Acta Biochimica Polonica

Vol. 48 No. 4/2001

995–1002

QUARTERLY

Influence of Me_2SO and incubation time on papain activity studied using fluorogenic substrates✪

Mariusz Szabelski, Krystyna Stachowiak and Wiesław Wiczk✉

Faculty of Chemistry, University of Gdańsk, Gdańsk, Poland

Received: 27 July, 2001; revised: 19 September, 2001; accepted: 27 November, 2001

Key words: papain, fluorogenic substrate, enzyme activity, Me_2SO

Papain activity in a buffer containing Me_2SO was studied using fluorogenic substrates. It was found that the number of active sites of papain decreases with increasing Me_2SO concentration whereas the incubation time, in a buffer containing 3% Me_2SO does not affect the number of active sites. However, an increase of papain incubation time in the buffer with 3% Me_2SO decreased the initial rate of hydrolysis of Z-Phe-Arg-Amc as well as Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. Moreover, an increase of Me_2SO concentration in working buffer decreased the initial rate of papain-catalysed hydrolysis of both substrates. A rapid decrease of the initial rate (by up to 30%) was observed between 1 and 2% Me_2SO. Application of the Michaelis-Menten equation revealed that at the higher Me_2SO concentrations the apparent values of k_{cat}/K_m decreased as a result of K_m increase and k_{cat} decrease. However, Me_2SO changed the substrate binding process more effectively (K_m) than the rate of catalysis k_{cat}.

The activity of proteolytic enzymes is usually measured with synthetic chromogenic or fluorogenic peptide substrates [1]. These compounds usually contain from two to six amino acid residues. Their sequence is often strictly defined and imitates the binding fragments of natural substrates or inhibitors of the proteases. The catalytic pocket of the majority of these enzymes has a hydrophobic rather than hydrophilic character. Therefore the amino-

✪This work was supported by the State Committee for Scientific Research (KBN, Poland) under grant 1410/T09/2001/21.

✉Corresponding author: J. Sobieskiego 18, 80-952 Gdańsk, Poland; phone: (48 58) 345 0353; fax: (48 58) 341 0357; e-mail ww@chemik.chem.univ.gda.pl

Abbreviations: Amc, 7-amino-4-methylcoumarin; Boc, *t*-butoxycarbonyl; BOP, 1-benzotriazolyloxy-tris-dimethylamino-phosphonium hexafluorophosphate; Bzl, benzyl; Dabcyl, 4-(4′-dimethylaminophenylazo)benzoyl; DCC, dicyclohexylcarbodiimide; Me_2SO, dimethyl sulphoxide; DMF, dimethyformamide; E-64, *trans*-epoksysuccinyl-L-leucylamido(4-guanidino)butane; Edans, 5-[(2-aminoethyl)amino]naphthalene; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

acid sequences of "good" synthetic substrates are dominated by hydrophobic residues. The hydrophobicity of the substrates is additionally increased by their chromophoric and fluorophoric groups. As a consequence, the synthetic peptide substrates of proteases are poorly soluble in water and their application in enzymatic tests requires buffers containing an addition of an organic solvent. Unfortunately, the organic solvent may change the enzyme conformation as a result of water replacement or by disrupting hydrophobic internal interactions. Organic solvents may also disturb substrate desolvation necessary for its penetration into the enzyme catalytic pocket, and block the enzyme active center or destabilize enzyme–substrate transition state as well as reduce the conformational mobility of the enzyme [2]. Therefore, the general opinion that 5–10% of an organic solvent in the assay does not inhibit the enzyme activity needs to be checked in each case [3].

The most commonly used organic solvent in enzymatic assays is Me_2SO, less often DMF, methanol, ethanol or acetonitrile. In standard measurements of the papain family enzymes activity Me_2SO concentration is 1–2% and it is assumed not to influence the kinetics [4]. However, Me_2SO, like many other organic solvents, dissolves proteins [5], changes their conformation [5, 6] and often modifies enzyme activity in water/Me_2SO mixtures of both low and high Me_2SO concentration [5, 7–10].

