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COVALENT METHODS OF BINDING POLYGALACTURONASE TO POROUS GLASS CARRIERS

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Key words: enzymes, immobilization, immobilized polygalacturonase, porous glass, derivatives of porous glass.

Tests were carried out with the aim to bind polygalacturonase to carboxy-, alkylo- and aryloamine derivatives of porous glass. The influence of the type of carrier surface on the quantity and activity of the immobilized enzyme and the role of substrate in the process of enzyme binding were examined.

INTRODUCTION

The last few years, have brought an increase in the number of papers dealing with the immobilization of enzymes. Researchers have been especially interested in enzymes which have an industrial significance.

In 1975, Datunaszwili [2] published the results of his work on the immobilization of polygalacturonase and pectin methylesterase by the methods of covalent bonds and the adsorption technique.

Fleming [3] examined the suitability of carriers, Sepharose and cellulose type and of non-porous glasses for binding endo- and polygalacturonase.

Weibel [13] immobilized pectinesterase on the surface of porous glass, using benzoyl azide as an activator.

Hanish [5] bound polymathoxygalacturonide lyase to DEAE-cellulose and porous glass. The immobilization and properties of the pectinolytic enzymes were studied by Steren [11] and Sheiman [10].

Test aimed at immobilizing polygalacturonase on collagen were conducted by Krakowiak [6].

MATERIAL AND METHODS

CARRIERS

For immobilization of enzyme on the surface of porous glass of a 100-120 mesh granulation, various types of glass with the following characteristic parameters were used:

Symbol of carrier	Specific surface m^2/g	Porosity $A(10^{-10}m)$
A	13-10	2000
B	80-60	400
C	120-100	200

Carriers manufactured by Corning Glass and one obtained from the Department of Physical Chemistry of the Maria Skłodowska-Curie University in Lublin, were used in the experiments.

ENZYMATIC PREPARATIONS

The pectinolytic preparation with an activity of polygalacturonase (PG) equal to 90 Units PG/mg protein, was immobilized on carriers. The preparation was obtained by a lyophilisation of active fractions of the chromatographic separation of a preparation with the activity PG 9 U/mg, produced by Sigma Chemical Company.

PECTIN AND POLYGALACTURONIC ACID

Citrus pectin with estrification degree = 67% and polymerisation degree = 143, produced by Koch Light Laboratories was used in the experiment. Polygalacturonic acid obtained from pectin according to the method given by Lifszyc [7] was also applied.

ANALYTICAL METHODS

a. The protein content in the soluble preparations was determined by Lowry's method, as modified by Brothertone [1].

b. The activity of polygalacturonase was determined parallelly by two methods: titration method elaborated by Lifszyc [7] and viscosimetric method, modified by Hanckock [4].

MODIFICATION OF CARRIERS SURFACE

a. **Silanisation of the surface.** The silanisation of the surface of glass with the use of 3-aminopropyltriethoxysilane (APTS) in toluene solutions was carried out by the method described by Weetall [12].

b. **Obtaining of carboxyl derivatives of porous glass.** A 10 g sample of silanised glass was introduced into a 300 ml solution containing 20 g of succinic acid anhydride. The reaction was carried on for 12 h at pH 6. The strong acidification appearing in the initial phase of reaction was neutralized by an addition of 10% NaOH.

c. **Obtaining of arylmine derivatives of porous glass.** A 10 g sample of silanised glass was introduced into a 300 ml solution containing 1 g of p-nitrobenzoyl chloride and 1 ml of triethylamine in toluene. The reaction mixture was allowed to stand for 12 h at 45°C. Then the obtained carrier was washed out with toluene and dried. The reduction of the nitro group into amino group was conducted using sodium dithionate.

METHODS OF OBTAINING THE IMMOBILIZED ENZYME

a. **Immobilization of the enzyme on alkylamine derivatives of porous glass.** The 10 g sample of silanised glass was introduced into 150 ml of 2% solution of glutaraldehyde at pH 7 and allowed to stand for 1 h. Then the carrier was filtrated and washed with water. The washed carrier was immediately introduced into a solution containing 200 mg of enzyme dissolved in 30 ml of 0.05 m buffer at pH 4 and 5 ml of 1% pectin solution. The reaction mixture was allowed to stand for 12 h at 4°C. Then the carrier with the immobilized enzyme was washed with citrate buffer till the pectinolytic activity dissapeared in the filtrate.

b. **Immobilization of enzyme on the carboxyl derivative of glass.** The 10 g sample of carboxyl glass derivative was introduced into 50 ml solution containing 1 g of 1 cyclohexylo-3-(2 morpholino-ethyl) carbodiimide meta-p-toluenosulphonate, and it was left for 2 h at 4°C. Then the carrier was washed with ice water and introduced to the enzyme solution in which 500 mg of enzyme and 5 ml of 1% pectin, had been dissolved. The mixture was allowed to stand for 12 h at 4°C and then the immobilized enzyme was filtrated by washing it with water until the pectinolytic activity dissapeared in the filtrate.

