Immobilization of thiol peptides and proteins on poly(thioglycoloyloxyethyl methacrylate) gel

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Poly(thioglycoloyloxyethyl methacrylate) gel has been prepared. The reactivity of its SH groups was checked with [^{14}C]phenyl isothiocyanate. Immobilization of thiol peptides and proteins to a carrier can be performed after its activation with 6,6′-dithiodinicotinic acid. The attached compounds can be released with 50 mmol dm^{-3} dithiothreitol or can be hydrolyzed with enzymes or acids without decomposition of the carrier.

Был приготовлен политиогликолоилоксиэтилметакрилатный гель. Реактивность его группы SH была исследована при помощи [^{14}C]фенилизотиоцианата. Иммобилизирование тиоловых пептидов и протеинов носителем наступает после его активации 6,6′-дитиодиникотиновой кислотой. Фиксированные вещества можно освободить с 50 ммоль дм$^{-3}$ раствором дитиотреитола или возможно осуществить их энзиматический или кислотный гидролиз, без разложения носителя.

One of the most studied methods of immobilization of different compounds, particularly proteins, is the formation of covalent bonds between specific functional groups of the support and the compound to be immobilized. Covalent bonds can be formed also by interaction of the SH groups of the matrix and those of the binding compound, which results in a heterodisulfide. At the temperature and pH adequate to physiological conditions, the heterodisulfide bonds can be created only after activation of the SH groups of the carrier. For this purpose mostly 2,2-dipyridyl disulfide [1, 2] is used. Thiol enzymes, namely papain, ficin, and urease [3—5], mercaptoalbumin [6], peptides containing cysteine [7, 8], and lately even interferon [9] have been isolated on agarose activated in this way.

Macroporous hydroxyethyl methacrylate gels (Spheron) are successfully utilized in affinity chromatography and immobilization of enzymes [10—12]. It is due to their excellent mechanical properties, large inner surface, resistance to microorganisms, and stability in concentrated acids and alkalies even at higher temperatures. These properties could be well utilized also in immobilization of proteins through the cysteine SH groups of the peptide chain. For this purpose we focused

our attention on the preparation of polythiol derivative of Spheron and its activation with 6,6′-dithiodinicotinic acid.

Experimental

Chemicals and equipments

Spheron 100 (40—63 and 63—100 μm) was produced by Lachema, Brno, Enzacryl-Polythiol by Koch-Light.

6,6′-Dithiodinicotinic acid (CPDS), dithiothreitol (DTT), and human albumin were purchased from Calbiochem, thioglycolic acid, glutathione (GSH), and the other chemicals used were of anal. grade (Lachema, Brno). Methyl thioglycolate was produced by Fluka.

[^{14}C]Phenyl isothiocyanate (1.92×10^3 MBq/mmol) and *N*-ethyl-2,3[^{14}C]maleimide (1.48×10^2 MBq/mmol) were purchased from Radioactive Centre Amersham. Albumin was labeled with Na[^{125}I] after [13].

Elemental analyses were carried out on a Hewlett—Packard CHN analyzer, sulfur was determined after [14]. The incorporated activities were measured on an NZQ 714-T Tesla set ([^{125}I]) and on an LKB-Wallac 81000 Liquid Scintillation Counter with an SLD-31 Scintillator ([^{14}C]).

Amino acids were analyzed on an automatic HD-1200 E amino acid analyzer (SNP Works, Žiar nad Hronom) according to the method of *Spackman, Moor,* and *Stein* [15].

Poly(thioglycoloyloxyethyl methacrylate)

Spheron 100 (35.2 g), methyl thioglycolate (46.3 g), tetrahydrofuran (130 cm^3), and concentrated sulfuric acid (10 cm^3) were put into a flask and the mixture was refluxed for 4 h. The carrier was washed with tetrahydrofuran and dried at 80°C after filtration. Thiol-Spheron (2 g) was washed with the mixture (50 cm^3) of acetone—0.05 mol dm^{-3} sodium carbonate (6 : 4). Then the mixture was put into the solution (acetone—0.05 mol dm^{-3} Na_2CO_3 = 6 : 4) which contained CPDS (20 mg). After 1 h activation, the carrier was washed with 60% acetone in distilled water. Enzacryl-Polythiol was activated similarly. The concentration of SH groups in the prepared carriers and in Enzacryl-Polythiol was established with CPDS after *Grassetti* [16]. Their presence was proved also in the reactions of the carriers with *N*-ethyl-2,3[^{14}C]maleimide (NEM) at pH 7.3 and with [^{14}C]phenyl isothiocyanate at pH 7.0 [17]. The results are presented in Table 1.