The measurements are often performed with the dipeptide Z-Phe-Arg-Amc as a substrate, which is specific for all papain-like cysteine proteases. Determination of the activity of particular enzymes present in (samples from) cell homogenates or physiological fluids requires the use of complex indirect methods, for instance with the use of specific inhibitors [11, 12]. A different, simpler way would be to employ substrates specific for the chosen enzyme. However, they have to possess longer amino-acid sequences to be selectively recognized and, what usually goes with it, a more hydrophobic character. Enzyme reactions with such substrates would require buffers containing more than 2% of an organic solvent. Our research on synthetic peptide substrates for chosen lysosomal cysteine proteases [13] prompted us closer to examine the influence of Me_2SO on the enzyme kinetics. The experiments were performed for papain, a model enzyme of the papain-like family of cysteine proteases. We used both a short, standard Z-Phe-Arg-Amc substrate and a longer one, proposed by Garcia-Echeverria & Rich [14], displaying high specificity for papain. Papain activity was measured as an increase of fluorescence intensity of liberated amino-coumarin when Z-Phe-Arg-Amc was used as the substrate. In the other fluorogenic substrate used quenching of Edans fluorescence by distance-dependent resonance energy transfer to the Dabcyl quencher is eliminated upon cleavage of the intervening peptide linker between glycine residues (see Fig. 1 for details).

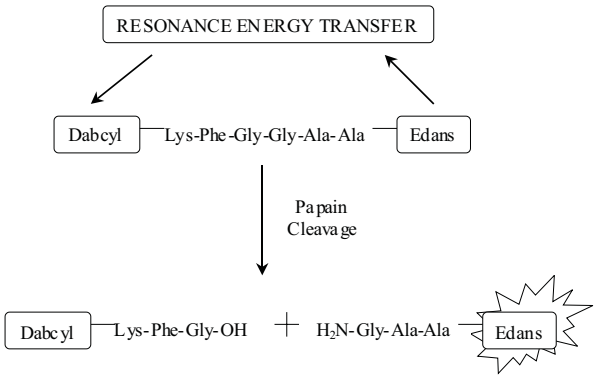

Figure 1. Principle of the fluorogenic response to papain cleavage of the Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans substrate.

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 [15]. Z-Phe-Arg-Amc, Amc, Boc- and Fmoc-amino acids were purchased from Bachem

AG, Me_2SO (BioChemika MicroSelect for molecular biology) from Fluka.

Peptide synthesis. The substrate Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans was synthesized and characterised using the procedure described by Garcia-Echeverria & Rich [14].

The fluorescent peptide Trp-Val-Ala-Edans was obtained by means of classical solution methods, according to the following outline: Boc-Trp-Val-Ala + Edans → Boc-Trp-Val-Ala-Edans → Trp-Val-Ala-Edans. Boc-Trp-Val-Ala was synthesized using the DCC/HOBt method [16] beginning with the coupling of Boc-Val and Ala-OBzl. The product obtained was joined, after removing the Boc group, with Boc-Trp. The C-terminal benzyl group was removed by hydrogenolysis. The coupling of Boc-Trp-Val-Ala with Edans was accomplished using the BOP/HOBt method [17]. All Boc protecting groups were removed with a TFA/phenol/triispropylsilane/H2O (88 : 5 : 2 : 5) mixture. The crude product was purified by means of HPLC on a Kromasil 7-μm C_8 column (25 × 250 mm) using two step gradient elution (10 min 0–10% and 120 min 10–30% aqueous CH_3CN containing 0.1% TFA) at a 5 ml/min flow rate and 223 nm detection wavelength. The final product was evaluated by analytical HPLC on a Kromasil 5-μm C_8 column (4.6 × 250 mm), 20 min linear gradient (15–30% CH_3CN with 0.1% TFA), 1 ml/min flow rate, λ = 223 nm; t_R = 12.3 min and identified by FAB MS; *m/z* 624 $(M+H)^+$.

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 and Trp-Val-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.* [18], partly modified by us. 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, Me_2SO (total concentration of Me_2SO in the cuvette was from 0.95 to 10% depending on the experiment) and was filled to 3000 μl of total volume with 0.1% Brij 35. Because of the lower sensitivity of our spectrofluorimeter than used by Barrett *et al.* [18], it was necessary to use a higher concentration of the substrates poorly soluble in water. The stock solution of the substrate prepared before the experiments was 870 μM Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans in Me_2SO or 100 μM Z-Phe-Arg-Amc in Me_2SO/H_2O (1 : 2, v/v) mixture. Thus, the lowest final concentration of Me_2SO in the reaction mixture was 0.95%.

