

ON SOME REACTIVE POLYMERS AND IMMOBILIZED ENZYMES

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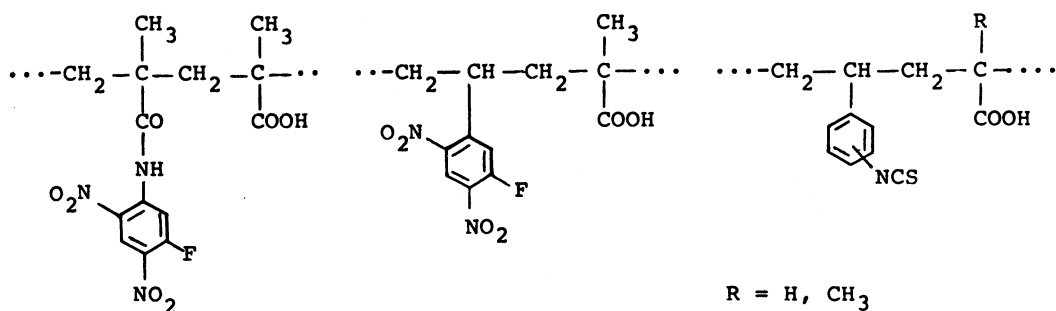
Abstract - New reactive polymer carriers with ionic or neutral hydrophilic matrices have been synthesized. Effective were the copolymers of 3- or 4-isothiocyanato styrene and acrylic acid, copolymers of 1-vinyl-2-pyrrolidone and maleic anhydride or acrylic acid but also with acrylic acid/styrene modified with maleimide groups. As effective reactive carriers were also used polyethyleneimine, polyethyleneimine carbonic acids, poly(allyl alcohol), polyvinylethers and various carriers of different polymer combinations containing poly(vinyl alcohol) in form of crosslinked gels, coated tubes and synthetic pulp. The ability of these individual carriers to bind enzymes was studied. Various parameters which affect the immobilization reaction and the properties of the immobilized enzymes were investigated.

Enzymes can be immobilized by different methods, one of the most important is the covalent binding of the enzymes to polymer carriers (Ref. 1). In this paper results from the author's laboratory concerning this immobilization technique will be given.

For the immobilization of enzymes on synthetic polymer carriers by covalent binding we prepared a large number of reactive carriers (Ref. 2 & 3). The reactive groups were formed either by polymer analogous reactions or by copolymerization of reactive monomers. Depending on the composition of the polymer matrix good hydrophilic properties were obtained from ionic or neutral structures.

We have investigated several polymer carriers with acrylic acid or methacrylic acid as the comonomer. Good results were achieved with copolymers of methacrylic acid and 3-fluoro-methacryl-anilide, which were crosslinked with 1% divinylbenzene (DVB). These products contained the 3-fluoro-4,6-dinitro-anilido group as the reactive group (Ref. 4).

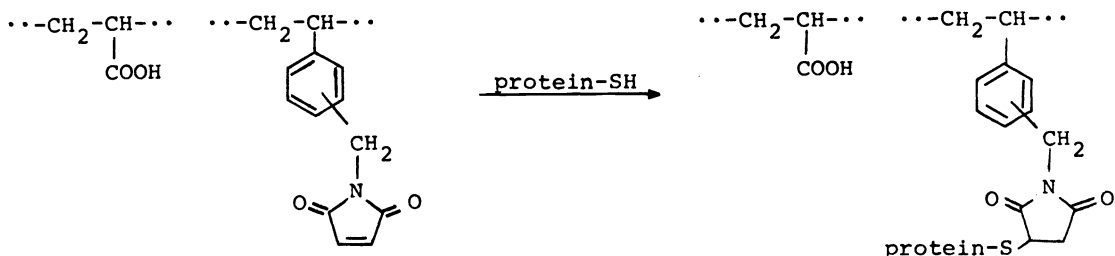
The comparable high binding ability of enzymes to these carriers could also be achieved by nitrated copolymers of methacrylic acid with the three fluorostyrene isomers (Ref. 5). In all cases copolymers with a macroreticular structure were especially effective.



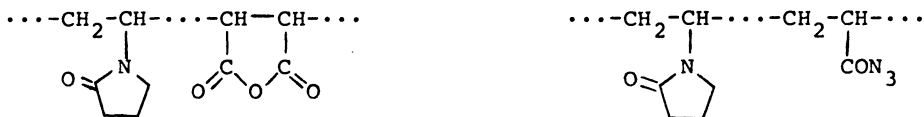
Other efficient polymer carriers were synthesized by copolymerization of acrylic acid with 3- or 4-isocyanato styrene and DVB as crosslinking agent (Ref. 6). Variations of the component ratio gave carriers with different reactivity and hydrophilicity. Also here the properties of the reactive carriers were improved by the macroreticular structure. The immobilization of enzymes gave active products with good mechanical and chemical stabilities. In the case of papain up to about 1000 mg papain per g carrier could be bound. In some cases immobilized papain showed a retained activity of more than 80% towards N^{ϵ} -benzoyl-L-arginine ethyl ester (BAEE) as substrate at the pH optimum (Ref. 3, 6c).

