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Influence of solvent viscosity on the rate of hydrolysis of dipeptides by carboxypeptidase Y

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ABSTRACT: The influence of solvent viscosity on the rate of enzymatic hydrolysis of a series of dipeptides (Z-Phe-Gly, Z-Phe-Sar, Z-Phe-Ala, Z-Phe-NMeAla, Z-Phe-Aib and Z-Phe-Pro) by carboxypeptidase Y was investigated. The effect of solvent viscosity on the enzymatic hydrolysis revealed that whereas all k_{cat} values decreased with viscosity, those of the *N*-alkyl peptides decreased more than those of the *N*-H peptides. The kinetic behaviour implies the involvement of conformational changes of the enzyme in terms of the 'induced-fit' process. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: viscosity; induced fit process; carboxypeptidase-Y; dipeptides; hydrolysis

INTRODUCTION

Solvent viscosity constitutes an informative medium variable, whose utility is of prime importance for understanding the nature of solvent–solute interactions.[1] Consequently, the diversity of physical models developed to rationalize the viscosity dependence of molecular transformations has reached an impressive level. Furthermore, viscosity has played a definitive role in the detection of reaction intermediates and the elucidation of their chemical behaviour. The classical cage-effect studies constitute an excellent example of the use of solvent viscosity as a powerful mechanistic tool. In pioneering work, Kramers[2] proposed a chemical reaction model by considering the Brownian motion of a particle along the reaction coordinate in the presence of a potential-energy barrier. There have been many investigations on the influence of solvent viscosity on rate in various chemical reactions, such as stereochemical inversion upon nitrogen extrusion from cyclic azoalkanes,[3] electron transfer between a platinum electrode and $Fe(CN)_6^{3-/4-}$ or ferrocene$^{0/+}$,[4] and the isomerization reactions of stilbene,[5] to name just a few.

Our approach is based on the conceptually simple free-volume model of viscosity (η), in which the fractional-power viscosity dependence ($\eta^{-\alpha}$) provides the effective volume available for the enzymatic conformational change under investigation. The free-volume model of viscosity was suggested by Doolitle,[6] who intuitively pointed out that the translational motion of a molecule in a liquid is only possible when sufficient free volume (V_f) is available, i.e. when V_f is larger than some 'critical' value V_0. The fluidity (η^{-1}) is proportional to the probability factor [$\exp(-V_0/V_f)$] for the translational motion of an ensemble of molecules. Therefore, the free-volume dependence of the viscosity may be expressed by Eqn (1), where A is a proportionality factor:

$$\eta = A \exp(V_0/V_f) \tag{1}$$

In contrast to translational diffusion, enzymatic conformational changes involve only a portion of the molecule. Thus, only a fraction αV_0 ($\alpha < 1$) of the critical volume V_0 is required to execute the conformational motion, for which the catalytic rate constant k_{cat} of the reaction from enzyme–substrate complex to product in the enzymatic pocket is given by Eqn (2). Substitution of Eqn (1) into Eqn (2) results in Eqn (3). From Eqn (3), the higher the solvent viscosity, the smaller the reaction rate becomes.

$$k_{cat} = k_0 \exp(-\alpha V_0/V_f) \tag{2}$$

$$k_{cat} = k_0 (A/\eta)^{\alpha} \tag{3}$$

Carboxypeptidase-Y (CPD-Y) (EC 3.4.16.5) from baker's yeast is a lysosomal serine exopeptidase, which catalyses the hydrolysis of the C-terminal peptide bond of proteins. It has been suggested that a Ser-His-Asp catalytic triad is involved in the process as found in the trypsin and chymotrypsin families of endopeptidases.[7] An interesting feature is that a peptide bond consisting of a proline residue can be hydrolysed by catalysis of

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CPD-Y, whereas this bond is resistant to cleavage by α-chymotrypsin. CPD-Y is synthesized in the nucleus as a preproenzyme and transported to vacuoles. CPD-Y is a popular model protein for studying protein sorting in yeast.[8] Initial studies have focused on applications such as C-terminal sequence analysis. Recently, the structure of monomeric CPD-Y has been determined by x-ray diffraction studies.[9]

In general, enzyme catalysis is initiated by interaction between the enzyme and the substrate prior to reaction, which gives rise to what is known as the enzyme–substrate (E–S) complex or Michaelis complex. The 'induced-fit' process has been suggested to account for the reactivity in enzymatic reactions.[10–12] According to this assumption, the enzyme reaction proceeds by catalytic conformations induced by interaction between the enzyme and the substrate. The flexible active site opens up to allow the incorporation of the substrate, but once the substrate is incorporated, the amino acid residues of the active site become fixed around the substrate.

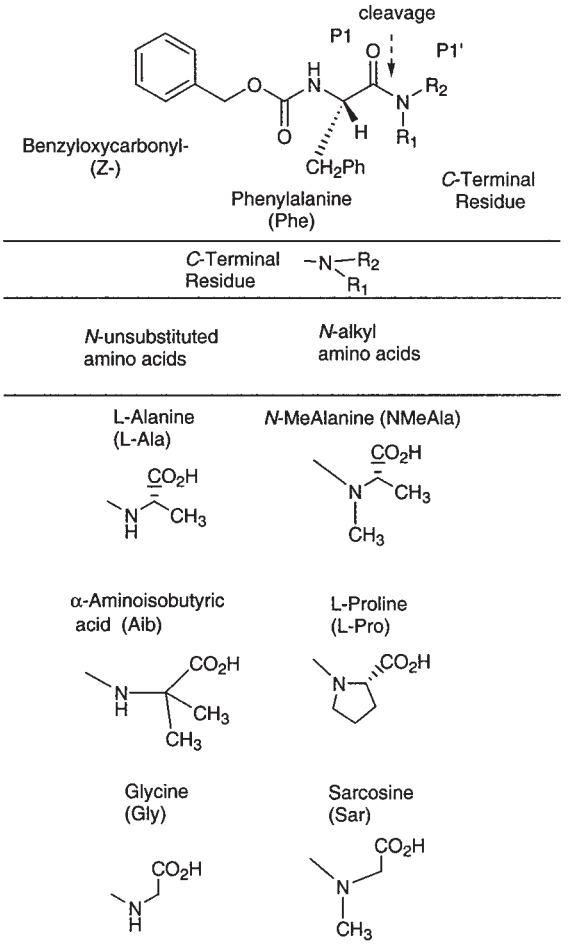
Increasing solvent viscosity generally results in stronger friction forces between solvent and solute. Likewise, structural fluctuations of enzymes are also expected to be diminished.[13] Therefore, the rate constant of a step where the conformational effect of an enzyme is significant should be strongly affected by solvent viscosity. In fact, there are several examples where the rate of an enzymatic reaction or isomerization of a protein depends on the solvent viscosity.[14–17] For instance, it has been reported that an increase in solvent viscosity causes a decrease in the rate in the photocycle of bacteriorhodopsin,[18] in the diffusion of atom groups CO or O_2 in myoglobin[19] and in enzymatic reactions by carboxypeptidase A.[16] In the case of the diffusion of CO or O_2, the relation between viscosity and rate could be correlated by the value of $0.4 < \alpha < 0.8$. In another example, from the dependence on solvent viscosity of kinetic parameters for the hydrolysis of *p*-nitroanilides catalyzed by α-chymotrypsin, it was concluded that the formation of a tetrahedral intermediate in the acylation of the enzyme is significantly more susceptible to conformational effects of the enzyme than the breakdown of the intermediate.[20]

As a step towards gaining an understanding of the mechanism of the hydrolysis of peptides by enzymes, we have pursued this issue in terms of solvent viscosity by comparing the difference in hydrolytic behaviour of *N*-alkyl and *N*-H peptides catalysed by carboxypeptidase Y.

