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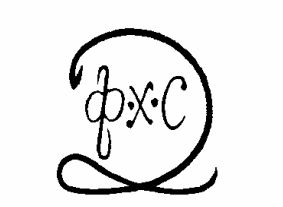

PHYSICAL CHEMISTRY 2022

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IMMOBILIZATION OF α-AMYLASE FROM *BACILLUS PARALICHENIFORMIS* ON BENTONITES

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ABSTRACT
α-Amylase from *Bacillus paralicheniformis* (*Bli*Amy) is a highly efficient raw starch digesting enzyme. Starch is an inexpensive source of many food industrial products. Naturally occurring clay are non-toxic, environmentally friendly and inexpensive. Therefore, immobilization of *Bli*Amy by adsorption on three differently modified bentonites was studied. Modifications included common Na-exchange procedure, acid activation, and alkali activation. The modified clays were characterized by X-ray powder diffraction, mercury intrusion porosimetry and the points of zero charge were determined. The adsorption of the enzyme was significantly influenced by the type of modification of bentonite, being the highest for the acid-activated bentonite with the highest porosity. On the other hand, the highest enzyme activity for immobilized α-amylase was obtained with alkali-modified bentonite (98 U/g), suggesting it as a good candidate for immobilization of α-amylase for application in the food industry.

INTRODUCTION
α-Amylases (1,4-α-D-glucan glucohydrolases) are one of the most important commercial enzymes and represent about 25% of the enzyme market. It catalyzes the hydrolysis of α-1,4-glucosidic bonds in starch, glycogen and related polysaccharides and oligosaccharides in a random manner while releasing reducing groups in the α-configuration. Starch is one of the most important food ingredients and a substrate for the production of many industrial products such as bioethanol, or could be used in drug delivery, as well as in the paper and textile industry, for example [1]. α-Amylase from *Bacillus paralicheniformis* (*Bli*Amy) was proven to be a highly efficient raw starch digesting enzyme [2], and due to various industrial and technological applications, the study of its stability through immobilization is attractive.

Due to high specificity and selectivity, high efficiency and yield, mild reaction conditions and lower energy costs of enzyme catalysis, industrial application of these biocatalysts deserve more and more attention compared to traditional chemical processes. There are no side reactions and product degradation, and the procedures for separation and purification of final products are cheaper. The main disadvantages of using enzymes in the native state are insufficient stability, high cost and uneconomical way of application. Immobilization uses the same amount of enzyme as in the case of catalysis with an enzyme in its native state, but a larger amount of product is obtained. The reaction can be carried out continuously which makes it easier to control it, the enzyme is stabilized and does not lose activity during long-term use and storage.

Clay minerals, primarily smectites (main constituents of bentonites), modified in different manners, have been used as enzyme supports [3, 4]. Smectites are low cost, chemically inert, thermostable and are easily modified into materials with tailored properties. Bentonite organomodified with hexadecyl-trimethylammonium bromide was used for immobilization of α-amylase from *Bacillus subtilis* by adsorption [5]. The optimal support was proven to be organobentonite having a bilayered arrangement of surfactant in the interlamellar space of smectite.

Enzymes immobilized on this support did not deform in the presence of microorganisms and proteolytic enzymes and no change in pH and temperature optimum of the enzyme after immobilization was found. On the other hand, the activity was preserved in a wider pH range and storage stability was improved. Amylase from *Aspergillus oryzae* was covalently immobilized on chitosan-montmorillonite nano support, with no change in pH optimum and with improved thermal and storage stability [6].

In this paper the influence of surface acidity of modified bentonites on their ability to immobilize *Bli*Amy amylase was tested.

METHODS

The enzyme supports were prepared from bentonite from Coal mine "Bogovina", Serbia where clay, although valuable resource, is still considered to be tailings. The clay fraction of < 2μm (B-2) was obtained by hydroseparation. Three types of simple modification were performed: Na-exchange, acid activation, and alkaline activation.

The B-2 was subjected to the common Na-exchange procedure [7] by stirring in a 1 M NaCl solution and Na-B was obtained. The acid activated bentonite (B-Ac) was obtained by activation with 4.5 mol dm^{-3} HCl at 90 °C for 2 h [8]. The alkali activated bentonite (B-Al) was obtained by adding Na_2CO_3 in water suspension of B-2 [9]. The excess of Na^+, Cl^- and exchangeable cations were removed by dialysis.

The identification of the crystalline phases was performed using the X-ray powder diffraction (XRPD) technique (Rigaku SmartLab automatic multipurpose X-ray diffractometer) at the scanning rate of 3° min^{-1} and using CuKα radiation (λ = 0.15418 nm). Mercury intrusion porosimetry (MIP) was applied using the fully automated conventional apparatus Carlo Erba 2000 porosimeter (pressure range: 0.1–200 MPa; pore size (diameter) range: 7.5–15000 nm). Data acquisition was carried out using the Milestone 200 software package. The point of zero charge (pH_{PZC}) of the samples was determined by the common method [10].

*Bli*Amy was overproduced in high yield in *E. coli* and partially purified after heat treatment of the fermentation broth at 60 °C for 1 h [2].

The immobilization procedure was performed as follows. The 15 mg of modified bentonite sample was dispersed in 1.5 ml of *Bli*Amy (1.5 mg/mL; 285 U/mL) suspended in 50 mM phosphate buffer pH 6.5 and shaken continuously at 25 °C for 24 h. Thereafter, reactions were stopped by centrifugation of enzyme adsorbed on support and subsequent washing with 50 mM phosphate buffer, pH 6.5 five times.