Other new polyanionic carriers were investigated starting from a copolymer of acrylic acid and styrene which was crosslinked with DVB. Under Friedel-Crafts conditions this polymer was

allowed to react with N-chloromethylmaleimide (Ref. 7). The maleimide groups showed a specific reactivity towards mercapto compounds and enzymes with free SH-groups could be immobilized, e.g. up to 90 mg catalase/g carrier which showed a retained activity of 0.5 %. Enzymes without SH-groups, such as trypsin, could be bound only after the thiolation of the enzyme by reacting it with N-acetylmethionine lactone. In this way up to 80 mg trypsin could be immobilized which showed retained activities up to 7.5 %.

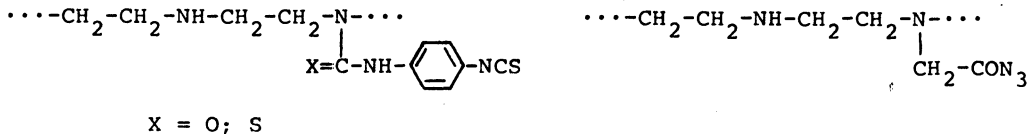


Other types of reactive carriers were obtained by copolymerization of 1-vinyl-2-pyrrolidone with maleic anhydride or acrylic acid respectively in the presence of DVB (Ref. 8). The latter copolymer was activated by polymer analogous reaction and azide groups were introduced.



The maleic anhydride copolymer bound up to 450 mg papain/g carrier (retained activity up to 7 %), the carrier containing azide groups bound up to 260mg papain/g carrier (retained activity up to 5%).

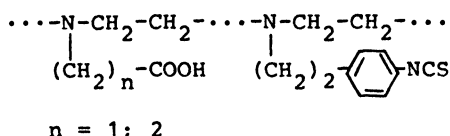
Reactive carriers with a basic matrix were prepared from polyethyleneimine (Ref. 9). This polymer could be crosslinked by reacting it with diisothiocyanates resp. diisocyanates. Nitro groups could be introduced by reaction with 4-nitrophenyl isothiocyanate or isocyanate. The nitro groups were reduced and allowed to react with thiophosgen and gave reactive carriers containing isothiocyanato. With 4 mole % of a crosslinking agent carriers with different contents of reactive groups were prepared which bound 30 - 140 mg papain/g carrier. The retained activities determined by BAEE at pH 6 were in the range of 2 - 20 %, at the pH optimum of the immobilized papain derivatives they were about twice as high (Ref. 9).



Furthermore ethyl chloroacetate was allowed to react with crosslinked polyethyleneimine, and azide groups were formed via the hydrazide. Compared with the polyethyleneimine carriers containing isothiocyanato groups the carriers with azide groups were more hydrophilic which led to a better binding ability for enzymes; up to 250mg papain could be immobilized per g of carrier

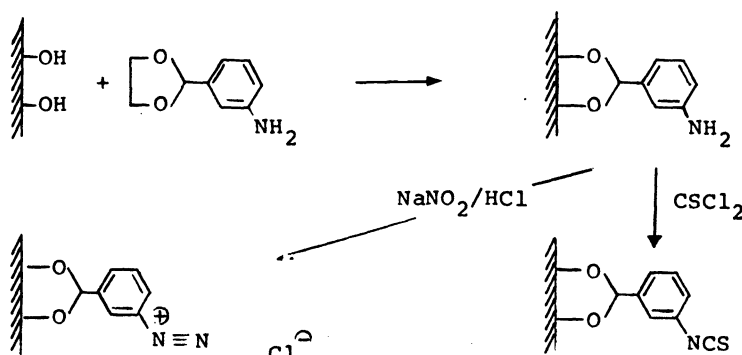
Amphoteric carriers with good properties were synthesized by copolymerization of β -(1-aziridinyl)-ethyl-4-nitrobenzene with ethyl(1-aziridinyl)-acetate or ethyl (1-aziridinyl)-propionate in the presence of bis-aziridino compounds as crosslinking agent (Ref. 10). After the hydrolysis of the ester groups an amphoteric structure of the polymer was obtained. The

nitro groups were reduced to amino groups and transformed to isothiocyanato groups.



