Immobilization of α-Chymotrypsin onto Hydrolyzed Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate

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Synopsis

α-Chymotrypsin has been immobilized onto partially hydrolyzed poly(ethylene)-*g*-co-hydroxyethyl methacrylate (120% graft), using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-*p*-toluene-sulphonate (CMC) as the activating agent. The influence of the enzyme concentration, the carbodiimide concentration, and the coupling medium on the immobilization reaction was studied. A system with 160 mg of coupled enzyme per gram copolymer, providing 126 mg of active enzyme per gram of copolymer was obtained. The K_M, V_{max}, and the optimum temperature profile and optimum pH value for the free and immobilized α-chymotrypsin were determined.

INTRODUCTION

In earlier work,[1,2] different methods were used for the immobilization of various enzymes onto poly(ethylene)-based graft copolymers. More recently,[3,4] it has been shown that the systems based on a graft copolymer comprising poly(ethylene)-*g*-co-hydroxyethyl methacrylate provided very promising support for the immobilization of trypsin and papain. In this article, we report some work concerned with the immobilization of α-chymotrypsin onto poly(ethylene)-*g*-co-hydroxyethyl methacrylate. α-Chymotrypsin, after being immobilized, offers great advantages in its use in cosmetics,[5] food science,[6] analytical chemistry,[7] organic synthesis, and peptide synthesis.[8]

It is clear from our earlier studies, that more attention needs to be given to the nature of the copolymeric support.[1-4] Such attention needs to consider not only the criteria for success in immobilization but also the application properties of the immobilized composite,[5-9] including processability, flow and melt properties, formulation capability, and so on.

Table I gives an indication of levels of achievement attained by earlier workers in the immobilization of α-chymotrypsin.[10-14] The general conclusion drawn from these citations is that the activity of the immobilized biocatalyst is related to the degree of hydration of the total product rather than to the amount of enzyme coupled. This has been shown by comparison between two insoluble immobilized composites that have equal amounts of protein coupled to the substrate. A derivative that has a tendency to swell in the continuous medium

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TABLE I
Immobilization of α-Chymotrypsin: The More Successful Recorded Systems

Support	Immobilization coupling agent	mg Enzyme per gram of dry conjugate	mg Active enzyme per gram conjugate	Activity ratio of bound to free enzyme (%)	Substrate	Ref.
Sepharose 6B	CNBr	72	NR	78	ATEE	10
Sepharose 4B	*p*-Bq	72	NR	66	ATEE	11
Hydrolyzate	Acidic pH	32	NR	50	BTEE	
				14	HB	—
Lignin	Formaldehyde			52	BTEE	12
	Alkaline pH	52	NR	11	HB	
Agarose	CNBr	326	NR	16	ATEE	
			NR	13	CASEINE	13
Cellulose	CNBr	295	NR	11	ATEE	
			NR	0	CASEINE	13
Sephadex	CNBr	280	NR	6	ATEE	
			NR	0	CASEINE	13
Low DS	Azide coupling	95	30	NR	ATEE	
High DS		350	86	NR	ATEE	14

gives two to three times greater activity than a derivative that does not possess such a swelling tendency.

EXPERIMENTAL

Materials

The low-density poly(ethylene) was obtained in powder form from Telcon Plastics, Ltd, U.K. The α-chymotrypsin (from bovine pancreas), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene-sulfonate (CMC), *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA), and hemoglobin were ob-

TABLE II
Immobilization of α-Chymotrypsin (E) onto Partially Hydrolyzed Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate[a]

Activating agent	mg E coupled per gram copolymer	Coupling yield (%)	mg Active E per gram copolymer	Retention of activity (%)
	Substrate: hemoglobin			
p-Benzoquinone	100	25	2.2	2.2
p-Toluene sulphonyl chloride	200	50	8.0	4.0
Epichlorohydrin	0	0	0.0	0.0
Cyanuric chloride	63	15.8	2.6	4.1

[a] 120% graft, 50% hydrolysis by using various methods of activation, in 0.1 *M* $NaHCO_3$ at 277 K for 18 h.