For the determination of 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 an adequate amounts of Me_2SO and Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans were added. The reaction was monitored for 10 min.

To study the influence of Me_2SO concentration on the activity of papain we carried out a series of kinetic measurements for different Me_2SO concentrations and constant substrate and papain concentrations using Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans or Z-Phe-Arg-Amc as the substrate.

To estimate the influence of incubation time with Me_2SO on papain activity, Me_2SO was added to the activating buffer at the same time as the enzyme and the duration of the initial incubation was increased to 30 min. In this experiment Z-Phe-Arg-Amc was used as substrate.

Influence of Me_2SO on Amc and Edans fluorescence intensity. A series of solutions containing a constant concentration of Amc or Trp-Val-Ala-Edans, 750 μl of activating

buffer and different amounts of Me_2SO was prepared. These solutions were filled up with 0.1% Brij 35 to of 3000 μl and later fluorescence spectra were measured. The fluorescence intensity of Trp-Val-Ala-Edans and Amc at the chosen wavelength as a function of Me_2SO concentration was estimated.

Active sites titration. The molar concentration of papain active sites was assessed by titration with E-64. 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 H_2O containing 0.1% (v/v) Me_2SO were filled with 0.1% Brij 35 to 2900 μl. After 60 min of incubation at 40°C, 100 μl of 100 μM Z-Phe-Arg-Amc solution in a water/Me_2SO mixture (1 : 2.5, v/v) was added to each tube. Thus, the total concentration of Me_2SO reached 0.95%. 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 the fluorescence of the liberated Amc was measured. To examine the influence of Me_2SO concentration on the number of papain active sites the titration with E-64 was also carried out at 4.95% and 9.95% Me_2SO, according to the procedure mentioned above, but after 60 min incubation of the enzyme with E-64 incubation 100 μl of 100 μM Z-Phe-Arg-Amc and an appropriate amount of Me_2SO were added. The total volume of the reaction mixture was kept constant at 3000 μl by lowering the volume of 0.1% Brij 35 used. To estimate the influence of the incubation time with Me_2SO on papain active sites number, 50 μl of the enzyme solution was initially incubated at 40°C for 30 min in a series of tubes containing 750 μl of the activating buffer, 60 μl of Me_2SO (3%) and an appropriate amount of 0.1% Brij 35. Then a suitable amount of 0.1 μM E-64 solution was added to each tube and the incubation was continued for 30 min and further procedure was performed as described above. The total incubation time of papain in this case was one hour. In another experiment the organic solvent (3% of Me_2SO) was added to the reaction mixture after 60 min of incubation of papain with E-64.

RESULTS AND DISCUSSION

The addition of Me_2SO to the buffer solution containing Amc or Trp-Val-Ala-Edans, in both cases caused a blue-shift of their fluorescence spectra and simultaneously an increase of the fluorescence intensity of fluorophores (not shown). The observed increase of the fluorescence intensity for Edans as well as for the Amc with increasing Me_2SO concentration was non-linear. Such behaviour is frequently observed for fluorophores whose fluorescence derives from the charge-transfer excited state [19]. Thus, the calibration curve used for enzyme activity calculation based on the fluorescence intensity changes has to be obtained in the presence of an appropriate Me_2SO concentration. Such calibration curves were used for papain activity calculations presented in our report.

The finding that E-64 reacts rapidly and stoichiometrically with the cysteine residue essential for the catalytic activity of the papain family enzymes leads to the common usage of this covalent-type inhibitor for the active site titration of these enzymes [18]. The stock solution of E-64 for kinetic measurements is usually 1.0 nM in 1% Me_2SO in water. This means that during the standard procedure of titration, at the time of incubation of the enzyme with the inhibitor the concentration of Me_2SO is far below 1%, i.e. much lower than the concentration used during the measurements of kinetics of hydrolysis synthetic peptide substrates. To establish whether the Me_2SO concentration used during these measurements influences the number of papain active sites we titrated the enzyme according to the standard procedure in buffers containing 0.95%, 4.95% and 9.95% Me_2SO using 60 min incubation of papain with E-64.