These carriers were able to immobilize up to 1000 mg papain/g carrier and showed retained activities towards BAEE at pH 6 in the range of 12 - 17 % and at pH 8 up to about 35 %.

Beside these carriers we were also interested in investigating the immobilization of enzymes on neutral hydrophilic polymers such as poly(vinyl alcohol) (PVA), poly(allyl alcohol) and polyvinylethers. The reaction of PVA with 2-(*m*-aminophenyl)-1,3-dioxolane gave a polymer containing amino groups (Ref. 11). The amino groups could be transformed into reactive groups either by diazotization or by reaction with thiophosgen.



These activation reactions could be applied to the following PVA derivatives (Ref. 12):

- gels of PVA crosslinked with terephthalaldehyde
- PVA containing fiber material (SWP synthetic pulp, Mitsui Zellerbach K.K. Japan)
- tubes consisting of vinylacetate-ethylene copolymers, coated with PVA.

Immobilization reactions have been carried out with papain, trypsin, and glucose oxidase. Diazotized PVA gels were able to bind up to 685 mg papain and 400 mg trypsin, respectively, per g carrier (Ref. 12a). In comparison with these results the carrier containing isothiocyanato groups showed lower binding abilities (up to 170 mg papain per g carrier but in some cases they had a better retained activity (up to 40% at pH6) (Ref. 12b)

Synthetic fiber material which contained small amounts of PVA could be activated with different amounts of reactive groups. The content of reactive groups was in the range of 5 - 20 μmole per g pulp (determined by coupling of tyrosine). In Table 1 some of the enzymes which are immobilized on pulp are described (Ref. 12b).

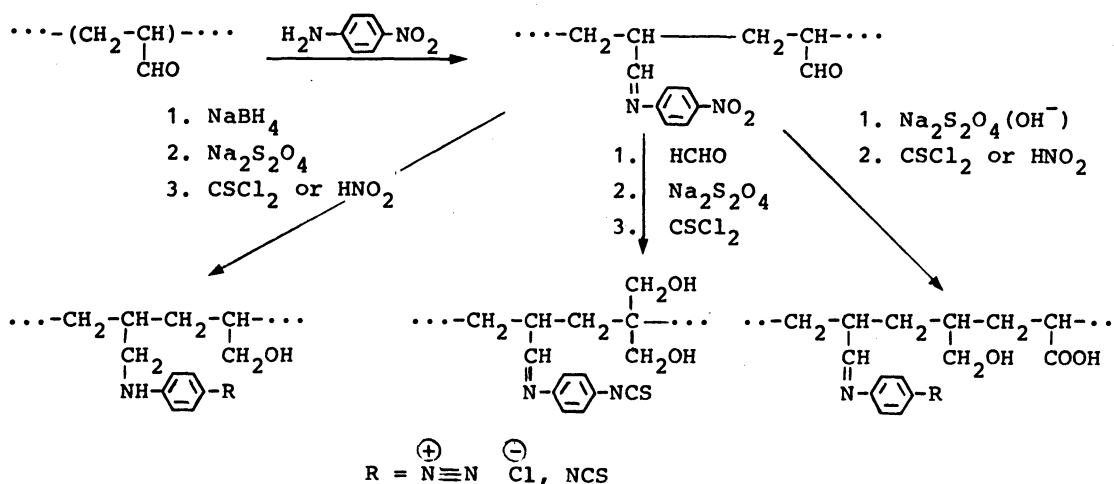
TABLE 1. Immobilization of enzymes on synthetic pulp (SWP R830)

Reactive groups	Immobilized enzyme type	amount mg/g pulp	Activity U ⁺ /g	Ret. activity %	Substrate
NCS	trypsin	12	10-15	3-4	BAEE, pH 8
N ₂ ⁺	trypsin	46	90-100	7	BAEE, pH 8
N ₂ ⁺	papain	147	60	16	BAEE, pH 6
N ₂ ⁺	glucose oxidase	11	10-20	0.4-0.8	glucose, pH 7

⁺) 1 U = 1 $\mu\text{mole min}^{-1}$

The inner surface of tubes consisting of vinylacetate-ethylene copolymers could be hydrolyzed to vinylalcohol units and be coated with partially crosslinking PVA by covalent binding. The coating procedure affected an enlarged and hydrophilized tube surface. Diazonium groups could be introduced by this method. Trypsin and glucose oxidase were successfully immobilized at the inner surface (Ref. 12b). Trypsin, immobilized on a tube with an inner diameter of 1.5 mm showed an activity of 0.15 U per 1 m tube material using N^{α} -benzoyl-DL-arginine-4-nitroanilide (BAPA) ($2.5 \cdot 10^{-3}$ M, pH 7,8, 25°C) as substrate. The stability of the trypsin tubes with regard to repeated applications was very good. After allowing to react the tube 15 times with the substrate there was no detectable loss in tryptic activity. Glucose oxidase was immobilized on tube surfaces in the same way and gave products with an activity of 0.2 U per 1 m tube material. Better results could be achieved by coupling glucose oxidase via a Schiff's base reaction. In this case activities of about 1 U per 1 m tube material could be reached.

