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THE EFFECT OF METHANOL, UREA AND OTHER SOLUTES ON THE ACTION OF PAPAIN*

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SUMMARY

The activity of papain (EC 3.4.4.10) decreases with increasing methanol concentration. At relatively low concentrations (2–4%), methanol, a few other solvents and urea (2 M) exhibit apparently competitive inhibition. However, the extent of inhibition by methanol and urea is not the same for the two substrates benzoylglycine ethyl ester and benzoylarginine ethyl ester.

Partial methanolysis of benzoylarginine amide was observed in 30% methanol by a titration method. Methanol was a 5–6 times more effective nucleophile than water. In 50% methanol almost no activity remained.

This loss of activity is believed not to be due to conformational change for the following reasons: (1) There was no change in optical rotatory dispersion up to 70% methanol. (2) There was no change in viscosity in 50% methanol. (3) Papain is an enzyme of unusual stability, since in 8 M urea it retains considerable activity and exhibits no essential change in optical rotatory dispersion.

It is considered possible that the inhibitory effects are to a large extent due to interference with hydrophobic interactions between enzyme and substrates. Certain consequences for the X-ray analysis of papain crystals grown from water–methanol mixtures are pointed out.

INTRODUCTION

In the preparation of papain crystals of sufficient size to allow X-ray analysis, it was found to be necessary to crystallize the enzyme from media containing 50–70 % (by vol.) methanol[1]. Therefore it was of interest to know whether or not the

Abbreviations: BGEE, benzoylglycine ethyl ester; BA, benzoylarginine; BAA, benzoylarginine amide; BAEE, benzoylarginine ethyl ester; BAME, benzoylarginine methyl ester; BAPA, (+, −)-benzoylarginine *p*-nitroanilide; BAL, 2,3-dimercaptopropanol; EDTA, ethylenediaminetetraacetate; ORD, optical rotatory dispersion.

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enzyme is active in such solvent mixtures. STOCKELL AND SMITH[2] investigated the effect of up to 50% (by vol.) methanol on the hydrolysis of BAA, and found that a decrease in activity occurs which is due mainly to an increase in K_m.

The present paper reports further investigations on the effect of methanol. Attention has been paid to reaction kinetics, to the possible occurrence of papain-catalyzed methanolysis and to some physical properties of the enzyme. Some data concerning other organic solvents and denaturing agents have been included.

EXPERIMENTAL

Materials

Substrates and activators were those of a previous paper[3]. BAPA was prepared according to ERLANGER[4]. Two kinds of methanol were used: an ordinary grade, carefully redistilled, and reagent grade (*pro analysi*) (for ultraviolet spectrometry, from Merck, Darmstadt). The other solvents, dimethylsulphoxide (Stepan Chem. Comp., Chicago), dimethylformamide (Merck) and acetonitrile (Schuchard, Munich), were redistilled. The purity of all solvents was checked by gas chromatography.

Activity determinations

The automatic titration equipment and the general procedures for the estimation of ester hydrolysis were those described previously[3]. For the standardization of the glass electrode 0.3 M histidine buffer(pH 7.0) was triple-diluted either with water alone or with water *plus* the amount of methanol necessary to obtain the final methanol concentration required. The pH of the methanol–water buffer was then assumed to be equal to the pH of the aqueous buffer, since the pK of the imidazole group of histidine is known to be little affected by organic solvents[5]. The glass electrode was adapted to the methanol–water mixture to be used by immersion overnight.

The hydrolysis of BAPA was followed spectrophotometrically at 410 mμ on a home-made recording spectrophotometer, provided with thermospacers (40°), in 0.03 M ethylenediamine buffer (pH 6.5).

The activator concentration in the reaction mixture was either 5 mM cysteine *plus* 1 mM EDTA or 10 mM BAL alone. The latter activator was used when cysteine might complicate analysis (thin-layer chromatography and formaldehyde titration). The KCl concentration was 0.3 M except in the thin-layer chromatography experiments, where no salt was added.

