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Preparation and Enzymatic Hydrolysis of Polymers Anchoring Biphenyl-2-ol

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Polymers anchoring fungicidal biphenyl-2-ol(BPOL) were prepared by the polymerization and copolymerization of 2-biphenylyl methacrylate(BPMA) with various comonomers such as styrene, vinyl acetate, 2-hydroxyethyl methacrylate, *N*-methacryloylglycine, and *N*-methacryloyl-DL-alanine. The reactivity ratios of BPMA with the comonomers were evaluated. The α-chymotrypsin-catalyzed hydrolysis of the polymers was also studied. The release of BPOL from the polymers was represented by means of the Michaelis constant K_m and the catalytic-rate constant k_{cat}. The BPMA homopolymer gave higher K_m and k_{cat} values than the copolymers, and the copolymers with a higher content of substrate units underwent cleavage more readily. It was found that the hydrolysis of the polymeric substrates is affected by the structure and character of the comonomer rather than the arrangement order of the repeating monomer units in the polymer sequence.

Recently, the usefulness of biodegradable poly(α-amino acid)s in drug delivery systems has been reported. The rate of *in vivo* enzymatic degradation of the polymers can be controlled, and the drugs stored in matrix rods and capsules[1,2] or covalently bonded to polymer matrixes[3] are released with nearly zero-order kinetics. Nakajima and co-workers[4] studied the hydrolysis of poly(*N*-hydroxyalkyl-L-glutamine) by papain to simulate the *in vivo* degradation. On the other hand, Kopecek and co-workers[5-8] reported the cleavage of free 4-nitroaniline from amino acid 4-nitroanilide terminal groups in the origopeptidic side chains of the copolymers by α-chymotrypsin. In a previous paper[9] we reported on the hydrolytic process of homopolymer and copolymers of 2-biphenylyl acrylate under physiological conditions. If the polymers are attacked by a microorganism such as fungus, the ester gruops linking fungicidal biphenyl-2-ol (BPOL) will also be hydrolyzed enzymatically. In this work we describe the polymerization and copolymerization of 2-biphenylyl methacrylate (BPMA) and a study of the hydrolytic release of BPOL from the polymers by α-chymotrypsin. It is known that α-chymotrypsin effectively cleaves ester linkages as well as peptide linkages, but no data are available about the hydrolysis of a polymeric substrate such as poly(2-biphenylyl methacrylate) anchoring bulky and hydrophobic biphenyl groups.

Experimental

Materials. 2-Hydroxyethyl methacrylate (HEMA), styrene (St), vinyl acetate (VAc), and BPOL were distilled under reduced pressure prior to use. α-Chymotrypsin (51 ATEE units, Tokyo Chem. Ind. Co.) was used without further recrystallization. Solvents were dried by the usual methods and distilled. Other chemicals were of reagent grade.

Monomer Preparation. BPMA was prepared by the reaction of BPOL with methacryloyl chloride in the presence of triethylamine in benzene by a method similar to that described in a previous paper.[9] Purification was achieved by distillation under reduced pressure in the presence of 4-*t*-butylcatechol to yield BPMA (71.1%): bp 123°C (133Pa); ^{1}H-NMR ($CDCl_3$) δ=1.85 (3H, s, CH_3), 5.51 (1H, d, CH=); 6.06 (1H, d, CH=) and 6.93—7.53 (9H, m, Aromatic).

N-Methacryloylglycine (MAG) was obtained by the reaction of glycine with methacryloyl chloride in an aqueous sodium hydroxide solution according to Ref.[5] Yield 60.8%: mp 101—102°C (lit, 101—102°C); ^{1}H-NMR(DMSO-d_6) δ= 1.85 (3H, s, CH_3), 3.78 (2H, d, CH_2), 5.35 (1H, d, CH=), 5.70 (1H, d, CH=), 7.70—8.30 (1H, t, NH) and 10.80—12.40 (1H, s, OH); IR (KBr) 3330 (NH), 1715 (C=O), 1640 (C=O) and 1570 (C=C).

N-Methacryloyl-DL-alanine (MAA) was obtained from DL-alanine with methacryloyl chloride according to Ref.[5] Yield 84.5%; mp 116—117.5°C (lit, 117—118°C); IR (KBr) 3290 (NH), 1695, 1640 (C=O) and 1600 (C=C); ^{1}H-NMR (DMSO-d_6) δ=1.32 (3H, d, CH_3), 1.85 (3H, s, CH_3), 4.17 (1H, m, CH), 5.33 (1H, d, CH=), 5.70 (1H, d, CH=), 7.70—8.20 (1H, d, NH) and 9.00—10.00 (1H, broad, OH).