The results presented in Fig. 2 indicate that papain activity, expressed as the fluorescence intensity of liberated Amc, decreases linearly with increasing E-64 concentration for all

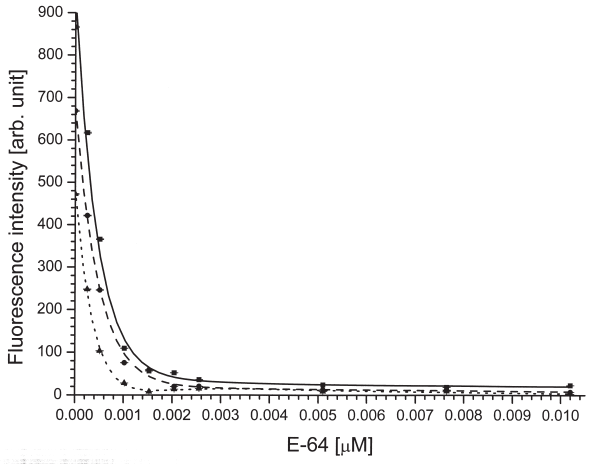

Figure 2. Influence of Me_2SO concentration on titration of papain with E-64.

a) ■ 0.95% Me_2SO, number of active sites = 8.1656×10^{-10} mol/dm^3; b) ● 4.95% Me_2SO, number of active sites = 6.6401×10^{-10} mol/dm^3; c) ▲ 9.95% Me_2SO, number of active sites = 5.1286×10^{-10} mol/dm^3. The ordinate presents Amc fluorescence intensity after 10 min of the enzymatic hydrolysis of Z-Phe-Arg-Amc.

Me_2SO concentrations studied but does not reach zero. Below 20% activity (depending on Me_2SO concentration) the curves deviate markedly from linearity, tending to become asymptotic with the abscissa. Such behaviour was observed by Barrett *et al.* [18] for cathepsin H. Despite this, we found that the inactivation fitted a linear relationship down to at least 50% of inactivation, and a straight line could be drawn through the upper points and extrapolated to the base line. Such procedure for determination of the number active sites was applied by Barrett *et al.* [18] for cathepsin H.

When calculated based on the results presented in Fig. 2 the active sites number of papain decreases with increasing Me_2SO concentration; for the lowest Me_2SO concentration used (0.95%) it is 8.17×10^{-10} mol/dm^3 and decreases to 5.13×10^{-10} mol/dm^3 for 9.95% Me_2SO. The incubation time, in buffer containing 3% Me_2SO, has no influence on the number of active sites (not shown), but further shifts of the equilibrium between active and inactive form of papain can not be excluded. Kinetic studies of reaction between E-64 and papain in the presence of Me_2SO (which are in progress), similar to those conducted by Barrett *et al.* [18] should give information about such equilibrium.

Basing on the crystal structure of papain complexes with E-64 and with its analogue E-64c a great importance of the hydrophobic interactions of P_2 with S_2 and P_3 with S_3 was established for the inhibitory activity of these compounds [20, 21]. The binding process can be disrupted by the competitive hydrophobic Me_2SO interactions with both the inhibitor and the enzyme catalytic pocket. Our results suggest that such interactions could be responsible for the change of the effective concentration of the active form of papain in the presence of Me_2SO.

Another parameter that can influence the proteolytic activity of enzymes is the time of their incubation in buffers containing an organic solvent. It should be taken into account in at least two cases. The first is the measurement of the activity of cysteine proteases after different time of incubation with synthetic inhibitors poorly soluble in water (time-dependent inhibition) [22, 23]. The second is the measurement of the activity of enzymes found in tissue homogenates. In some cases the test for cathepsins activity includes incubation of homogenates with the substrate Z-Phe-Arg-Amc in buffers containing up to 23% Me_2SO from 10 to 60 min [11]. Then the enzyme reaction is stopped and after adequate dilution the fluorescence of the released coumarin is measured.

