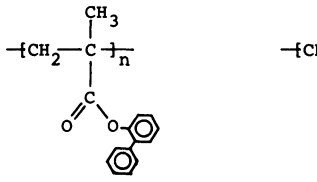
  Bull. Chem. Soc. Jpn., 59, 3621—3624 (1986)

Enzymatic Esterolysis of Polymers Containing 2-Biphenylyl Ester Bonds in Side Chains

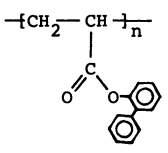
Tomomichi Ishikawa,* Makoto Otsuki, and Toshiaki Iwatsuki
Department of Industrial Chemistry, Fucalty of Technology, Tokyo Metropolitan University, Fukasawa, Setagaya-ku, Tokyo 158
(Received June 4, 1986)

The preparation and α-chymotrypsin-catalyzed esterolysis of polymeric substrates anchoring fungicidal biphenyl-2-ol are described. In order to obtain information regarding the influence of one type of polymeric backbone and the distance between a cleavable bond and a polymer main chain, poly(2-biphenylyl acrylate), poly(2-biphenylyl methacrylate), and poly(methacryloylamino acid 2-biphenylyl ester)s were studied. The esterolysis were evaluated by means of Michaelis constant K_m and the catalytic reaction rate constant k_{cat}. Poly(2-biphenylyl methacrylate) was a more suitable substrate than poly(2-biphenylyl acrylate). Poly[6-(methacryloylamino)hexanoic acid 2-biphenylyl ester] was the most suitable substrate in a series of polymers containing amino acid side chains with 2-biphenylyl ester terminal groups. The release rate of biphenyl-2-ol could also be controlled by using copolymers with an appropriate comonomer.

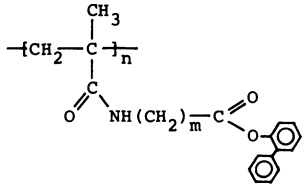
The use of polymers in drug delivery systems has recently received a great deal of attention.[1-5] The mildew defacement of water-soluble paints and their coatings has been a serious problem in the paint industry and for keeping of a good life environment. In connection with this problem, Pittman and co-workers[6,7] examined the biological properties of film-forming polymers with chemically attached biocides using microbiological accelerated fungus-growth tests. In a previous paper[8] we reported on the kinetics of the chymotrypsin-catalyzed hydrolytic cleavage of free biphenyl-2-ol (fungicides, abbreviated to BPOL) from ester groups in the side chains of homopolymer and copolymers with various comonomers in order to simulate microbiological degradation. Kopecek and co-workers[9] also reported on the kinetics of the enzymatic cleavage for the copolymers of various 4-nitroanilides of methacryloylamino acids or origopeptides with *N*-(2-hydroxypropyl)methacrylamide. On the other hand, Kloss and Schröder[10] reported on the hydrolysis of 51 kinds of peptides alkyl esters by chymotrypsin and trypsin. These results suggest that one type of carboxylic acid which forms cleavable ester bonds affects the enzymatic cleavage rates. Therefore, this paper describes the influence of the structure of a polymer main chain and the distance between a cleavable bond and the main chain upon the kinetics of the enzymatic cleavage of polymers **1**, **2**, and **3a—c**.



1



2



3a m=1
3b m=2
3c m=5

Experimental

Materials. Methacryloyl chloride was distilled prior to use. BPOL and dicyclohexylcarbodiimide (DCC) were purified by distillation. Solvents were dried by the usual methods and distilled. The α-chymotrypsin was the same as in a previous paper,[8] and its activity was checked at the bigining of the experiments. Other chemicals were of reagent grade.

Monomer Preparation. 2-Biphenylyl acrylate (BPA) was prepared according to a method described in a previous paper.[11]

2-Biphenylyl methacryloylaminoacetate (BPMAA-1) was prepared by the esterification of *N*-methacryloylglycine[9,10] with BPOL in the presence of DCC in a way similar to that of a known procedure.[12] Yield 12.3%; mp 131.5—132.9 °C; IR (KBr) 3310 (NH), 1765 (C=O), 1650 (C=O), 1610 (C=C), 760, 730, and 700 cm^{-1}; ^1H NMR (DMSO-d_6) δ=1.90 (3H, s, CH_3), 4.02 (2H, d, CH_2), 5.39 (H, s, CH=), 5.83 (H, s, CH=), 7.30—7.75 (9H, m, Aromatic) and 8.54 (H, broad, NH).

