# IMMOBILIZATION OF SOME HYDROLASES ON POLYMETHACRYLATE BY THE METHOD OF REACTIVE ESTERS

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The immobilization of chymotrypsin (EC 3.4.4.5), papain (EC 3.4.4.10), α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3), and of a technical pectolytic enzyme on various reactive polymethacrylate esters (Ostion KM) was examined and compared with the immobilization of the same enzymes on carboxymethyl-cellulose. There is no difference in the individual reactive esters (2,4-dinitrophenyl, N-hydroxysuccinimidyl, ethyl-1-hydroxyacetyl) when enzymes reacting fast (papain, chymotrypsin) are immobilized. The ethyl-1-hydroxyacetyl ester of the support is most suitable for slowly reacting enzymes. The reaction does not proceed at pH-values lower than 7·5. The specific activity of immobilized amylolytic and pectolytic enzymes decreases with the increasing pH because of inactivation of the enzyme.

Derivatives of polysaccharides, of cellulose or agarose, or custom-made synthetic polymers are the carboxylic supports widely used for the immobilization of enzymes. The reactive esters of carboxylic polysaccharide derivatives are prepared by the action of dicyclohexylcarbodiimide in organic solvents[1] or are produced commercially[2]. The reactive esters of synthetic carboxylic polymers are mostly prepared by polymerization of esterified monomers; this makes the hydrophobic ester groups oriented inside the polymers and thus less accessible to the reaction with proteins[3]. Polymers of carboxylic acids, such as polyacrylic acid, polymethacrylic acid and others are produced on a large scale as synthetic, weakly acidic anion exchangers whose prices are convenient even for industrial use of immobilized enzymes.

In this study we investigated the use of the commercial polymethacrylate cation exchanger, Ostion KM, for the immobilization of certain hydrolases by the method of reactive esters. The course of the immobilization was compared with the immobilization of the same enzymes by the identical method on Whatman CM-70 carboxymethyl-cellulose.

## EXPERIMENTAL

Carboxymethyl-cellulose CM-70 was a product of Whatman. The polymethacrylate used in this study was a commercial weakly acidic cation exchanger, Ostion KM (Spolek pro chemickou a hutní výrobu, Ústí n. L.), porosity 62% and particle size 0·6 to 1·2 mm

The following enzymes were immobilized: Crystalline chymotrypsin (Spofa, Praha), specific activity 33·5 c.u./mg, papain (Lachema, Brno), specific activity 3·87 c.u./mg; mold α-amylase, pure (Lachema, Brno), specific activity 225 EU/mg; amyloglucosidase AMG 150L (Novo Industri, Denmark), specific activity 1·87 E.U./mg, and a pectolytic preparation (Pektofoetidin G3X), specific activity 0·87 E.U./mg.

The following compounds were used for the preparation of esters: 2,4-Dinitrophenol, 2,4-dinitrochlorobenzene, N-hydroxysuccinimide, N-bromosuccinimide, and ethyl-1-chloroacetate, all products of Lachema, Brno.

*Preparation of carboxymethyl-cellulose esters*[1]. Carboxymethyl-cellulose (50 g, Whatman CM-70) was suspended in 250 ml of dioxane together with 0·1 mol of the hydroxy compound. After the latter had completely dissolved, 3 g of dicyclohexylcarbodiimide was added and the suspension was stirred 4 h at room temperature. The product was filtered off, washed with dioxane and water and dried *in vacuo* at 35°C. The final product thus prepared contained on the average 0·19 millimol of reactive ester groups per 1 g of dry weight.

*Preparation of polymethacrylate esters.* Polymethacrylate (10 g) was suspended in 10 ml of dioxane and treated (with stirring and cooling by ice) with 3·58 g of triethanolamine (24 millimol) in 5 ml of dioxane, subsequently with 24 millimol of the reactive halogenide in 10 ml of dioxane, and finally with 0·1 g of NaBr. The reaction mixture was stirred under a reflux 6 h. The polymethacrylate ester was filtered off, washed with dioxane and water, and dried *in vacuo* at 35°C. The product contained on the average 2·08 millimol of reactive ester groups per 1 g of dry weight.

*Determination of concentration of ester groups.* The activated support was equilibrated in 1M-NaCl to neutralize the unsubstituted carboxyls; subsequently 0·5 g of the activated support was suspended in 20 ml of 0·1M-NaOH and shaken overnight at room temperature. The unreacted sodium hydroxide was back-titrated with 0·1M-HCl.

