SELF-DEGRADABLE HYDROGEL WITH COVALENTLY BOUND PROTEOLYTIC ENZYME

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Dedicated to Dr Blahoslav Sedlacek on the occasion of his 70th birthday.

Biodegradable hydrogel as a self-degradable system for controlled release of biologically active macromolecules was prepared from the biodegradable poly[N^5–(2-hydroxyethyl)-L-glutamine-*stat*-N^5-(2-methacryloyloxyethyl)-L-glutamine-*stat*-L-glutamic acid], to which a proteolytic enzyme, papain, was covalently bound. The polymer–enzyme conjugate was crosslinked by radical copolymerization with acrylamide. Significantly improved thermal stability and increased pH optimum of the catalytic activity of conjugated papain was observed. Self-degradation of the hydrogel by the action of encapsulated papain could be triggered by the addition of a low-molecular-weight enzyme activator, such as dithioerythritol.

Hydrogels are biocompatible materials capable of retaining and releasing in a controlled way high-molecular-weight compounds, such as biologically active peptides and proteins[1]. The permeability of peptides and proteins with respect to their molecular size can be controlled by the crosslinking density of the gel. A biodegradable hydrogel as a model for controlled delivery of macromolecular agents was prepared from a biodegradable polymer, poly[N^5–(2-hydroxyethyl)-L-glutamine] (PHEG). PHEG with partially methacryloylated side chains was used as a macromonomer crosslinker in crosslinking copolymerization with a low-molecular-weight acrylic monomer, acrylamide. The resulting three-dimensional network (hydrogel) was enzymatically degradable by papain and its degradation was controllable by the gel network density[2]. This technique affords hydrogels formed under conditions when the crosslinking reaction does not impair biological activity of proteins, which can thus be incorporated into the gel by adding them to the polymerization mixture. The network density can be varied[3] to a sufficient extent to permit a controlled permeability (release) of incorporated macromolecular substances in the molecular weight range of 100–100 000.

For certain applications it would be desirable to trigger the release of incorporated compounds by causing the gel to swell. The expansion of the network can be accom-

plished either by increasing its interaction with water (e.g., due to ionization of the polymer) or by decreasing the crosslinking density by degradation of crosslinks. This article deals with the preparation of a self-degradable hydrogel based on PHEG–acrylamide network containing a proteolytic enzyme, papain (m.w. 21 000), covalently attached inside the gel. Papain is known to be highly efficient in degradation of PHEG and its derivatives[4,5]. If the enzyme was activated by a low-molecular-weight activator, such as cysteine or dithioerythritol, which can easily penetrate into the gel, the enzyme would degrade the chains of PHEG. Thus the network will become "open", more permeable and, eventually, the gel dissolves. Such a "triggered" degradation could facilitate the release of the incorporated compound in a relatively short time after an external stimulus. The paper presents the data on the synthesis of PHEG–enzyme conjugate, characterization of the enzyme activity in the gel and demonstrates, in a model system, the feasibility of a "triggered" degradation of the gel.

EXPERIMENTAL

Materials

2-Aminoethanol, methacryloyl chloride, L-cysteine, sodium *N*-hydroxysulfosuccinimide and disodium ethylenediaminetetraacetate (EDTA) were purchased from Fluka, Switzerland. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMED), dithioerythritol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N^{α}-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BANA) were products of Serva, Germany. Papain (405 mU/mg) was a kind gift of Dr Torchilin, Institute of Experimental Cardiology, Moscow, Russia. Solvents were purified using standard laboratory procedures.

N-carboxyanhydride of γ-benzyl L-glutamate was prepared according to the procedure of Blout and Karlson[6]. Poly(γ-benzyl L-glutamate) (PBG; *I*) was prepared by solution polymerization of the *N*-carboxyanhydride in dioxane with sodium methoxide as an initiator[7].