RESULTS AND DISCUSSION

Preparation and hydrolysis of *N*-benzyloxycarbonyl-protected dipeptides

A series of dipeptides (Z-Phe-Gly, Z-Phe-Sar, Z-Phe-Ala, Z-Phe-NMeAla, Z-Phe-Aib and Z-Phe-Pro) as shown in Scheme 1 were prepared by typical procedures.

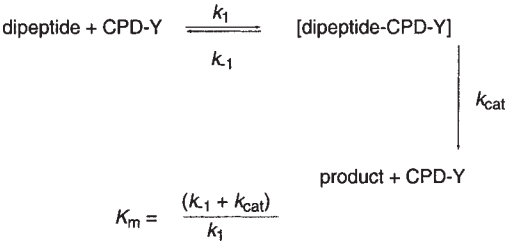


Scheme 1

Kinetics

All kinetic experiments were performed in pH 6.5 at 37 °C. The progress of the reactions was determined by monitoring spectrophotomerically (230–240 nm) the relative amount of the released products. Kinetic parameters as designated in Scheme 2 for the hydrolysis of these dipeptides by CPD-Y are summarized in Table 1. The listed values are the average of 3–5 runs.

In addition, the enzymatic reaction of six substrates was carried out in glycerol or sucrose at four or five different concentrations and the kinetic data were calculated by non-linear fitting of the Michaelis–Menten equation to plotted experimental data.



Scheme 2

Table 1. Kinetic parameters for hydrolysis of dipeptides by CPD-Y

Dipeptide Z-Phe-Xaa	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
-NMeAla	0.273 ± 0.033	0.657 ± 0.027	2.40 ± 0.30
-L-Pro	0.338 ± 0.018	45.0 ± 0.8	133 ± 7
-L-Ala	0.939 ± 0.099	187.7 ± 11.4	200 ± 24
-Sar	1.94 ± 0.058	25.9 ± 0.55	13.3 ± 0.49
-Gly	3.36 ± 0.12	189 ± 5.5	56.4 ± 2.6
-Aib	2.86 ± 0.15	147 ± 9.66	51 ± 4.9

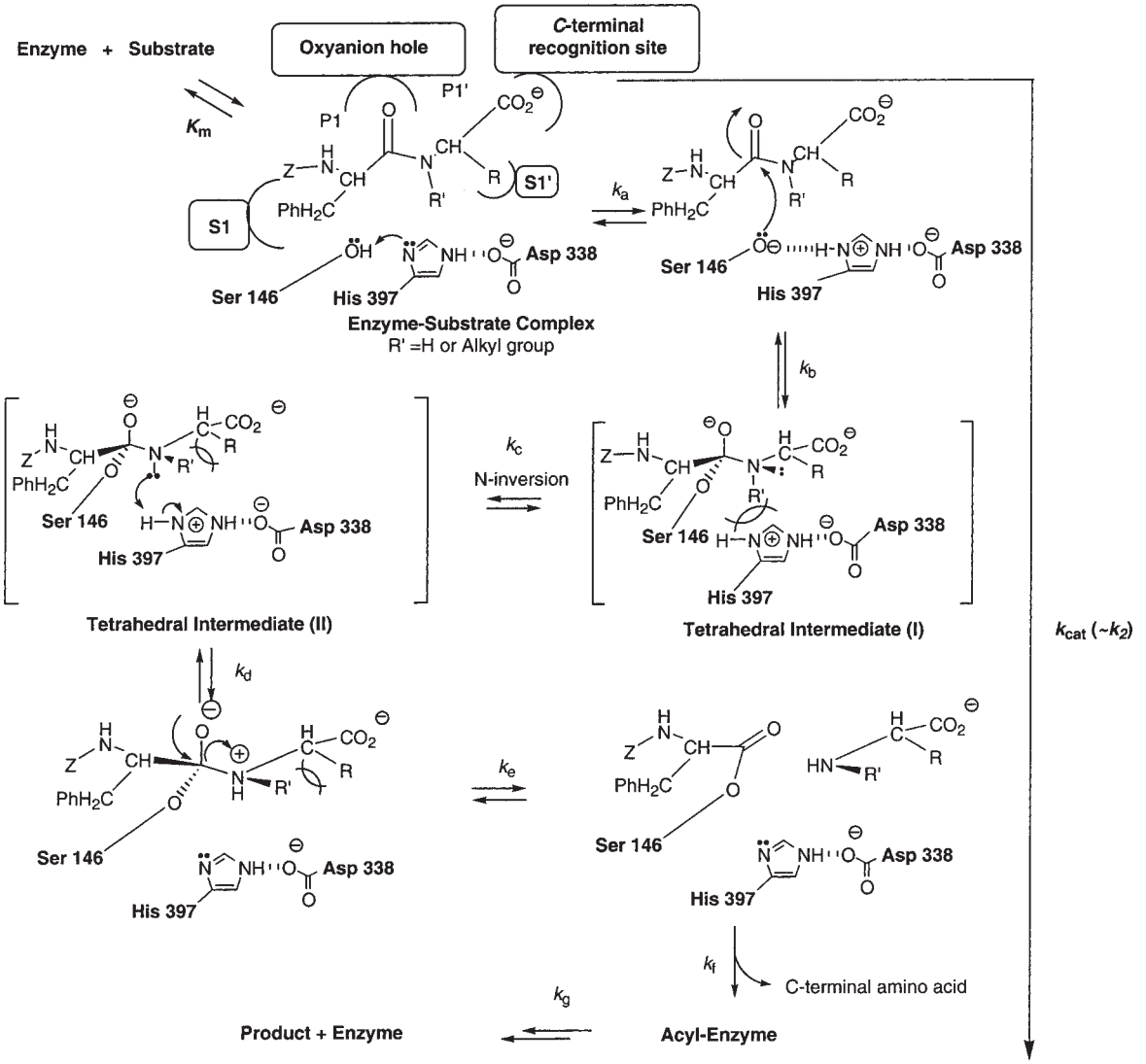
Hydrolysis of dipeptides by carboxypeptidase Y

The order of the overall reactivity (k_{cat}/K_m) of the C-terminal residue in the dipeptides was Ala > Pro > Gly≈Aib > Sar > NMeAla, as shown in Table 1.

