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Communication

Influence of organic solvents on papain kinetics★✪

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Papain activity was studied in water-organic solvent mixtures using the fluorogenic substrate Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. The increase of organic solvent (MeOH, EtOH, iPrOH, TFE, MeCN, $(MeO)_2Et$ and DMF) concentration in the mixture caused a substantial decrease the initial rate of papain-catalyzed hydrolysis. Moreover, the number of papain active sites decreased with the increase of DMF and MeOH concentration.

Since the beginning of the 1980s it has been clearly shown that enzymes can be used in organic solvents with great efficiency [1]. Nonaqueous enzymology is one of the major fields in biotechnological research [2]. At low organic solvent concentrations, the activity can be as high as in aqueous solution, or even slightly enhanced [3, 4]. At moderate concentrations of miscible solvents, usually in the 30–50% volume range, the catalytic rate is often close to zero [5, 6]. However, at higher organic solvent concentrations, typically in the range 70–99%, the catalytic activity can often be observed again [7–9]. After extensive re-

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Abbreviations: Amc, 7-amino-4-methylcoumarin; Boc, *t*-butoxycarbonyl; Dabcyl, 4-(4′-dimethylaminophenylazo)benzoyl; DMF, dimethyformamide; Me_2SO, dimethyl sulphoxide; E-64, L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; Edans, 5-[(2-aminoethyl)amino]naphthalene; Fmoc, 9-fluorenylmethoxycarbonyl; iPrOH, 2-propanol; MeCN, acetonitrile; $(MeO)_2Et$, 1,2-dimethoxyethane; TFE, 2,2,2-trifluoroethanol; Z, benzyloxycarbonyl.

search in various laboratories, molecular basis of the behaviour of enzymes in water-organic solvent mixtures is nowadays understood [10–14].

Papain, a cysteine protease, can be used to synthesize protected dipeptides in an aqueous-organic two phase system [15], but it loses its proteolytic activity in neat glycerol [16]. Our previous study on papain activity [17] revealed that an increase of Me_2SO concentration in a water mixture decreases the active site number of the enzyme as well as the initial rate of hydrolysis of Z-Phe-Arg-Amc and Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. In this communication we present the influence of seven organic solvents: MeOH, EtOH, iPrOH, TFE, MeCN, $(MeO)_2Et$ and DMF on papain activity measured with the fluorogenic substrate Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans displaying a high specificity for papain [18] as well as the standard substrate Z-Phe-Arg-Amc.

MATERIALS AND METHODS

Materials. E-64 and papain (EC 3.4.22.3) were purchased from Sigma. The commercial enzyme was purified as described previously [19]. Z-Phe-Arg-Amc, Amc, Boc- and Fmoc-amino acids were purchased from Bachem AG, all solvents used were from Aldrich and were HPLC grade or the highest grade available (TFE, $(MeO)_2Et$).

Peptide synthesis. The substrate Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans was synthesized and characterized using the procedure described in [17, 18].

Fluorescence measurements. Fluorescence was monitored on a Perkin-Elmer LS 50B spectrofluorimeter using the time drive option of the Fl WinLab software provided by the manufacturer. The measurements for Amc and Z-Phe-Arg-Amc were done using 380 nm as the excitation wavelength and 460 nm as the observation wavelength. In the case of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans the excitation and observation wavelengths were 336 nm and 490 nm, respectively.

Enzymatic assays. All kinetic experiments were performed according to the method described by Barrett *et al.* [20], partially modified by us [17]. The activating buffer was 0.4 M sodium potassium phosphate, pH 6.8, containing 8 mM dithiothreitol and 4 mM EDTA. During the measurements the cuvette contained 750 μl of the activating buffer, 50 μl of papain (1.68×10^{-7} M solution in 0.1% Brij 35), substrate, organic solvent (total concentration of organic solvent in the cuvette was from 0.2 to 10% depending on the experiment) and was filled up with 0.1% Brij 35 to 3000 μl. The stock solutions of the substrates prepared before the experiments were 870 μM Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans in the organic solvent.

In order to determine the Michaelis-Menten kinetic parameters, the enzyme was preincubated for 10 min at 40°C in cuvettes containing the activating buffer and 0.1% Brij 35 and then appropriate amounts of organic solvent and Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans were added. The reaction was monitored for 10 min.