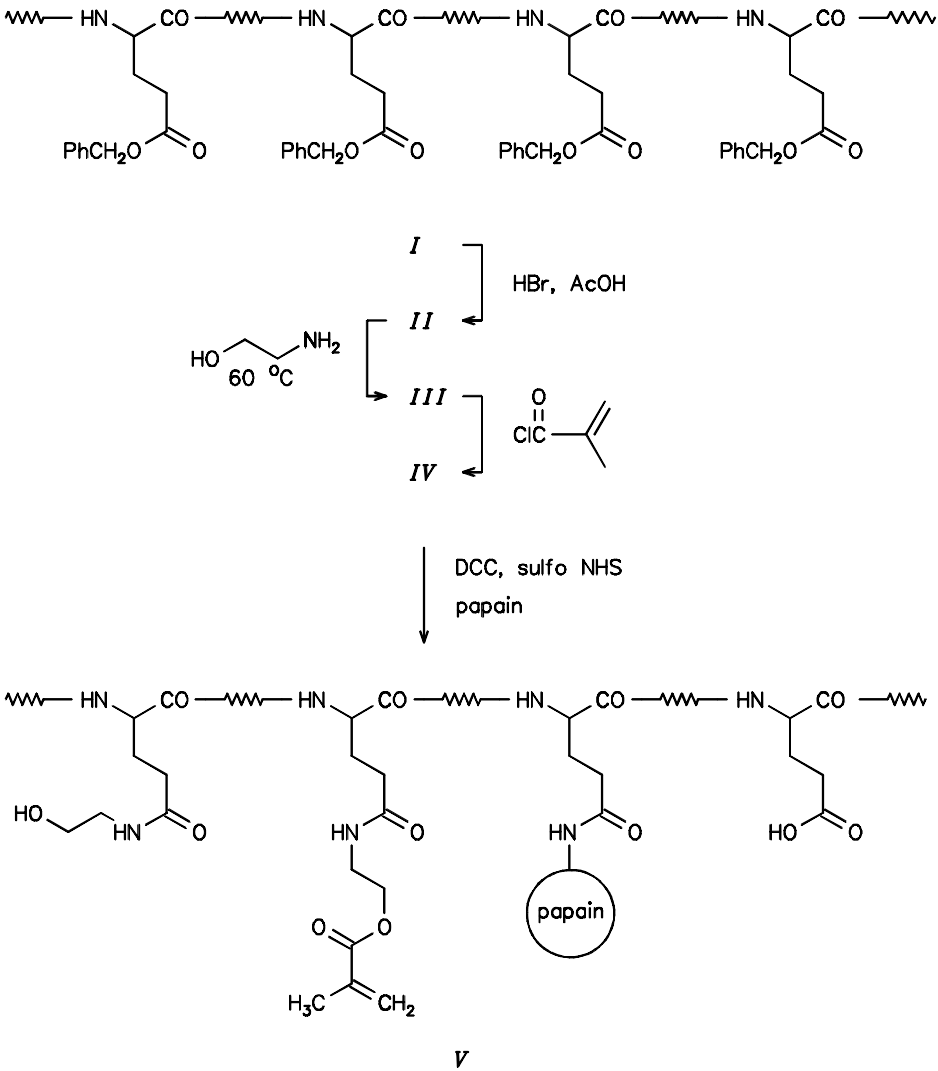
Copolymer of γ-benzyl L-glutamate and L-glutamic acid (poly(BG-stat-Glu), II*).* The copolymer was prepared by partial debenzylation of PBLG by the reaction with HBr. Typically, to an 8% solution of PBLG in benzene a 33% solution of HBr in acetic acid was added in excess and the mixture was stirred for 90 min at 30 °C. The product was precipitated in ethanol, washed, dissolved in dioxane and lyophilized. The content of glutamic acid in the copolymer was determined by titration with 0.1 M sodium methoxide in benzene–methanol (85 : 15 v/v).

Poly[N^5-(2-hydroxyethyl)-L-glutamine-stat-L-glutamic acid], (poly(HEG-stat-Glu); III*).* The copolymer was prepared by the aminolysis of poly(BG-*stat*-Glu) with 2-aminoethanol[8]. The reaction was carried out for 48 h at 60 °C. The resulting copolymer was purified by dialysis and freeze-dried from water.