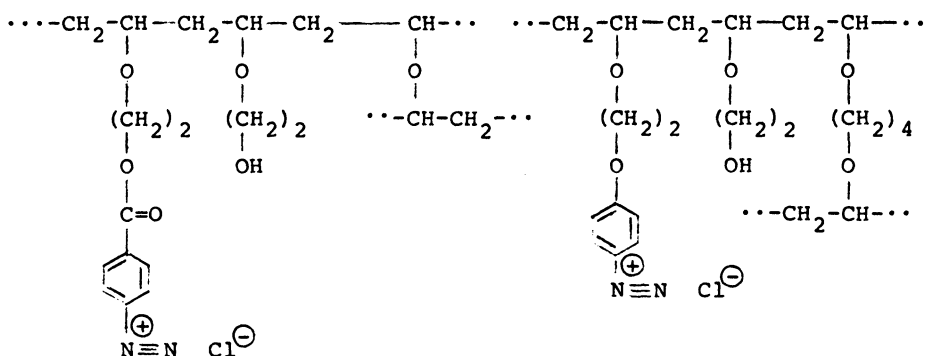
Reactive poly(allyl alcohol) carriers were synthesized starting from polyacrolein (Ref. 13). The reaction of polyacrolein with amines, e.g. 1-amino-4-nitrobenzene or 2-amino-5-nitro-pyridine gave Schiff's bases which could be transformed by different routes to reactive carriers (Ref. 13b).



In one case the azomethine and also the residual aldehyde groups were hydrogenated with NaBH_4 . Subsequent reduction of the nitro groups with sodium dithionite gave amino groups, which could be activated by conversion to diazonium or isothiocyanato groups. Alternatively in the case of the azomethines with 1-amino-4-nitrobenzene substitution the residual aldehydic functions were allowed to react with formaldehyde to give alcoholic groups, and then the reduction of the nitro groups to the amino groups was carried out. Beside the reduction of the nitro groups the direct reaction of the polymeric Schiff's bases with dithionite in alkaline media led to the formation of carboxylic groups by intramolecular Cannizzaro reactions.

Furthermore polyacrolein was allowed to react with glycine ethyl ester (Ref. 13b). After the hydrogenation of unreacted aldehyde groups with NaBH_4 , azide groups could be introduced by reaction with hydrazine and nitrous acid. The binding ability for papain was generally in the range of 100-200mg per g carrier. But in the case of the carriers containing carboxylic groups up to about 1200mg papain per g carrier could be immobilized.

The novel monomers vinyloxyethyl-4-nitrobenzoate and vinyloxyethyl-4-nitrophenyl ether were cationically copolymerized with 2-hydroxyethyl vinyl ether using divinyl ether or 1,4-di-(vinyl)oxy butane as crosslinking agent (Ref. 14). By reduction of the nitro groups with phenylhydrazine and subsequent diazotization carriers were produced which bound up to 200 mg papain per g carrier and showed a retained activity of up to 3.5%. Better results could be obtained with $\text{Na}_2\text{S}_2\text{O}_4$ instead of phenylhydrazine as reducing agent. In this case up to 450mg papain could be immobilized per g carrier, which showed a retained activity of 10% at pH 6 (Ref. 14).



Although up to now a large number of reactive polymers has been investigated for the immobilization of enzymes, the compositional and structural diversity of proteins makes it impossible to get an ideal support of universal applicability. Because of the numerous factors which influence the immobilization, each enzyme requires an individual solution. In the following some of the factors which affect the immobilization as well as the properties of the immobilized enzymes will be discussed.

As heterogeneous reaction the immobilization of enzymes depends on the extent and the constitution of the surface of the polymeric support. Generally carriers with an enlarged surface bind larger amounts of enzyme. An enlarged surface can be achieved by carriers with a macroreticular structure which can be obtained by copolymerization in the presence of inert solvents. As it has been reported already in previous papers (Ref. 6b) carriers with a macroreticular structure could immobilize about twice the amount of enzyme as the comparable carriers with gel-like structure.

The porosity could be widely modified by variation of the ratio of the comonomers (including the crosslinking agent) and by variation of the amount of the inert solvent. Information about the structure of the surface and the pore size could be obtained by scanning electron microscopy.