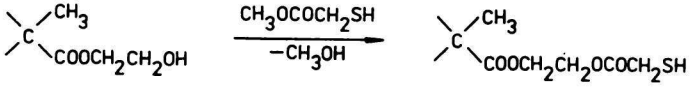
Immobilization of albumin and glutathione

Immobilization of [^{125}I]albumin, present in the reaction solution in 2—500 μg per 1 g carrier, proceeded in 0.05 mol dm^{-3} tris-HCl buffer (2 cm^3) containing 1 mmol dm^{-3} EDTA at pH 8.0 under shaking for 30 min. The activity of the carrier was measured after washing with buffer.

Immobilization of glutathione, present in the reaction medium in 100 mg per 1 g carrier, proceeded under the same conditions as that of albumin. After washing with buffer, the dried carrier (200 mg) was hydrolyzed in a sealed ampule (6 mol dm^{-3} HCl; 18 h).

Results and discussion

The polythiol derivatives of Spheron were prepared by reesterification of methyl thioglycolate with Spheron (Scheme 1). The formed polythiols were characterized



Scheme 1

by elemental analysis, establishing the concentration of SH groups by the method of *Grassetti* [16] and by using *N*-ethyl-2,3[^{14}C]maleimide [17]. Polyacrylamide polymer Enzacryl-Polythiol (Table 1) was used for comparison.

The binding properties of the prepared carriers are dependent on the availability and reactivity of SH groups. The reactivity, however, depends not only on nucleophilicity but also on the concentration of R—S^- forms. The course of the reaction of [^{14}C]phenyl isothiocyanate with thiol-Spheron (63—100) in dependence on pH is illustrated in Fig. 1. In this reaction, which is often used for characterization of the reactivity of SH groups [17], polymeric forms of esters of dithiocarbamic acid are formed. Comparison of the reactions of thiol-Spheron with those of Enzacryl-Polythiol at pH 6.0 points to higher reactivity of the former carrier (Fig. 2).

Table 1

Results of elemental analyses and concentration of SH groups of the carriers

Substance	% C	% H	% S	Concentration of SH groups*	
				After [16]	[^{14}C]NEM
Spheron 100 (40—63)	57.63	7.69	—	—	—
Thiol-Spheron 100 (40—63)	53.98	7.19	7.19	1300	2200
Spheron 100 (63—100)	58.35	7.68	—	—	—
Thiol-Spheron 100 (63—100)	55.44	7.19	5.56	1900	3800
Enzacryl-Polythiol	45.66	5.78	4.62	1400	2900

* Expressed in μmol g^{-1}.

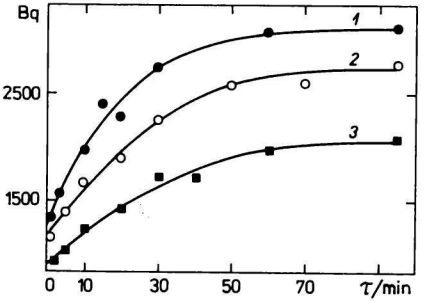


Fig. 1. Reaction of thiol-Spheron 63—100 (50 mg) with [^{14}C]phenyl isothiocyanate (4.92×10^{-2} mol dm^{-3}) in dependence on pH; 0.2 mol dm^{-3} McIlvaine, 25°C.
1. pH 10; *2.* pH 8; *3.* pH 4.
The reaction was stopped in time intervals by acidifying with concentrated HCl to pH 1.5.

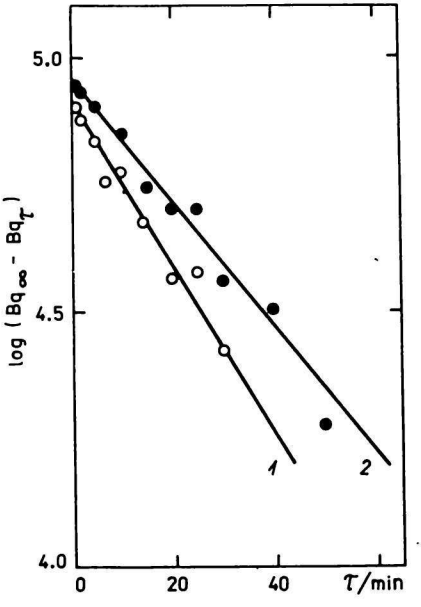
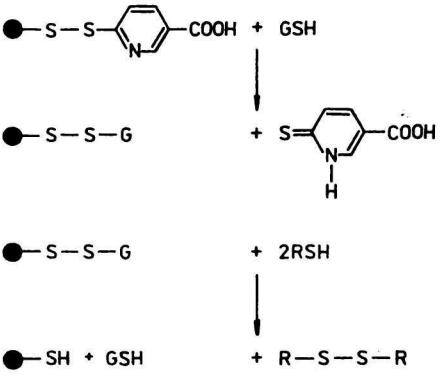


Fig. 2. Kinetics of the reactions of [^{14}C]phenyl isothiocyanate with thiol-Spheron 63—100 (*1*) and Enzacryl-Polythiol (*2*) at pH 6.0.
The reaction conditions are presented in Fig. 1.