TABLE III
Immobilization of α-Chymotrypsin (E) onto the Partially Hydrolyzed Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate[a]

Activating agent	Coupling medium	mg E/g	Coupling yield (%)	mg Active E/g	Retention of activity (%)
	Substrate: hemoglobin				
p-Toluene sulphonyl chloride	Distilled water	34	8.5	4.5	13.2
	0.1 *M* $NaHCO_3$	200	50.0	8.0	4.0
	Acetate buffer 0.1 *M*, pH 5.0	60	15.0	10.8	18.8
	Phosphate buffer 0.1 *M* pH, 7.0	60	15.0	26.0	43.3
	Cyanuric chloride				
	Distilled water	54	13.5	3.9	7.2
	0.1 *M* $NaHCO_3$	63	15.8	2.6	4.1
	Acetate buffer 0.1 *M*, pH 5.0	148	37.0	14.6	9.9
	Phosphate buffer 0.1 *M*, pH 7.0	176	44.0	15.3	8.9

[a] 120% graft, 50% hydrolysis, using two different methods of activation, in different coupling media, at 277 K for 18 h.

tained from Sigma Biochemical Co, Poole, Dorset, U.K. All the other chemicals were obtained from BDH Ltd., Poole, Dorset, U.K., in analytical grades.

Preparation of the Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate

A portion (20 g) of powdered poly(ethylene) was immersed in a methanolic solution of hydroxyethyl methacrylate (HEMA) (10% w/w in methanol), 300 cm^3, and irradiated at 18.3 rad/s in a Co(60) source for 72 h at 298 K, in the presence of air. The resulting suspension comprising unreacted monomer, ho-

TABLE IV
Immobilization of α-Chymotrypsin (E) onto Partially Hydrolyzed Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate[a]

Coupling medium	mg E/g	Coupling yield (%)	mg Active E/g	Retention of activity (%)
		Substrate: hemoglobin		
0.1 *M* $NaHCO_3$	116	29	1.4	1.2
Acetate buffer 0.1 *M*, pH 5.0	28	7.0	4.0	14.3
Phosphate buffer 0.1 *M*, pH 7.0	34	8.5	3.9	11.4

[a] 120% graft, 50% hydrolysis, using CMC as activating agent, for different coupling media at 277 K for 18 h.

TABLE V
Immobilization of α-Chymotrypsin (E) onto Partially Hydrolyzed Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate[a]

Coupling medium	mg E/g	Coupling yield (%)	mg Active E/g	Retention of activity (%)
		Substrate: GPNA		
0.1 *M* $NaHCO_3$	135	33.8	11.3	8.4
Acetate buffer 0.1 *M*, pH 5.0	20	5.0	3.0	15.0
Phosphate buffer 0.1 *M*, pH 7.0	35	8.8	30.0	85.7

[a] 120% graft, 50% hydrolysis, using CMC as activating agent, for different coupling media at 277 K for 18 h.

mopolymer, and copolymer was poured into an excess of methanol, filtered, washed thoroughly with methanol to remove homopolymeric poly(HEMA) and residual monomer, and dried under vacuum at 313 K. This procedure does not remove homopolymeric poly(ethylene).

Hydrolysis of Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate

Portions of poly(ethylene)-*g*-co-hydroxyethyl methacrylate PE–*g*-co–HEMA (120% graft) (2 g) were refluxed for specified times (in a range between