On the basis of the K_m values (NMeAla < Pro < Ala < Sar < Aib < Gly), the affinity of the Pro substrate towards the CPD-Y enzyme is favourable for making the E–S complex relative to those of Ala, Sar, Aib and Gly substrates. It was found that there is a high correlation between hydrophobicity of the dipeptide substrates and K_m, suggesting that the hydrophobicity of the C-terminal amino acid is a major factor in governing the stability of the E–S complex.[21] Therefore, the higher reactivity (k_{cat}/K_m) of the Pro substrate relative to the Sar, Aib and Gly substrates could be attributed to its stronger affinity induced by hydrophobic interactions and to the difference in steric demands to which it is subjected in the transition state compared with the other substrates.

Examination of the x-ray structure of CPD-Y[9] led to the proposal that in CPD-Y the segment of the enzyme bearing the amino acid residues Ser146, Asp338 and His397, which could constitute the catalytic triad or charge relay system, is the active site or catalytic cavity, as illustrated in Scheme 3.

Near these catalytic residues are two hydrophobic pockets consisting of the residues Thr60, Phe64, Glu65, Tyr256, Tyr269, Leu272, Met398 and disulfide 56–298



Scheme 3. Acylation step in the hydrolysis by serine proteinase

designated the S1′ binding site, and Tyr147, Leu178, Tyr185, Tyr188, Leu245, Trp312, Ile340 and Cys341 designated the S1 binding site. It would be possible for these pockets to accommodate hydrophobic side-chains at the P1 (corresponding to the N-terminal amino acid moiety) and P1′ (corresponding to the C-terminal amino acid moiety) positions, respectively. Another important region at the active site, which could be assumed to be the oxyanion hole consisting of Gly53 and Tyr147, is also present.

The mechanism of bond cleavage can be rationalized as proceeding via tetrahedral intermediates formed by the attack of Ser146 upon the dipeptide substrates, which go on to collapse to acyl-enzyme intermediates by extrusion of the amino group of the cleaved peptide bond following inversion upon the amide nitrogen atom and protonation, as illustrated in Scheme 3. According to the x-ray structure, there seems to be higher steric hindrance at the tetrahedral intermediate (**I**) in the enzymatic hydrolysis by α-chymotrypsin than carboxypeptidase Y.[22,23] When the C-terminal residue is NMeAla and Sar, which have acyclic structures, different steric interactions are expected to arise upon conformational change of the enzyme as compared with the Pro substrate in the tetrahedral intermediate (**II**). This is based on the principle of microscopic reversibility and the stereoelectronic control theory.[22] The role of the His397 and Asp338 residues is thought to be to maintain the Ser146 residue in a state capable of reacting with the incoming peptide chain, and also to stabilize the tetrahedral intermediate formed during catalysis. The imidazole group of His397, which is essential in the enzymatic catalysis,[7b] assists as a strong general base; the functional group abstracts the alcoholic proton of Ser146 and shuttles it to the amino leaving group. The negatively charged Asp338 stabilizes the positive charge developed on His397.

The order of the catalytic rate constants (k_{cat}) in the present work was Gly > Ala > Aib > Pro > Sar > NMeAla and happens to be approximately the same as the order of pK_a values of the corresponding amino acids [Gly (9.60), Ala (9.69), Aib (10.21), Pro (10.60) and Sar (10.01)].[24] Since the electron-donating ability of the amino acid nitrogen atom is expected to correlate with the pK_a of the amino acid, the experimental results can be rationalized as the more basic and thus the more electron-donating amino acid being less capable of being attacked by the oxide anion of the Ser146, thus leading to lower k_{cat} values.

Since *N*-H substrates are similarly hydrolysed by both α-chymotrypsin and carboxypeptidase-Y, whereas *N*-alkyl substrates are only readily hydrolysed by the latter, it can be assumed that whereas the sizes of the cavity of both enzymes are similar, carboxypeptidase-Y is much more flexible and large conformational changes facilitate the formation of E–S complexes even for *N*-alkyl substrates in the hydrolysis reactions.

Influence of solvent viscosity on the kinetic parameters K_m and k_{cat}

Since more conformational change of the enzyme is expected to be necessary in the enzymatic hydrolysis of *N*-alkyl peptides relative to the *N*-H substrates, we expected that the k_{cat} of *N*-alkyl substrates would be more sensitive than *N*-H substrates to changes in solvent viscosity. Thus, the relation between solvent viscosity and parameters K_m and k_{cat} was next examined. In order to ascertain that viscosity did not affect the structure of the enzyme, circular dichroism (CD) spectra in the wavelength range 200–300 nm were recorded for the enzyme dissolved in Tris–HCl buffer containing 50% glycerol (6 M), and compared with spectra of a reference solution containing just Tris–HCl buffer and of a solution containing 8 M urea with buffer. As shown in Fig. 1, since the spectral profiles of the 50% glycerol and reference solutions were essentially identical, it was assumed that the presence of glycerol did not greatly affect the enzyme secondary structure.

In the hydrolysis of dipeptides by carboxypeptidase-Y, K_m increased only slightly with viscosity and the maximum variation was within 1.28 times the value with no viscogen (Fig. 2, Table 2).

Generally, the effects of viscogen on enzymatic reactions have been explained by excluded volume effects,[25] secondary and tertiary structual change,[26] changes in bulk water concentration[27] and inhibition by the added viscogen.[26] As the additives (glycerol and sucrose) are very hydrophilic hydroxy compounds, these compounds have no excluded volume effect but produce a stabilization effect of proteins in aqueous solution.[28] The comparison of the CD spectra (Fig. 1) indicates that secondary and tertiary structure changes by excluded volume effects did not occur substantially in the enzyme itself. Changes in bulk water concentration and inhibition by the additive is concerned with the changes in K_m values. On the other hand, it is considered that the catalytic rate constants k_{cat} from the E–S complex to the product are not directly affected by the additive but are indirectly influenced through the enzymatic conformational changes since the active site of CPD-Y is isolated from the surrounding solvent.[9]

Since K_m was little influenced by the presence of viscogens, we could assume that the association of the enzyme and a substrate does not require appreciable conformational changes of the enzyme in the present series of reactions. The small increase in the K_m values could be attributed to the loss of entropy upon the incorporation of the substrate into the enzyme interior by the partial destruction of the water cluster structure caused by the intervention of viscogen molecules and the competitive inhibitor by the viscogen. When substrates are more difficult to incorporate into the enzyme by competitive inhibitor, the values of K_m increase.