On the other hand enlarged surfaces could easily be prepared by decreasing the grain size of the polymeric carrier (Ref. 2, 4c, 6c). Besides a better binding ability the advantage of smaller particles was a higher retained activity of the immobilized enzymes due to an enhanced accessibility for the substrate. The advantage of bigger particles was a better separation behaviour from solutions and the possibility of applications in columns. Most of our investigations were performed with grain sizes in the range from 0.1 - 0.2 mm diameter. An enhanced swellability of the carriers also enlarged the surface and led to higher amounts of bound protein.

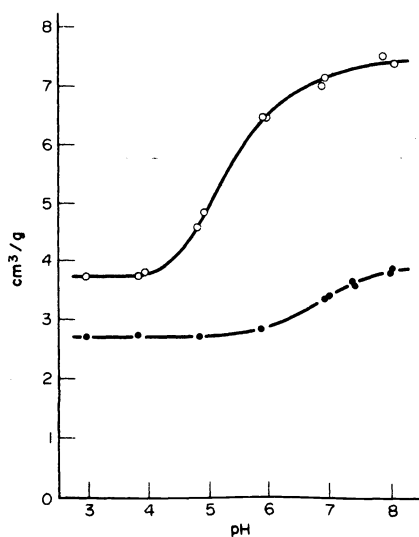


Fig. 1. pH dependence of the swelling of the carrier 4-isothiocyanato styrene/acrylic acid (molar ratio 1 : 3, crosslinked with 1 mole% (○) or 10 mole% (●) DVB) (Ref. 6c)

As it is shown in Fig. 1 the swelling was obviously affected by the degree of crosslinking and, in the case of the given example of a polyanionic carrier, was strongly pH-dependent. The reactive carrier with higher crosslink density showed a lower binding ability but an improved mechanical stability.

Furthermore, an increase of the hydrophilic component (higher swellability) of the carrier gave a distinct improvement of the bound amount of enzyme though the number of reactive groups was decreased.

TABLE 2. Influence of the composition of the carrier on the amount of bound papain and its esterase activity. (4-isothiocyanato styrene/acrylic acid copolymers, cross-linked with 10 mole-% DVB) (Ref. 6c).

Molar ratio NCS : COOH	Reactive groups mmole/g carrier	Bound papain ^{a)} mg/g carrier	Retained activity ^{b)} %
1 : 2	2.9	45	36
1 : 3	2.3	139	36
1 : 6	1.5	347	23
1 : 9	1.1	294	44

a) immobilized at pH 5

b) N^{α} -benzoylarginine ethyl ester (BAEE) as substrate; pH 6, 30° C

As it is shown in Table 2 the improvement of the binding ability by dilution of the reactive groups reached a maximum which is accompanied by a minimum of the retained activity. The dependence between the content of the reactive groups of the carrier and the binding ability has been investigated for many carriers. Figure 2 shows this effect for a poly(vinyl alcohol) carrier with diazonium groups (Ref. 12a).

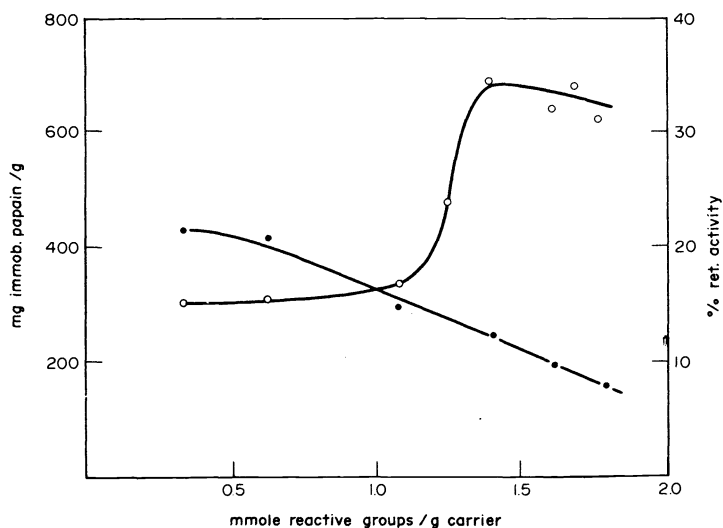


Fig. 2. The dependence of the amount of bound papain (o) and the retained activities of the immobilized papain (●) on the content of reactive groups (carrier: diazotized PVA-3-aminobenzaldehyde acetal, 5% crosslinked; substrate N^{α} -benzoyl-L-arginine ethyl ester (BAEE), pH 6, 30° C)

A maximum of binding corresponding to a maximum of coating of the carrier with enzyme was achieved when the carrier contained 1.4 mmole reactive groups per g. When the concentration of reactive groups was too high the amount of bound papain decreased. This may be caused by

hydrophobization of the carrier. The retained activities decreased with increasing contents of reactive groups. If the amino groups of the carrier were activated to isothiocyanato groups instead of the more hydrophilic diazonium groups the binding ability drastically decreased. The maximum binding was shifted to lower contents of reactive groups (≤ 0.3 mmole/g carrier) (Ref. 12b).