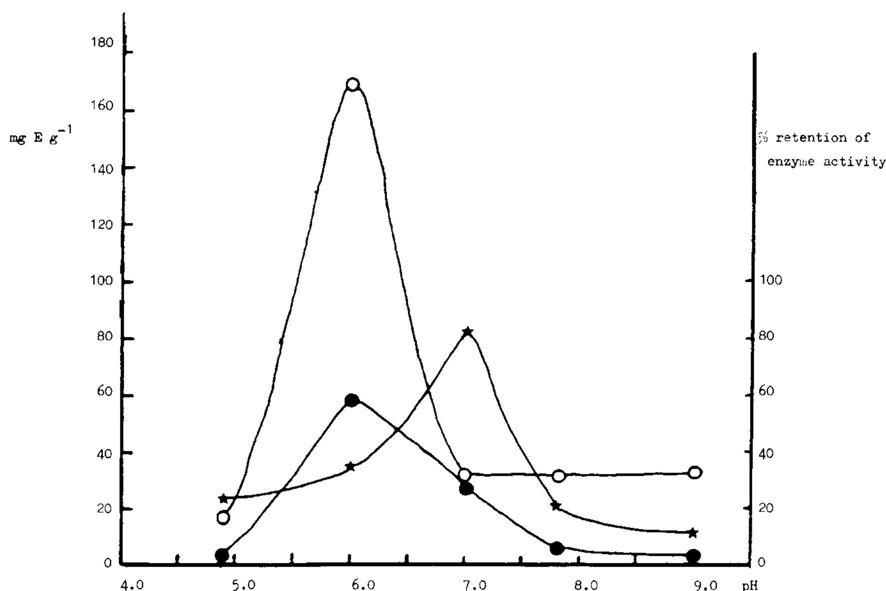

Fig. 1. Determination of the optimum pH for the immobilization of α-chymotrypsin on hydrolyzed poly(ethylene)-*g*-co-hydroxyethyl methacylate (120% graft, 50% hydrolysis): – ○ – mg coupled enzyme/g copolymer; – ● – mg of active enzyme/g copolymer; – × – % retention of the enzyme activity.

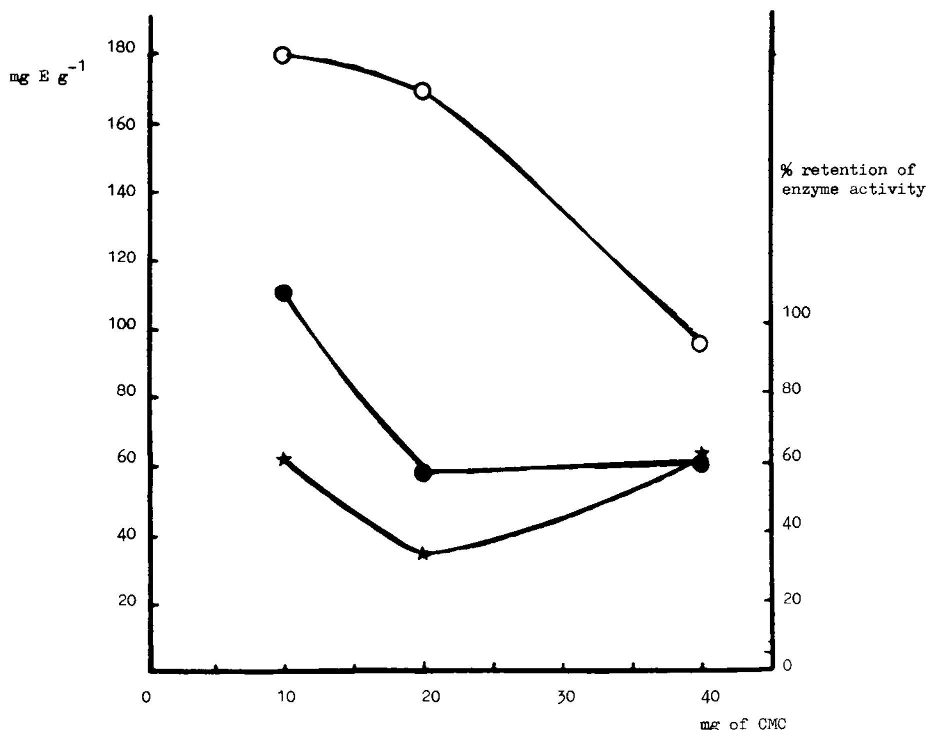

Fig. 2. Influence of the CMC concentration on the immobilization of α-chymotrypsin on poly(ethylene)-g-co-hydroxyethyl methacrylate (120% graft, 50% hydrolysis): – O – mg of coupled enzyme/g copolymer; – ● – mg of active enzyme/g copolymer; – X – % retention of the enzyme activity.

1 and 3 h) in 100 cm^3 of 1 *M* NaOH. The copolymers were then filtered and washed with water (500 cm^3). The carboxyl group content of the hydrolyzed copolymers was determined by titration with 0.1 *M* NaOH.[9]

Immobilization of α-Chymotrypsin

The enzyme was immobilized through the hydroxyl groups and through the carboxyl groups of the support. For attachment through the hydroxyl groups, the activation was carried out using each of the following: *p*-benzoquinone,[11] *p*-toluenesulphonyl chloride,[15] cyanuric chloride,[16] or epichlorohydrin.[17] To couple α-chymotrypsin through the carboxyl groups of the hydrolyzed copolymers, CMC was used as the activating agent.[18]

The ratio of the amount of enzyme presented to the amount of support in the coupling reaction was 40/100 mg, unless otherwise stated. The amount of coupled enzyme was determined by the Lowry method.[19] It is expressed as milligram of enzyme per gram of dry support and as the coupling yield (percent of coupled enzyme/enzyme presented for reaction).