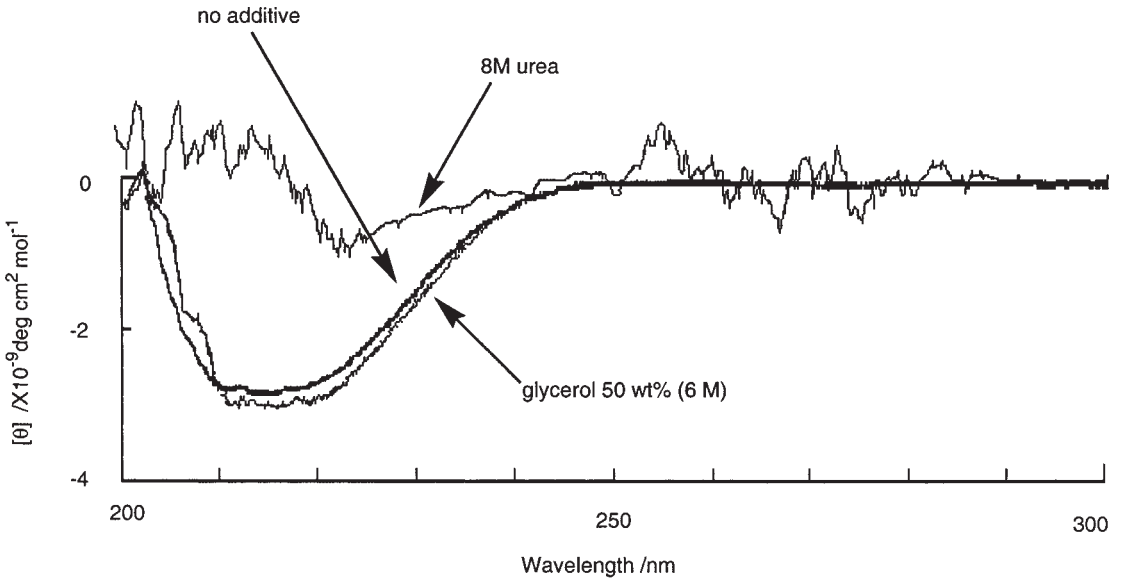


Figure 1. CD spectra of carboxypeptidase-Y in Tris–HCl buffer at pH 6.5 (no additive), in 50 wt% glycerol in Tris–HCl buffer at pH 6.5 and in 8 M urea in Tris–HCl buffer

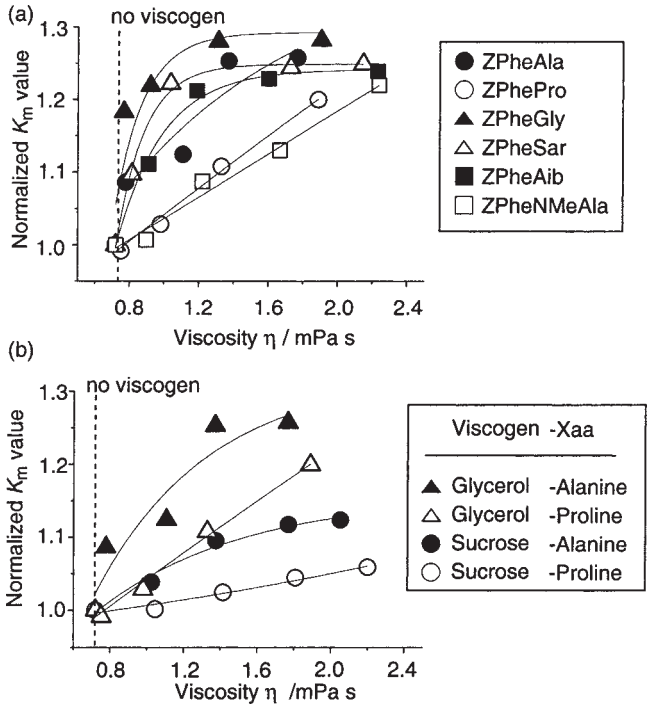


Figure 2. (a) Dependence of the K_m of CPD-Y-catalysed hydrolysis of dipeptides Z-Phe-Xaa on viscosity. (b) Dependence of K_m of the dipeptide Z-Pha-Xaa hydrolysis on viscosity with sucrose or glycerol as viscogen

Figure 3(a) shows that the k_{cat} values of the *N*-alkyl peptides were more strongly affected by viscosity than those of the *N*-H peptides. Figure 3(b) indicates that the influence of viscosity on the k_{cat} value in the sucrose–water system is smaller than that in the glycerol–water system.

The tendency for the smaller viscogen to be more influential has also been observed in the inhibition of adenosine deaminase-catalysed hydrolysis of adenosine by transition-state analogue inhibitors,[15] where sucrose or Ficoll (Ficoll 400 is a neutral, highly branched, hydrophilic polymer of sucrose which dissolves readily in aqueous solution) was used as the viscogen. The association and dissociation rates of the inhibitor showed a decrease in the presence of the small viscogen sucrose whereas the large viscogen Ficoll had no effect.[15] Furthermore, in the hydrolysis of benzoylglycyl-L-phenyllactate by carboxypeptidase A, where sucrose and glycerol were used as viscogens,[16] the reaction rate constant k_{cat} in the glycerol–water system was found to be smaller than that in the sucrose–water system at the same viscosity, revealing that glycerol affects the reaction more than sucrose.

Here, the reaction rate constant k_{cat} decreased as the solvent viscosity increased for all the substrates studied. It can be assumed that the decreases are due to the loss of conformational mobility of the enzyme and can be associated with the conformational change of enzymes known as the 'induced-fit' process, induced by the interaction between an enzyme and the substrate. Hence it is suggested that the k_b step involving the formation of tetrahedral intermediate (**I**) is the rate-determining step.[20] Furthermore, as described above, the dependence of the electron-donating ability of the amino acid moiety at the C-terminus on the k_{cat} values is consistent with this step being rate determining.