Titration of liberated ammonia

A mixture of 5.0 ml of 0.087 M BAA and 0.3 M KCl, 3.0 ml of methanol and 10 mg of BAL was introduced into the reaction vessel of the titrator. After temperature equilibration (40°) and adjustment to pH 6.0, the reaction was started by addition of 1 ml of papain stock solution (approx. 0.3 mg) at 40°. After a suitable interval 2.0 ml of neutralized 35% formaldehyde (Merck) was added. The reaction ceased immediately, and the pH decreased. Titration with 0.1 M NaOH was carried out up to pH 7.0. The amount of base added is equal to the amount of liberated ammonia after subtraction of a small blank value. The method was checked by titration of known amounts of NH_4Cl.

Thin-layer chromatography

After application, the adsorbent layers of Al_2O_3–$CaSO_4$ (Type D5 from Fluka, Switzerland) were dried at room temperature. Samples of 0.2 ml were taken from the reaction mixture at suitable intervals and added to 0.02 ml of 70 mM $HgCl_2$, which effectively stopped the reaction. A small precipitate quickly settled to the bottom of the tube. The supernatant provided enough material for application to several plates. The plates were run in methylethylketone–acetone–water–formic acid (120:6:18:3, by vol.) as solvent. The staining procedure of REINDEL AND HOPPE[6] was used with slight modifications. The dry plates were moistened by spraying with alcohol–ether (1:1) and drying superficially. After the recommended treatment in a dilute chlorine atmosphere the plates were sprayed with the toluidine–KI solution instead of being immersed in it. It appeared to be unnecessary to wash away the excess reagent. Photographs were taken immediately.

Substrate binding on non-activated papain (ref. 7)

The activation was carried out in 15% (by vol.) of methanol at 25°. During the determination of both K_m and of K_s the pH was 6.5 instead of 6.0, in view of the slight shift in pH optimum (Fig. 1A). Activator concentration was 5.0 mM cysteine and 1 mM EDTA. Five different BAEE concentrations in the range of 10–100 mM (at least two determinations for each concentration) were used.

Optical rotation

Solutions of 1% protein in 0.06 M ethylenediamine buffer[8] (pH 6.0) were measured at 365, 436, 546, 578 and 589 mμ in a Perkin–Elmer photoelectric polarimeter Model 141, equipped with the appropriate filters and lamps for this limited number of wavelengths, in a cuvette of 10 cm path length. The values were plotted according to MOFFITT[9]; $\lambda_0 = 210$ was found to give a somewhat better fit than $\lambda_0 = 212$. From the amino acid composition of papain[10] the mean residue weight M was calculated as 111. The refractive indices of water were taken from a paper of FASMAN[11]. The refractive indices of water–methanol mixtures were taken as equal to those of water, because the dispersion data for methanol[12] differ very little from those of water. For 6 and 8M urea solutions and 5M guanidine–HCl solution the refractive indices of water were multiplied by factors of 1.389/1.333, 1.408/1.333 and 1.415/1.333, respectively, 1.389 and 1.408 being n_D for 6M and 8M urea as interpolated from the data of FASMAN's Table VI, 1.415 being n_D for 5M guanidine–HCl as interpolated from Table VI of ref. 13 and 1.333 being n_D for water. Papain concentration was measured by absorption at $\lambda = 280$ mμ, assuming $E^{1\%}_{1\,cm} = 25$ (ref. 14).

Viscometry

An Ubbelohde viscometer was used with two bulbs above the capillary. Flow from the upper one alone was used for the more viscous methanol–water (1:1, by vol.) solvent (flow time 7.5 min), and flow from both bulbs for water (flow time 6 min). The temperature of the water-bath, 24.9°, was controlled to within $\pm$0.01°. The specific weight of the protein solutions was calculated assuming a specific volume of 0.73 (ref. 15). The NaCl in the papain preparations was taken into account by adding equal amounts of NaCl to the blank.

