COMPARISON OF THE STRUCTURE AND THE STABILITY OF SINGLE ENZYME NANOPARTICLES

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Single enzyme complex nanoparticles were synthesized following the method of Kim et al [1]. The enzyme complex used was cellulosome that is a large spatial association of enzymes. Single enzyme nanoparticle of celluclast BG cellulase enzyme complex (SEN-CK) can degrade as great substrates as natural cellulose. It was proved that the stability of SEN-CK enzyme complex is significantly better than that of native enzyme complex (enzyme without pretreatment). Significant portion of the activity of SEN-CK is remaining at 80 °C for 1 hour time.

Keywords: enzyme activity, improvement of enzyme stability, single enzyme nanoparticle, effects of temperature on enzyme stability

Introduction

The enzymatic fermentation of cellulose to glucose under industrial conditions is a crucial point in industrial bioethanol synthesis [2]. The most interesting step of the bioconversion is the enzymatic fermentation of cellulose molecules under industrial conditions. Cellulose is one of the most stable biopolymers. The polymer chains in natural cellulose build up crystalline and amorphous regions (*Fig. 1*), [2].
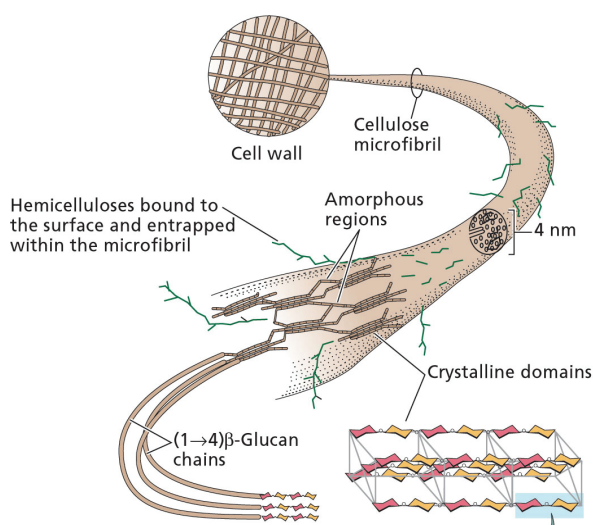

Figure 1: The spatial structure of natural cellulose. Polymer chains build up crystalline domains

Cellulases comprise three types of enzymes: endoglucanases, which cleave internal β-1,4-glucosidic bonds; exoglucanases, which processively act on the reducing and non-reducing ends of cellulose chains to release short-chain cello-oligosaccharides; and β glucosidases, which hydrolyze soluble cellooligo-saccharides (e.g. cellobiose) to glucose (*Fig. 2*) Plant cell-wall degrading enzymes exist either in complexed or non-complexed systems [2, 3].

Cellulosome is one type of hyperstructures. A hyperstructure is a large, spatial association of cellular constituents such as molecules, macromolecules, and ions that performs a particular function because the constituents are associated with one another [4]. A hyper-structure interacts with other hyperstructures at a level of organization intermediate between the macromolecule and the bacterial cell (Fig. 2).
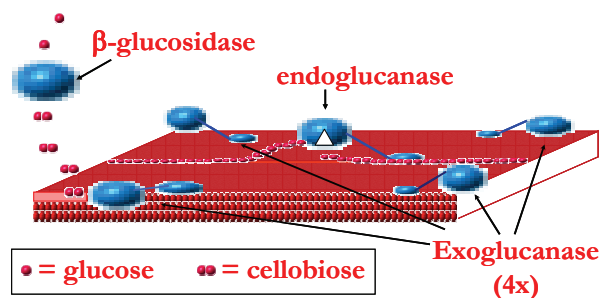