*Protein determination.* The concentration of proteins was measured by the method of Hartree[4]. The method was modified for the determination of immobilized enzymes: a six-times larger quantity of all reagents was used and the quantity of the immobilized enzyme taken for the assay was 20 mg. All incubations were carried out with shaking.

*Determination of enzyme activity.* The amylolytic activity was determined by using a reagent kit "Biolatest" (Lachema, Brno) with a colored substrate. The proteolytic activity was measured by the method of Slavík and Smetana[5]. Amyloglucosidase activity was determined according to Ruttloff[6]. The enzyme activities were determined in a thermostated shaker. The activity of the proteases was expressed in casein units[5] (c.u.); one unit is defined as the quantity of enzyme which will digest 2% casein at 30°C and optimal pH to 1 mg of fragments soluble on 0·1M trichloroacetic acid. All the remaining enzyme activities were expressed in micromol of product per 1 min (EU).

The results were evaluated by methods of mathematical statistics[7].

*Coupling of enzymes to reactive esters of polycarboxylic supports.* The reactive ester (100 mg) was suspended in 5 ml of 0·2M buffer of the chosen pH, containing 20 to 40 mg of proteins in 1 ml (with pectolytic enzymes up to 50 mg of proteins in 1 ml) and the suspension was shaken at room temperature for the desired period. The immobilized enzyme was washed with 1M-NaCl, water, and finally with buffer optimal for the activity of the enzyme. The immobilized enzymes were stored in buffer at 4°C.

## RESULTS AND DISCUSSION

When intensive stirring, such as in this study is applied, the diffusion barrier of the boundary between the liquid and the solid phase is without effect on the activity of the enzyme. Any drop of the activity of the enzyme after immobilization is therefore caused, besides inactivation, exclusively by diffusion inside the support. The effect of the support was also weakened by a large excess of the support[8]. We measured the specific activity of the enzymes immobilized under these conditions and compared it with the specific activity of native soluble enzymes. The results are given in Table I. By using the Student t-test we found that with the exception of α-amylase there is no statistically significant difference at the 0·95 confidence level in the specific activity of the two types of enzymes. The specific activity of α-amylase was decreased by ca 90%; this value corresponds to the reaction of the immobilized enzyme with the insoluble substrate and is in agreement with the results of other authors[9–12]. The results given in Table I were obtained with supports activated by esterification with N-hydroxysuccinimide. We obtained the same results when the other esters were applied to α-amylase (Table II). It is obvious that the type of the ester is without effect on the specific activity of the immobilized enzyme; this was also confirmed by evaluation of the results by the t-test. This finding also shows that the byproducts formed during the immobilization (dinitrophenol *etc.*) do not inactivate enzymes. At the same time we were not able to show any difference in the specific activity of the enzyme attached to carboxymethyl-cellulose and to polymethacrylate.

TABLE I

Specific Activity of Enzymes Immobilized on Whatman CM-70 (CMC) Carboxymethyl-cellulose N-Hydroxysuccinimidyl Ester and on Technical Polymethacrylate Ostion KM (PMA)

The specific activities are given in EU (cat.u.) per 1 mg of immobilized protein; $n$ number of independent measurements, $v$ variation coefficient ($= s^2$), $s$ standard deviation.

| Enzyme | Spec. activity | $n$ | $v$ | $s$ |
|---|---|---|---|---|
| α-Amylase-CMC | 19·91 | 14 | 214·4 | 14·6 |
| α-Amylase-PMA | 24·23 | 14 | 180·2 | 13·4 |
| Pectolytic complex-CMC | 1·02 | 13 | 0·227 | 0·477 |
| Pectolytic complex-PMA | 0·89 | 14 | 0·142 | 0·202 |
| Amyloglucosidase-CMC | 1·56 | 14 | 1·72 | 1·31 |
| Amyloglucosidase-PMA | 1·71 | 13 | 2·09 | 1·44 |
| Papain-CMC | 3·53 | 14 | 6·25 | 2·50 |
| Papain-PMA | 3·82 | 14 | 3·33 | 1·82 |
| Chymotrypsin-CMC | 34·38 | 14 | 277·1 | 16·6 |
| Chymotrypsin-PMA | 32·14 | 14 | 312·2 | 17·7 |

