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TWO-STEP COVALENT IMMOBILIZATION OF ENZYMES AS A WAY FOR STUDY OF EFFECTS INFLUENCING CATALYTIC ACTIVITY[1]

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Isothiocyanatopropyl derivatives of trypsin and pepsin obtained by treatment with 3-isothiocyanato-1-propyl isocyanate (in the first step) can be covalently bound to carriers having amino groups. Besides all the general principles valid for reactions of isothiocyanates with amines, prerequisite for the satisfactory coupling properties of isothiocyanatopropyl derivatives are (a) a sufficiently high modification degree with isothiocyanate, and (b) a concentration excess of NH_2-carrier groups. With respect to the coupling properties, nonspecific sorptions and the catalytic properties of the immobilized trypsin isothiocyanatopropyl derivatives, the better carrier appears to be the cross-linked polyethyleneimine. Isothiocyanatopropyl, 3-isothiocyanato-bromopropyl, and isothiocyanatopentyl derivatives of leucine, insulin, and albumin also have good coupling properties. The mode in which the amidolytic and caseinolytic activity of isothiocyanatopropyl derivatives of trypsin was influenced after its immobilization on polyethyleneimine clearly indicated that the functional groups of the enzyme did not participate in the coupling to the carrier. Moreover, no intermolecular reactions were observed during the immobilization process. On the other hand, the conformational changes of the protein molecule are important, since they probably influence the changes in the catalytic properties of modified enzymes after their immobilization.

INTRODUCTION

Studies of physicochemical properties of covalently immobilized enzymes face serious shortcomings in their characterization by both physical and chemical methods. Immobilized enzymes, in contrast to the native water-soluble enzymes, show problems regarding the precise determination of such basic parameters as the mode and site of attachment of enzyme to a carrier, the number of these attachments, etc. Since the effects of chemical modification through the immobilization of enzymes are nevertheless some of the most significant factors (1–3), this might be considered as a serious

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disadvantage. This problem has been also confirmed in works where the number of bonds between an enzyme and matrix was an important criterion in the characterization of physicochemical properties of the immobilized enzymes (4,5).

The investigation of a suitable model system has shown several analogies that might help solve this problem (6,7). The results of these studies have clearly shown the need of elaborating a method of covalent immobilization, which will enable (a) the direct characterization of the immobilized enzyme by the methods of chemical modification of proteins, and (b) exclude the effect of chemical modification of enzyme during its immobilization. In this respect, the two-step covalent immobilization using heterobifunctional reagents appears to be the most suitable method for this purpose (8–10). Though its principles have been known for a long time (11), the real contribution to physicochemical characterization could be only obtained after elaboration of the procedures that could guarantee its controlled course.

The existing methods of covalent immobilization led to the simultaneous chemical modification and immobilization of enzyme. Therefore, it seemed likely that the procedure carried out in two steps should enable us to separate the modification of enzyme (first step) from its immobilization (second step). Procedures used for two-step covalent enzyme immobilization till now have not, however, ensured such a controlled course. The present paper reports more thoroughly on the two-step controlled covalent immobilization of enzymes using heterobifunctional reagents.

MATERIALS AND METHODS

Chemicals

O-(aminoethyl)-cellulose AE-50 (1.0 meq/g) was obtained from Whatman Ltd, Maidstone, *Nα*-benzoyl-DL-arginine-4-nitroanilide hydrochloride (DL-BAPA) from Fluka, Buchs. Cross-linked polyethyleneimine was prepared from ethyleneimine-polymer (Fluka, Buchs) using 20% w/w 1-chloro-2,3-epoxypropan (12).

3-Isothiocyanato-1-propyl isocyanate, 5-isothiocyanato-1-pentyl isocyanate, 3-isothiocyanatopropionyl chloride, and 5-isothiocyanâtovaleryl chloride were prepared according to Kricheldorf (13,14), and 2,3-dibromopropyl isothiocyanate was prepared by bromination of allyl isothiocyanate (Koch–Light, Colnbrook), by a slightly modified procedure (15).