Poly[N^5-(2-hydroxyethyl)-L-glutamine-stat-N^5-(2-methacryloyloxyethyl)-L-glutamic acid] (poly(HEG-stat- MEG-stat-Glu); IV*).* The copolymer was prepared by the reaction of poly(HEG-*stat*-Glu) with methacryloyl chloride in dimethylacetamide solution (polymer concentration 5%). For the synthesis, see Scheme 1.

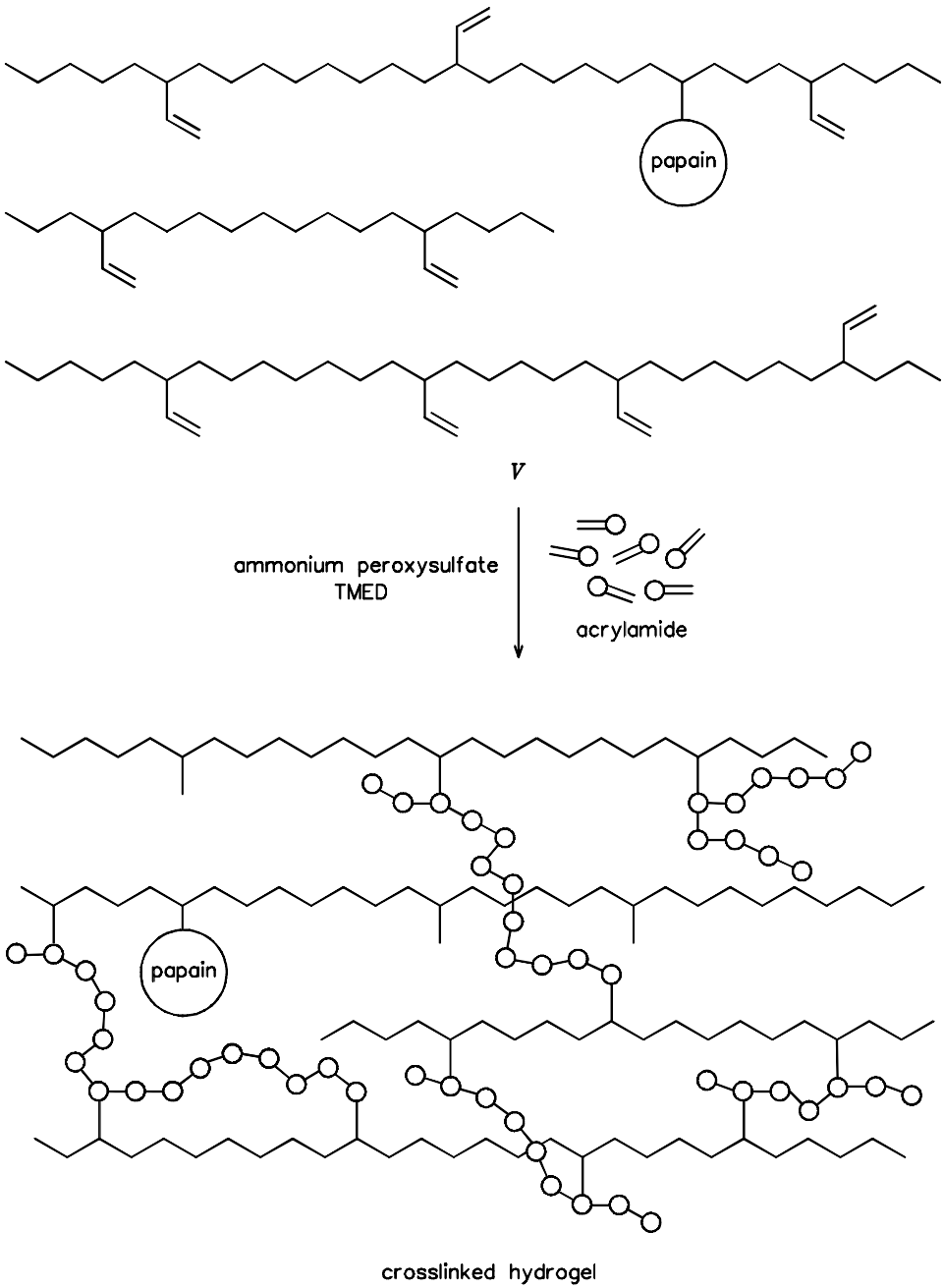
*Poly(HEG-stat-MEG-stat-Glu)–papain conjugate (*V*).* The conjugate was prepared by a covalent binding of papain to poly(HEG-*stat*-MEG-*stat*-Glu) (IV) using the activation of carboxylic groups in the copolymer by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in the presence of *N*-hydroxysulfosuccinimide and L-cysteine[9]. Activation at pH 3.7 in a salt-free solution (15 min) was followed by the papain binding reaction in 0.05 M phosphate buffer (pH 7.8) for 18 h at 4 °C. Con-