RESULTS

The activity of papain in high concentrations of methanol

The overall activity of papain at a fixed (11.0 mM) concentration of BGEE was determined as a function of methanol concentration at a few pH values (Fig. 1A). The activity decreased sharply with increasing methanol concentration. The pH of maximal activity was shifted slightly to higher values, but the variation in activity within the range pH 6 to pH 8 was always small compared with the decrease caused by methanol. The activity at the optimal pH values is plotted *versus* methanol concentration in Fig. 1B.

The results suggest that at 50% (by vol.) of methanol little activity of papain is left. However, the results obtained may be misleading, since it is conceivable that hydrolysis is gradually replaced by methanolysis (see below). The latter reaction would remain undetected by automatic titration. Experiments were therefore carried out with BAPA. From the latter substrate, nitroaniline is liberated and can be detected by spectrophotometry regardless of whether hydrolysis or methanolysis occurs.

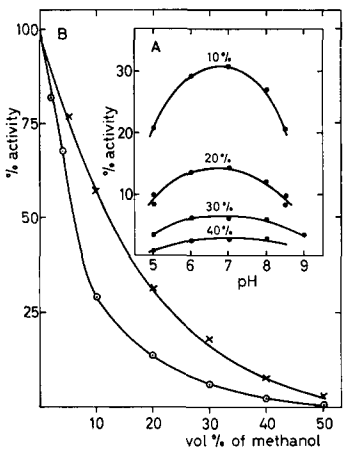


Fig. 1. A. Activity of papain as a function of pH and of methanol concentration, relative to activity at pH 6.0 (optimum activity) in water. Substrate 11.0 mM, methanol concentration as indicated. B. Relative activity of papain *versus* methanol concentration. ○, 11.0 mM BGEE at optimum pH values; ×, 0.6 mM BAPA at pH 6.5.

Fig. 1B shows that with this substrate too the activity of papain in 50% methanol is very low. Complete and immediate reactivation was found when a papain solution in 50% methanol was diluted with water.

Methanol, owing to air oxidation, might contain traces of formaldehyde responsible for the observed inhibition. This, however, cannot be the explanation. Gas chromatography did not reveal any formaldehyde, although the amount needed to bring about comparable inhibition appeared to be plainly evident on a gas chromatogram in the presence of the required excess of methanol. Moreover, the activity of papain in the presence of formaldehyde greatly depended upon the concentration of the activator. Under the conditions employed (11.1 mM BGEE, 0.3 M KCl, pH 6.0, 40°, approx. 4 mM formaldehyde) there was a 2.5-fold increase in activity when the

activator concentration was increased from 2 to 20 mM BAL. No such variation was obtained in the presence of 10% methanol. Therefore the inhibitory action in the latter conditions may be considered as due to methanol itself.

The occurrence of methanolysis

For the detection of methanolysis the substrate BAA was used. The hydrolysis of BAA does not cause any change in pH in neutral media, because equal amounts of acid and base are liberated (Eqn. 1). In water–methanol mixtures methanolysis might occur, leading to formation of BAME and ammonia. Because BAME is neutral and ammonia is basic the pH will increase (Eqn. 2).

$$R\text{–}CONH_2 + H_2O \rightarrow R\text{–}COO^- + NH_4^+ \quad (1)$$

$$R\text{–}CONH_2 + CH_3OH + H_2O \rightarrow R\text{–}COOCH_3 + NH_4^+ + OH^- \quad (2)$$

$$R\text{–}COOCH_3 + H_2O \rightarrow R\text{–}COO^- + CH_3OH + H^+ \quad (3)$$

Since the ester is a substrate too, it will be hydrolyzed with liberation of protons (Eqn. 3), resulting in a decrease in pH. Therefore, if methanolysis occurs, one may expect the following phenomena on incubation of BAA in say 30% of methanol in the presence of papain. At first the pH is expected to rise rather quickly by the formation of BAME up to a certain limit. This limit is reached when the rate of formation and the rate of hydrolysis of BAME are equal. When the conversion of BAA proceeds further, the concentration of BAA and therefore the rate of formation of BAME gradually decrease. The rate of hydrolysis of BAME then somewhat exceeds its rate of formation, resulting in a slow decrease in pH. Such a quick rise and slow decrease have actually been observed (Fig. 2A).