Strajbl *et al.* have demonstrated that in the event of nucleophilic attack of ROH such as serine protease on an amide carbonyl group, the alcohol is assisted by base by deprotonation.[29] From the principle of microscopic reversibility, the tetrahedral intermediate must therefore eliminate RO^- instead of ROH. A comparison of the pK_a values of methanol and ethanol (15.6 and 16,

Table 2. Kinetic parameters for the hydrolysis of the dipeptides Z-Phe-Xaa by CPD-Y in glycerol–water or sucrose–water mixed solvent

Viscogenic agent	Concentration (wt%)	Viscosity (mPa s)	k_{cat} (s^{-1})	K_m (mM)
Z-Phe-Ala	0	0.7197	187.7 ± 11.4	0.9386 ± 0.099
Glycerol	5.008	0.7776	181.4 ± 9.3	1.020 ± 0.054
	18.11	1.107	169.9 ± 3.9	1.055 ± 0.025
	25.29	1.370	164.8 ± 3.2	1.176 ± 0.023
	33.73	1.766	154.8 ± 5.1	1.180 ± 0.391
Z-Phe-Pro	0	0.7197	44.97 ± 0.78	0.3387 ± 0.0180
Glycerol	3.236	0.7514	41.09 ± 0.36	0.3359 ± 0.0039
	14.16	0.9791	36.16 ± 0.72	0.3483 ± 0.0088
	23.39	1.329	33.96 ± 0.86	0.3752 ± 0.0113
	34.38	1.888	30.84 ± 1.00	0.4062 ± 0.0149
Z-Phe-Gly	0	0.7197	189.4 ± 5.5	3.358 ± 0.121
Glycerol	4.514	0.7665	190.8 ± 16.1	3.971 ± 0.337
	13.18	0.9195	184.8 ± 5.4	4.091 ± 0.121
	24.13	1.311	171.3 ± 7.9	4.298 ± 0.198
	34.51	1.903	162.1 ± 7.4	4.303 ± 0.216
Z-Phe-Sar	0	0.7197	25.93 ± 0.55	1.944 ± 0.058
Glycerol	7.048	0.8128	23.76 ± 0.92	2.134 ± 0.084
	16.47	1.036	21.36 ± 1.62	2.375 ± 0.182
	31.81	1.727	19.69 ± 0.84	2.418 ± 0.104
	38.36	2.146	18.33 ± 0.59	2.429 ± 0.078
Z-Phe-Ala	0	0.7197	187.7 ± 11.4	0.9386 ± 0.099
Sucrose	14.08	1.026	182.6 ± 1.0	0.9745 ± 0.0057
	23.13	1.376	173.5 ± 14.5	1.028 ± 0.089
	28.69	1.77	166.8 ± 13.3	1.049 ± 0.086
	32.13	2.053	163.5 ± 4.2	1.055 ± 0.028
Z-Phe-Pro	0	0.7197	44.97 ± 0.78	0.3387 ± 0.0180
Sucrose	13.47	1.046	40.01 ± 0.26	0.3392 ± 0.0028
	21.82	1.416	35.45 ± 0.42	0.3469 ± 0.0052
	27.55	1.809	34.38 ± 0.34	0.3537 ± 0.0040
	31.00	2.201	34.12 ± 1.07	0.3586 ± 0.0134
Z-Phe-NMe-Ala	0	0.7197	0.6569 ± 0.0270	0.2740 ± 0.0325
Glycerol	9.51	0.8955	0.5937 ± 0.0039	0.2759 ± 0.0026
	20.47	1.219	0.5406 ± 0.0058	0.2979 ± 0.0041
	30.19	1.666	0.5147 ± 0.0099	0.3095 ± 0.0073
	39.56	2.236	0.4803 ± 0.0126	0.3341 ± 0.0102
Z-Phe-Aib	0	0.7197	147.3 ± 9.7	2.864 ± 0.147
Glycerol	9.554	0.9075	135.8 ± 4.6	3.182 ± 0.107
	20.01	1.186	129.2 ± 3.9	3.470 ± 0.104
	29.74	1.600	118.2 ± 4.6	3.518 ± 0.136
	39.61	2.228	117.2 ± 2.4	3.549 ± 0.363

respectively)[30] with those of the ammonium group of amino acids also indicates that the RO^- moiety has a poorer leaving group ability from the tetrahedral intermediate (**I**) than the counterpart protonated amino acid. Here, the increase in the pK_a values of the corresponding amino acid makes the addition of RO^- even harder. In other words, the addition of ROH assisted by base is more difficult than the elimination of the corresponding amino acid moiety. Since the activation energies of the *N*-protonation and the *N*-inversion steps are generally much smaller than those of the addition of alkoxide ion and the elimination of amino acid steps, the rates of *N*-protonation (k_a, k_d) and *N*-inversion (k_c) should be faster than those of addition (k_b) and elimination (k_e). If the step involving C-terminal amino acid product release (k_f) were rate determining, a relationship between k_{cat} and the hydrophobicity of the corresponding amino acid moieties would be expected since there are two hydrophobic pockets in CPD-Y. However, for the six substrates in this study, no relationship (correlation factor $r = 0.12$) between k_{cat} and hydrophobic parameter ΔG_{ret},[21] which is correlated with the hydrophobicity of the corresponding amino acid moiety, was observed. As the *N*-terminal residues of all the substrates are the same and k_{cat} differs for all the substrates examined, the final k_g step could not be rate determining. This would leave the step involving the formation (k_b) of the tetrahedral intermediate (**I**) and the step involving breakdown (k_e) of the protonated tetrahedral intermediate as candidates for the rate-determining step. The fact that k_{cat} diminished with

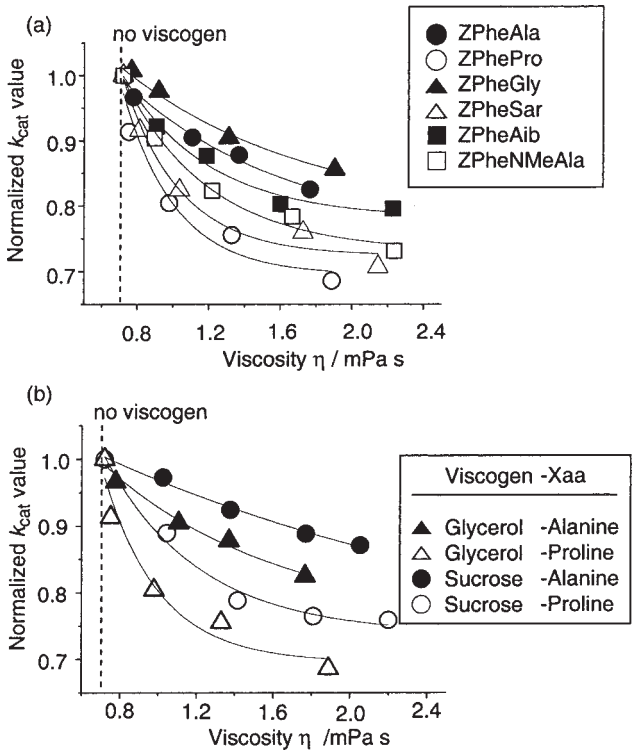


Figure 3. (a) Dependence of the k_{cat} of CPD-Y-catalysed hydrolysis of dipeptides Z-Phe-Xaa on viscosity in glycerol–Tris–HCl buffer mixed solvent. (b) Dependence of k_{cat} of the dipeptide Z-Phe-Xaa hydrolysis on viscosity with sucrose or glycerol as viscogen

higher amino acid basicity and higher solvent viscosity implies that the former formation step is the overall rate-determining step.