2-Biphenylyl 3-(methacryloylamino)propionate(BPMAA-2) was prepared by the esterification of 3-(methacryloylamino)propionic acid with BPOL in the presence of DCC.[12] Yield 16.3%; mp 88—88.5 °C; IR (KBr) 3340 (NH), 1740 (C=O), 1645 (C=O), 1610 (C=C), 770, 740, and 700 cm^{-1}; ^1H NMR (DMSO-d_6) δ=1.81 (3H, s, CH_3), 2.53 (2H, t, CH_2), 3.39 (2H, q, CH_2), 5.15 (H, s, CH=), 5.45 (H, s, CH=), 6.00 (H, broad, NH) and 7.00—7.40 (9H, s, Aromatic).

2-Biphenylyl 6-(methacryloylamino)hexanoate(BPMAA-5) was prepared by the esterification of BPOL with 6-(methacryloylamino)hexanoic acid[9] in the presence of DCC.[12] Yield 57.1%; mp 64.7—66.0 °C; IR (KBr) 3350 (NH), 1710 (C=O), 1650 (C=O), 1600 (C=C), 750 and 700 cm^{-1}; ^1H NMR (DMSO-d_6) δ=0.90—1.80 (6H, m, $3CH_2$), 1.83 (3H, s, CH_3), 2.23 (2H, t, CH_2), 3.05 (2H, q, CH_2), 5.25 (H, s, CH=), 5.61 (H, s, CH=), 7.10—7.60 (9H, m, Aromatic) and 8.13 (H, broad, NH).

General Procedure for Polymerization. All polymerization experiments were carried out in a hot oil bath at 60 °C using a solution-polymerization technique (in toluene) in the presence of 1 mol% AIBN. The mixtures were poured into methanol. The obtained polymers were dissolved in toluene and precipitated into methanol. The precipitation was repeated, and the polymer was dried in a vaccum oven at 40 °C.

Synthesis of Model Compounds. 2-Biphenylyl propionate (BPP) was obtained by a reaction of BPOL with propionyl chloride.[8]

2-Biphenylyl isobutyrate(BPIB) was obtained by the reaction of BPOL with isobutyryl chloride.[8] Yield 19.0%; bp 155—160 °C (665 Pa); ^{1}H NMR (DMSO-d_6) δ=0.99 (6H, d, 2CH$_3$), 2.41 (1H, m, CH) and 6.95—7.50 (9H, m, Aromatic).

2-Biphenylyl pivalate (BPPV) was obtained by the reaction of BPOL with pivaloyl chloride.[8] Yield 69.3%; bp 114.0 °C (133 Pa); ^{1}H NMR ($CDCl_3$) δ=1.11 (9H, s, 3CH$_3$), 7.00—7.60 (9H, m, Aromatic).

Rate of Esterolysis with α-Chymotrypsin. An esterolysis of monomeric and polymeric substrates was carried out in 50 cm^3 of a buffer solution (0.08 mol dm^{-3} 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) and 0.1 mol dm^{-3} $CaCl_2$ adjusted with HCl to pH=8.0) in the presence of 2.8 mg α-chymotrypsin. For each experiment a substrate converted into 0.15—1.0 mmol dm^{-3} BPOL was used. The reactions were carried out at 25 °C with a regulated water bath. The reaction mixture was continuously shaken. After a given time, some part of this mixture was removed and then the detection of released BPOL was performed by UV at 240.0 nm. The degree of conversion was evaluated by observing the difference between the absorbance at time zero and t.

Results and Discussion

Polymeric substrates anchoring BPOL were obtained by the polymerization of vinyl monomers in proportion to the structure of their polymer units. In order to observe the influence of the structure of a polymer backbone on the rate of esterolysis of a 2-biphenylyl ester bond in a side chain, polymer **2** was obtained by the polymerization of BPA,[11] and its reactivity during esterolysis was compared with that for polymer **1**.[8] On the other hand, it is known that the hydrolysis rate for a cleavable bond at the end of a side chain is affected by the distance between the cleavable site and the polymer backbone.[9,13] Therefore, some methacryloylamino acid 2-biphenylyl esters were synthesized and polymerized; then their polymers were hydrolyzed. We chose an amino acid such as glycine, 3-aminopropionic acid, and 6-aminohexanoic acid as a spacer of side chains in polymeric substrates. Some monomeric model compounds were also synthesized for a comparison with the polymeric substrates. The abbreviation of the monomers and model compounds are shown in Fig. 1.