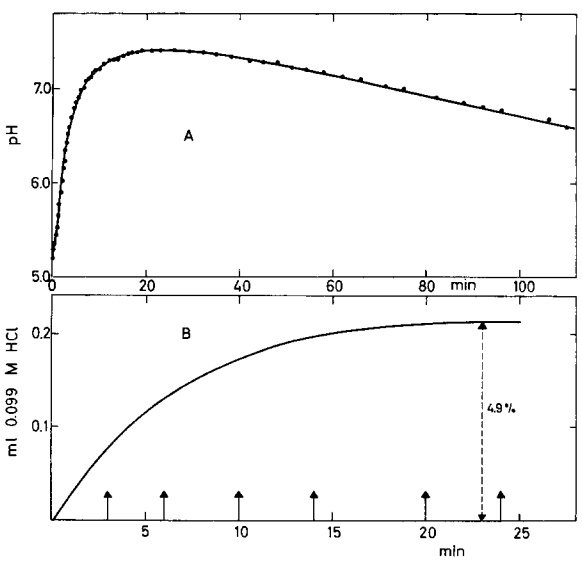


Fig. 2. A. Change in pH caused by converison of 3.6 mM BAA in 30% methanol in unbuffered medium. Activation by 10 mM BAL. B. Acid consumption of 43 mM BAA in 30% methanol at pH 6.0, 40°. Activation by 10 mM BAL. In a duplicate run aliquots for chromatographic analysis were taken at the intervals indicated by the arrows.

If, on the other hand, the pH is kept constant by automatic titration, acid consumption occurs at first (Fig. 2B). The acid consumed equals the amount of BAME formed *minus* the amount of BAME hydrolyzed. The titrator stops when the rates of formation and hydrolysis are equal. Hence the final level of acid consumption indicates the maximal amount of BAME present in the reaction mixture. This was found to be no more than 5% of the initial BAA concentration under the conditions used.

Since the evidence for methanolysis up to this point was rather indirect, a chromatographic identification of the products was desirable. In view of the very low concentration of BAME the utmost sensitivity was needed. The requirements were met by the combination of thin-layer chromatography and the chlorine–toluidine reagent for NH groups. The result is shown in Fig. 3. It confirms both the appearance of BAME and the general trend in the concentration of the products (*cf.* Figs. 2B and 4)*.

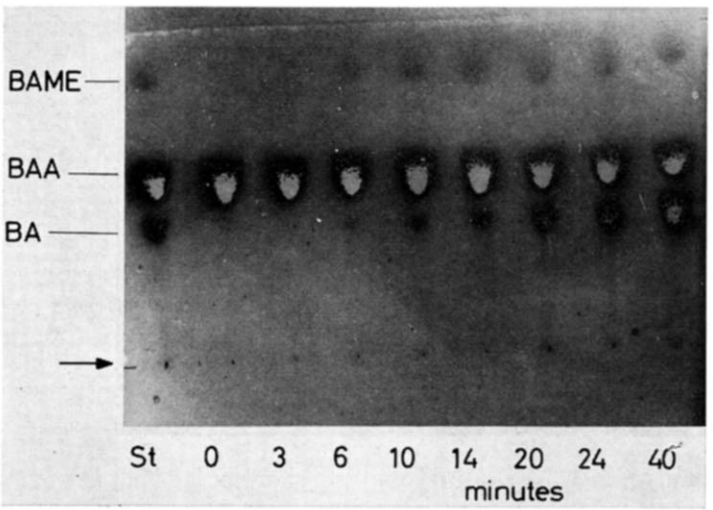


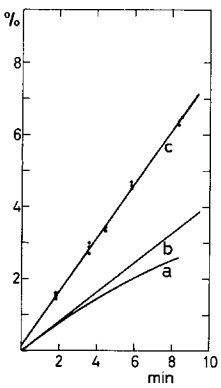
Fig. 3. Thin-layer chromatography of the formation of BAME and BA from 43 mM BAA in 30% methanol at pH 6.0. Samples were taken from a run similar to that represented in Fig. 2B at the intervals indicated. The standard (St) was 30 mM in BAA, 2 mM in BAME and 10 mM in BA.