centrations of reagents in the reaction mixture were: poly(HEG-*stat*-MEG-*stat*-Glu) 0.56 . 10^{-3} mol l^{-1}, papain 0.56 . 10^{-4} mol l^{-1}, L-cysteine 5 . 10^{-3} mol l^{-1}, *N*-hydroxysulfosuccinimide 3 . 10^{-3} mol l^{-1}, the carbodiimide 2 . 10^{-2} mol l^{-1}. The polymer–enzyme conjugate was separated from the unbound papain by gel permeation chromatography on a Sephacryl S-400 column. Fractions containing the conjugate were dialyzed against distilled water at 4 °C, concentrated in a vacuum evaporator and lyophilized. The yield of bound enzyme activity was measured after the bonding reaction and after GPC isolation.



V

SCHEME 1


papain
V
ammonium peroxysulfate
TMED
acrylamide
papain
crosslinked hydrogel

SCHEME 2

Hydrogel containing the polymer–papain conjugate. The hydrogel was prepared by radical cross-linking polymerization of the *V*–acrylamide mixture (2 : 1 w/w) in water solution at 4 °C in the way analogous to that described elsewhere[2]. The procedure is depicted in Scheme 2. The volume fraction of the monomers in gel formation was 0.15. Ammonium peroxysulfate as an initiator (mole ratio peroxysulfate/total double bonds 0.01) and TMED as a co-catalyst were used in equimolar amounts. The gel was prepared in the form of a 2 mm thick sheet by the polymerization in a mold consisting of two glass plates separated by a silicone rubber dilation insert. After the polymerization, the gel was washed and equilibrium-swollen in redistilled water for 3 days at 4 °C; the washing water was changed several times.

Methods

Mole fraction of MEG units in copolymer *IV* was determined from the ^{1}H NMR spectra[2] and its molecular weight by gel permeation chromatography using calibration with PHEG standards[10].

Papain activity measurement. The measurements were carried out at 25 °C in the incubation buffer of pH 7.5 containing 0.05 mol l^{-1} KH_2PO_4, 0.005 mol l^{-1} dithioerythritol and 0.002 mol l^{-1} EDTA using BANA as a substrate. The activity was determined spectrophotometrically by measuring the absorption of released *p*-nitroaniline at 410 nm using Varian SuperScan3 Spectrophotometer.

pH optimum for the activity of covalently bound and free papain was determined with BANA in phosphate incubation buffers in the pH range of 5.5–9.0.

For determination of thermal stability, the enzymatic activity of free papain and the conjugate in the incubation buffer was measured at 37 and 50 °C in 1 h intervals.

To measure the enzyme activity of the incorporated papain the gel sample was equilibrated in the incubation buffer at 25 °C, and then mechanically disintegrated. Upon adding BANA the mixture was stirred and the absorbance of the solution at 410 nm was measured after 20 min.

"Triggered" degradation of the gel. The degradation was followed in the equilibrium-swollen gel. Two gel samples were immersed in phosphate buffers (0.05 M KH_2PO_4, pH 8.0) at 4 °C. The weight of the gel samples was monitored in 12 h intervals for 5 days. The samples were then equilibrated at 25 °C and dithioerythritol and EDTA were added to one of the solutions to make their final concentrations 0.005 mol l^{-1} and 0.002 mol l^{-1}, respectively. The weight of the samples was followed for another 4 days. After a complete dissolution of the activated sample, the residual enzymatic activity in the solution was measured using the BANA substrate.

