) with no hydrolysis product.[15]

Table 2. Peptide Synthesis Catalyzed by Soluble Chymotrypsin[a]

ester donor	nucleophile	reactn time (h)	conversn[b] (% of ester donor)	yield[c] (%)
Bz-Tyr-OEt[c]	Phe-NH_2	2	96	99
Bz-Tyr-OEt	Tyr-NH_2	2	93	98
Bz-Tyr-OEt	Leu-NH_2	2	91	97
Bz-Tyr-OEt	Pro-NH_2	4	87	85
Ac-Trp-OEt	Phe-NH_2	2	94	98
Bz-Ala-OMe	Phe-NH_2	2	92	98
Bz-Ala-OMe	Pro-NH_2	6	65	84
Ac-Phe-OEt	Phe-NH_2	2	98	99
Ac-Phe-OEt	Leu-NH_2	2	96	94

[a] The reactions were performed in 1 mL of isooctane containing 30% (v/v) THF (to aid in solubility) and 5 mM L-ester, 7.5 mM L-amino acid amide, and 0.15 mg/mL soluble CT. The reactions were terminated by addition of 0.1 mL of glacial acetic acid and dried. The residue was redissolved in 25 mM triethylammonium phosphate buffer (pH 3.0) containing 30% (v/v) CH_3CN and analyzed by reversed-phase HPLC (C_{18} μBondapak, Waters) with the same buffer as eluant. Ac and Bz represent N-acetyl and N-benzoyl, respectively. [b] Total conversion of ester substrate. [c] With respect to dipeptide formed versus total product (dipeptide + hydrolysis) formed.

In conclusion, the simple ion-pairing of a common surfactant to α-chymotrypsin results in a highly active biocatalyst soluble in anhydrous organic solvents. We are in the process of extending our findings to other enzymes and surfactants. To that end, we have found that subtilisin Carlsberg dissolved in isooctane is also highly active for the transesterification of Ac-L-Phe-OEt with 1-propanol (k_{cat}/K_m = 4160 M^{-1} s^{-1}, ca. 68% of the value in aqueous buffer). Thus, the approach described herein appears to be general and predictable, yet simple to perform, and results in highly active enzyme preparations for fundamental and applied uses.

Acknowledgment. This work was supported by grants from the Army Research Office (DAAL03-91-G0224) and the National Science Foundation (PYI award to J.S.D.).

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(13) Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145.
(14) Ala-Pro is an intermediate in the synthesis of the antihypertensive drug enalapril (Patchett, A. A.; et al. *Nature* **1980**, *288*, 280.)
(15) Analysis of the dipeptide indicated the presence of 93 μg of AOT/g of peptide. Thus, very little excess surfactant is entrained in the final product.