TABLE 3. The influence of the content of isothiocyanato groups of the carrier on the bound amount and the activity of papain
(carrier: PVA-3-isothiocyanato benzaldehyde acetal, 5% crosslinked)

mmole NCS- groups/g carrier	mg bound papain/ g carrier	activity ^{+) in U/g carrier (retained activity in %)}		
		1. assay	2. assay	3. assay
1.3	110	11.6 (5.0)	13.6 (5.9)	13.5 (5.9)
0.9	130	22.5 (8.4)	36.7 (13.7)	40.2 (15.0)
0.3	170	79.9 (25.8)	89.2 (25.8)	90.9 (26.3)

<sup>+) substrate: BAEE (0.05 M), pH 6.0, 30° C
coupling conditions: carrier (50 mg), papain (50 mg in 10 cm³
phosphate buffer pH 8, I = 0.15), 25° C, 16 h</sup>

As it is also seen in Table 3, at high contents of reactive groups there is a drastical decrease of activity of the immobilized enzyme which may be caused by multiple fixation of the same enzyme molecule to the matrix.

The binding ability is strongly affected by the pH of the coupling reaction. Neutral carriers do not show great differences in the volume in the swollen state within a wide pH range. Therefore, reactive poly(allyl alcohol) carriers are useful to study the dependence of the reactivity of isothiocyanato and diazonium groups over a wide pH range.

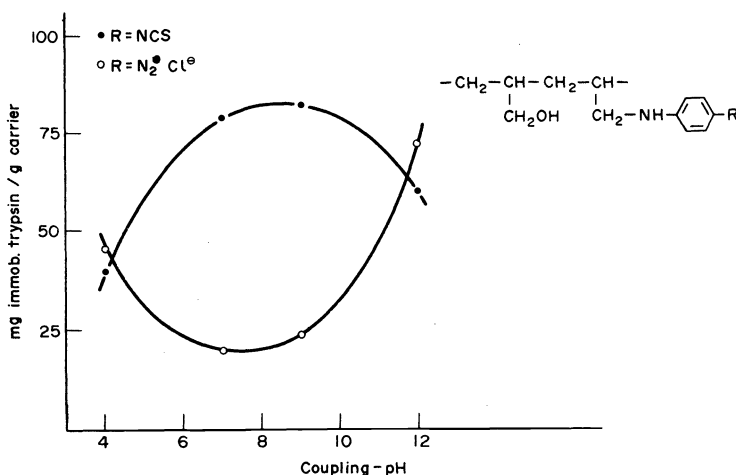


Fig. 3. The dependence of the coupling pH on the bound amount of trypsin (Ref. 13b)

Figure 3 shows this dependence for the example of the immobilization of trypsin. In the range of pH 7-9 isothiocyanato carriers showed maximum, whereas diazonium carriers showed minimum of reactivity (Ref. 13b). The latter effect is comparable to the reactivity of monomeric diazonium compounds. In the case of the NCS-coupling the maximum can be explained by the increased reaction rate of the hydrolysis of the NCS-groups at higher pH values which is in concurrence with the immobilization reaction.

In the case of polyanionic carriers, e.g. 3-isothiocyanato styrene/acrylic acid copolymers, at higher pH-values the swellability as well as the reactivity of the NCS groups were enhanced. As a result distinctly more papain is bound at higher pH values.

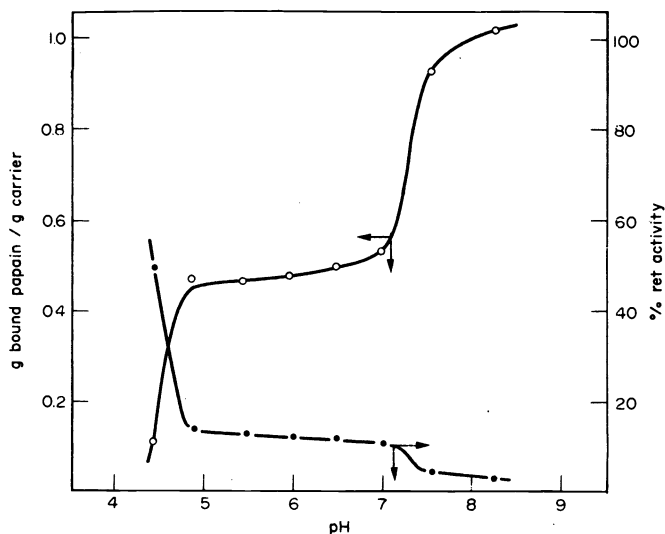


Fig. 4. Binding ability for papain at different pH-values (o) and retained activities of the immobilized papain (●) (carrier: 3-isothiocyanato styrene/acrylic acid (1:10), 4 mole% DVB; substrate: BAEE, pH 6, 30°C) (Ref. 6c).

