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PROTEIN IMMOBILIZATION ON SILICA SUPPORTS

A LIGAND DENSITY STUDY

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SUMMARY

The immobilization of proteins on diol-bonded silica matrices containing carboxyl groups (spacer arms) was studied. It was found that the activated ester coupling method worked best with N,N′-dicyclohexylcarbodiimide as the condensing agent in the activation step. During protein coupling, the amount of protein immobilized was highest below pH 6. The optimum pore size of the silica was 300–1000 Å. The spacer arm ligand density was varied over as much as a 100-fold range and the effects on the total activities and specific activities of several proteins were studied. Two proteins exhibited up to two-fold increases in specific activity at low ligand densities. However, the total amounts of activity and protein immobilized decreased at low ligand densities.

INTRODUCTION

Immobilized proteins have found important applications over the past several decades. These applications include biochemical purification by affinity chromatography, biochemical analysis by immunoassay methods, and the use of immobilized enzymes for the catalysis of reactions of industrial interest.

An immobilized protein may have a lower specific activity than the soluble protein. This may be due to multipoint attachment, which causes distortion of the three-dimensional structure of the immobilized protein, or to an immobilized orientation which results in steric hindrance of the binding site[1]. By using a low surface concentration of spacer arms or activated sites for immobilization, multipoint attachment can be minimized and the steric hindrance diminished to some extent, so that the specific activity of the immobilized protein should be increased. This has been observed for several proteins immobilized on hydroxyalkyl methacrylate gels[2], Sepharose CL[3], and aminoethylcellulose[4].

In a previous report[5], silica was silanized to produce diol-bonded silica and further derivatized with diglycolic anhydride to yield a matrix of variable ligand

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density, *i.e.*, variable surface concentration of spacer arms. This has made it possible to investigate the effects of spacer arm concentration on the activity of immobilized proteins over a very broad surface concentration range. In this study, several proteins were immobilized on silica supports of varying spacer arm concentration by first activating the carboxylate spacer arms with a carbodiimide and N-hydroxysuccinimide. The specific activities of these immobilized proteins were studied as a function of spacer arm surface concentration. The influence of the carbodiimide, pore size, and pH on the immobilization yield were also examined. In this paper, the term "ligand density" is defined as the initial surface concentration of the carboxylate spacer arms.

EXPERIMENTAL

Reagents

Eel acetylcholinesterase, soybean trypsin inhibitor, bovine pancreas α-chymotrypsin, bovine pancreas trypsin, *Staphylococcus aureus* protein A, rabbit immunoglobulin G (IgG), goat anti-human serum albumin (anti-HSA, immunoglobulin fraction), bovine pancreas ribonuclease A, porcine pepsin, human serum albumin (HSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), acetylcholine iodide, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide, and N-acetyl-L-phenylalanyl-3,5-diiodo-1-tyrosine were from Sigma (St. Louis, MO, U.S.A.). Ninhydrin, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC), dithio bis-2-nitrobenzoic acid, 1,1′-carbonyldiimidazole (CDI), hydrindantin, and 2-methoxyethanol were from Aldrich (Milwaukee, WI, U.S.A.). N,N′-dicyclohexylcarbodiimide (DCC), Triton X-100, and phenol reagent solution were from Fisher (Fair Lawn, NJ, U.S.A.). Nucleosil 1000-5 (1000 Å pore size, 25 m^2/g surface area, 5 μm particle diameter) and 300-5 (300 Å, 100 m^2/g, 5 μm) were obtained from Alltech (Deerfield, IL, U.S.A.). LiChrospher SI-4000 (4000 Å, 10 m^2/g, 10 μm) and SI-1000 (1000 Å, 30 m^2/g, 10 μm) were from Rainin (Woburn, MA, U.S.A.). N-hydroxysuccinimide (NHS) was from Eastman Kodak (Rochester, NY, U.S.A.).