DL-leucine and casein (Cas) according to Hammarsten were obtained from Lachema Brno, and ovalbumin (Ova) flakes from BDH, Poole. Fatty

acids were removed (16) from bovine serum albumin (freeze-dried, Sevac, Prague). Insulin-Spofa (Léčiva Prague) (23 U/mg) was purified by gel chromatography on Sephadex G-50 (17) (Pharmacia Fine Chemicals, Uppsala), freeze-dried, and along with DL-leucine, was used for blending the corresponding radioactive materials. Crystalline bovine pancreas trypsin (E.C. 3.4.21.4), 1.0 TU Cas/mg, and pepsin (E.C. 3.4.23.1) 0.75 PU Ova/mg were preparations of Léčiva Prague.

L-[1-^{14}C] leucine (12.9 μCi/mmol) was obtained from the Institute for Research, Production and Application of Radioisotopes, Prague, and [^{125}I]-labeled human serum albumin (0.7 mCi/ml) from INR Swierk TN/Otwock. [^{125}I]-labeled insulin (7.5 μCi/ml) and [^{125}I]-labeled rat prolactin (72.1 μCi/ml) were the kind gifts of Dr. O. Földeš. 2,3-Dibromopropyl [^{35}S] isothiocyanate (15.5 μCi/mmol) was prepared in a similar manner as unlabeled 2,3-dibromopropyl isothiocyanate. Allyl [^{35}S] isothiocyanate (60 μCi/mmol) was a gift of Dr. J. Augustín.

Coupling Properties of Bifunctional Isothiocyanates

Coupling properties of bifunctional isothiocyanates, using leucine, insulin, trypsin, and albumin were examined. Leucine was coupled to the AE-cellulose in the following way: In the first step, a 0.5 to 1.5 M dioxane solution of bifunctional isothiocyanate was adjusted to 1% (v/v) concentration in the reaction mixture containing 0.5 M borate buffer (pH 8–9) and 1 mM [^{14}C] leucine (0.12 μCi/ml). The control had no isothiocyanate. The mixture was stirred continuously at room temperature. At appropriate time intervals, aliquots (1.5 ml) were taken and extracted with benzene (1.5 ml). In the second step, the aqueous layer (1 ml) was added to AE-cellulose (25 mg) at an appropriate pH value. The suspension was shaken for 24 h at room temperature. The conjugates were washed with distilled water (50 ml), borate buffer-saline containing 1 M NaCl and 0.1% Tween 20 (100 ml), distilled water (50 ml), followed by water-ethanol mixtures of increasing ethanol concentration, and finally with acetone (50 ml). The amount of leucine in the product was determined radiometrically.

The experiments with insulin and trypsin were carried out as follows. To the reaction mixture containing 0.2 M borate buffer and [^{125}I] insulin (1.5 mg/ml, 0.04 μCi/ml), a 1 M dioxane solution of bifunctional isothiocyanate was added. In the case of trypsin, the reaction mixture was prepared in the following way: bifunctional isothiocyanate (2.1 μmol/ml) was added to the solution containing 0.2 M borate buffer, trypsin (5.0 mg/ml), and [^{125}I] prolactin (0.15 μCi/ml) as a radioactive marker. In both experiments, the coupling was completed as previously described in the case of leucine; however, ether (1.5 ml) was used for the extractions.

The experiments with albumin were carried out as follows. To the reaction mixture containing 0.2 M borate buffer (50%), dimethyl sulfoxide (DMSO) (50%), bovine serum albumin (5.0 mg/ml), and [^{125}I] human serum albumin (0.1 μCi/ml) as a radioactive marker, isothiocyanate (45.6 μmol/ml) was added. The aliquots (1 ml) were added to AE-cellulose (25 mg).

Preparation and Immobilization of Isothiocyanate Derivatives of Trypsin and Pepsin

In the case of trypsin, the reaction conditions were the same as in the preceding experiment. For pepsin, the following conditions were used: borate buffer (0.2 M) with pepsin (5.0 mg/ml) and bifunctional isothiocyanate (1.25 μmol/ml). The reactions were stopped by adjusting the pH to 5.7, under continuous stirring, at room temperature. The samples were dialyzed and then freeze-dried.