The same specific activity of native and soluble enzymes can be explained either by a high porosity of the support, which is not the case here, or by supposing that the immobilization takes place on the surface of the support only. A simple indirect proof of the assumption that the immobilization takes place only on the surface of the polymethacrylate is the determination of the activity per surface unit of different particle size. The polymethacrylate particles used in this study can be to a good approximation regarded as spherical and thus their surface area can easily be calculated. Ostion KM was divided into four fractions: Larger than 1 mm (4·16% of the original material); 0·63 – 1·00 mm (79·68%); 0·40 – 0·63 mm (13·05%), and 0·18 to 0·40 mm (3·12%). The support does not contain particles of diameter below 0·18 mm. We immobilized the amyloglucosidase on the support sorted out as described and found that the quantity of the immobilized enzyme was in better agreement with the total surface of the support than with its weight. The correlation coefficient of the dependence of the quantity of immobilized enzyme on the surface area of the support was 0·89 whereas the corresponding coefficient for the weight dependence was 0·62. The coefficient of linear correlation of the weight dependence does not exclude completely the volume effect and neither does the coefficient of linear correlation of the surface area correspond to an exact dependence. Nevertheless, the results obtained permit us to conclude that the immobilization takes place predominantly, if not exlusively, on the surface of the support.

The total number of reactive (unprotonated) amino groups in the protein is determined by the pH of the medium. We examined therefore the effect of pH on the immobilization of the enzyme. Since a pH higher than 9·0 cannot be used with the amalyses and pectolytic enzymes, we did not study the pH-range exceeding this value. We examined the quantity of immobilized enzyme in the pH-range 5·0 to 9·0. The

TABLE II

Comparison of Specific Activity of α-Amylase Immobilized on Various Esters of Carboxymethyl--cellulose and Polymethacrylate

| Ester | Spec. activity | $v$ | $s$ |
|---|---|---|---|
| N-Hydroxysuccinimidyl-CMC | 19·91 | 214·4 | 14·6 |
| N-Hydroxysuccinimidyl-PMA | 24·23 | 180·2 | 13·4 |
| 2,4-Dinitrophenyl-CMC | 24·35 | 214·4 | 14·6 |
| 2,4-Dinitrophenyl-PMA | 19·40 | 70·7 | 8·4 |
| Ethyl-1-hydroxyacetyl-PMA | 21·18 | 172·4 | 13·1 |

results are shown in Fig. 1. We attached the enzyme to a support activated by esterification with N-hydroxysuccinimide at room temperature for 60 h. A considerable quantity of papain and chymotrypsin is immobilized at pH 5·0. The reactivity of the amino groups at this pH, however, is very low and immobilization proceeds *via* adsorption only; this is evidenced by the fact that we were able to elute the enzyme by the substrate and by denaturing agents. We observed the same effect to a lesser degree also with the pectolytic enzymes. In all cases the concentration of the enzymes on the support decreases with the increasing pH from 5·0 to 7·2–7·5 where it is minimal. Starting from this value the concentration of the immobilized enzymes again goes up abruptly. The results shown in Fig. 1 provide evidence that immobilization *via* covalent bonding cannot be effected at pH lower than 7·5; even at pH 8·0 the concentration of the protein on the support is relatively low.

Since the enzymes are also inactivated at higher pH-values we also examined the specific activity of the immobilized enzymes as a function of pH during the immobilization. We measured the concentration of the enzymes immobilized on the support as well as their activities after 4 h of the reaction in order to obtain measurable enzyme activities even under unfavorable conditions. The results are shown in Fig. 2. It is obvious that the amylases and the pectolytic enzymes undergo inactivation in the pH-range optimal for their immobilization.

The results given in Table I were obtained as limit values after a very long immobilization time, *i.e.* after the concentration of the protein had leveled off at a value which

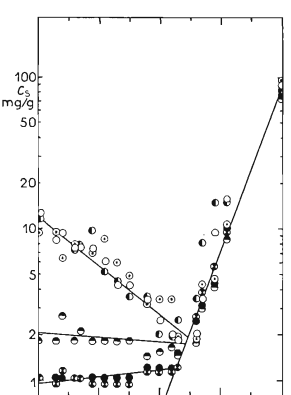


FIG. 1

Concentration of Proteins on Support ($C_s$) as Function of pH during Long-term Immobilization (60 h)

○ Chymotrypsin-PMA, ⊙ chymotrypsin-CMC, ◐ papain-PMA, ◓ pectolytic-complex-PMA, the point with full × vertical amyloglucosidase-PMA, ● α-amylase-PMA. The N-hydroxysuccinimidyl esters of the supports were used.