Methods

Carboxylated silica of variable ligand density was prepared and quantitated as described previously[5]. The NHS ester was synthesized according to a published procedure[6] with the following modifications: the silica was suspended in dioxane and degassed by sonication under aspirator vacuum for 10 min prior to reaction, and the NHS ester silica was dried under vacuum at room temperature overnight. The activated silica (0.1 g) was suspended in 2 ml of the coupling buffer (0.1 *M* sodium phosphate of the desired pH) and degassed for 10 min. The protein was added and the suspension shaken in a wrist action shaker. In the pH study, the trypsin (10 mg) and acetylcholinesterase (2 mg) reaction mixtures were shaken at 4°C for one day, trypsin inhibitor (30 mg) at 4°C for 6 h, and anti-HSA (30 mg) at room temperature for 18 h. In the pore size study, the reactions with trypsin inhibitor (30 mg protein, pH 3.0 buffer) and anti-HSA (30 mg, pH 5.0) were shaken at room temperature for 18 h. In the activity study, the α-chymotrypsin (14 mg, pH 4.0, 7 days), pepsin (10 mg, pH 4.0, 7 days), ribonuclease A (20 mg, pH 5.0, 7 days), anti-HSA (30 mg, pH 7.0, 24 h), acetylcholinesterase (10 mg, pH 6.0, 18 h), and trypsin inhibitor (30 mg, pH 3.0, 5 days) were shaken at 4°C, and protein A (20 mg, pH 5.0, 18 h) at room temperature.

In the pore size study, 0.1 g diol-bonded silica was also activated with CDI as described previously[7] and used for the immobilization of anti-HSA at 4°C (30 mg, pH 5.0, 6 days), or oxidized with periodate[8] and used for the immobilization of the anti-HSA at 4°C (30 mg, pH 5.7, 6 days) by the Schiff base method[8,9]. The protein-silica products were washed with 2 *M* sodium chloride, followed by water in the pH and pore size studies, or with 1 *M* sodium chloride in 0.1 *M* sodium phosphate buffer of the appropriate pH in the activity study.

Immobilized glucosamine was quantitated as described previously[5]. Immobilized protein was determined by a modified Lowry protein assay[10] in which all of the reaction times were increased by 50% and the silica was removed by centrifugation prior to absorbance measurement. In both of these assays, blank values were determined using diol-bonded silica.

The activities of immobilized acetylcholinesterase and pepsin were determined by enzyme assay[11,12]. Sodium arsenate was used as the buffer rather than triethanolamine for the assay of α-chymotrypsin[13]. The activities of immobilized trypsin inhibitor, protein A, and anti-HSA were determined from chromatographic breakthrough curve[14] analyses using trypsin, IgG, and HSA, respectively.

RESULTS AND DISCUSSION

Selection of carbodiimide

It was reported in the case of a succinylated agarose matrix that some of the carbodiimide reagents were more effective than others in the formation of NHS esters[15]. Nucleosil 300-5 (carboxyl group content: 310 μmol/g) was similarly examined using the carbodiimides DCC, CMC, and EDC at concentrations of 0.2 *M* and NHS at a concentration of 0.12 *M*. Glucosamine (0.1 *M*) was immobilized at pH 7.4 and assayed by the ferricyanide method[11] as a measure of the effectiveness of each carbodiimide in forming the activated ester. The quantities of glucosamine immobilized with each carbodiimide were: DCC, 124 ± 10 μmol/g; CMC, 61 ± 5 μmol/g; EDC, 54 ± 5 μmol/g. Therefore, DCC was chosen for use in this study. The overall immobilization yield for glucosamine using DCC was 40%.

Comparison of the activated ester method with other immobilization methods

The immobilization of anti-HSA by three different immobilization methods was compared using 4000 Å (LiChrospher) and 300 Å (Nucleosil) pore size silicas. As shown in Table I, the amount of protein immobilized was approximately the same regardless of the coupling method. Since both the CDI and Schiff base methods result in excellent coupling efficiency of immunoglobulins[7–9], it is clear that the N-hydroxysuccinimide method is similar in coupling efficiency. In the case of the carboxylate-silica, it should be noted that high ligand density matrices were used (Table I). These tend to favor high coverage of immobilized protein.

Effect of pore size

The two silicas described above plus silica of 1000 Å pore size (LiChrospher) were used to immobilize trypsin inhibitor and anti-HSA (Table I). The protein content decreased for the largest pore size matrix. However, when corrected for the surface area of the silica (Table I), the amount immobilized per unit of surface area

TABLE I

EFFECT OF PORE SIZE AND COUPLING METHOD ON PROTEIN IMMOBILIZATION

Protein	*Silica*	*Pore size (Å)*	*Carboxyl content* (μmol/g)*	*Protein (mg/g silica)*			*Protein* (mg/m²)*
				NHS	*CDI*	*Schiff base*	
STI	LiChrospher	4000	40	15			1.5
		1000	90	39			1.3
	Nucleosil	300	310	38			0.4
Anti-HSA	LiChrospher	4000	40	30	32		3.0
		1000	90	80			2.7
	Nucleosil	300	310	81	71	66	0.8

* NHS coupling method only.

was actually higher for the larger pore diameter. This is explained by the distribution of pore diameters in each silica[16]. Especially for the 300-Å pore size silica, a fraction of the pores were too small or were blocked by protein molecules, thereby diminishing the available surface area of the matrix. In this case, either the 300-Å or 1000-Å pore size materials were optimal in terms of total protein immobilized.