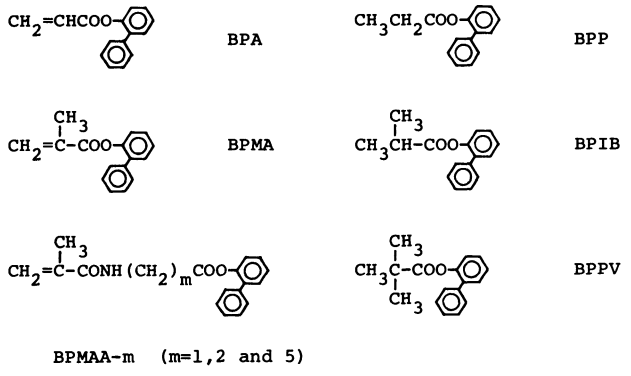


Fig. 1. Abbreviation of 2-biphenylyl esters.

The monomeric and polymeric substrates were hydrolyzed with α-chymotrypsin in a Tris-buffer solution (pH 8). The amount of released BPOL was periodically determined using UV.[8] It is well-known that a chymotrypsin-catalyzed reaction can be expressed by the equation of Michaelis-Menten[14]; thus, the Michaelis constant K_m and the maximum reaction rate V_{max} were determined by a method of Lineweaver and Burk[15] from the relation between the initial reaction rate and the initial concentration of the substrate.

The results for polymer **1** and **2** and the model compounds are summarized in Table 1. It is quite evident from Table 1 that the values of K_m and k_{cat} for the model compounds are in the order BPIB>BPP>BPPV. The order of BPIB and BPP agree with those of the Hammett-Taft's σ^* values. However, no systematic structural correlation was found between BPPV and the others. The small values of K_m and k_{cat} for the BPPV indicate that an enzyme-substrate complex formed is more stable in comparison with those for the others. This can be attributed to the significantly large hydrophobic and steric properties of the t-butyl group. The enzyme was also inactivated

Table 1. α-Chymotrypsin-Catalyzed Esterolysis of Monomeric and Polymeric Substrates

Substrate	Yield / %	K_m 10^3 / mol dm^{-3}	V_{max} 10^5 / mol dm^{-3} h^{-1}	k_{cat}[a] / h^{-1}	k_{cat}/K_m / l mol^{-1} h^{-1}
BPP	64.0	0.89	74	330	370000
BPIB	19.0	1.2	83	370	310000
BPPV	69.3	0.14	9.4	42	300000
1[b]	61.9	1.9	1.7	7.4	3800
2	64.0	1.3	0.82	3.7	2800
3a	12.3		No change		
3b	95.3	0.93	1.0	4.5	4800
3c	57.1	0.63	7.2	32.1	51000

a) Calculated by using a value of 25000 as the molecular weight of α-chymotrypsin. b) Reported in the previous paper (Ref. 8).

owing to a so-called product inhibition[16,17] of pivalic acid which was formed while hydrolyzing BPPV. This result suggests that the reactivity of the substrates is affected by the alkyl groups adjoining to the cleavable bond. As shown in Table 1, both K_m and k_{cat} for polymer **1** is larger than those for polymer **2**, and that the reactivity of the polymeric substrates with α-chymotrypsin varies with the structure of the polymeric main chain. Both K_m and k_{cat} indicate that the formation of the enzyme-polymer **2** complex is easier than that for the polymer **1**; however, the reactivity of the polymer **2**-complex is lower than that of the polymer **1**-complex. When the kinetic parameters for the polymeric substrates is compared with those for the monomeric substrates, the former gave almost the same K_m value as with the latter; a remarkably large difference was observed in the k_{cat} values. The reactivity of chymotrypsin-catalyzed hydrolysis can be estimated by a k_{cat}/K_m value.[18] The high k_{cat}/K_m value for polymer **1** indicates that this polymeric structure is more useful than that of polymer **2**.