Activation of the SH groups of the carrier with 6,6′-dithiodinicotinic acid is advantageous because, in consequence of the electron-accepting effect of nitrogen and carboxyl group, instable disulfide bonds are formed to which proteins, peptides, and other free thiols are easily attached through the free SH groups. Further advantage is that the forming 6-thiopyridone does not contain a free SH group and thus, it cannot be bonded to the free activated functional groups of the carrier. It is possible to establish the amount of the bound peptide or protein ($\log \varepsilon_{340} = 3.951$) from the released amount of 6-thiopyridone. The bonded substance can be released from the carrier by excess amount of a low-molecular thiol, such as cysteine, mercaptoethanol, DTT, *etc.* Afterwards, the carrier can be regenerated by activation with CPDS (Scheme 2).



Scheme 2

In order to find out the binding capacity of the activated carriers, we performed the immobilization of [^{125}I]albumin. The immobilized glutathione was prepared with purpose to ascertain the stability of the carrier under the conditions of acid hydrolysis.

Immobilization of proteins or peptides to the activated thiol-Spheron is dependent on their concentration in the reaction medium. The amount of [^{125}I]albumin bound on the activated carriers thiol-Spheron 100 (40—63) and thiol-Spheron 100 (63—100) as well as on Enzacryl-Polythiol is illustrated in concentration dependence in Fig. 3. From these relationships it is evident that thiol-Spheron 100

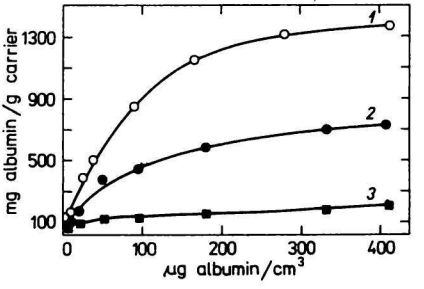


Fig. 3. Amount of the bound [^{125}I]albumin in dependence on its concentration in the reaction mixture.
1. Thiol-Spheron 100 (63—100); *2.* thiol-Spheron 100 (40—63); *3.* Enzacryl-Polythiol.
The reaction conditions: 0.05 mol dm^{-3} tris-HCl buffer, pH 8.0, 100 mg carrier, 25°C, reaction time 30 min.

(63—100) has the highest binding ability which corresponds to the highest concentration of SH groups (Table 1). The relatively low affinity of Enzacryl-Polythiol to albumin can be explained by inaccessibility of the activated disulfide bonds of the carrier for the SH groups of the binding protein.

Methods describing enzymic hydrolysis of immobilized proteins on agarose, after which the formed peptides are freed from the carrier, are known from the literature

[7]. This material, however, cannot be used for acid hydrolysis of the immobilized proteins because, in strong acids and bases, it undergoes decomposition. Therefore, we performed the immobilization of the tripeptide glutathione on the activated thiol-Spheron and the obtained preparation was subjected to acid hydrolysis (Table 2). Glutamic acid and glycine were released during hydrolysis while cysteine remained attached to the carrier through the SH group. Cysteine was released with 50 mmol dm^{-3} DTT after the hydrolysis was completed. DTT did not interfere in establishing the amino acids.

Table 2

Amino acid composition of the hydrolyzate (6 mol dm^{-3} HCl; 18 h) of the immobilized glutathione Displacement with 50 mmol of DTT. The results are calculated for 1 μmol GSH

Substance	Glu	Gly μmol	Cys
Thiol-Spheron 100 (40—63)—GSH			
Before displacement	0.51	0.50	—
After displacement	—	—	0.48
Thiol-Spheron 100 (63—100)—GSH			
Before displacement	0.82	0.82	—
After displacement	—	—	0.80
Enzacryl-Polythiol		not detectable	
GSH (control)	1.00	1.00	0.96

The amounts of the individual amino acids released were almost equimolar, which proves that only peptide bonds were cleaved during the hydrolysis, while the disulfide bond between cysteine and Spheron remained intact. The situation was different with Enzacryl-Polythiol where the peptide bonds of the gel itself were cleaved during the hydrolysis.

The fact that decomposition of thiol-Spheron has not been observed during the acid hydrolysis is important from the view-point of its utilization in establishing the number of bonded cysteine residues through which the protein is attached to the carrier. Poly(thioglycoloyloxyethyl methacrylate) gels are supposed to be useful also in isolation of SH peptides and proteins as well as in the investigation of structure and function of the active centres of enzymes.

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