The activity of the enzyme was determined using either GPNA[20] or hemoglobin[9] as substrates, at the optimum pH for the enzyme (7.8) and at room temperature.

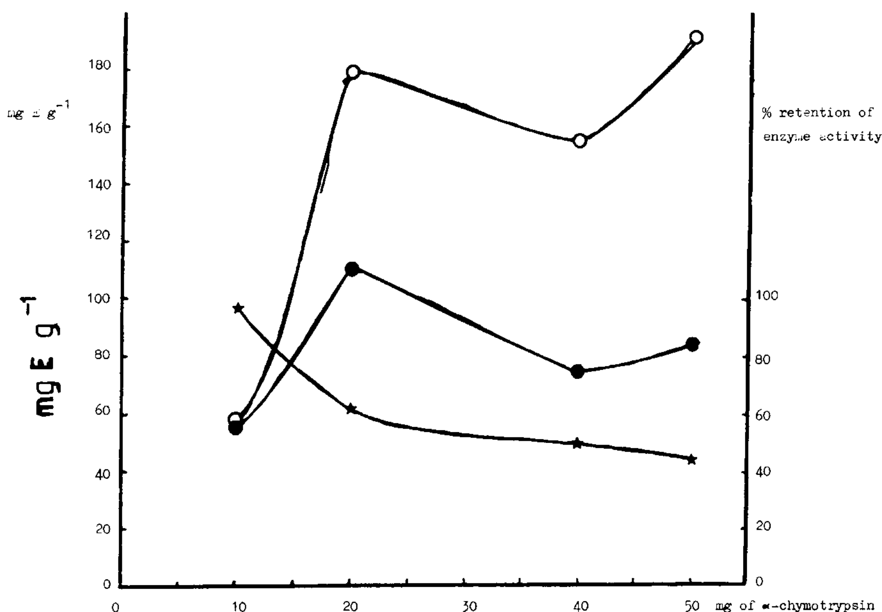

Fig. 3. Influence of the enzyme concentration on the immobilization of α-chymotrypsin on poly(ethylene)-*g*-co-hydroxyethyl methacrylate (120% graft, 50% hydrolysis): – O – mg of coupled enzyme/g copolymer; – ● – mg of active enzyme/g copolymer; – × – % retention of the enzyme activity.

In the assay of the enzyme in its immobilized form, the enzyme was incubated with its substrate solution. At the required time, the reaction was stopped by filtering off the composite particles, containing the immobilized enzyme, under vacuum. The enzymatic activity obtained was converted to milligrams of active enzyme through use of a calibration curve of native enzyme activity against milligram of native enzyme present. The activity yield is expressed as milligram of active enzyme per gram of dry copolymer.

RESULTS AND DISCUSSION

Previous work[3] has shown partially hydrolyzed PE–g-co–HEMA (120% graft, 50% hydrolysis) to be a promising support for the immobilization of trypsin. This support has since been used to couple α-chymotrypsin using either the available hydroxyl groups or carboxyl groups.

Initial attempts at coupling α-chymotrypsin to PE–*g*-co–HEMA (120% graft, 50% hydrolysis) involved use of the hydroxyl groups of the support after these groups had been activated. Different methods of activation were used, as indicated in Table II. In these cases, hemoglobin was the substrate. The results (Table II) show that good yields of coupling were obtained when the activation of the supports was carried out with *p*-benzoquinone, *p*-toluene sulphonyl chloride, and cyanuric chloride. However, the higher retentions of activity were observed when *p*-toluene sulphonyl chloride or cyanuric chloride were the activating agents. Consequently, the influence of the coupling medium on the