In order to determine the ratio of methanolysis and hydrolysis the conversion of BAA was followed by two methods simultaneously: (1) By automatic titration with acid, as described above. The initial slope of the acid–consumption curve is a direct measure of the rate of formation of BAME, because at zero time the concentration and hence the rate of hydrolysis of BAME is still zero. (2) By formaldehyde titration of liberated ammonia, the amount of which equals the amount of BAA hydrolyzed *plus* the amount of BAA methanolyzed.

* STOCKELL AND SMITH[2] and GLAZER[16] looked in vain for alcoholysis of BAA. In view of the low concentration of ester now known to occur (Fig. 2B), their analytical methods (paper chromatography and Sakaguchi reaction) may have been insufficiently sensitive. In keeping with our results LOWE AND WILLIAMS[17] report a slight incorporation of [^{14}C]methanol into non-labelled hippuric methyl ester in the presence of papain.

The result of such an experiment is given in Fig. 4. From the slope of Lines b and c, and the molar ratio of water and methanol in the medium, one can calculate the ratio of the rates of methanolysis and hydrolysis per mole of these solvents. This ratio was found to be 5.6 ± 0.3, indicating that methanol is 5 to 6 times more reactive than water.

Similar ratios have been reported for the phosphate bond-splitting enzymes ribonuclease[18] (8.0 for methanolysis, 2.6 for ethanolysis) and phosphatase[19] (2–3 for



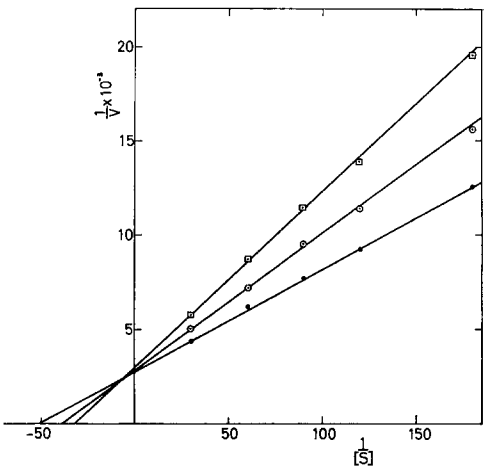


Fig. 4. Acid consumption (Curve a), initial slope of acid consumption (Curve b) and ammonia liberation (Curve c) during the conversion of 48 mM BAA in 33% methanol.

Fig. 5. Lineweaver–Burk plot of the inhibition of the hydrolysis of BGEE by methanol. Uninhibited reaction (●); inhibition by 2% methanol (○); by 4% methanol (□). v in $M \cdot min^{-1}$. $[S]$ in units of M.

alcoholysis by ethanolamine) and for non-enzymatic alcoholysis of *N*-acetylimidazole[20] (ratio of 4–6) and acetylphenylalanine esters (ratio of 1.6–2.4). On the other hand, a ratio of about 100 has been reported for chymotrypsin substrates[20,22]. The reason for this high ratio is not yet known.

Kinetic measurements

Determination of the kinetic parameters has been limited mainly to experiments in the presence of low concentrations of organic solvent, in order to restrict effects due to change in the dielectric constant in the bulk of the solvent. Even at such low concentrations inhibitory effects were plainly evident. Examples of $1/v$ *versus* $1/[S]$ plots are presented in Fig. 5. Virtually the same results were obtained with two brands of methanol, a common one and a highly purified one (Table I). The main effect of methanol is a decrease in $1/K_m$ (Fig. 5), *i.e.* the inhibition is of the competitive type. v_{max} is at most slightly decreased (about 7%) and the extent of this decrease is of the order of magnitude to be expected for the replacement of some of the water by methanol. The inhibition, however, does not conform to conventional competitive inhibition, because the extent of inhibition is not the same for the two substrates. Similar results were obtained with urea: the inhibition was competitive but not the

TABLE I

INHIBITION CONSTANTS

The constants are calculated from the abscissal intercepts of [S]/v *versus* [S] plots. Reaction conditions: pH 6.0, 0.3 M KCl, temperature 40° unless indicated.