The results of the enzymatic hydrolysis of the polymeric substrates containing amino acid side chains with 2-biphenylyl ester terminal groups are also presented in Table 1. It can be seen from Table 1 that no BPOL is released from the glycine 2-biphenylyl ester terminals in polymer **3a**. This is in agreement with the resistance of glycine ethyl ester,[10] *N,N*-succinylglycine 4-nitroanilide and the polymer with glycine 4-nitroanilide terminals.[9] Although the k_{cat} values are in the order of polymer **3b**<**1**<**3c**, the values of K_m and k_{cat}/K_m indicate that the general influence of the spacer become greater with an increase in the number of methylene groups. From these results, it seems that the reactivity of the substrates depends on the stability of enzyme-substrate complexes. In the polymeric substrates, the complex of polymer **3c** with enzyme is easily formed and shows a high reactivity: Namely, that the Michaelis constant K_m is the lowest and the reaction rate constant k_{cat} is the highest value of the series. Kopecek and co-workers[9] examined the necessity of a spacer having a certain length for the cleavage of polymers with amino acid 4-nitroanilide terminals. He concluded that in the case of chymotrypsin the spacer must be at least 6-atoms long. In this study the result for polymer **3d** was in agreement with their conclusion.

Table 2. Additional Effect of 3-(Methacryloylamino)-propionic Acid (MAA-2) in Esterolysis of BPMAA-2

BPMAA-2 / mol dm^{-3}	MAA-2 / mol dm^{-3}	v_0 / mol dm^{-3} h^{-1}
9.99×10^{-4}	5.01×10^{-4}	0.370×10^{-4}
9.86×10^{-4}	—	1.79×10^{-4}
3.96×10^{-4}	11.0×10^{-4}	0.216×10^{-4}
4.09×10^{-4}	—	1.43×10^{-4}
2.78×10^{-4}	—	1.16×10^{-4}
1.36×10^{-4}	13.6×10^{-4}	0.105×10^{-4}
1.55×10^{-4}	—	0.709×10^{-4}

The substrates anchoring BPOL through the amino acid residues are competitively degraded in both the amide and the ester bonds with α-chymotrypsin. Therefore, some mixtures of the BPMAA-2 and 3-(methacryloylamino)propionic acid were hydrolyzed in order to obtain information on the influence of amides regarding the esterolysis rate. All the experiments were carried out under the conditions of the same initial concentration on the amide group. The results are shown in Table 2. It is evident from Table 2 that all the initial rates for the mixtures were slower than that for a single substrate, BPMAA-2. In the case of mixtures, values of K_m=0.52×10^{-3} mol dm^{-3} and k_{cat}=20 h^{-1} were obtained. The K_m and k_{cat} values for BPMAA-2 were 0.52×10^{-3} mol dm^{-3} and 143 h^{-1}, respectively. These results suggest that 3-(methacryloylamino)propionic acid acts as a so-called noncompetitive inhibitor, because the Michaelis constant K_m for the mixture gives the same value as that for mixture, whereas the maximum reaction rate V_{max} decrease with an increase in the content of the amide groups. If the cleavage of the ester and amide bonds proceeds competitively, the K_m value for the mixture ought to be different from that for the substrate BPMAA-2. Therefore, the same K_m value for the mixture and for the BPMAA-2 can be assumed to be caused by a very slow cleavage rate of the amide bonds with α-chymotrypsin.

Generally, water-soluble paints are prepared from vinyl acetate (VAc) and methyl methacrylate (MMA). Film-forming polymers with chemically attached BPOL can be used as a vehicle for fungicidal paints. Therefore, copolymers of BPMAA-2 with VAc or

Table 3. Preparation and Esterolysis of Copolymers of BPMAA-2 with VAc and MMA[a]

Comonomer	Molar fraction BPMAA-2 in monomer	Time / h	Yield[b] / %	Molar fraction BPMAA-2 in polymer[c]	$[\eta]$[d] / dm^3 g^{-1}	K_m 10^3 / mol dm^{-3}	k_{cat} / h^{-1}	k_{cat}/K_m / l mol^{-1} h^{-1}
—	1.000	44	95.3	1.000	0.12	0.93	4.5	4800
VAc	0.700	71	51.6	0.650	0.05	1.2	9.8	8170
MMA	0.500	43	86.8	0.456	0.22	0.81	23.6	29100

a) Reacted in toluene at 60 °C in the presence of AIBN(1—1.4 mol%). b) Reprecipitated with hexane. c) Determined by ^{1}H NMR. d) Measured in $CHCl_3$ at 25 °C.