Immobilization reactions at lower pH values had the advantage of a better retained activity of the immobilized enzyme. In the case of the hydrolases papain and trypsin this was not only caused by the smaller amount of bound enzyme but also by the better stability of these enzymes in buffer solutions of lower pH values during the coupling reaction. At higher pH values the enzymes lose most of their activity within a few hours. Therefore shorter immobilization periods were often more effective.

Furthermore, the amount of immobilized enzymes depends on the amount and the concentration of the offered enzyme during the immobilization reaction. This effect has been studied in several cases (Ref. 6c, 12a, 13b) and is shown in Fig. 5.

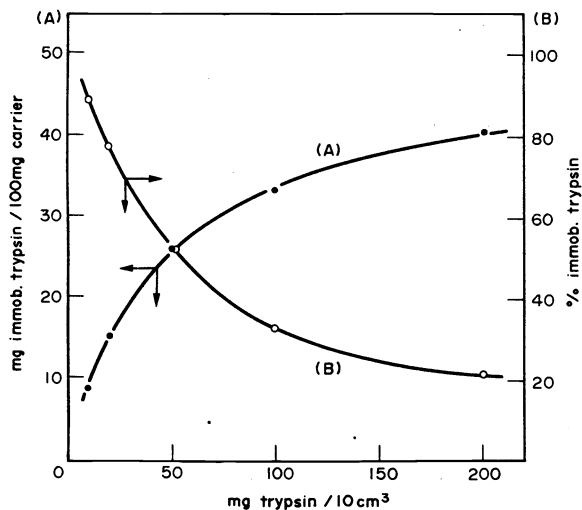


Fig. 5. Amount and yield of bound trypsin in dependence on the concentration of the enzyme in solution (carrier: diazotized PVA-3-aminobenzaldehyde acetal, 1.4 mmole reactive groups/g carrier, 5 % crosslinked) (Ref. 12a)

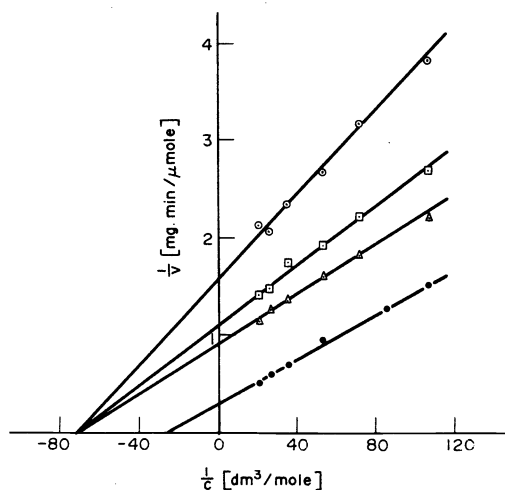


Fig. 7. Lineweaver-Burk plot of immobilized and native papain (carrier: 4-isothiocyanato styrene/acrylic acid, 10 mole% DVB; substrate: BAEZ, pH 6, 30°C) (Ref. 6c)

	NCS : COOH	bound papain [mg/g]	V_{\max} [U/mg]	K_M [mole/dm ³]
□	1 : 3	139	0.92	0.014
○	1 : 6	347	0.65	
△	1 : 9	294	1.11	
●	native papain		3.45	0.040

TABLE 4. Michaelis constants of native and immobilized trypsin (carrier: diazotized PVA-3-aminobenzaldehyde acetal, 1.4 mmole reactive groups/g, 5% crosslinked; substrate: N^ε-benzoylarginine-4-nitroanilide (BAPA), pH 7.8, 25°C) (Ref. 12a)

mg bound trypsin per g carrier	$K_{M(\text{app})}$ [mmole·dm ⁻³]
86.5	0.3
273	1.4
321	1.7
400	3.1
native trypsin	2.7

In other cases diffusion restriction may play a more important role. As it can be seen in Tab. 4 the $K_{M(\text{app})}$ values of trypsin immobilized on diazotized PVA-3-aminobenzaldehyde acetal depended on the amount of bound enzyme; At small amounts of immobilized trypsin electrostatical attraction to the substrate may have a certain importance (Ref. 12a). But if more enzyme was immobilized the supply with substrate becomes more and more diffusion controlled leading to higher $K_{M(\text{app})}$ values. This is also indicated by deviations of the Michaelis-Menten kinetics in the case of the sample with the highest amount of immobilized trypsin.