To study the influence of the organic solvent concentration on the activity of papain we carried out a series of kinetic measurements for different organic solvent concentrations using Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans as a substrate.

Active sites titration. To examine the influence of the organic solvent concentration on the papain number of active sites per dm^{-3} titration with E-64 was carried out at 0.95%, 4.95% and 9.95% of organic solvent, according to the procedure described in [20].

A series of tubes containing 750 μl of the activating buffer, 50 μl of the enzyme solution and a suitable amount (from 0 to 400 μl) of 0.1 μM E-64 solution in 0.1% Brij 35 were filled up with 0.1% Brij 35 to 2900 μl, 2780 μl or 2630 μl for the total concentration of the organic solvent (DMF or MeOH) equal to 0.95%, 4.95% and 9.95%, respectively. After 30 min of

incubation at 40°C 100 μl of 100 μM Z-Phe-Arg-Amc solution and organic solvent were added to each tube. After 10 min the reaction was stopped with 1500 μl of 100 mM sodium monochloroacetate solution in 100 mM sodium acetate (pH = 4.3) and then the fluorescence of the liberated Amc was measured.

RESULTS AND DISCUSSION

In this work we examined the influence of some organic solvents on the initial rate of papain-catalyzed hydrolysis of the papain specific substrate Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. We carried out these reactions at fixed substrate and enzyme concentrations changing only the organic solvent content within the range from 0.2 to 10% in reaction mixtures. The results for seven organic solvents (MeOH, EtOH, iPrOH, TFE, MeCN, $(MeO)_2Et$ and DMF) and Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans as a substrate are presented in Fig. 1. The curves show that the increase of organic solvent concentration in the working buffer decreased the initial rate of papain-catalyzed hydrolysis of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. The fastest decrease of the initial rate was observed for $(MeO)_2Et$ and DMF between 1 and 3%. Papain loses about 80% of the initial activity in a buffer containing 2–3% of DMF or $(MeO)_2Et$. For MeOH the initial rate of the reaction decreases very slow.

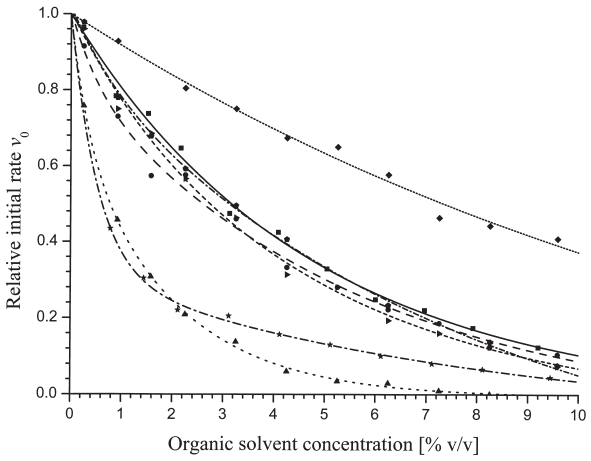

Figure 1. Dependence of the initial rate of papain catalyzed hydrolysis of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans on organic solvent concentration: a) ■ EtOH, b) ● MeCN, c) ▲ $(MeO)_2Et$, d) ★ DMF, e) ⬟ iPrOH, f) ◆ MeOH, g) ▶ TFA.

In order to examine the influence of organic solvents concentration on the number of papain active sites we titrated the enzyme according to the procedure described in [17] in buffers containing 0.95%, 4.95% and 9.95% DMF (Fig. 2) or MeOH (not shown) using 30 min incubation of papain with E-64, a covalent-type inhibitor for active sites titration of the papain family enzymes [20]. The results presented in Fig. 2 indicate that the active sites number depends on DMF concentration in buffers and decreases with increasing DMF concentration. We observed the same effect for buffers containing MeOH but the changes were smaller (Table 1).