Protein concentration was determined by the Bradford method [11]. α-Amylase activity (free or immobilized) was determined as previously reported [2] in 50 mM phosphate buffer pH 6.5 at 25 °C. The amount of liberated reducing sugar was determined by the DNS acid method [12]. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute at 25 °C. Maltose was used to construct a standard curve.

RESULTS AND DISCUSSION

In this paper, three different types of bentonite modification were tested as a support matrix for *Bli*Amy. The results of XRPD analysis confirmed the presence of smectite as the dominant mineral in all samples. The associated minerals like quartz and feldspar were present in small amounts. The modification process only affected the intensity and position of d_{001} diffraction peak of smectite as expected [7, 8, 13].

The shape of mercury intrusion curves (Fig. 1) indicated the presence of interparticle and intraparticle porosity [14], which would mean that the real porosity is somewhat lower. The average pore diameter for all samples is centred in the macropores region (6.72 μm – 7.77 μm). However,

from the pore size distribution curves (Fig. 1), the presence of pore in the mesopore region (about 20 nm) is obvious. This is also indicated by the specific surface area, which values are associated with this pore size. The main textural parameters obtained from MIP measurements are given in **Table 1.**

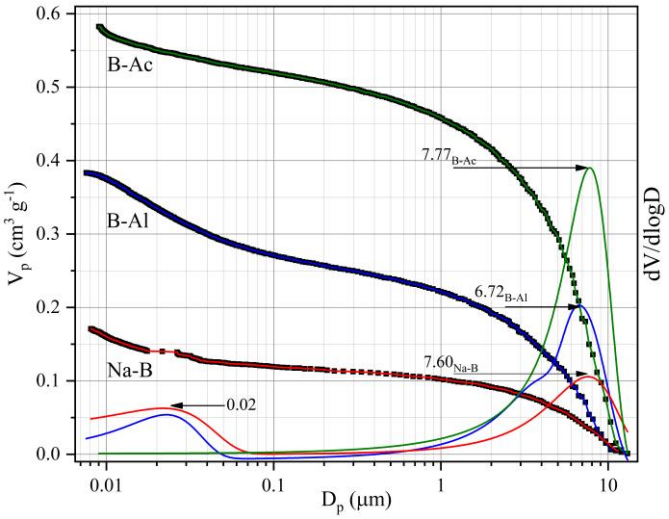

Figure 1. Total intruded volume of Hg and pore size distribution of Na-B, B-Ac, and B-Al samples

Table 1. Main features of texture from Hg-porosimetry

Sample	V_p (cm^3 g^{-1})	S_{Hg} (m^2 g^{-1})	D_p (μm)	BD (g cm^{-3})	BD_{Corr} (g cm^{-3})	P (%)
Na-B	0.17	13.84	9.78	1.86	2.73	31.82
B-Ac	0.58	15.43	9.78	1.03	2.58	60.01
B-Al	0.38	23.54	7.85	1.13	1.99	43.33

V_p-*total intruded Hg volume obtained at the pressure of 200 MPa*; S_{Hg}-*specific surface area obtained for a cylindrical pore model*; D_p-*average pore diameter*; BD-*bulk density*; BD_{Corr}-*corrected bulk density*; P-*sample porosity*.

The porosity and total intruded Hg volume increased in the following order Na-B< B-Al< B-Ac.

The results for pH_{PZC} are given together with results of amylase immobilization, desorption of immobilized amylase and the specific activity (U per g of support) of the bentonite-amylase system toward starch (**Table 2**.).

Table 2. The parameters of α-amylase immobilization on modified bentonite clays

Sample	pH_{PZC}	Adsorption (%)	Desorption (%)	Activity (U/g)
Na-B	8.2	33	3	20
B-Ac	5.2	74	1	14
B-Al	9.7	22	0	98

The surface of the bentonite clay supports Na-B and B-Al with pH_{PZC} 8.2 and 9.7, respectively, was positively charged at pH 6.5, while B-Ac (pH_{PZC} 5.2) was negatively charged. The amylase surface at pH 6.5, which is optimal for enzyme activity and used for adsorption, is slightly negatively charged since its pI value is 6.02 [15]. The highest adsorption observed with B-Ac (74%) implies that the dominant adsorption interactions between *Bli*Amy and bentonite are not ionic. It is known that amylase can form electrostatic and hydrophobic interactions with the supports [5]. However, the highest adsorption was achieved for the sample with the highest porosity. The highest activity of immobilizate is achieved with amylase adsorbed on B-Al, and five times higher than amylase

adsorbed on Na-B. This might seem a bit counterintuitive since both bentonite clays, Na-B and B-Al, are Na clays, however, alkaline modification caused morphological changes that resulted in different pore size distribution and consequently higher specific surface area. It can be speculated that the change in porous structure enabled better accessibility of active sites of B-Al-amylase biocatalyst toward starch. Access to the substrate is very important, especially for large polymer substrates such as starch. Further structural characterization of alkali-modified bentonite with immobilized α-amylase would provide better insight into the nature of adsorption interaction and immobilizate activity.

CONCLUSION

It was shown that modified low-cost material, bentonite clay can be used as efficient support for amylase immobilization and starch hydrolysis. The type of simple chemical modification of bentonite significantly affects the adsorption of enzyme and the activity of immobilized amylase. The highest adsorption of amylase was obtained with the acid-activated bentonite. On the other hand, the highest activity was achieved for immobilizate obtained with alkali-activated bentonite. Further characterization of immobilizates including the thermal, pH, storage, and operation stability would provide better insight into possible applications in the food industry.

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