The conjugates of bifunctional isothiocyanates with trypsin and pepsin were dissolved (5.0 mg/ml) in 0.2 M borate buffer (pH 8). Aliquots (1 ml) were added to the carrier (25 mg), and the suspensions were shaken at room temperature for 24 h. After two washings with distilled water (50 ml), the resuspended product in aliquots corresponding to 40–50 mg of the dry weight were sampled for enzyme assay. The solutions of trypsin containing [^{125}I] prolaction or pepsin (5.0 mg/ml) in 0.2 M borate buffer (pH 8) for the determination of nonspecific sorptions were used. For the procedures used to determine the amount of covalently coupled enzymes, see above under "Coupling properties of bifunctional isothiocyanates." In samples containing pepsin, the protein content was determined by amino acid analysis.

Introduction of [^{35}S] Isothiocyanatopropyl Structures into Proteins by 2,3-Dibromopropyl [^{35}S] Isothiocyanate

Albumin. To 0.2 M borate buffer, pH 9 (0.32 ml), or to a mixture of the same buffer (50%) and DMSO (50%), pH 9 (0.32 ml), containing 1.6 mg of albumin, 2,3-dibromopropyl [^{35}S] isothiocyanate (2 μl, 15.2 μmol, 0.025 μCi) was added. The reaction was carried out with stirring at room temperature. At appropriate time intervals, the reactions were terminated by adding ether (1 ml), and after extraction (twofold) and removing of the organic layer, the aqueous layer was used for radiometric assay.

Trypsin. 2,3-dibromopropyl [^{35}S] isothiocyanate (1 μl, 7.5 μmol, 0.012 μCi) was added to a solution of 0.2 M borate buffer, pH 9 (0.5 ml), containing trypsin (1.825 mg). Further procedure was as described above under albumin.

Other Analytical Methods

The proteolytic activity of trypsin and pepsin was determined according to Bergmeyer (18) with casein and ovalbumin as substrates. The amidolytic activity of trypsin toward DL-BAPA was carried out according to Erlanger et al. (19). In a similar manner, the activity of the immobilized enzymes was determined by analysis of the supernatants after centrifugation (Specord UV VIS and Spekol, VEB Carl Zeiss, Jena, GDR).

The samples containing [^{14}C] radionuclids (ca. 5 mg) were dissolved in concentrated $HClO_4$ (0.2 ml). The solutions were decolorized by addition of 30% hydrogen peroxide (0.1 ml). Ethylene glycol monoethyl ether (5 ml) was then added, together with 0.5% w/v of PPO in toluene (5 ml). After being stirred thoroughly, the samples were measured on a Packard Tri-Carb, Model 3330 scintillator.

Where necessary, the samples containing [^{35}S] (0.3–0.5 ml) were clarified by the addition of concentrated sulphuric acid (up to 0.5 ml). After ethylcellosolve (10 ml) and scintillation liquid (5 ml) were added, the measurements were performed as described previously. The dry samples labeled with [^{125}I] were measured directly using a scintillation probe Well-type NaI/Tl, Well (England) and a scintilation counter Tesla NZQ 717-T, Tesla (Czechoslovakia).

Polyacrylamide slab gel electrophoresis was performed on an SDS polyacrylamide gel (10%, w/v) (20) without addition of thiol. The samples were applied from the reaction mixture, simulating two-step covalent immobilization. In the first step, the protein was modified by bifunctional isothiocyanate. The reaction was then stopped through extraction with ether and ethylamine was added in the second step. Aliquots of the reaction mixture from both steps were diluted: 50 with 25 mM tris and 192 mM glycine buffer (pH 8.3) containing 1% SDS and 10% saccharose, and applied (20 μl) on a gel.