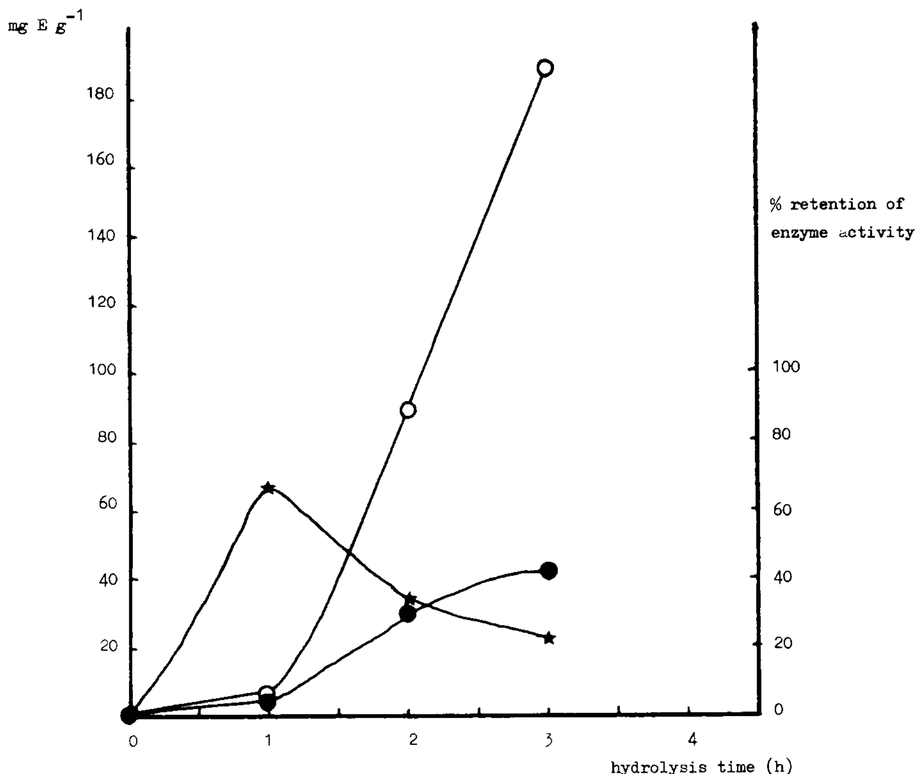

Fig. 4. Influence of the percent of hydrolysis of the graft copolymer, poly(ethylene)-*g*-co-hydroxyethyl methacrylate, on the immobilization of α-chymotrypsin: – O – mg of coupled enzyme/g copolymer; – ● – mg of active enzyme/g copolymer; – X – % retention of the enzyme activity.

immobilization efficiency for these two activating agents was studied. Good yields of coupling were obtained and the retention of activity was satisfactory in some cases (Table III).

The carboxyl groups of the support, after activation with CMC, were used in the coupling of the α-chymotrypsin. The influence of the coupling medium was studied. There is evidence of improvement in the results obtained (Table IV) relative to the immobilization of the enzyme onto the hydrolyzed PE–*g*-

TABLE VI
Optimum Temperature, Optimum pH, and Michaelis–Menten Constant for the Free and Immobilized α-Chymotrypsin onto Partially Hydrolyzed Poly(ethylene)-*g*-co-hydroxyethyl Methacrylate (120% Graft, 50% Hydrolysis)

Sample	Optimum pH value	Optimum temperature (K)	K_m (M)	V_{max} (μmol/min mg)
Free enzyme	7.8	298	7.3×10^{-3}	8.2×10^{-3}
Immobilized enzyme	7.8	298	9.6×10^{-3}	11.1×10^{-3}

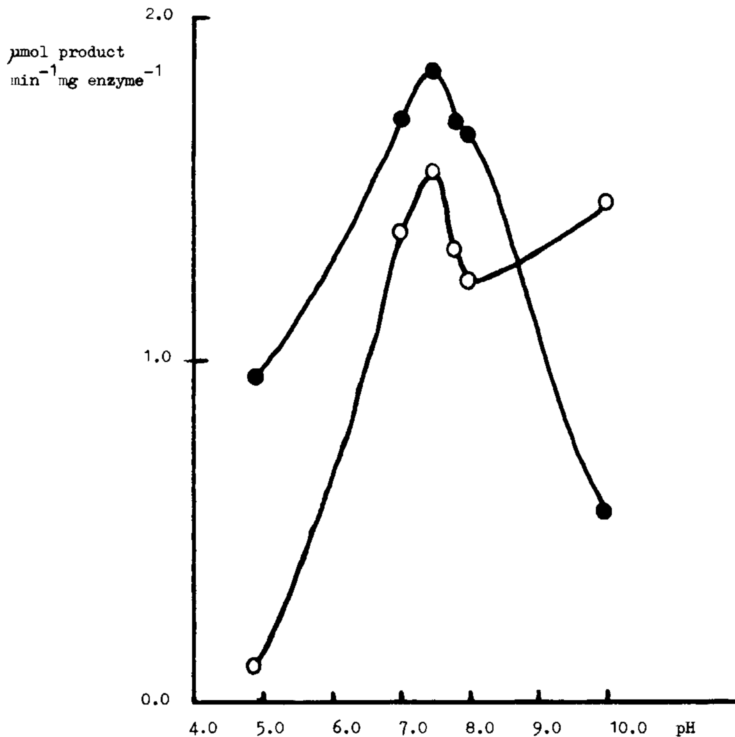

Fig. 5. Variation of the specific activity of free and immobilized α-chymotrypsin on poly(ethylene-*g*-co-hydroxyethyl methacrylate (120% graft, 50% hydrolysed) with different pH values: – ● – free enzyme; – ○ – immobilized enzyme.