Support for this conclusion in seen in *ab initio* calculations of the base-catalysed methanolysis of formamide, which can be considered a simplified model system of an enzymatic hydrolysis reaction. The nucleophilic attack of an alkoxide anion is indicated to be the rate-determining step and the calculated activation energy of this step is consistent with experimental values for the general base-catalysed hydrolysis of several amides.[29]

Further support for the rate-determining step was provided by Fersht and Jencks, who showed that the rate constant k_2 ($M^{-1}s^{-1}$) for the nucleophilic attack of $R''O^-$ on amide groups increases with decreasing electron-donating ability of the amine moiety according to the relationship $\log k_2 = 7.2 - 0.67pK_a(R'NH_3^+)$, where k_2 represents the rate constant for the step involving acylation of the enzyme in the hydrolysis by serine proteases.[31] Furthermore, it is feasible for the catalytic His to abstract a proton from the catalytic Ser simultaneously as the latter attacks the peptide bond to be cleaved, because CPD-Y is known to be highly active even at low pH where other serine endopeptidases are inactive.[8] In the alcoholysis of amides at low pH, the rate-determining step is generally the formation of a tetrahedral intermediate.[31]

In order to evaluate the α values in Eqn (3), substitution of $k_{cat}{}^0$ and η^0, which are the values for no viscogen, into Eqn (3) results in Eqn (4). Equation (3) divided by Eqn (4) produces Eqn (5).

$$k_{cat}^0 = k_0(A/\eta^0)^\alpha \quad (4)$$

$$k_{cat}/k_{cat}^0 = (\eta/\eta^0)^{-\alpha} \quad (5)$$

The α values for the substrates employed here were calculated from normalized k_{cat}/k_{cat}^0 and normalized η/η^0 with Eqn (5) by non-linear fitting (Fig. 4, Table 3).

The α values correspond to the degree of dependence of the rate on viscosity and here may be regarded as the degree of solvation around the active site which is located on the inside of the surface of the enzyme; $\alpha = 1$ corresponds to a case where the variations in the solvent mobility are fully transferred to the reaction site of the

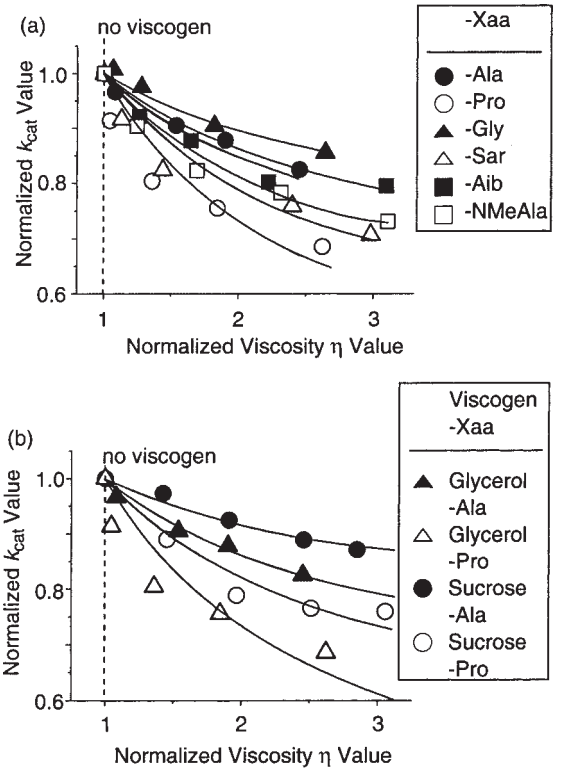


Figure 4. (a) Normalized k_{cat} value against normalized viscosity of the dipeptide Z-Phe-Xaa hydrolysis in glycerol–Tris–HCl buffer mixed solution. (b) Normalized k_{cat} value against normalized viscosity of the dipeptide Z-Phe-Xaa hydrolysis with sucrose or glycerol as viscogen

Table 3. α Values calculated from Eqn (5)

	Glycerol	Sucrose
Z-Phe-L-Ala	0.21 ± 0.01	0.12 ± 0.01
Z-Phe-Gly	0.16 ± 0.01	
Z-Phe-Aib	0.24 ± 0.02	
Z-Phe-L-Pro	0.45 ± 0.06	0.29 ± 0.02
Z-Phe-NMeAla	0.30 ± 0.02	
Z-Phe-Sar	0.34 ± 0.04	

enzyme. From the α values in Table 3, the hydrolysis reaction of the *N*-alkyl peptides was more sensitively affected than the *N*-H peptides by variations of viscosity.

According to the free-volume model of viscosity,[6] as viscosity increases, the stronger an effect free volume has, resulting in a larger α value. In other words, the hydrolysis reaction of the *N*-alkyl peptides accompanied larger structural fluctuations (larger free volume) than the *N*-H peptides. These results are consistent with the 'induced-fit' process model in the enzymatic reactions, induced by the interaction between the enzyme and the substrate.

Examination of the x-ray structure[9] of the main chain of CPD-Y [Fig. 5(a)] reveals that the catalytic His397 is near the C-terminal helix domain (Phe401–Ile415). This domain is not bound by a surrounding peptide chain and not located within a β-strand structure [Fig. 5(c)] and may therefore fluctuate with ease. On the other hand, the catalytic His of α-chymotrypsin is located within a β-strand structure (Trp51–Thr54 and Val65–Ala68) and is fixed to a surrounding peptide chain [Fig. 5(b)]. For this reason, we believe that the hydrolysis reaction of the *N*-alkyl peptides accompanied more fluctuation of the domain including His397.

Scheme 4 illustrates the effect of viscosity on the enzymatic reaction by the free-volume model.

With the conformational change of the substrate during the conversion from the Michaelis complex to a tetrahedral intermediate as depicted, the geometry of the amide bond nitrogen changes from planar to pyramidal with the accompaniment of a conformational change of the enzyme. As in Scheme 4(b), it is expected that a larger conformational change of the enzyme (larger free volume) is required in the case of the *N*-alkyl substrates during the reaction, and therefore is more susceptible to solvent effects. The decrease in k with increasing η implies that the increase in friction forces exerted by the viscous solvent leads to retardation of the fluctuation of the enzyme.

It has also been reported that most proteins are effectively hydrated on the addition of glycerol, this being the origin of structure stabilization of proteins in aqueous glycerol.[28] As shown in Fig. 3, although there is a slight difference in the degree of change, the profiles were similarly shaped for the two different viscogens. This supports our conclusion that viscosity is directly related to the speed of conformational change and that the phenomenon is independent of the nature of the viscogen.

EXPERIMENTAL

Instrumentation. CD spectra were recorded on Jasco J-500CH instrument. A Shimadzu UV-240 UV–visible recording spectrophotometer was used for kinetic measurements. Viscosity was measured by using a stalagmometer at 310 K.