RESULTS AND DISCUSSION

Preparation and Characterization of the Conjugate

The copolymer poly[HEG-*stat*-MEG-*stat*-Glu] was prepared by modifying the procedures described previously[11]. The resulting copolymer contained 15 mole % of L-glutamic acid and 8 mole % of methacryloylated HEG units (MEG). The weight-average molecular weight of the copolymer was 32 000, as determined from GPC using the column calibration for PHEG. Papain was covalently bound to the copolymer, using a water-soluble carbodiimide as an activation agent for carboxylic groups of glutamic acid residues. The amount of papain taken into the reaction was about 10% of that of the polymer carrier, assuming that, theoretically, no more than one tenth of polymer molecules could carry the enzyme. The yields of papain activity after the binding reac-

tion is presented in Table I. It can be seen that a larger decrease in the enzyme activity occurred during the reaction, which can be primarily attributed to the effect of chemical modification of the enzyme and/or to changes in its microenvironment. A further decrease in the enzyme activity in the sample caused by the purification of the conjugate can be related to the amount of unbound enzyme removed during the GPC purification. The data indicate that papain can be bound to the carrier with a good yield and that a fairly significant fraction of its catalytic activity can be retained. Specific papain activity of the preparation used for further experiments with gelation was 26.2 mU (BANA) per 1 mg of the conjugate.

The binding of a polymer to an enzyme can change the microenvironment of the enzyme active site and affect the thermodynamic stability of the protein molecule. These effects are reflected in the change of pH optimum and thermal stability of the covalently bound papain.

TABLE I
The activity of papain in the binding reaction to *IV*

Conditions	Total activity, mU	Activity, %
Taken into the reaction	4 050	100.0
After the reaction	2 576	63.6
After purification of *V*	2 228	55.0

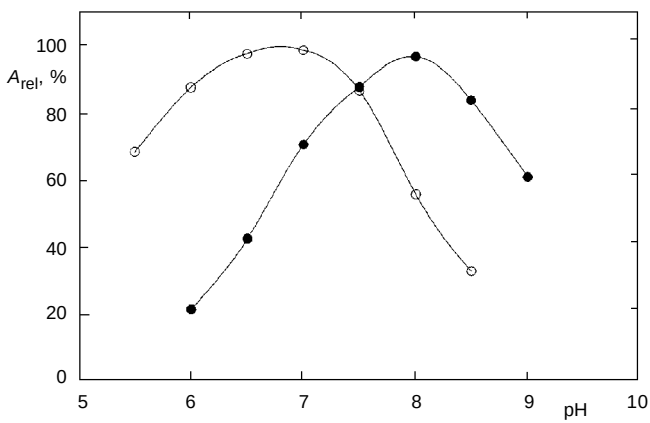


FIG. 1
The pH dependences of relative enzymatic activity A_{rel} of the covalently bound (●) and free (○) papain

Figure 1 presents the pH dependence of relative enzymatic activity of the free and covalently bound papain. pH optimum of the covalently bound papain (around pH 8) is significantly higher than that of the free enzyme (pH 6.5–6.8). The shift of the pH optimum can be explained by a change in the microenvironment of the enzyme molecule caused by carboxylic groups of the polymer coil bound to and/or surrounding the enzyme. The actual pH inside the coil could be lower than that in the surrounding bulk solution.

The results showing thermal stability of the covalently bound and free papain are presented in Fig. 2. Thermal stability of papain increased considerably by the covalent binding to the polymeric carrier. While the activity of free papain decreased to 36% of its original value after 4 h of incubation at 50 °C, about 78% of the activity of covalently bound enzyme was retained under the same conditions. The covalently bound enzyme appears to be better protected against autodegradation.