In order to examine whether the duration of papain incubation in solutions containing Me_2SO influences the rate of Z-Phe-Arg-Amc hydrolysis we followed the kinetics of this reaction in a buffer containing 3% Me_2SO. Me_2SO was added after 0, 10, 20 and 30 min of papain incubation in buffer only. The sub-

strate was added after 30 min of preincubation at 40°C. During the measurements both the enzyme and substrate concentrations were constant. Figure 3 presents the increase

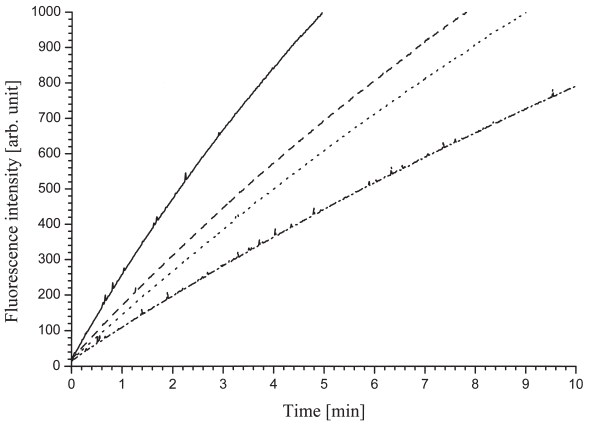

Figure 3. Influence of papain incubation time with 3% Me_2SO on the enzymatic hydrolysis of Z-Phe-Arg-Amc (30 min total incubation time).

a) — 0 min, b) – – 10 min, c) - - - 20 min, d) - · - 30 min.

of fluorescence intensity during the first 10 min of the reaction. The curves indicate that the longer the incubation time with Me_2SO, the smaller the initial rate of the reaction, reaching approximately half of the initial activity of the longest time of incubation with Me_2SO. This suggests that, during incubation in a buffer containing 3% Me_2SO, slow papain conformational changes occur. These changes influence the initial rates of Z-Phe-Arg-Amc hydrolysis, but have no impact on the evaluation of the active sites by E-64 titration.

In the present work we also examined the influence of Me_2SO concentration on the initial rate of papain-catalysed hydrolysis of the standard cysteine protease substrate Z-Phe-Arg-Amc and the papain specific substrate Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. Figure 4 presents the results of experiments for Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans. The curve displays a rapid decrease of the initial rate between 1 and 2% Me_2SO. Papain loses about 30% of activity in the buffer containing 2% Me_2SO. For higher Me_2SO concentrations the initial rate of the reaction decreases more slowly. Similar results of papain activity changes were obtained using Z-Phe-Arg-Amc as substrate (not shown). These observations are important because in many papers it is assumed that Me_2SO concentration up to 2% does not change the enzyme activity [4]. This suggests that for a particular content Me_2SO, the concentration of papain in the active conformation may not necessarily be equal to the concentration of papain active sites found by E-64 titration.

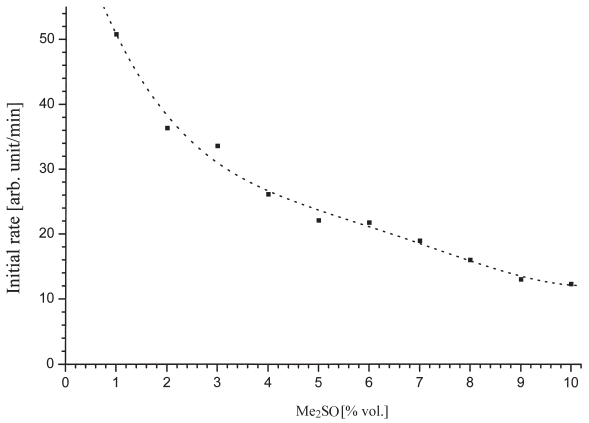

Figure 4. Dependence of the initial rate of papain catalysed hydrolysis of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans on Me_2SO concentration.

A simple model of one-substrate enzymatic reaction is described by the Michaelis-Menten equation. This approach was used to compare the kinetics of papain-catalysed hydrolysis of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans in buffers containing 1% and 5% Me_2SO. The dependence of the initial rate on substrate concentration is presented in Fig. 5. The apparent kinetic parameters calculated basing on the Michaelis-Menten equation (taking into account the different numbers of active sites at different Me_2SO concentration) are: for 1% Me_2SO $K_m = 5.06 \pm 0.61\ \mu M$, $k_{cat} = 15.91\ s^{-1}$, thereby $k_{cat}/K_m = 3.14\ \mu M^{-1}s^{-1}$, and $K_m = 31.58 \pm 2.47\ \mu M$, $k_{cat} = 24.85\ s^{-1}$ and $k_{cat}/K_m = 0.79\ \mu M^{-1}s^{-1}$ for 5% Me_2SO. These results indicate that Me_2SO affects the binding process as well as the rate of catalysis. However, its influence on the binding is higher than on the rate of catalysis. The most probable reason of that