MMA were also prepared, and hydrolyzed with α-chymotrypsin. The results are given in Table 3. As shown in Table 3, a small difference between the homopolymer and the copolymers was observed in the K_m values, whereas the k_{cat} values were apparently in the order of polymer **3b**<VAc-BPMAA-2 copolymer< MMA-BPMAA-2 copolymer. On the other hand, we have reported in a previous paper[7] that the reaction rate of a VAc-BPMA copolymer is slower than that of a BPMA homopolymer, and that the K_m value for both the homopolymer and the copolymer was equal to 1.9 mol dm^{-3}. The contrast between the two cases could readily be explained in terms of differences in a steric conditions at the cleavable bond, which is surrounded with acetoxyl groups. In the case of a VAc-BPMAA-2 copolymer, the 2-biphenylyloxycarbonyl groups that are sensitive to chymotrypsin attack a row of acetoxyl groups and can easily form a complex with the enzyme. In contrast to this case, the 2-biphenylyloxycarbonyl groups on the VAc-BPMA copolymer are buried into the acetoxyl groups. Azori and co-workers[19] examined the chymotrypsin catalyzed hydrolysis in the range 1 to 20 mol% for side chains containing *p*-nitroaniline bound to poly(*N*-vinyl-2-pyrrolidone-co-maleic anhydride), and reported that the maximum reaction rate V_{max} varies with the mole fraction of the side chains. These results indicate that the release rate of BPOL from the polymers can be controlled by using copolymers with an appropriate comonomer. Although the hydrolysis rate constant k_{cat} for the MMA-BPMAA-2 copolymer showed a considerably large value among the polymeric substrates used in this work, only 4% of total BPOL was released during a four-hour period. However, the conversion of the same substrate in hydrolysis without chymotrypsin was 0.9% during a four-day period.

References

1) H. Lingsdorf, *J. Polymer Sci., Symposium*, **51**, 135 (1975).
2) C. G. Gebelein, *Polymer News*, **4**, 163 (1978).
3) J. Feijen, D. Gregonis, C. Anderson, R. V. Petersen, and J. Anderson, *J. Pharm. Sci.*, **69**, 871 (1980).
4) S. K. Chandrasekaran, Editer, *Am. Inst. Chem. Eng., Symposium Series*, No. 206, vol. **77** (1981).
5) T. Hayashi, Y. Tabata, and A. Nakajima, *Rep. Pro. Polym. Phys. Jpn.*, **26**, 578 (1983).
6) C. U. Pittman, Jr., G. A. Stahl, and H. Winters, *J. Coatings Tech.*, **50**, 49 (1978).
7) C. U. Pittman, Jr., *J. Coatings Tech.*, **48**, 31 (1976); A. Stahl, and C. U. Pittman, Jr., *J. Coatings Tech.*, **50**, 62 (1978).
8) T. Ishikawa, and Y. Tanaka, *Bull. Chem. Soc. Jpn.*, **58**, 1182 (1985).
9) J. Drobnik, J. Kopecek, J. Labasky, P. Rejmanova, J. Exner, V. Saudek, and J. Kalal, *Makromol. Chem.*, **177**, 2833 (1976).
10) G. Kloss and E. Schröder, *Hoppe-Seyler.*, **336**, 248 (1964).
11) T. Ishikawa and S. Asai, *Bull. Chem. Soc. Jpn.*, **56**, 2177 (1983).
12) P. Rejmanova, J. Labsky, and J. Kopecek, *Makromol. Chem.*, **178**, 2159 (1977); H. D. Jakubke, and A. Voigt, *Chem. Ber.*, **99**, 2948 (1966).
13) J. Pato, M. Azori, K. Ulbrich, and J. Kopecek, *Makromol. Chem.*, **185**, 231 (1984).
14) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).
15) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
16) W. W. Cleland, *Biochem. Biophys. Acta*, **67**, 173 (1963).
17) R. Y. Hsu, W. W. Cleland, and L. Anderson, *Biochem.*, **5**, 799 (1966).
18) F. E. Brot and M. L. Bender, *J. Am. Chem. Soc.*, **91**, 7187 (1969).
19) M. Azori, J. Pato, F. Fehervari, and F. Tudos, *Makromol. Chem.*, **187**, 303 (1986).