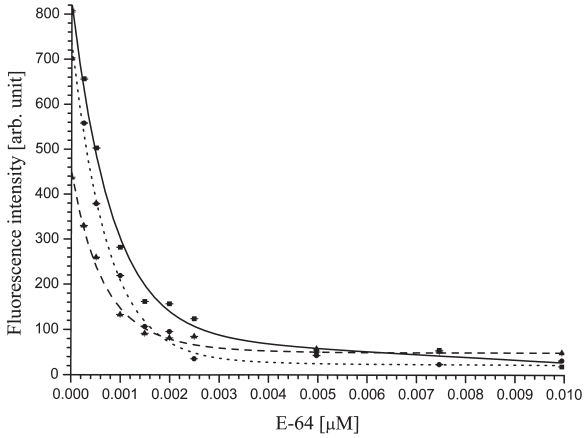

Figure 2. Influence of DMF concentration on titration of papain with E-64:

a) ■ 0.95% DMF, number of active sites = 1.2168 × 10^{-9} mol/dm^3; b) ● 4.95% DMF, number of active sites = 1.0010 × 10^{-9} mol/dm^3; c) ▲ 9.95% DMF, number of active sites = 0.7967 × 10^{-9} mol/dm^3.

The Michaelis-Menten equation was used to compare the kinetics of papain-catalyzed hydrolysis of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans in buffers containing DMF or MeOH. The initial rate dependence on substrate concentration for 0.6% and 5% DMF is presented in Fig. 3. For 5% DMF it was impossible to cal-

Table 1. Kinetic parameters calculated from the Michaelis-Menten and Hill equations

Organic solvent	Number of papain active sites × 10^9 [mol/dm^3]			C_p	Enzymatic assays								
					Michaelis-Menten equation					Hill equation			
	C_p 0.95%	C_p 4.95%	C_p 9.95%		V_{max} [μM/min]	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\mu M^{-1}s^{-1}$]	R^2	V_{max} [μM/min]	$K_{0.5}$ [μM]	b	R^2
DMF	1.2168	1.0010	0.7967	0.6%	0.87 ± 0.03	20.75 ± 1.33	11.92	0.57	0.9988	–	–	–	–
				5%	19 ± 58	1765 ± 5313	–	–	0.9982	–	–	–	–
MeOH	1.3843	1.2833	1.0317	1%	0.23 ± 0,02	3.85 ± 0.52	2.77	0.72	0.9965	0.15 ± 0.01	1.65 ± 0.13	1.40 ± 0.07	0.9992
				5%	0.57 ± 0.03	12.43 ± 1.10	6.86	0.55	0.9980	0.45 ± 0.03	7.69 ± 0.86	1.21 ± 0.07	0.9990

culate with reasonable errors K_m and V_{max}. The kinetic parameters were calculated basing on the Michaelis-Menten equation for both organic solvents and are presented in Table 1. In the case of MeOH, Hill equation gave a better fit to the experimental data. The results obtained suggest that Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans shows higher affinity for

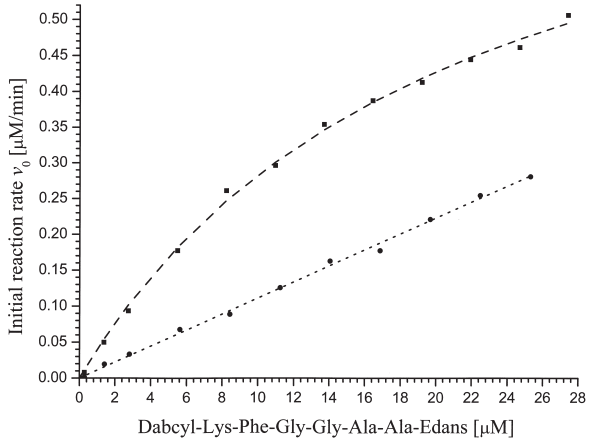

Figure 3. Dependence of the initial rate of papain catalyzed hydrolysis on substrate concentration in buffer containing ■ 0.6% and ● 5% DMF.

papain in buffers containing MeOH. The k_{cat}/K_m value is the same within experimental error, for 0.6% DMF, 1% and 5% MeOH. However, the hydrolysis products are probably released more slowly from the enzymatic pocket in buffers containing MeOH.

The results presented in this paper indicate that organic solvents seriously affect papain activity. Therefore the results of any kinetic study using this enzyme will depend very much on the content of organic solvents used in the assay. This should be taken into account especially when data from different papers are being compared.

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