is a serious disturbance of hydrophobic (but not electrostatic) interactions in the binding of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans with papain caused by Me_2SO. At low concentrations Me_2SO does not substantially affect the dielectric constant of water. The hydrophobicity of Me_2SO is also responsible for the k_{cat} decrease since hydrophobic intramolecular interactions are an essential source of protein stability [24, 25]. The decrease of k_{cat} with Me_2SO concentration increase reflects the weakening of these interactions. The active conformation of papain becomes less stable and some conformational changes may occur.

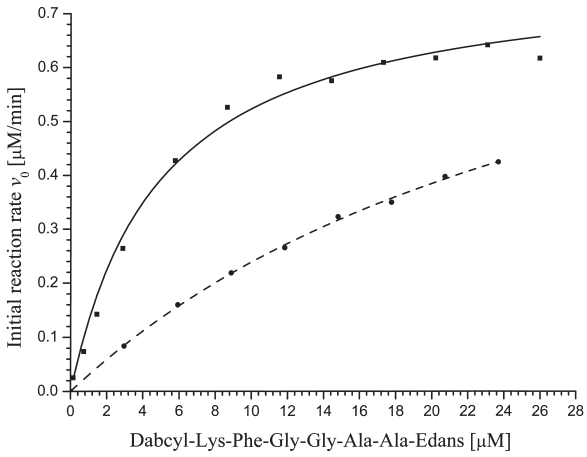

Figure 5. Dependence of the initial rate of papain catalysed hydrolysis of Dabcyl-Lys-Phe-Gly-Gly-Ala-Ala-Edans on substrate concentration.

The non-linear least-squares fit to the Michaelis-Menten equation is shown for 3% ■ Me_2SO and ● 10% Me_2SO.

The results presented in this paper indicate that Me_2SO seriously affects papain activity, especially in the low concentrations range (up to about 2%). The time of incubation in buffers with Me_2SO also influences papain activity. Therefore the results of any kinetic study using this enzyme will depend very much on the assay method. This should be taken into account especially when the data from different works are being compared.

REFERENCES

1. Gershkovich, A.A. & Kholodovych, V.V. (1996) Fluorogenic substrates for proteases based on intramolecular fluorescence energy transfer (IFETS). *J. Biochem. Biophys. Methods* **33**, 135–162.

2. Klibanov, A.M. (1997) Why are enzyme less active in organic solvents than in water? *Trends Biotechnol.* **15**, 97–101.

3. Sarath, G., De La Motte, R.S. & Wagner, F.W. (1989) Protease assay methods; in *Proteolytic Enzymes: a Practical Approach* (Beynon, R.J. & Bond, J.S., eds.) pp. 25–55, IRL Press at Oxford University Press, Oxford, New York, Tokyo.

4. Barrett, A.J. & Kirschke, H. (1981) Cathepsin B, cathepsin H and cathepsin L. *Methods Enzymol.* **80**, 535–561.

5. Zaks, A. & Klibanov, A.M. (1988) Enzymatic catalysis in nonaqueous solvent. *J. Biol. Chem.* **7**, 3194–3201.

6. Sirotkin, V.A., Zinatulin, A.N., Solomonov, B.N., Fazillin, D.A. & Fedotov, V.D. (2001) Colorimetric and Fourier transform infrared spectroscopic study of solid proteins immersed in low water organic solvents. *Biochim. Biophys. Acta* **1547**, 359–369.

7. Grard, E. & Legoy, M.-D. (1999) Activity and stability of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F in the presence of organic solvents. *Enzyme Microb. Technol.* **24**, 425–432.

8. Potier, P., Bouchu, A., Descotes, G. & Queneau, Y. (2000) Proteinase N-catalyzed transestrification in Me_2SO-water and DMF-water; preparation of sucrose monomethacrylate. *Tetrahedron Lett.* **41**, 3597–3600.