General synthetic method. Treatment of L-phenylalanine (Phe) with benzyloxycarbonyl chloride (Z-Cl) and L-alanine (Ala) with di-*tert*-butyl dicarbonate (Boc_2O) afforded Z-Phe and Boc-Ala, respectively. Methylation of Boc-Ala with methyl iodide gave the *N*-methylalanine derivative (Boc-MeAla). Reaction of α-amino acid (Xaa = L-Ala, Gly, NMeAla, L-Pro, Aib and Sar) with thionyl chloride followed by MeOH gave the corresponding methyl ester (HCl·Xaa-OMe). The coupling reactions

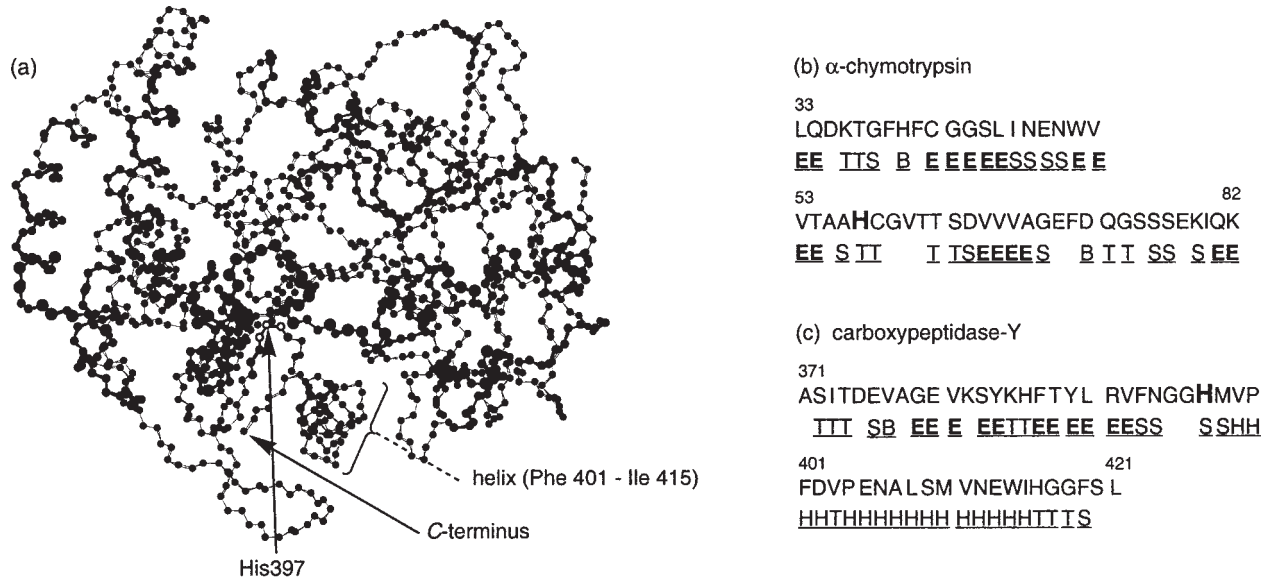
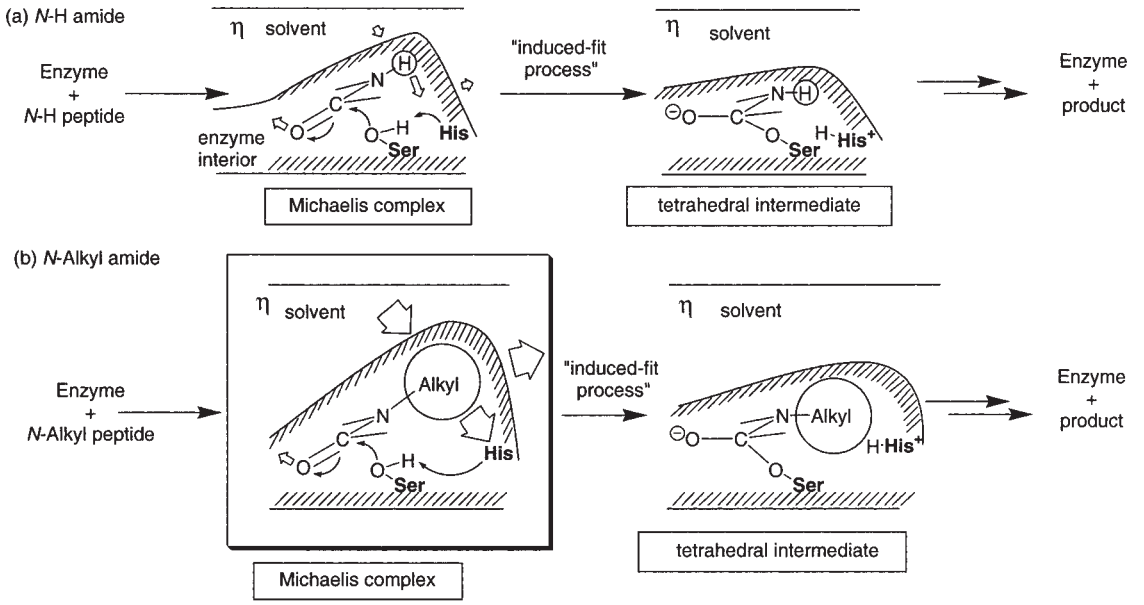


Figure 5. The main chain of CPD-Y x-ray structure (PDB code: 1YSC).[9] (b), (c) Sequence and secondary structure of α-chymotrypsin [(PDB code: 4CHA),[23] Leu33–Lys82, (b)] and carboxypeptidase-Y [Ala371–Leu421, (c)]. The sequence is shown as a single letter code and **H** is the catalytic histidine of each enzyme. The assignments of secondary structure are: H = helix; B = residue in the isolated beta bridge; **E** = extended β-strand; T = hydrogen-bonded turn; S = bend



Scheme 4. The viscosity effect on the enzymatic reaction

were mediated by dicyclohexylcarbodiimide to give the corresponding *N*- and *O*-protected dipeptides (Z-Phe-Xaa-OMe). Deprotection of the C-terminus of the dipeptides was carried out by treatment with an equivalent volume of 1 M NaOH for 0.5–3 h at room temperature to give the dipeptide substrates Z-Phe-Xaa.

N-Benzyloxycarbonyl-L-phenylalanyl-2-methylalanine (Z-Phe-Aib). M.p. 158 °C (lit.[32] 161 °C), $[\alpha]_D^{20} = -9.7$ ($c = 0.10$, MeOH) (lit.[32] $[\alpha]_D^{20} = -9.5$, $c = 0.1$, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$), δ 7.47–7.05 (10H, m), 6.48 (1H, s), 5.69 (1H, s), 5.08 (2H, s), 4.48 (1H, m), 3.05 (2H, m), 1.55 (3H, s), 1.42 (3H, s).