Effect of pH on the immobilization of proteins

The amount of each protein immobilized was found to increase as the pH of the reaction mixture decreased (Table II). It is well-known that hydrolysis of the ester bond of the activated silica is substantially faster at higher pH so that coupling of proteins should decrease with an increase in pH. On the other hand, higher pH values promote the deprotonation of amino groups of proteins, which would favor the nucleophilic attacking reaction and thus produce a higher coupling yield[15]. The data in Table II indicate that hydrolysis was the more important factor in determining the amount of protein immobilized.

TABLE II

EFFECT OF pH ON THE IMMOBILIZATION OF SEVERAL PROTEINS
Nucleosil 300-5 with a carboxyl content of 310 μmol/g as used.

Protein	*Protein immobilized (mg/g silica)*					
	pH					
	3	*4*	*5*	*6*	*7*	*8*
Trypsin inhibitor	24	–	11	5	4	2
Acetylcholinesterase	32	–	27	–	14	–
Trypsin	9	8	9	8	–	6
Anti-HSA immunoglobulin	90	–	89	–	26	–

Activities of immobilized proteins

Several proteins were immobilized on matrices of spacer arm densities covering a range of up to 100-fold (Table III). At the lowest ligand densities, the average distance between spacer arms was approximately the same as the diameters of the proteins, thus multipoint attachment should have been minimized. Examining first the specific activities of the proteins, it is apparent that the specific activities of several of the proteins (acetylcholinesterase, trypsin inhibitor, protein A, and anti-HSA) were independent of ligand density. However, α-chymotrypsin and pepsin increased in specific activity as the ligand density decreased. A previous fluorescence study of α-chymotrypsin immobilized on Sephadex G-200 has shown that this can be attributed to multiple covalent attachment of the enzyme molecule to the matrix, which disturbs the tertiary structure of the protein[17]. Also, the enzyme molecule may be oriented on the surface in a way that renders it less accessible to the substrate. This may explain the partial loss of enzyme activity even at the lowest ligand density.

TABLE III

SPECIFIC ACTIVITY AND COUPLING YIELD AS A FUNCTION OF LIGAND DENSITY

Protein	*Pore size (Å)*	*Ligand density (μmol/g)*	*Average distance* (Å)*	*Protein coupled (mg/g)*	*Specific activity***
α-Chymotrypsin	1000	3	40	1.5	0.39
		10	19	14.7	0.25
		50	9	31.8	0.19
Pepsin	1000	3	40	0.8	0.59
		10	19	1.5	0.51
		50	9	6.1	0.42
Acetylcholinesterase	300	3	75	1.1	0.97
		50	18	8.7	1.01
		310	7	14.5	1.04
Ribonuclease A	1000	3	40	22.1	–
		20	15	10.2	–
		50	9	0.7	–
Trypsin inhibitor	300	3	75	2.9	0.92
		310	7	38.4	0.92
Protein A	1000	3	40	1.0	3.4
		10	19	3.6	3.5
		50	9	22.1	3.6
Anti-HSA immunoglobulin	300	3	75	13.1	0.044
		50	18	20.8	0.041
		310	7	20.9	0.041

* Average distance between spacer arms based on surface area and ligand density.

** Listed as the ratio of immobilized/native as determined by enzyme assay for the enzymes, or as mg test solute adsorbed/mg immobilized protein as determined by breakthrough curve analysis for the other proteins.

Alternatively, since the carboxylate groups on the silica may not be evenly distributed on the surface, it may be that there is still significant multipoint bonding even at the lowest ligand density.

Although the specific activity increased as much as two-fold at low ligand density, the amount of protein coupled decreased two- to twenty-fold (Table III). Therefore, the total immobilized activity was in all cases lower at low ligand densities. Although the spacer arm density is high enough at the low densities to potentially immobilize a monolayer of protein, hydrolysis of the active ester groups prevents the coupling yield from being high. Therefore, from a practical point of view, it appears that the conventional method of using a high density of activated groups will usually yield a material of the highest total activity. This is usually a more important concern than the specific activity.

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