9. Simon, L.M., László, K., Vértesi, A., Bagi, K. & Szajáni, B. (1998) Stability of hydrolytic enzymes in water–organic solvent systems. *J. Mol. Catal. B: Enzymatic* **4**, 41–45.

10. Okazaki, S., Goto, M. & Furusaki, S. (2000) Surfactant-protease complex as a novel biocatalyst for peptide synthesis in hydrophilic organic solvents. *Enzyme Microb. Technol.* **26**, 159–164.

11. Schmid, H., Koop, M., Utermann, S., Lambacher, L., Mayer, P. & Schaefer, L. (1997) Specific catalytic activity of cathepsin S in comparison to cathepsins B and L along the rat nephron. *Biol. Chem.* **378**, 61–69.

12. Kirschke, H., Clausen, T., Göhring, B., Günther, D., Heucke, E., Laube, F., Löwe, E., Neef, H., Papesch, H., Peinze, S., Plehn, G., Rebmann, U., Rinne, A., Rüdrich, R. & Weber, E. (1997) Concentrations of lysosomal cystein proteases are decreased in renal cell carcinoma compared with normal kidney. *J. Cancer Res. Clin. Oncol.* **123**, 402–406.

13. Dwojakowska, D., Dąbrowska, A., Łankiewicz, L., Wiczk, W. & Stachowiak, K. (1999) Fluorogenic substrates of papain with structural resemblance to the inhibitory center of family 2 cystatins; in *Peptides 1998* (Bajusz, S. & Hudecz, F., eds.) p. 632, *Proc. 25th Europ. Peptide Symp*., Akademiai Kiado, Budapest.

14. Garcia-Echeverria, C. & Rich, D.H. (1992) New intramolecularly quenched fluorogenic peptide substrates for the study of the kinetic specificity of papain. *FEBS Lett.* **297**, 100–102.

15. Blumberg, S., Schechter, I. & Berger, A. (1970) The purification of papain by affinity chromatography. *Eur. J. Biochem.* **15**, 97–102.

16. Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Chemistry*. Springer-Verlag, New York.

17. Jou, G., González, I., Albericio, F., Lloyd-Williams, P. & Giralt, E. (1997) Total synthesis of dehydrodidemnin B. Use of uranium and phosphonium salt coupling reagents in peptide synthesis in solution. *J. Org. Chem.* **62**, 354–366.

18. Barrett, A.J., Kembahavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. & Hanada, K. (1982) L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **201**, 189–198

19. Lakowicz, J.R. (1999) *Principles of Fluorescence Spectroscopy*; 2 edn., chap. 6, pp. 185–210, Kluwer Academic/Plenum Publisher, New York, Boston, Dordrecht, London, Moscow.

20. Matsumoto, K., Murata, M., Sumiya, S., Kitamura, K. & Ishida, T. (1994) Clarification of substrate specificity of papain by crystal analyses of complexes with covalent-type inhibitors. *Biochim. Biophys. Acta* **1208**, 268–276.

21. Matsumoto, K., Murata, M., Sumiya, S., Mizoue, K., Kitamura, K. & Ishida, T. (1998) X-Ray crystal structure of papain complexed with cathepsin B-specific covalent-type inhibitor: Substrate specificity and inhibitory activity. *Biochim. Biophys. Acta* **1386**, 93–100.

22. Guo, Z., Ramirez, J., Li, J. & Wang, P.G. (1998) Peptidyl N-nitrosoanilines: A novel class of cysteine protease. *J. Am. Chem. Soc.* **120**, 3726–3734.

23. Iguchi, S., Kawasaki, K., Okamoto, H., Umezawa, C. & Okada, Y. (1999) Synthesis of some pseudo-peptide analogs of tiol proteinase inhibitors. *Chem. Pharm. Bull.* **47**, 423–427.

24. Maurel, P. (1978) Relevance of dielectric constant and solvent hydrophobicity to the organic solvent effect in enzymology. *J. Biol. Chem.* **253**, 1677–1683.

25. Turk, B., Dolenc, I., Lenarčič, B., Kri aj, I., Turk, V., Bieth, J.G. & Björk, I. (1999) Acidic pH as a physiological regulator of human cathepsin L activity. *Eur. J. Biochem.* **259**, 926–932.