N-Benzyloxycarbonyl-L-phenylalanyl-L-alanine (Z-Phe-L-Ala). M.p. 160–162 °C (lit.[33] 165 °C), $[\alpha]_D^{20} = -10.8$, ($c = 0.0198$, MeOH) (lit.[33] $[\alpha]_D^{20} = -11.0$, $c = 2$, alcohol);^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47–7.05 (10H, m), 6.47 (1H, s), 5.41 (1H, s), 5.08 (2H, s), 4.52 (1H, q, $J = 7.5$ Hz), 4.50 (1H, t, $J = 7.5$ Hz), 3.10 (1H, q, $J = 7.5$ Hz), 3.06 (1H, q, $J = 7.5$ Hz), 1.38 (3H, d, $J = 7.5$ Hz).

N-Benzyloxycarbonyl-L-phenylalanylglycine (Z-Phe-Gly). M.p. 144–147 °C (lit.[34] 154 °C), $[\alpha]_D^{20} = -9.9$ ($c = 0.025$, AcOH) (lit.[34] $[\alpha]_D^{18} = -10.2$, $c = 2.73$, AcOH); ^{1}H NMR (500 MHz, $CDCl_3$), δ 7.47–7.05 (10H, m), 6.52 (1H, s), 5.39 (1H, s), 5.07 (2H, s), 4.54 (1H, dd, $J = 6.5$, 12.5 Hz), 4.06 (1H, d, $J = 17.9$ Hz), 3.92 (1H, d, $J = 17.9$ Hz), 3.96–3.92 (1H, m), 3.08 (2H, m). Found: C, 63.94; H, 5.57; N, 7.92%. Calculated for $C_{19}H_{20}N_2O_5$: C, 64.04; H,5.66; N; 7.68%.

N-Benzyloxycarbonyl-L-phenylalanyl-N-methyl-L-alanine (Z-Phe-NMeAla). M.p. 39–40 °C, $[\alpha]_D^{20} = -33.2$ ($c = 0.100$, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$), δ 7.33–7.16 (10H, m), 6.23 (1H, d, $J = 9.0$ Hz), 5.17 (1H, q, $J = 7.5$ Hz), 5.12 (1H, d, $J = 8.5$ Hz), 5.09 (1H, d, $J = 8.5$ Hz), 4.95 (1H, quintet, $J = 7.4$ Hz), 3.09–2.94 (2H, m), 2.86 (3H, s), 1.39 (3H, d, $J = 7.5$ Hz). Found: C, 65.47; H, 6.46; N, 7.19%. Calculated for $C_{21}H_{24}N_2O_5$: C, 65.63; H, 6.25; N, 7.29%.

N-Benzyloxycarbonyl-L-phenylalanyl-L-proline (Z-Phe-L-Pro). M.p. 107–108 °C (lit.[35] 106.5 °C), $[\alpha]_D^{20} = -43.7$ ($c = 0.130$, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.120 (10H, m), 5.57 (1H, d, $J = 9.0$ Hz), 5.11 (1H, d, $J = 12.5$ Hz), 5.06 (1H, d, $J = 12.5$ Hz), 4.69 (1H, dd, $J = 8.0$, 15.1 Hz), 4.58 (1H, d, $J = 5.5$ Hz), 3.51 (1H, dd, $J = 8.5$, 15.9 Hz), 3.06–3.03 (2H, m), 2.85–2.80 (1H, m), 2.34–2.30 (1H, m), 2.01–1.93 (1H, m), 1.89–1.83 (1H, m), 1.78–1.71 (1H, m). Found: C, 66.64; H, 6.13; N 6.90%. Calculated for $C_{22}H_{24}N_2O_5$: C, 66.65; H, 6.10; N, 7.06%.

N-Benzyloxycarbonyl-L-phenylalanylsarcosine (Z-Phe-Sar). M.p. 61–63 °C (lit.[36] 46–50 °C), $[\alpha]_D^{20} = -9.4$ ($c = 0.40$, EtOH) (lit.[36] $[\alpha]_D^{20} = -9.5$, $c = 4.0$, EtOH); ^{1}H NMR (500 MHz, $CDCl_3$), δ 7.34–7.15 (10H, m), 5.91 (1H, d, $J = 9.0$ Hz), 5.08 (1H, d, $J = 12.5$ Hz), 5.02 (1H, d, $J = 12.5$ Hz), 4.95 (1H, q, $J = 8.2$ Hz), 4.20 (1H, d, $J = 17.5$ Hz), 3.94 (1H, d, $J = 17.5$ Hz), 3.07–2.94 (2H, m), 2.88 (3H, s). Found: C, 64.71; H, 6.24; N, 7.45%. Calculated for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.56%. FABMS: *m/z* 371.1639. Calculated for $C_{20}H_{23}N_2O_5$: 371.1608.

Solvent viscosity. The viscosity of the reaction solvent was controlled by adding an appropriate amount of glycerol or sucrose to Tris–HCl buffer (50 mM, pH 6.5).

When an equal volume of viscous fluid is measured by using the same thin tube, the viscosity (η) is given by

$$\eta = (\rho t \eta^0)/(\rho_0 t_0) \quad (6)$$

where t is the time required for passage through the tube, ρ is the solution density and η^0, t_0 and ρ_0 are the values for water as the standard fluid. The time for passage of 5 ml of solutions and water was measured five times with the stalagmometer. The average passage times were used to calculate the solution viscosities relative to water.

CD spectra. The effect of solvent viscosity on the secondary structure of the enzyme was investigated by CD spectroscopy in the wavelength range 200–300 nm. The enzyme was dissolved in solutions controlled at pH 6.5 with Tris–HCl buffer (50% glycerol, 8 M urea and a reference solution without either additive).

Enzyme CPD-Y. CPD-Y prepared by Oriental Yeast was purchased from Wako Pure Chemical Industries and used without purification.

Kinetic measurements. A spectrophotometer was used for kinetic measurements. Kinetics were measured by following the decrease in absorbance at 230 nm. The reaction conditions were kept constant over all kinetic measurements: temperature 310 K, concentration of the buffer 50 mM, pH 6.5.

As a typical procedure of these experiments, carboxypeptidase-Y (4.68 mg, ca 7.8×10^{-8} mol) was dissolved in Tris–HCl buffer (1 ml) and the solution was diluted appropriately. A stock solution of substrate was prepared (ca 1 mM) by dissolving the substrate in Tris–HCl buffer and diluting appropriately. The solution of substrate (3 ml) was placed in a thermostated compartment of the spectrophotometer and incubated for 5 min. After 20 µl of enzyme solution had been added, the mixture was shaken to make it homogeneous. The absolute absorbance of this solution was then measured. One set of measurements was composed of data from five different substrate concentrations and the initial velocity of each substrate concentration was calculated as the average of 3–5 runs. The dipeptide and the hydrolysis product of the dipeptide Z-Phe-Xaa of carboxypeptidase-Y were checked by reversed-phase HPLC and no diastereomer was detected. The mobile phase (0.5 ml min^{-1}) consisted of acetonitrile–water (3:7) containing 60 mM AcONa buffer (pH 6.0).

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