no longer practically changed. The immobilization rate, however, is not the same for all the enzymes during the initial stage of the reaction. This may be the effect of the actual concentration of free amino groups in the enzyme molecule, their accessibility, and other factors. The coupling of the enzyme to the support follows the $(S_{N2})_{O-Ac}$ mechanism, *i.e.* that of a bimolecular nucleophilic substitution with cleavage of the oxygen-acyl bond. If a large excess of one component is used the kinetics of the reaction can be reduced to first order, *i.e.* to transform the reaction to pseudomonomolecular. We used therefore in the subsequent experiments a ten-fold excess of the support and measured the apparent rate constant of the first order for the immobilization of various enzymes on supports esterified to different degrees. The results of these measurements are given in Table III. In order to completely eliminate the effect of the support it is necessary to carry out the experiments at the same concentration of reactive groups of all ester types. This condition could be fulfilled both with carboxymethyl-cellulose and polymethacrylate. The concentration of the reactive groups of carboxymethyl-cellulose was, however, by one order lower than the concentration of these groups in polymethacrylate and therefore the results obtained with both supports cannot be directly compared. However, the values of rate constants given in Table III permit us to conclude that polymethacrylate is at least as suitable for the immobilization of enzymes as carboxymethyl-cellulose. Our results show that there is no difference between the individual esters if fast reacting proteins such as chymotrypsin or papain are concerned. The best results

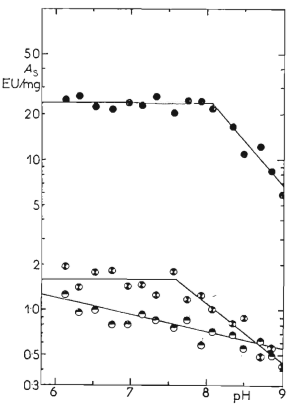


FIG. 2

Specific Activity of Immobilized Amylolytic and Pectolytic Enzymes as Function of pH of Coupling

$A_s$ Specific activity of immobilized enzyme (EU/mg); the remaining symbols are identical with those given in the legend to Fig. 1.

TABLE III

Kinetic ($k$) Constants of Immobilization of Enzymes on Various Carboxymethyl-cellulose and Polymethacrylate Esters

The measurement was carried out under the conditions of pseudomonomolecular reaction at a high excess of the support.

| Enzyme | $k$ | $n$ | $s$ |
|---|---|---|---|
| | N-Hydroxysuccinimidyl-CMC | | |
| α-Amylase | $1{\cdot}34 \cdot 10^{-5}$ | 8 | $6{\cdot}22 \cdot 10^{-6}$ |
| Amyloglucosidase | $8{\cdot}9 \cdot 10^{-6}$ | 8 | $4{\cdot}13 \cdot 10^{-6}$ |
| Pectolytic complex | $1{\cdot}09 \cdot 10^{-7}$ | 8 | $5{\cdot}02 \cdot 10^{-8}$ |
| Papain | $1{\cdot}73 \cdot 10^{-5}$ | 8 | $8{\cdot}03 \cdot 10^{-6}$ |
| Chymotrypsin | $2{\cdot}82 \cdot 10^{-5}$ | 4 | $1{\cdot}49 \cdot 10^{-5}$ |
| | N-Hydroxysuccinimidyl-PMA | | |
| α-Amylase | $3{\cdot}24 \cdot 10^{-3}$ | 4 | $1{\cdot}71 \cdot 10^{-3}$ |
| Amyloglucosidase | $2{\cdot}81 \cdot 10^{-3}$ | 4 | $1{\cdot}48 \cdot 10^{-3}$ |
| Pectolytic complex | $2{\cdot}23 \cdot 10^{-5}$ | 4 | $1{\cdot}18 \cdot 10^{-5}$ |
| Papain | $3{\cdot}31 \cdot 10^{-3}$ | 4 | $1{\cdot}75 \cdot 10^{-3}$ |
| Chymotrypsin | $2{\cdot}02 \cdot 10^{-3}$ | 4 | $1{\cdot}07 \cdot 10^{-3}$ |
| | 2,4-Dinitrophenyl-CMC | | |
| α-Amylase | $6{\cdot}10 \cdot 10^{-6}$ | 4 | $3{\cdot}22 \cdot 10^{-6}$ |
| Amyloglucosidase | $1{\cdot}05 \cdot 10^{-7}$ | 4 | $5{\cdot}44 \cdot 10^{-8}$ |
| Pectolytic complex | $1{\cdot}51 \cdot 10^{-7}$ | 4 | $7{\cdot}84 \cdot 10^{-8}$ |
| Papain | $9{\cdot}50 \cdot 10^{-6}$ | 4 | $5{\cdot}61 \cdot 10^{-6}$ |
| Chymotrypsin | $3{\cdot}64 \cdot 10^{-6}$ | 4 | $1{\cdot}29 \cdot 10^{-6}$ |
| | 2,4-Dinitrophenyl-PMA | | |
| α-Amylase | $1{\cdot}47 \cdot 10^{-3}$ | 4 | $7{\cdot}67 \cdot 10^{-4}$ |
| Amyloglucosidase | $3{\cdot}32 \cdot 10^{-4}$ | 4 | $1{\cdot}52 \cdot 10^{-4}$ |
| Pectolytic complex | $1{\cdot}61 \cdot 10^{-5}$ | 4 | $8{\cdot}08 \cdot 10^{-6}$ |
| Papain | $1{\cdot}82 \cdot 10^{-3}$ | 4 | $9{\cdot}69 \cdot 10^{-4}$ |
| Chymotrypsin | $2{\cdot}98 \cdot 10^{-3}$ | 4 | $1{\cdot}34 \cdot 10^{-3}$ |
| | Ethyl-1-hydroxyacetyl-CMC | | |
| α-Amylase | $3{\cdot}48 \cdot 10^{-5}$ | 4 | $1{\cdot}73 \cdot 10^{-5}$ |
| Amyloglucosidase | $1{\cdot}22 \cdot 10^{-6}$ | 4 | $6{\cdot}16 \cdot 10^{-7}$ |
| Papain | $1{\cdot}02 \cdot 10^{-6}$ | 4 | $5{\cdot}56 \cdot 10^{-7}$ |
| Chymotrypsin | $1{\cdot}86 \cdot 10^{-6}$ | 4 | $9{\cdot}28 \cdot 10^{-7}$ |