Determination of the amount of pepsin and trypsin fixed was carried out by amino acid analysis (Amino Acid Analyzer AAA Hd 1200 E, Czechoslovakia) after acid hydrolysis (2). The protein content was calculated from the recovery of aspartic and glutamic acid. The circular dichroism (CD) spectra were obtained with a Roussel-Jouan Dichrograph, Model 185, at room temperature in 0.1 cm cells.

RESULTS AND DISCUSSION

The second step of the controlled two-step covalent immobilization should not cause any chemical modification of the enzyme. A modified enzyme used in this process is thus well characterized and contains new

reactive groups enabling the chemical coupling of the enzyme to a carrier. New reactive groups are introduced by a reaction of the enzyme with a heterobifunctional reagent. Reactive groups of a carrier must then react with the new reactive groups of the enzyme and must not react with the polypeptide chain.

Coupling Properties of Bifunctional Isothiocyanates

The bifunctional isothiocyanates used in this work are known to have the second functional group more reactive (21–24).

In order to evaluate the coupling properties of bifunctional isothiocyanates, all isothiocyanate derivatives of leucine were coupled to the AE-cellulose (Table 1), which is a carrier with a high content of NH_2-groups, and which has good coupling properties toward isothiocyanates (24). The reaction conditions given in Table 1 are optimal for both steps, corresponding to our knowledge of reaction kinetics of isocyanates and isothiocyanates with NH_2-groups of proteins and/or the water-insoluble carriers (21,24,25). The rates and ranges of the conversion are controlled by pH and by the concentration of the resulting leucine isothiocyanates. The reaction courses strongly indicate that the reaction takes place at the NH_2-group of leucine.

In both steps, an excess of one of the two reagents was used. While in the first step, a 10–15-fold excess of bifunctional isothiocyanates toward leucine was used, in the second step, a 12–40-fold excess of NH_2-groups of a carrier was present in the reaction with the isothiocyanate derivative of leucine. Excess of the carrier in the second step should exclude any competitive intra- and intermolecular side reactions. At these concentration ratios, 85% of leucine was coupled to the AE-cellulose, but only 4% of

TABLE 1. **Coupling of Leucine to AE-Cellulose Using Bifunctional Isothiocyanates**

	Coupling conditions					
	First step		Second step		Amount of coupled leucine (μmol/g)	Yield of coupled leucine[a] (%)
Bifunctional isothiocyanates	pH	Time (h)	pH	Time (h)		
$OCN(CH_2)_3NCS$	9	8	8	24	32.8	82
$OCN(CH_2)_5NCS$	9	8	9	24	30.7	77
$ClCO(CH_2)_3NCS$	8	24	9	24	18.8	47
$ClCO(CH_2)_5NCS$	8	24	9	24	6.5	16
$BrCH_2(Br)CHCH_2NCS$	9	24	9	24	1.7	4

[a] Based on amount of added leucine.

NH_2-groups were occupied (Table 1). Under the same reaction conditions (in the control experiments), the underived leucine was bound to AE-cellulose in amounts less than 1%.

Though coupling of the leucine 3-isothiocyanato-bromopropyl derivative (1.7 μmol/g) does not reach the extent of isothiocyanatopropyl and those of the isothiocyanatopentyl derivatives of leucine (Table 1), application of their *N*-alkyl derivative has certain advantages. These are evident by forming and coupling the 3-isothiocyanato-bromopropyl derivatives of proteins (Table 2, insulin and trypsin). The *N*-alkyl derivatives preserve the charge of the parent NH_2-groups. Moreover, full preservation of the conformation and of the catalytic activity of *N*-alkylated proteins after reductive alkylation is known (26). On the other hand, the reaction products of proteins with bifunctional isothiocyanates, e.g., the substituted ureas, lose their parent charge on the nitrogen of the NH_2-group.