At lower temperatures the dependence of the enzymatic activity can be described by the Arrhenius law. At higher temperatures there are deviations caused by heat denaturation. In the case of trypsin or papain which were immobilized on carriers derived from polyacrolein maximum reaction rates were reached at temperatures of 60°C or 70°C. (Fig. 9, Ref. 13b). Repeated determinations of the activity at these temperatures showed hardly any loss of activity.

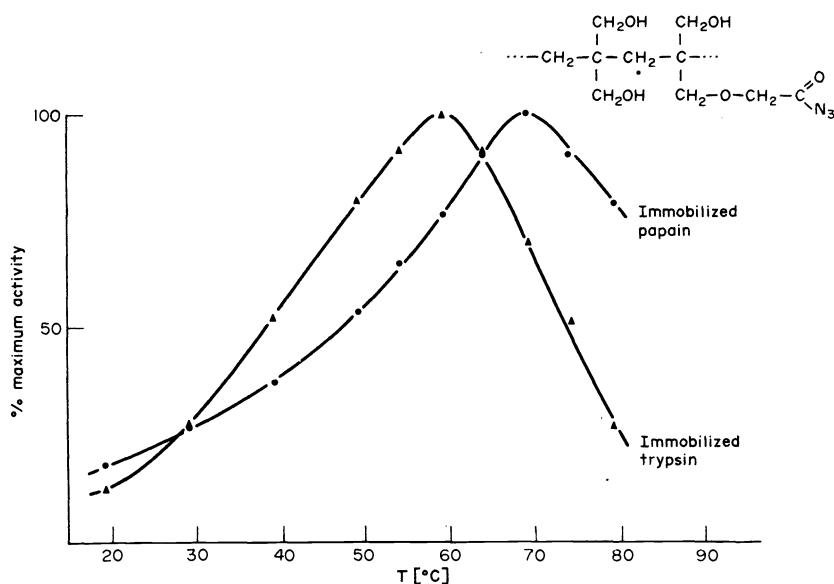


Fig. 8. Influence of the temperature on the activity of immobilized papain and immobilized trypsin (substrate: BAEE)

Without the stabilizing effect of the substrate, immobilized enzymes as well as the native enzymes are more temperature sensitive if they are incubated in solution at higher temperatures.

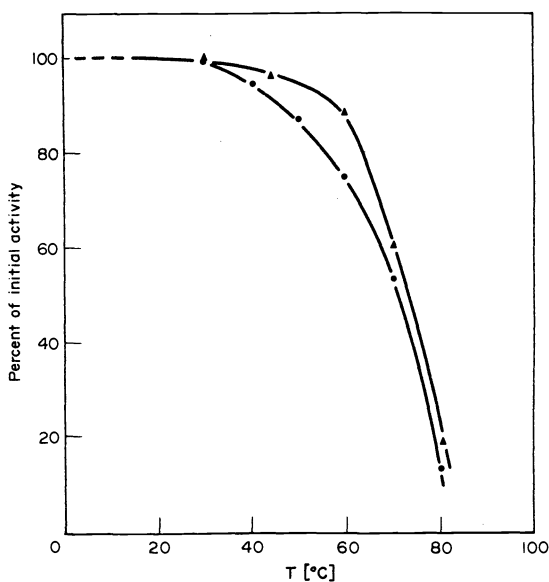


Fig. 9. Thermostability of native papain (●) and immobilized papain (▲) in aqueous media; incubation time 30 min. (carrier: 3-isothiocyanato styrene/acrylic acid 1:4, 2 % crosslinked, macroreticular structure; substrate: BAEE) (Ref. 6c)

The activity of papain samples which was determined at a standard temperature (30° C) showed already a slight decrease in activity at an incubation temperature of 40° C. The thermostability of the immobilized enzyme in some cases was higher as compared to the native enzyme (Fig. 10).

The stability with regard to repeated applications and also the storage stability at 4° C over a prolonged period in many cases was possible without any or without significant loss of activity.

In summary the application of immobilized enzymes instead of native enzymes in solution introduced a new dimension in the use of these materials. Various reactive carriers for the covalent immobilization of enzymes have been in the meantime synthesized and investigated. These carriers can be selected and specially tailored on the basis of the specific need and can therefore have a great deal of selectivity under a variety of conditions.

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