Gel Preparation and Degradation

The hydrogel was prepared by crosslinking polymerization of the polymer–papain conjugate and acrylamide in water solution. Physical properties of hydrogels prepared by this technique were investigated previously and are reported elsewhere[2,3]. About 40% of the original activity of the papain added to the crosslinking system was retained in the gel. Actually, the activity in the gel could be somewhat higher than that measured because the reaction with chromogenic substrate could be slowed down due to the diffusion of the substrate into gel particles.

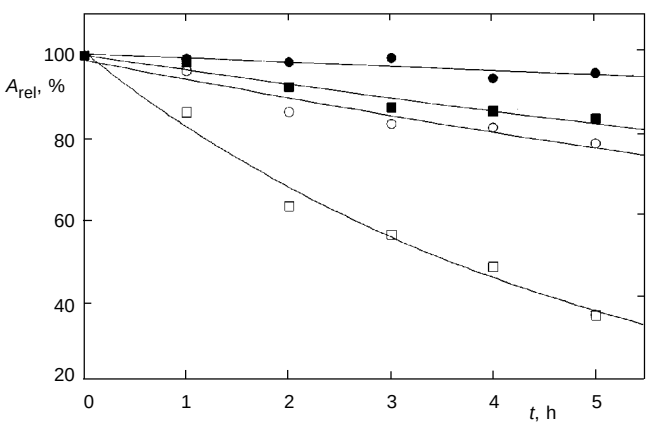


FIG. 2
Time course of relative enzymatic activity of covalently bound (filled points) and free (open points) papain on the incubation at 37 °C (❍) and 50 °C (❑), respectively

The degradation of gel was followed after the addition of enzyme activators to the equilibrium-swollen gel sample. The progress of the degradation was determined by measuring the change in the gel weight caused by the additional swelling due to degradation of PHEG chains in the gel. The results are presented in Fig. 3. The sample without enzyme activators showed only insignificant increase in the gel weight during the incubation at 25 °C. The weight of the gel with activators added increased, as papain bound inside the gel degraded the peptide bonds in the poly(HEG-*stat*-MEG-*stat*-Glu) chains. After four days of incubation, the gel sample was completely dissolved. The residual enzymatic activity in the obtained solution was about 20 to 30% of the papain activity in the gel sample before the degradation. This indicates that the gel structure effectively shields the enzyme molecules and thus protects them from autodegradation, until the gel completely dissolves.

The gel containing covalently bound papain was stable in a buffer solution void of enzyme activators and can be stored under these conditions and at a low temperature for a long time. The activation of the enzyme can be accomplished with low-molecular-weight compounds, such as dithioerythritol and EDTA, which can freely penetrate into the gel. As a result of the enzymatic degradation, the concentration of effective cross-links decreases, the polymeric network expands and finally the gel dissolves. The mechanism of "triggered" degradation can be used for a controlled release of macromolecular biologically active compounds, entrapped in the gel. The stability of the gel in the absence of activators is important for the stability of the preparation during the storage. The activation could be accomplished during the administration of the controlled-release system or by the action of endogenous thiol compounds in vivo.

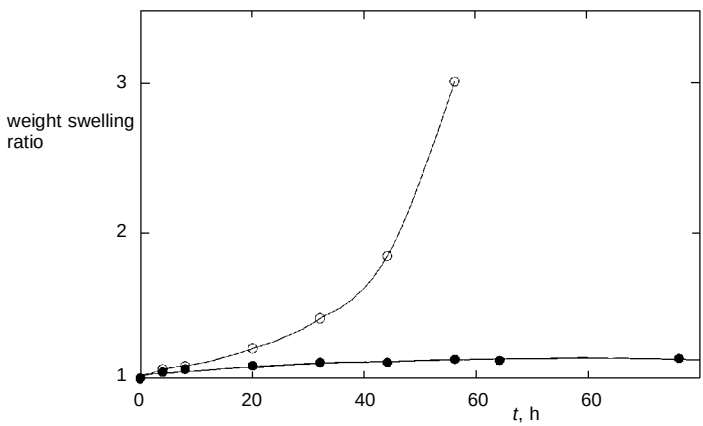


FIG. 3
Swelling of the gel during its incubation in the presence (○) and absence (●) of enzyme activators

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