General Procedure for Polymerization. All the polymerization experiments were carried out using a solution polymerization technique at 60°C in the presence of 1 mol% AIBN. The polymers obtained were purified by repeated precipitation from a solvent system into a non-solvent system. The purified polymers were dried at 40°C (under vaccum) to constant weight. Analyses of the copolymers obtained were carried out using ^{1}H-NMR and an elemental analysis.

General Procedure for Hydrolysis with α-Chymotrypsin. All the hydrolysis experiments were 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 α-chymotrypsin. The temperature was maintained at 25°C with a regulated water bath. The progress of the hydrolysis was followed by recording any increase in the absorbance at 240.0 nm. The activity of α-chymotrypsin was checked by the hydrolysis of a model compound, 2-biphenylyl propionate[9] at the beginning and the end of the experiments. They gave the same value.

Results and Discussion

Copolymerization. The solution copolymerization of VAc with BPMA was carried out in toluene. St, HEMA, MAG, and MAA were copolymerized with BPMA in ethanol. The contents of each component in the monomer and the compositions of the copolymers obtained by ^{1}H-NMR or elemental analysis are summarized in Tables 1—5. The monomer reactivity ratios

Table 1. Copolymerization of VAc with BPMA in toluene at 60 °C in the presence of AIBN

Molar fraction BPMA in monomers	Molar fraction[a] BPMA in copolymer	Time min	Yield %
0.100	0.656	60	4.8
0.300	0.863	28	2.5
0.500	0.934	27	10.0
0.700	0.970	25	11.6
0.900	0.992	25	9.1

a) Determined by ^{1}H-NMR.

Table 2. Copolymerization of St with BPMA in ethanol at 60 °C in the presence of AIBN

Molar fraction BPMA in monomers	Molar fraction[a] BPMA in copolymer	Time min	Yield %
0.100	0.271	190	6.0
0.300	0.455	75	5.0
0.500	0.589	73	6.6
0.700	0.743	70	8.0
0.900	0.908	70	6.3

a) Determined by elemental analysis.

Table 3. Copolymerization of HEMA with BPMA in ethanol at 60 °C in the presence of AIBN

Molar fraction BPMA in monomers	Molar fraction[a] BPMA in copolymer	Time min	Yield %
0.300	0.398	23	4.5
0.500	0.642	17	14.9
0.700	0.842	14	9.0
0.900	0.960	15	2.9

a) Determined by ^{1}H-NMR.

Table 4. Copolymerization of MAG with BPMA in ethanol at 60 °C in the presence of AIBN

Molar fraction BPMA in monomers	Molar fraction[a] BPMA in copolymer	Time min	Yield %
0.300	0.465	52	1.7
0.500	0.683	54	2.6
0.700	0.851	25	2.9
0.900	0.963	56	4.8

a) Determined by ^{1}H-NMR.

Table 5. Copolymerization of MAA with BPMA in ethanol at 60 °C in the presence of AIBN

Molar fraction BPMA in monomers	Molar fraction[a] BPMA in copolymer	Time min	Yield %
0.100	0.214	45	3.2
0.300	0.531	45	5.7
0.500	0.718	21	2.4
0.700	0.869	20	2.8
0.900	0.962	40	3.2

a) Determined by ^{1}H-NMR.

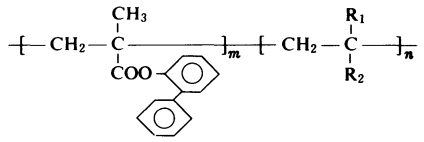
of these compounds were estimated (using the Fineman-Ross equation) from these data. The results are given in Table 6.

From Table 6 it appears that the polymer derived from BPMA with St shows a tendency towards alternation since r_1r_2 is relatively small (0.22). On the other hand, from the very high r_2 and low r_1 values, BPMA shows a homopropagation tendency in the copolymerization with VAc. In the copolymerization of HEMA, MAG, and MAA with BPMA, the r_1r_2 values were larger than unity. In any case, BPMA shows a random-propagation tendency. The copolymers of St with BPMA are perhaps the most interesting of the series. When the copolymers are used as coating films, the above results suggest that the fungicidal BPOL residues are distributed equally on the films.