TABLE 2. **Coupling of Proteins to AE-Cellulose Using 3-Isothiocyanato-1-Propyl Isocyanate and 2,3-Dibromopropyl Isothiocyanate**

Protein[a]	Isothiocyanate[b]	Coupling conditions[c]; First step		Amount of bound protein (mg/g)	Yield of bound protein[d] (%)
		pH	Time (h)		
Insulin	2,3-Dibromopropyl isothiocyanate	9	2	26.1	44
	3-Isothiocyanato-1-propyl isocyanate	9	2	26.2	44
	None (control)	9	2	13.1	22
Trypsin	2,3-Dibromopropyl isothiocyanate	9	8	90.0	45
	3-Isothiocyanato-1-propyl isocyanate	9	1	119.5	60
	None (control)	9	1	7.5	4
Pepsin	3-Isothiocyanato-1-propyl isocyanate	9	0.5	108.6	54
	None (control)	9	0.5	15.1	8
Albumin[e]	2,3-Dibromopropyl isothiocyanate	9	8	28.3	14
	None (control)	9	8	2.2	1

[a] Except for insulin (1.5 mg/ml), the protein concentration was 5 mg/ml; pepsin was used as a ready isothiocyanatopropyl derivative.
[b] Control samples were without isothiocyanate.
[c] In the second step in all cases, pH 9 and time 24 h were used.
[d] Based on amount of added protein.
[e] Reaction mixture for both steps contained DMSO (50%).

The coupling properties of isothiocyanatopropyl- and 3-isothiocyanato-bromopropyl derivatives of proteins (insulin, trypsin, pepsin, and albumin) toward AE-cellulose are described in Table 2. Reaction conditions were selected to be optimal for both steps. In contrast to leucine, the participation of nonspecific sorptions in this case is very important. An amount of an underived protein determined on the AE-cellulose was assigned to nonspecific sorptions.

In the case of albumin, the addition of 50% DMSO was necessary to achieve satisfactory coupling in both steps. Under these reaction conditions, 2,3-dibromopropyl isothiocyanate appears as the most suitable bifunctional isothiocyanate. The use of DMSO considerably decreases the nonspecific sorptions of albumin to the AE-cellulose.

The influence of DMSO is already remarkable in the first step, since an increase in the degree of albumin modification can be observed. For instance, 53.9 mol of 2.3-dibromopropyl [^{35}S] isothiocyanate was introduced into albumin when the reaction was carried out in 50% DMSO at pH 9. In the absence of DMSO, only 24.2 mol was introduced. This "catalytic" effect of DMSO has been described in the reactions of other alkylating reagents with the NH_2-group of proteins (27).

Trypsin

For the characterization of catalytic properties of the two-stepwise immobilized trypsin, its isothiocyanatopropyl derivatives were prepared. Conditions of preparation and the catalytic properties are given in Table 3.

TABLE 3. **Conditions for the Preparation and the Catalytic Properties of the Isothiocyanatopropyl Derivatives of Trypsin**

Sample no.	Reaction conditions[a]		Catalytic activity		Residual[b] (%)
	pH	Reaction time (min)	Caseinolytic (ΔA_{280}/min/mg)	Amidolytic (ΔA_{405}/min/mg)	
I	7	5		2.94	46.2
II	8	30		0.90	14.2
III	9	5		3.78	59.4
IV	9	15	0.28	0.38	6.0
V	9	30		0.22	3.4
Trypsin			1.04	6.36	100.0

[a] Isothiocyanatopropyl groups were introduced into trypsin, using 3-isothiocyanato-1-propyl isocyanate.
[b] Residual activity was calculated after comparing with amidolytic activity of trypsin.

Though the coupling properties of the AE-cellulose used till now toward isothiocyanate derivatives of proteins were satisfactory, lower nonspecific sorptions and better catalytic properties were shown after immobilization on cross-linked polyethyleneimine. Isothiocyanatopropyl derivatives of trypsin thus prepared were then immobilized on cross-linked polyethyleneimine. Immobilized trypsin was characterized by its coupling properties and catalytic activities (Table 4).