c. **Immobilization of enzyme on aryloamine derivatives of glass.** The 10 g sample of arylamine derivative of glass was introduced into 100 ml of 1 M HCl in an ice bath, and 20 g of $NaNO_2$ were added: After 12 h, the carrier was washed with water at 4°C and immediately introduced to a solution containing 500 mg of the enzyme and 5 ml of 1% pectin dissolved

in 50 ml buffer at pH 7. Changes in pH caused by the course of reaction were eliminated by an addition of 0.1 m NaOH. After 12 h, the carrier was filtrated and washed with water and then dried at room temperature.

EXPERIMENTS AND RESULTS

OBTAINING THE IMMOBILIZED ENZYME

At the beginning, in the process of obtaining preparations of immobilized polygalacturonase, the method of binding the enzyme to the arylamine derivatives through formation of diazo bonds, was applied. The obtained insoluble enzymatic preparations were characterized by a high level of protein bound with the carrier. The results obtained are presented in Table 1. The enzymatic activity of protein bound to the surface was low and the immobilized enzyme revealed only 3-6% activity of free enzyme.

Table 1. Immobilization of polygalacturonase on the aryloamine derivatives of porous glass

Specific surface m^2/g	Diameter of pores 10^{-10} m	Bound protein mg/g	Activity of the immobilized enzyme Units/g	The preserved activity %
13-10	2000	30	100	4
80-60	400	43	230	6
120-100	200	69	200	3

The preserved activity was calculated as follows:

$$x = \frac{n}{n \cdot A} \cdot 100\%$$

n — amount of bound protein
A — activity of free enzyme
x — preserved activity

Considerably better results were obtained by binding the enzyme to the alkyl-amine derivatives of porous glass, activated by glutaraldehyde. The results of this series of experiments are summarized in Table 2. The protein bound by this method was characterized by a high enzymatic activity amounting to about 55-59% of activity in free enzyme. The amount of enzymatic protein bound to the surface of the carrier 9-17 mg/g was dependent on the size of the specific surface.

Polygalacturonase was also bound to the carboxyl derivatives of porous glass. The results of these experiments are presented in Table 3. When using this method, the smallest losses in activity due to binding process, took place. The amount of protein bound to the carrier was 14-32 mg/g of the carrier; the highest quantities of protein were immobilized on the carriers with the largest specific surface.

The influence of the substrate presence in the process of enzyme binding on its activity after immobilization was also investigated. The results are presented in Table 4. The presence of the substrate has a significant influence on the activity of the immobilized enzyme, without significantly

Table 2. Immobilization of polygalacturonase on the alkylamine derivatives of porous glass

Specific surface m^2/g	Diameter of pores 10^{-10} m	Bound protein mg/g	Activity of the immobilized enzyme units/g	Preserved activity %
13-10	2000	9	480	59
80-60	400	14	700	55
120-100	200	17	430	25

changing the quantity of the enzymatic protein bound to the carrier. After immobilization, polygalacturonase preparations bound to the alkylamine and carboxy derivatives of porous glass in the presence of substrate were characterized by an almost 2-3 times higher activity than that of preparations obtained during binding of the enzyme without any addition of substrate.

DISCUSSION

INFLUENCE OF THE SIZE OF CARRIER SPECIFIC SURFACE ON THE QUANTITY OF THE IMMOBILIZED ENZYMATIC PROTEIN AND ITS ACTIVITY

The amount of enzymatic protein bound to the carriers was distinctly dependent on the size of the specific surface of the carriers, irrespectively of the method applied for enzyme immoblization. These realtionships were of an approximately parallel character but no linear dependence between these parameters was found. A ten-fold magnification of the specific surface i.e. from 13 to 120 m^2/g corresponded to a 2-2,5 times in-

Table 3. Immobilization of polygalacturonase on the carboxy derivatives of porous glass

Specific surface m^2/g	Diameter of pores 10^{-10} m	Bound protein mg/g	Activity of the immobilized enzyme units/g	Preserved activity %
13-10	2000	14	850	66
80-60	400	27	1480	60
120-100	200	32	430	17

Table 4. Influence of the substrate presence in the process of binding on the activity of the immobilized enzyme

Type of derivative of porous glass	Preserved activity in the presence of substrate %	Preserved activity in the absence of substrate %
Carboxy	63	32
Alkylamine	55	16
Arylamine	6	trace activity

crease in the quantity of immobilized protein. A similar relationship was obtained by Flemming [3] who immobilized 0.07 mg of polygalacturonase protein on 1 g of the carrier — non-porous glass beads with a specific surface equalling 0.0025 m^2/g. Using silanised porous glass with specific surface = 10 m^2/g, he immobilized 6.4 mg of protein/g of the carrier, applying activation by isocyanates. The methods applied in the present work give better results in case of immobilization of enzymatic protein on the surface of glass carriers.