Solvent	*Substrate*	K_m'/K_m*	K_i *(M)*
2% methanol	BGEE	1.28	1.8
4% methanol	BGEE	1.63	1.6
4% methanol *p.a.*	BGEE	1.70	1.4
4% methanol *p.a.*	BAEE	1.33	3.0
15% methanol *p.a.*	BAEE	2.40	2.6**
10–50% methanol	BAA		6 ± 2***
20% dimethylsulphoxide	BGEE	2.10	2.5****
4% acetonitrile	BGEE	1.22	3.5
4% dimethylformamide	BGEE	1.33	1.6
4% dimethylformamide	BAEE	1.26	2.0
2.0 M urea	BGEE	1.29	6.9
2.0 M urea	BAEE	1.60	3.3

* Ratio of K_m values in solvent mixture and in water.
** Temperature 25°. There was a 33% decrease in $v_{max.}$.
*** Calculated from table VI of ref. 2, at pH 5.2, 38°.
**** A 15% increase of $v_{max.}$ was indicated.

same for the two substrates. With urea, contrary to the effect of methanol, the hydrolysis of BAEE was more inhibited than the hydrolysis of BGEE. On the other hand, dimethylformamide competitively inhibited both substrates to about the same extent. Acetonitrile and dimethylsulphoxide are poorer inhibitors; 20% instead of 4% of dimethylsulphoxide was applied in order to determine K_i with some accuracy.

In order to be certain that the increase in K_m caused by methanol is due to an increase in the dissociation constant K_s of the enzyme–substrate complex, substrate binding on non-activated papain in 15% methanol was studied by a method described earlier[7]. The simplified procedure, with one activator concentration and a limited number of substrate concentrations (10–100 mM BAEE), was used.

The results (Table II) show that K_m of the activated enzyme and K_s of the non-activated enzyme were affected to the same extent by methanol and thus confirm that the substrate binding is indeed affected by methanol and not one of the other rate parameters which the general equation of K_m contains.

TABLE II

DISSOCIATION CONSTANTS OF PAPAIN–BAEE COMPLEX

Medium	K_m *(mM)*	K_s *(mM) extrapolated*	K_s *(mM) complete analysis*
Water*	18	28**	19.5
15% methanol	43	70**	56***
Ratio	0.42	0.40	0.35

* Taken from ref. 7.
** Obtained by graphical extrapolation to the [S] axis of a $1/k$ *versus* [S] plot.
*** Calculated assuming the activation rate of the complex of inactive enzyme and substrate to be 6% of the activation rate of the free inactive enzyme (*i.e.* equal to the ratio observed in water). For details see ref. 7.

Physical measurements

In order to find out whether or not conformational changes of the enzyme are responsible for the decrease in activity, the ORD of papain dissolved in various water–methanol mixtures has been measured. The values, in terms of $[\alpha]$, and of the parameters a_0 and b_0 of the MOFFITT equation[9], are given in Fig. 6.

The values both of a_0 and of b_0, the latter generally considered to indicate the helix content, remained unchanged up to 66% (by vol.) of methanol. The value of $b_0 = -135$* (mean of three determinations in water; $\lambda_0 = 210$ mμ) indicates a helix content of 21%, if 100% helix is assumed to correspond to $b_0 = -630$. These results have been confirmed by more extensive ORD measurements by DRENTH**. Even in the conventional denaturing medium, 6–8 M urea, there was no change in b_0 and only a 10% drop in a_0. Only drastic conditions (5 M guanidine hydrochloride, 0.01 M BAL, one night at room temperature) reduced the helix content to zero.