With increasing degree of trypsin modification, an increase of coupling efficiency (%) with its isothiocyanatopropyl derivatives toward cross-linked polyethyleneimine was achieved. In this case, an increase of the modification degree was obtained by raising the pH value (7–9) or through extension of reaction time (5–30 min). Reaction carried out in both ways resulted in extensive modifications of trypsin. Reaction with 2,3-dibromopropyl [^{35}S] isothiocyanate gave way to introduction of 5.9 mol isothiocyanate per mole of trypsin (pH 9) after 5 min and another 3.2 mol after 10 min. Higher coupling efficiency (%) was achieved for isothiocyanatopropyl derivatives of trypsin than for the 3-isothiocyanato-bromopropyl derivatives, so a higher degree of modification is assumed for the former than for the latter.

Influence of the increased pH value on the coupling properties of trypsin isothiocyanatopropyl derivatives is remarkable between the preparations I (pH 7; 5 min) and III (pH 9; 5 min), considerably differing in their coupling properties (Table 4). Samples III, IV, and V, prepared at pH 9

TABLE 4. **Characterization of Trypsin Isothiocyanatopropyl Derivatives Immobilized on Cross-Linked Polyethyleneimine**

	Coupling properties			Catalytic properties	
Immobilized preparation[a] no.	Amount of bound trypsin (mg/g)	Coupling efficiency (%)	Specific activity (ΔA_{405}/min/mg)	Residual activity[b] (%)	Overall activity yield[c] (%)
I	23.6	11.8	1.32	44.9	20.8
II	47.2	23.6	0.45	50.0	7.1
III	46.6	25.3	1.56	41.3	24.5
IV	99.0	51.4	0.18	46.9	2.8
V	132.2	61.6	0.075	34.7	1.2
Trypsin[d]	9.6	4.7	3.84	60.4	60.4

[a] Samples from Table 3 after immobilization.
[b] Residual activity was calculated after comparing the amidolytic activity of the corresponding isothiocyanatopropyl derivative of trypsin (see Table 3).
[c] Calculated after comparing the amidolytic activity of the underived trypsin (see Table 3).
[d] Trypsin nonspecifically sorbed on cross-linked polyethyleneimine.

for 5, 15, and 30 min, differed substantially in their coupling properties (Table 4). Preparations with the best coupling properties (IV, V) showed that the nonspecific sorptions corresponded to less than 1/10 of chemically bound protein.

Amidolytic and caseinolytic activity of trypsin decreases during the immobilization. In accordance with our results (28), decrease in the activity was more evident in the proteolytic than amidolytic activity. Thus for preparation V, the proteolytic activity decreased to 33.6%, compared with 23.1% of the proteolytic activity of the nonspecifically adsorbed trypsin. In no case, however, was this decrease higher than with trypsin immobilized using one-step standard immobilization (29).

The average residual amidolytic activity of all immobilized preparations is about 45% (Table 4), though various trypsin isothiocyanatopropyl derivatives (Table 3) were immobilized. Thus, for example, the residual amidolytic activities of immobilized preparations III and IV comprise 41.3 and 46.9% (Table 4), while the residual amidolytic activities of parent trypsin isothiocyanatopropyl derivatives were 59.4 and 6.0% (Table 4).

The residual amidolytic activities of immobilized isothiocyanatopropyl derivatives of trypsin approach the residual amidolytic activity of nonspecifically adsorbed trypsin, which is about 60% (Table 4). At the nonspecific sorption of trypsin, there is no chemical modification of trypsin, and therefore, this is not involved in the changes of catalytic properties of an immobilized preparation. For these reasons, it is also possible to exclude any chemical modification of trypsin isothiocyanatopropyl derivatives in the course of their covalent immobilization to a carrier. The observed decrease of catalytic activity by the immobilized preparations should be assigned to effects other than a chemical modification during an immobilization.

TABLE 5. **Molar Ellipticities of Native and First and Second Step Modified Albumin at 220 nm**

Albumin sample[a]	$10^{-3}\,\Theta$ at 220 nm[b]
Native	−10.50
After first step (2,3-dibromopropyl isothiocyanate coupled)	−9.73
After second step (ethylamine mixture)	−9.51

[a] Conditions for preparation as in Fig. 1, diluted 250 times.
[b] Units: deg cm^2 $dmol^{-1}$.