co–HEMA where the hydroxyl groups of the support were involved. In these experiments, hemoglobin was used as substrate. The high molar mass of this protein would create difficulties of access of the enzyme to the substrate. This could provide an explanation for the lower activity of the enzyme after its immobilization. The coupling involving CMC was repeated but with GPNA as the substrate for the enzyme. The results, as indicated in Table V, indicate that not only does this method of immobilization show promise in both its effectiveness and its ease of application, but also that it is in need of further study. In view of these promising results, the influence of various factors on the immobilization of α-chymotrypsin onto the partially hydrolyzed PE–*g*-co–HEMA was studied. Here GPNA was used as the substrate.

Immobilization of α-Chymotrypsin onto Partially Hydrolyzed Poly(ethylene)-*g*-co-Hydroxyethyl Methacrylate

The influence of the various factors (pH, carbodiimide concentration, and enzyme concentration) on the coupling reaction was determined.

When the influence of pH of the medium in which the coupling reaction took place was studied, various enzyme solutions in buffering media of different pH values were used. The buffering media utilized were acetate buffer 0.1 *M* pH 5, acetate buffer 0.1 *M* pH 6, phosphate buffer 0.1 *M* pH 7, phosphate buffer

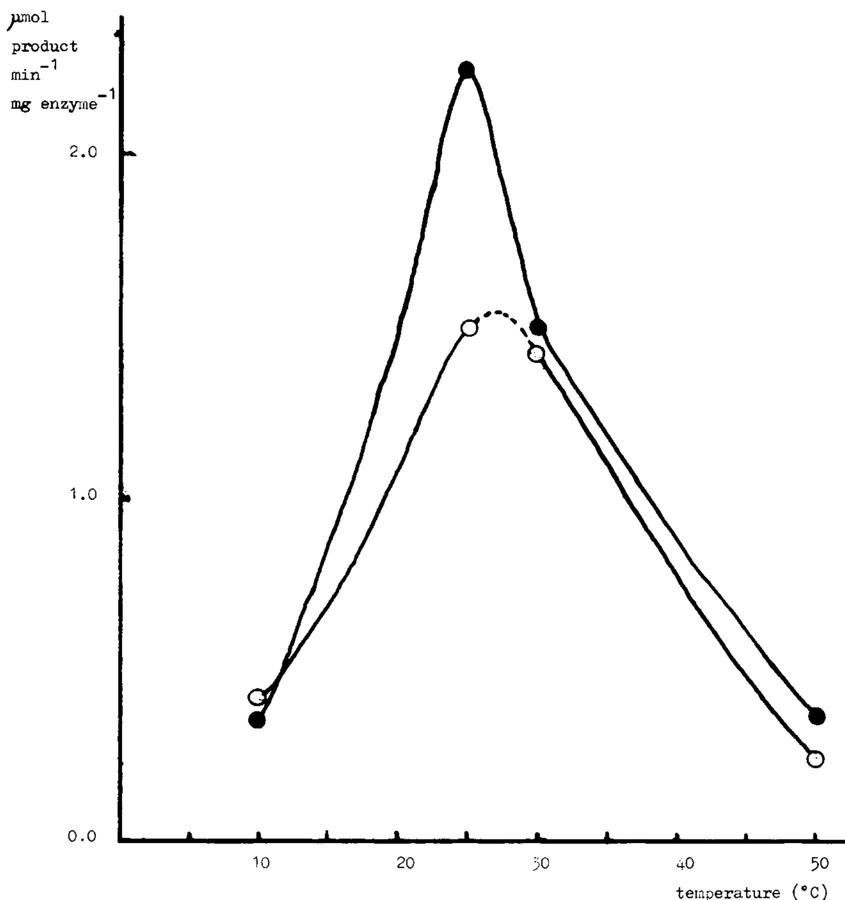

Fig. 6. Variation of the specific activity of free and immobilized α-chymotrypsin on poly(ethylene-*g*-co-hydroxyethyl methacrylate (120% graft, 50% hydrolysed) with temperature: – ● – free enzyme; – ○ – immobilized enzyme.