One example is known, namely the heat inactivation of chymotrypsinogen[25], in which denaturation gave no marked change in ORD but did cause a 1.6-fold increase in viscosity. No such change was found (Fig. 7) when the viscosity of papain in

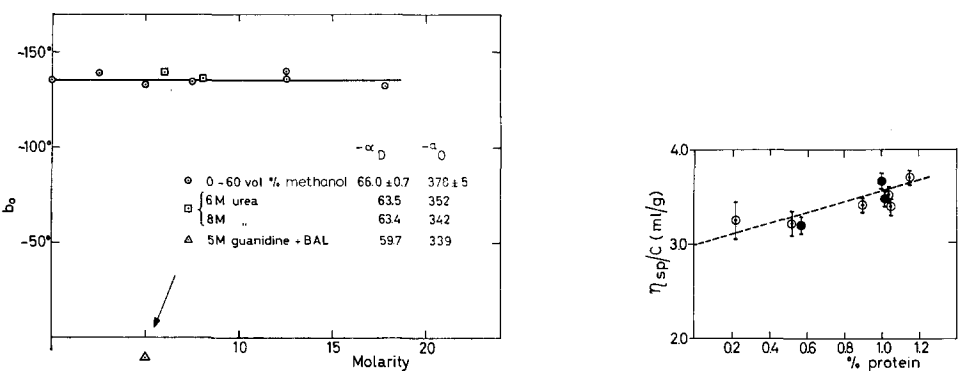


Fig. 6. Optical rotation of papain in various media, pH 6.0, 25°. Values of $-65.3°$ to $-70.3°$ for $[\alpha]_D$ of papain in water have been reported in the literature[23,24].

Fig. 7. Reduced viscosity (η_{sp}/c) of papain in water (○) and in 50% methanol (●), 0.01 M acetate (pH 5.5), 25°.

water was compared with that in 50% (by vol.) of methanol. This confirms the stability of papain in methanol–water mixtures.

The stability of papain was already indicated by the equality of $[\alpha]_D$ in water and in 8 M urea, as observed by HILL, SCHWARTZ AND SMITH[23]. On the other hand, HILL found a rapid loss of enzyme activity in urea solutions down to a certain final level of activity, both the rate of inactivation and the final level of activity depending upon the urea concentration. This phenomenon is now known to be due to reaction of cyanate[26], always present in low concentrations in urea solutions, with the active centre of papain. In freshly prepared 8 M urea solution the initial activity of papain

* For $\lambda_0 = 212$ mμ a value of $b_0 = -126°$ was calculated, corresponding to 20% helix.
** J. DRENTH, personal communication.

towards 30 mM BGEE (25°, 0.3 M KCl, 0.01 M BAL, pH 6.0) was found by the present author to be 50% of the activity in water (regardless of whether or not the non-activated papain had been preincubated in 8 M urea for 30 min). In view of the extent of competitive inhibition (Table I) an activity of 68% was expected. Hence intrinsically papain is at least 73% active in 8 M urea (*cf.* ref. 27).

It may be concluded from the results described in the last paragraph that papain is an enzyme of unusual stability. It is even more stable than ribonuclease, an enzyme which, in 8 M urea, is completely active but exhibits a marked change in optical rotation[28].

DISCUSSION

There are many papers in the literature concerning the effect of organic solvents upon enzyme action. Only a few can be cited. Experimentally competitive[2,29], non-competitive[30] and mixed[31] types of inhibition have been observed. The following explanations can be given: (1) change in dielectric constant of the medium[2,32]; (2) change in conformation of the enzyme; (3) ordinary competitive inhibition[29]; (4) change in partition coefficient of the substrate between the active site and the bulk of the medium, owing to better solubility of the substrate in the mixed solvent than in water[31].

None of these seems adequate.

(1) The shift of the pH optimum at higher methanol concentrations suggests that there may be some effect of the dielectric constant ε of the medium. However, the dielectric constant is incapable of explaining the marked increase in K_m which occurs even in low concentrations of methanol. If the substrate binding is aided by attraction between, say, two charges in the active centre and two dipoles in the substrate (the peptide bond and the ester group in BGEE), methanol should decrease and not increase the K_m, owing to a decrease in ε and hence an increase in charge interactions. In order to explain the observed increase in K_m, one has to assume that substrate binding occurs against opposing charge interactions (a rather unlikely assumption) or that the active site is available only after opposite charges in the active site have been separated. In the former alternative one would expect a larger increase in K_m for BAEE than for BGEE, because the former substrate has more points of interaction with the enzyme, owing to its (charged) side chain. Actually the opposite is true (Table I). In the case of charge separation in the active site one would expect an equal increase in K_m for both substrates. Moreover, the extent of the inhibition, especially with BGEE, is far larger than can be accounted for in terms of ε (*cf.* refs. 32, 33). Therefore the change of ε can have no more than a small effect.