THE INFLUENCE OF THE STRUCTURE OF CARRIER SURFACE ON THE QUANTITY AND ACTIVITY OF THE IMMOBILIZED ENZYME

Analysing the results presented in Tables 1, 2 and 3 and, first of all, values concerning the percentage of the preserved activity of the immobilized enzyme, it is possible to note distinct differences resulting not only from various methods of immobilization but also from the dependence on the size of the specific surface and its porous character. The results of the experiment allow to conclude that the porous structure of the carrier plays an important role in preservation of the enzyme activity. High losses in activity in case of application of carriers with porosity 200 A, were probably caused by the increased diffusion resistance of the narrowly porous structure of the carrier to the macromolecular substrate. Similar phenomena were observed by Weetall [12] who studied the properties of the immobilized glucoamylase on porous glass. Thus, the porous structure of the surface plays a very important role in the effectiveness of action of the immobilized enzymes which are the catalysts of changes of macromolecular substrates.

THE EVALUATION OF THE SUITABILITY OF THE METHODS FOR BINDING THE ENZYME

The choice of the methods for binding the enzyme to the carrier was dictated by the stability of the enzyme in weakly acid and neutral medium.

The methods applied for the immobilization of polygalacturonase enzymatic protein on porous glass cariers allowed to obtain highly active preparations. These preparations were characterized by a high level of bound protein and high stability. They were 3-4 times more stable than those obtained by Datunaszwili [2]. We may recognize as the only effective method of immobilization of polygalacturonase the binding to carboxyl and alkylamine derivatives of glass which yield preparations of high activity and a level of bound protein within the limits of 14-27 mg/g of the carrier.

Binding of the enzyme to the aryloamine derivatives of glass allowed to immobilize 30-69 mg protein/g of the carrier, however the enzyme bound in this way revealed only a small percent of its initial activity i.e. 4-6%. Such a considerable loss in activity results probably from the change in the configuration of the enzyme during the process of immobilization, or from the fact that the bond between the protein and the carrier is formed by the aminoacid residual, situated near the active centre.

The presence of the substrate in the process of immobilization is a very important factor, increasing the activity of the obtained enzyme-carrier complexes. The presence of the substrate in the process of enzyme binding has a decisive influence (irrespectively of the method applied) on the activity of the immobilized enzyme which revealed a 2-3 times higher activity than the preparations obtained without any addition of substrate. Similar phenomena connected with the influence of substrate or inhibitor on the activity of the immobilized enzymes were stated by Jaworek and Nelbock [9]. Such a significant influence of substrate on the preserved activity of the immobilized enzyme should be ascribed to the protective action of the substrate molecules in regard to the active centre. The active centre, isolated by the substrate, decreases probably the quantity of the bonds produced between protein and carrier. It does not influence, however, the stability of the preparations and their activity.

CONCLUSIONS

1. Active preparations of the immobilized polygalacturonase may be obtained by binding the soluble enzyme to the carboxyl derivatives of porous glass, activated with carbodiimide and with the alkylamine derivatives of porous glass, activated with glutaraldehyde.

2. The amount of the enzymatic protein of polygalacturonase immobilized on the surface of porous glass is dependent on the size of the specific surface of the carrier.

3. The structure of the carrier surface played an important role in the preservation of the activity of the immobilized enzyme. The width of pores reaching more than 4×10^{-8} m made it possible to obtain highly active preparations of the immobilized polygalacturonase.

4. The enzymatic preparations obtained in the presence of substrate were 2-3 times more active than those ones obtained when enzymes were immobilized without substrate.

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KOWALENCYJNE METODY WIĄZANIA POLIGALAKTURONAZY DO NOŚNIKÓW ZE SZKŁA POROWATEGO

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Streszczenie

Przeprowadzono próby wiązania poligalakturonazy do karboksylowych, alkilo- i aryloaminowych pochodnych szkła porowatego. Przebadano wpływ charakteru powierzchni nośnika na ilość i aktywność unieruchomionego enzymu. Najlepsze efekty dało zastosowanie jako nośników unieruchomionego enzymu pochodnych karboksylowych i alkiloaminowych, których średnice porów były większe niż 4×10^{-8} m. Unieruchomiony enzym wykazywał 55-66% aktywności enzymu wolnego przed unieruchomieniem. Nośniki o średnicy mniejszej niż 2×10^{-8} m pomimo największej ilości związanego białka przyczyniały się do znacznej utraty aktywności unieruchomionego enzymu. Bardzo istotnym czynnikiem wpływającym na aktywność unieruchomionego enzymu była obecność substratu w procesie wiązania enzymu. Preparaty otrzymane w obecności substratu były 2-3-krotnie aktywniejsze od tych jakie otrzymano wiążąc enzym bez substratu.