TABLE III
(*Continued*)

| Enzyme | $k$ | $n$ | $s$ |
|---|---|---|---|
| | Ethyl-1-hydroxyacetyl-PMA | | |
| α-Amylase | $6{\cdot}24 \cdot 10^{-3}$ | 4 | $3{\cdot}47 \cdot 10^{-3}$ |
| Amyloglucosidase | $5{\cdot}84 \cdot 10^{-3}$ | 4 | $3{\cdot}35 \cdot 10^{-3}$ |
| Papain | $2{\cdot}54 \cdot 10^{-3}$ | 4 | $1{\cdot}41 \cdot 10^{-3}$ |
| Chymotrypsin | $2{\cdot}22 \cdot 10^{-3}$ | 4 | $1{\cdot}27 \cdot 10^{-3}$ |

with slowly reacting proteins were obtained with supports esterified with ethyl--1-hydroxy acetate whereas the binding was slowest when supports esterified with 2,4-dinitrophenol were used. This finding was given supporting evidence by the Student t-test which showed that the measured differences in binding rates are statistically significant at the attained dispersion values.

The kinetic constants given permit us to calculate, again at an excess of the support, the optical concentration of the enzyme for immobilization during a defined period. The excess of the support, however, is not suitable for practical use since it decreases the degree of substitution. We were not able to determine a generally optimal ratio of the enzyme to the support for the binding. The course of the reaction is individual and must be determined experimentally for every single enzyme. The most suitable conditions of the immobilization of papain are 25 mg of protein (concentration 40 mg/ml) per 100 mg of support. If, however, a higher concentration of papain on the support is required an excess of the enzyme should be used. Practically the same conditions were also optimal for the immobilization of amyloglucosidase. The pectolytic enzymes were best immobilized at a ratio of 100 mg of enzyme per 10 mg of support or even higher.

The results obtained permit us to draw several general conclusions: *1*) The technical polymethacrylate cation exchanger Ostion KM is suitable for the immobilization of enzymes by the method of reactive esters; its properties are adequate to those of carboxymethyl-cellulose Whatman CM-70. *2*) The method of reactive esters is suitable for the immobilization of those enzymes which are stable in the alkaline pH-range, at least at pH 8·0. With less stable enzymes a partial inactivation during the coupling must be considered. *3*) The reactive polymethacrylate esters are synthetized best by alkylation with ethyl chloroacetate in the presence of a tertiary amine. This method is more suitable than all the remaining methods of preparation of the

esters. The 2,4-dinitrophenol ester was the least reactive of all the halogenides investigated. *4*) The reactivity of the individual enzymes considerably differs and the optimal conditions of binding must be determined individually for each enzyme. *5*) The immobilization of certain enzymes, especially in the acid pH-range is complicated by adsorption; it is therefore suitable to carry out the immobilization in the presence of high salt concentrations.

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