(2) A large change in conformation of the enzyme can be ruled out in view of the ORD and viscosity data. A very local change may remain undetected by these measurements and therefore cannot be excluded. On the other hand, a local change does not seem very likely in view of the great stability of papain, as evident from its considerable activity in 8 M urea.

(3) The inhibition of pepsin by methanol and other alcohols has been examined with only one synthetic substrate[29]. The extent of inhibition by methanol was similar to the present example. With pepsin ordinary competitive inhibition could explain the available data. With papain, however, three substrates were examined and were

found to have a different K_i value (Table I). Therefore classical competitive inhibition is not the right explanation. In the inhibition of papain by urea, too, unequal K_i values occur. On the other hand, RAJAGOPALAN, FRIDOVICH AND HANDLER report equal K_i values for two substrates in the competitive urea inhibition of xanthine oxidase and of aldehyde oxidase[34].

(4) Explanation 4 in its simplest form is inadequate. On the one hand the solubility of BGEE in 20% dimethylsulphoxide was found to be twice its solubility in water, an increase which corresponds very well with the observed 2.1-fold increase in K_m in this solvent mixture. On the other hand, the solubility of BGEE in 4% methanol was only 1.1 times its solubility in water, an increase which is much smaller than the observed 1.6–1.7-fold increase in K_m.

A tentative hypothesis, partially related to (4), is the following. The water of hydration on the surface of the enzyme and of the substrate is partially replaced by molecules of methanol or other solutes in a partition equilibrium. The solute molecules being more or less hydrophobic themselves, diminish the hydrophobic interaction between the substrate and the active site and thus increase K_m. This accounts for the difference in inhibition of BGEE and BAEE hydrolysis. BGEE binding relies greatly upon hydrophobic interaction between its phenyl group and unidentified groups in the active site. BAEE binding relies not only on these same groups but is further strengthened by interactions with the charge and the polar groups of the guanidine group of the side chain. The latter interactions will be somewhat increased by methanol (*cf.* (1)). Therefore BAEE binding will suffer to a lesser extent than BGEE binding in water–methanol mixtures.

Urea, on the other hand, is known to weaken both hydrophobic interactions and hydrogen bonds[35], and may therefore be expected to weaken the binding contribution of the arginine side chain too. Urea may even compete with the guanidine group for the pertinent part of the active site. Hence the greater effect of urea upon BAEE binding than on BGEE binding. Dimethylformamide, having something in common with both methanol and urea, fits in nicely, since it inhibits the two substrates to about the same extent.

From "solvent perturbation" experiments LASKOWSKI concluded that dimethylsulphoxide is more or less excluded from protein solvation layers[36]. This indicates that the inhibition by dimethylsulphoxide is due mainly to its interaction with the substrate, which may explain why there is correspondence here between inhibitory action and dissolving capacity as reported in (4).

A more detailed discussion of the hypothesis does not seem warranted at the moment. Only two points of practical interest may be mentioned.

(a) As discussed above, (2), the possibility of a local change of conformation of papain by methanol cannot be completely excluded. Even so, X-ray analysis of crystals grown from water–methanol mixtures will yield information which is valuable at least as a first approximation.

(b) The inhibitory action of methanol is due mainly to prevention of the formation of the enzyme–substrate complex. This precludes the preparation from water–methanol mixtures of a crystalline enzyme–substrate complex suitable for X-ray analysis, using either a "product" and active enzyme[37] or a substrate and non-activated enzyme[7]. For this purpose other conditions of crystallization are being examined.

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