0.1 *M* 7.8, and phosphate buffer 0.1 *M* pH 9.0. The protocol was as follows: 100 mg of copolymer were treated with 5.0 cm^3 of a solution of α-chymotrypsin (20 mg of enzyme) and 20 mg of CMC were added in the solid form. Figure 1 shows that the best result was obtained using a 0.1 *M* phosphate buffer, pH 6; 170 mg of enzyme coupled per g of copolymer (85% of the enzyme presented was coupled) with 65% of the α-chymotrypsin remaining active, i.e., there was a 35% retention of activity.

The influence of the carbodiimide concentration on the reaction was studied at pH 6. Here, 100 mg of copolymer were treated with 5.0 cm^3 of the enzyme solution (20 mg) in acetate buffer 0.1 *M* pH 6, for various CMC loadings (varying from 10 to 40 mg). Figure 2 shows that the amount of coupled enzyme and the amount of activity retained, decreased on increasing the CMC concentration. This last effect could be due to inactivation of the native enzyme by the carbodiimade and to coupling of inactive enzyme. In fact, when the native α-chymotrypsin was treated with CMC according to the immobilization protocol, but in the absence of the support, 25 and 98% of its initial activity was lost

when the continuous medium for the reaction was at pH 5.0 and 7.8, respectively. This is in accordance with the observation that CMC deactivates α-chymotrypsin (by reacting with the serine residue in its reactive site).[21] This is reported to cause the formation of an essentially inactive enzyme at pH 7 to 8 and of a partially inactive enzyme at pH 5.[21]

The decrease in the amount of coupled, active enzyme, which is seen on increasing the concentration of coupling agent beyond that needed for optimum coupling, might be due to the additional coupling agent causing involuntary crosslinking of the enzyme. Any noncoupling could arise from difficulties in access by the coupling agent to groups volumetrically located within the bulk of each particle.

Finally, the influence of the concentration of the enzyme used in coupling on the nature of the coupling reaction was studied. The optimum enzyme amount was 20 mg per 100 mg of copolymer and 10 mg of CMC, as shown in Figure 3.

It is concluded that the optimum conditions of the immobilization of α-chymotrypsin onto partially hydrolyzed poly(ethylene)-*g*-co-hydroxyethyl methacrylate (50% hydrolysis, 120% graft, 100 mg of copolymer) are 10 mg of CMC, 20 mg of enzyme in 5 cm^3 of acetate buffer, 0.1 *M*, pH 6. 180 mg of α-chymotrypsin coupled per gram of copolymer. Thus, 90% of the enzyme presented for coupling becomes coupled. Of the 180 mg of coupled enzyme, 112 mg were active, representing a 62% retention of activity.

The influence of the extent of hydrolysis of the copolymer on the immobilization efficiency was studied. Figure 4 shows that an increase of the yield of coupling and in the retention of activity results from increases in the percentage of hydrolysis. Both are due in part to the increase in the number of carboxylic acid groups present on the grafts, which are suitable for reaction with the enzyme. Hydrolysis of the ester groups of the copolymer branches increases the overall hydrophilicity of the support, thereby possibly providing a more compatible microenvironment for the α-chymotrypsin. Our early studies[1-3,9] have shown that effective swelling in a hospitable continuous medium is a prerequisite to successful immobilization.

After determining the best conditions for the immobilization of α-chymotrypsin onto the partially hydrolyzed PE–*g*-co–HEMA, the K_M, V_{max}, optimum temperature profile, and optimum pH value for the free and the immobilized α-chymotrypsin were determined.

In Table VI, the K_M and V_{max} for the free and immobilized α-chromotrypsin are indicated. The results suggest that the access of the substrate to the enzyme would be influenced by diffusional factors. However, this point needs to be substantiated by further studies. These are currently being undertaken.

The results indicated in Figures 5 and 6 show that there is no significant difference in the optimum pH and temperature values between the free and immobilized enzyme. However, the enzyme, after being immobilized, is more stable at higher pH values. In this case, the support adopts a negative charge due to the presence of COO^- group. Thus, the electrostatic field created could attract H^+, providing a better microenvironment for the enzyme.[22-24]

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