Table 6. Reactivity ratios of BPMA

Monomer M_1	Monomer M_2	r_1	r_2	r_1r_2
VAc	BPMA	0.04	14	0.56
St	BPMA	0.22	1.0	0.22
HEMA	BPMA	1.0	2.8	2.8
MAG	BPMA	0.72	2.9	2.1
MAA	BPMA	0.42	2.7	1.1

Table 7. Polymeric substrates anchoring BPOL



Substrate	R_1	R_2
1	CH_3	COO- 
2a, 2b	H	$OCOCH_3$
3a, 3b	H	C_6H_5
4a, 4b	CH_3	$COOCH_2CH_2OH$
5a, 5b	CH_3	$CONHCH_2COOH$
6a, 6b	CH_3	$CONHCHCOOH$ $\vert$ CH_3

Hydrolysis with α-Chymotrypsin. Polymeric substrates **1—6** anchoring BPOL were obtained by homopolymerization and copolymerization of BPMA. We have chosen hydrophobic and hydrophilic comonomers. The two classes of copolymers containing about 30 and 50 mol% BPOL residues were prepared for each comonomer. All the polymers were water insoluble. Kopecek and co-workers[5] reported on the water-soluble copolymers of various 4-nitroanilides of *N*-methacryloylorigopeptides with *N*-(2-hydroxypropyl)methacrylamide. In this paper, however, even the BPMA-HEMA copolymer contained 72 mol% of HEMA units was water insoluble. The polymeric substrates prepared are shown in Table 7. It is expected from the structure of the polymers **1—6** that the complete hydrolysis of the homopolymer and copolymers other than the St-BPMA copolymer gives a water-soluble polymer which can more readily be remove from biological systems.

The polymeric substrates were hydrolyzed with α-chymotrypsin in Tris-buffer solution (pH 8). The amount of BPOL released was determined periodically using UV. When the homopolymeric substrates were

Table 8. Hydrolysis of BPMA homopolymer and opolymers with α-chymotrypsin

Substrate[a]	Comonomer	Mol% of BPOL residue[b]	Yield %	$[\eta]$[c] $dm^3 g^{-1}$	$K_m 10^3$ mol l^{-1}	k_{cat} h^{-1}	k_{cat}/K_m l $mol^{-1} h^{-1}$
1	—	100.0	61.9	1.38	1.9	7.4	3830
2a	VAc	27.7	73.5	0.10	1.8	1.4	753
2b	VAc	54.4	30.0	0.13	1.9	2.2	1140
3a	St	23.5	76.6	0.24	0.38	0.64	1670
3b	St	46.0	88.3	0.52	0.47	1.0	2130
4a	HEMA	27.8	79.2	0.25	9.1	7.4	810
4b	HEMA	45.3	79.2	0.48	3.1	7.4	2350
5a	MAG	29.7	34.3	1.14	1.8	3.2	1740
5b	MAG	59.0	56.8	4.22	1.9	4.2	2250
6a	MAA	39.6	24.3	1.27	1.8	1.3	700
6b	MAA	63.8	58.4	2.60	1.9	2.3	1200

a) Prepared by using a solution polymerization technique at 60 °C in the presence of AIBN. b) Determined by ^{1}H-NMR and elemental analysis. c) Measured in $CHCl_3$ for substrates **1**—**4b** and in DMF for substrates **5a**—**6b** at 25 °C.

hydrolyzed for 3 and 24 h in the presence of 0.4 mol% α-chymotrypsin, the conversion was 0.9 and 7 mol%, respectively. In the case of a model compound such as 2-biphenylyl propionate, the conversion reached 13 mol% after 3 h of reaction under the same conditions. The release rate of BPOL from the copolymers was slower than that for the homopolymer. The enzymatic catalyzed hydrolysis was evaluated from Michaelis constant K_m and the catalytic rate constant k_{cat} determined by a method developed by Lineweaver and Burke.[10] The enzymatic hydrolysis data for the polymeric substrates were summarized in Table 8.

It can be seen from Table 8 that the k_{cat} for the polymer **1** indicates the highest value of the series, and that the copolymers with higher contents of the substrate units undergo cleavage more readily. The K_m values for the copolymers **3a** and **3b** are smaller than those for any other of the polymers. As described above, the values of the reactivity ratios of both BPMA and St indicate that the monomers in these pairs tend to alternate during its copolymerization. Therefore, these results suggest that the hydrophobic phenyl group adjacent to the BPOL ester group is contributed to the formation of chymotrypsin-substrate complex. However, the lower k_{cat} values for the copolymers **3a** and **3b** indicate that the reaction of the complex is slow, and that the BPOL is released very slowly from the St-BPMA copolymers. This is in agreement with the results of a study of the hydrolytic release rate of drugs made from copolymers containing 5-fluorouracil.[11] It can also be seen that both the K_m and k_{cat} for the St-BPMA copolymers decrease with an increase in the content of St units. This must be due to a dilution effect of the substrates with the St units, because of cross-propagation tendency in its copolymerization.