Gel Electrophoresis and CD Spectra

The high degree of modification of the protein or enzyme isothiocyanate derivatives exclude any intra- or intermolecular competitive reactions. This was confirmed when analyzing the reaction mixtures, simulating two-step covalent immobilization, in polyacrylamide gel electrophoresis (Fig. 1). In these reaction mixtures, the presence of water-insoluble carrier having NH_2-groups was simulated by ethylamine in the second step. On the other hand, modification of protein by bifunctional isothiocyanates and the proper conformational changes of native protein have already been observed in the first step. This reflects the decrease of intensity of the dichroic bands having a negative sign in the region of 200–22˜ nm of the CD spectra (Table 5). These changes continue in the second step, too (see the conditions in Fig. 1). In both cases, no changes of CD spectra could be

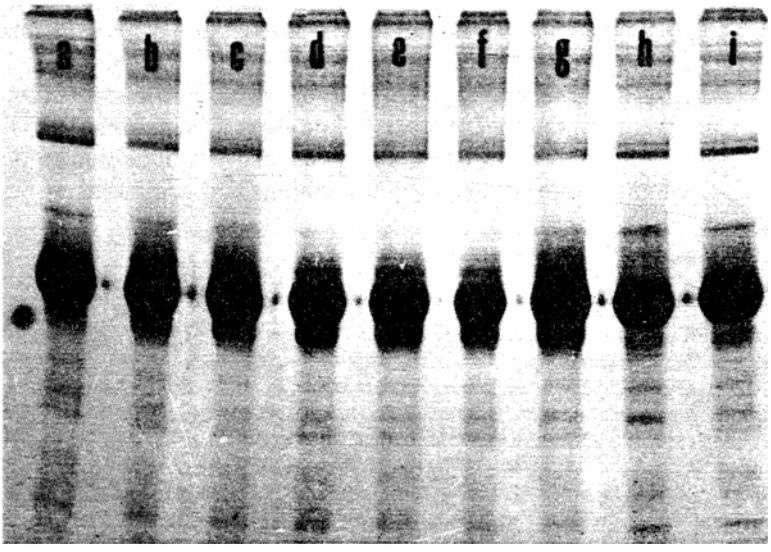

FIG. 1. Polyacrylamide gel electrophoresis of bovine serum albumin modified with 2,3-dibromopropyl isothiocyanate and further "bound" to ethylamine. First step: reaction mixture containing 74.6 μM of albumin, 6.13 mM of 2,3-dibromopropyl isothiocyanate and 1% v/v of dioxane in 0.5 M borate buffer, pH 9, was maintained with stirring at room temperature. At the defined time intervals (1–24 h, a–e; h) and after extraction with ether, the aliquots (5 μl) were taken for further workup (see the text under Materials and Methods). Second step: To the samples *e* (8 h), *f* (24 h), and to control *h* (24 h), ethylamine (37.5 mM) was added, and the mixture was stirred for 24 h at room temperature. The aliquots (5 μl) were withdrawn again for further workup (*f, g, i*). *a*, 1 h; *b*, 2 h; *c*, 4 h; *d*, 8 h; *e*, 24 h; 1 h, without 2,3-dibromopropyl isothiocyanate, 24 h; all first step. *f*, *g*, 24 h; *i*, without 2,3-dibromopropyl isothiocyanate, 24 h; all second step.

observed in this region. Therefore, the observed decrease of catalytic activity of the immobilized trypsin could be to some extent assigned to the conformational changes introduced during the enzyme immobilization.

The course of the two-step covalent immobilization of enzymes presented in this paper does not allow one to predict, regarding the relatively high degrees of modification, which parts of the enzyme molecule are oriented toward the matrix. Minimalization of the number of points of attachment between the enzyme and the carrier could permit one to define the region of the enzyme oriented toward the matrix, and consequently to decide which of the above-mentioned effects is decisive. Our investigation on this matter is in progress.

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