In the copolymers of VAc, HEMA, MAG, and MAA with BPMA, α-chymotrypsin cleaves the ester linkages in VAc or HEMA units and the amide linkages in MAG or MAA units as well as the ester linkages in BPMA units. In these cases, the comonomer units may act as inhibitors in the enzymatic hydrolysis of the substrate. The hydrolysis is affected by the intensity of affinity of α-chymotrypsin towards the inhibitor. For the copolymers **4a** and **4b** derived from HEMA and BPMA, the K_m value increases with an content increased of the HEMA units, while the k_{cat} values for the copolymers are similar to the value for the BPMA homopolymer. These results indicate that the hydroxyethyl ester groups and the substrate groups compete for the enzyme. Therefore, the hydroxyethyl ester groups become a competitive inhibitor in this reaction. On the other hand, the K_m values for the copolymers of VAc, MAG, and MAA with BPMA are similar to that for the BPMA homopolymer. However, the values of k_{cat} for the copolymers of same BPMA content are in the order, BPMA homopolymer>MAG-copolymer>VAc-copolymer>MAA-copolymer. The hydrolysis of these copolymers gives glycine, alanine and acetic acid, respectively. It is well known that chymotrypsin-catalyzed reaction rate decreases with an addition of various carboxylic acids, and that carboxylic acids with higher hydrophobic groups undergo a greater decrease.[12,13] Brot and co-workers[14] reported that a more suitable substrate exhibits a higher k_{cat}/K_m value. According to this conclusion, the BPMA homopolymer is the most suitable substrate of the series, and the copolymers with VAc are unsuitable substrates for chymotrypsin-catalyzed hydrolysis. As described above, BPMA shows a homopropagation tendency during copolymerization with VAc. Nevertheless, the values of k_{cat}/K_m for the copolymers **2a** and **2b** are smaller to those for the polymer **1**. These facts can be explained in terms of the product inhibition which is caused by the by-product in the hydrolysis of the copolymeric substrate.

From the above results, it is concluded that the chymotrypsin-catalyzed hydrolysis of polymeric substrates is affected by the structure and character of the comonomer rather than the arrangement order of the monomer unit in a polymer sequence.

References

1) K. R. Sidman, A. D. Schwope, W. D. Steber, S. E.

Rudolph, and S. B. Poulin, *J. Membrane Sci.*, **7**, 277 (1980).
2) M. Asano, M. Yoshida, I. Kaetsu, K. Nakai, H. Yamanaka, H. Yuasa, K. Shida, K. Suzuki, and M. Oya, *Makromol. Chem.*, **184**, 1761 (1983).
3) J. Feijen, D. Gregonis, C. Anderson, R. V. Petersen, and J. Anderson, *J. Pharm. Sci.*, **69**, 871 (1980).
4) T. Hayashi, Y. Tabata, and A. Nakajima, *Rep. Pro. Polym. Phys. Jpn.*, **26**, 578 (1983).
5) J. Drobnik, J. Kopecek, J. Labsky, P. Rejmanova, J. Exner, V. Saudek, and J. Kalal, *Makromol. Chem.*, **177**, 2833 (1976).
6) J. Kopecek, P. Rejmanova, and V. Chytry, *Makromol. Chem.*, **182**, 799 (1981).
7) P. Rejmanova, B. Obereigner, and J. Kopecek, *Makromol. Chem.*, **182**, 1899 (1981).
8) J. Pato, M. Azori, K. Ulbrich, and J. Kopecek, *Makromol. Chem.*, **185**, 231 (1984).
9) T. Ishikawa, and S. Asai, *Bull. Chem. Soc. Jpn.*, **56**, 2177 (1983).
10) H. Lineweaver, and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
11) P. Umrigar, S. Ohashi, and G. Butler, *J. Polym. Sci., Polym. Chem. Ed.*, **17**, 351 (1979).
12) W. W. Cleland, *Biochim. Biophys. Acta*, **67**, 173 (1963).
13) R. Y. Hsu, W. W. Cleland, and L. Anderson, *Biochem.*, **5**, 799 (1966).
14) F. E. Brot, and M. L. Bender, *J. Am. Chem. Soc.*, **91**, 7187 (1969).