Figure 2: The structure and function of cellulose multienzyme complex from *T. reesei*
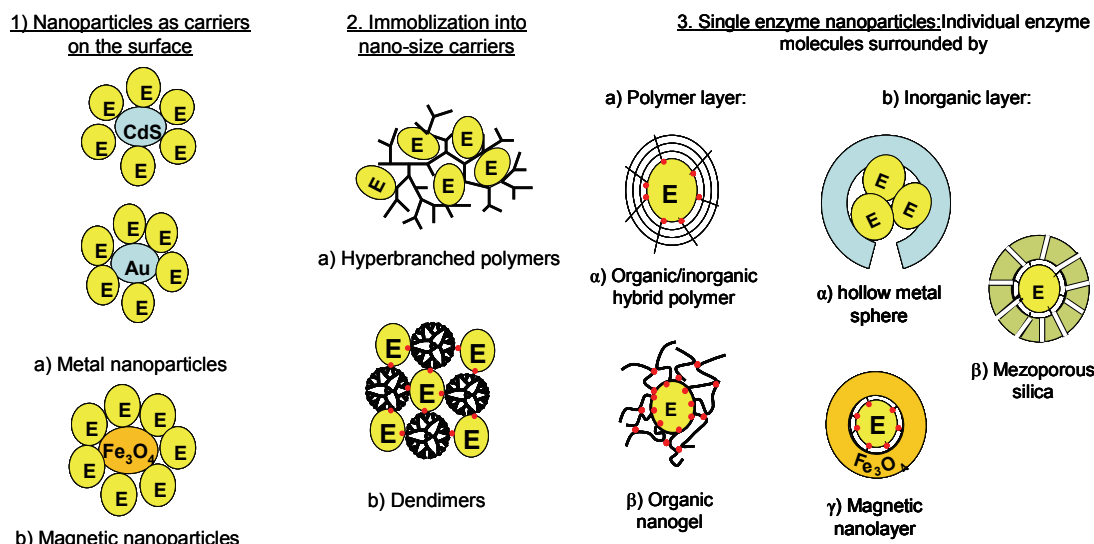
Figure 3: Nanotechnological methods for enzyme stabilization.
The new approach is the reducing the size of the enzyme carriers using 1) a) metal or b) magnetic nanoparticles as carriers 2) encapsulation into a) hyperbranched polymers b) dendrimers. 3) Single enzyme nanoparticles means single enzyme molecules encapsulated with a) polymer network [α)organic/inorganic hybrid polymer or β) nanogel] b) inorganic layer [α) hollow metal sphere, β) mezoporous silica or γ) magnetic nanolayer.]

We investigated Celluclast enzyme systems. This enzyme system not contains covalent linkage among different enzymes of the complex but each individual enzymes have a well defined spatial order and arrangement in the hyperstructure [3].

Improvement in enzyme stability enable further practical applications. Classical techniques for improving the stability of the enzymes are enzyme immobilization to the surface or inner cavity of the carrier, modification of the surface of the enzyme, protein engineering, reaction medium engineering and cross-linked enzyme crystals.

Recently growing interest has been shown in using nanoparticles as carriers to achieve enzyme immobilization in order to increase enzyme stability. The new approach is reducing the size of the enzyme carriers using metal [6, 7], or magnetic nanoparticles [8] as carriers, encapsulation into hyperbranched polymers [9] dendrimers [10, 11]. Single enzyme nanoparticles means single enzyme molecules encapsulated within polymer network (nanogel) [12] of inorganic layer. The inorganic layer could be hollowed metal or silica nano-sphere [13, 14] mezoporous silica [15] or magnetic nanolayer [16] (*Fig. 3*).

Single enzyme nanoparticles (SENs) represent a new approach in industrial enzyme research (Fig. 3) [1, 19]. Form of SEN means, that each enzyme molecule is surrounded by a nanometer-size polymer matrix layer, resulting in stabilization of enzyme activity without any serious limitation for the substrate transfer from solution to the active site of the enzyme. The synthesis of SEN particles is available via more or less simple laboratorial technique. Previously we have decided to apply this technique for industrial bioethanol synthesis. The steps of the synthesis of SEN-molecules comply with the

requirements of green chemistry. We would like to investigate how can the SEN-enzymes digest higher-size substrates.

We have tried to prepare single enzyme-complex nanoparticles where the functional level is not only a single enzyme, but a multifunctional supramolecular enzyme complex which can have a hyperstructure. We tried to investigate that supramolecular hyperstructures as single nanoparticles has similar behaviour as the monomolecular single enzyme nanoparticles, under extremely conditions.

Materials and methods

The following chemical compounds were used: Celluclast BG enzyme complex from *T: Reesei* (Novozymes), acryloyl chloride, 1,3-bis[tris(hydroxy-methyl)methylamino]propane or Bis-Tris propane (Sigma), sodium bis(2-ethylhexyl) sulphosuccinate or aerosol OT (AOT) (Fluka), disodium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, 2-propanol, n-hexane (Spektrum-3d, Scharlau), methacryloxypropyltrimetoxysilane (MAPS), 2,2-azobis-(2,4-dimethylvaleronitrile) (Fluka), N-acetyl-L-tyrosine ethyl ester (Sigma), 3,5-dinitro-salicylic acid (Sigma).

Julaba F12 cryostate was used to keep the reaction mixture at 0 °C. A gas chromatographic syringe (volume 5 μl) was used for the addition of a few microlitres of acryloyl chloride to the enzyme solution. The polymerization step in the synthesis of single enzyme nanoparticles of CK enzyme (SEN-CK) was carried out in a double-walled stirring vessel (*Fig. 4*). The solution was irradiated by a UV-lamp made by Vilber Lourmat. Filtration of the surface-polymerized

enzymes was carried out with a syringe filter (pore size 0.1 µm) made by Millipore. UV-spectra were recorded and enzyme activity measurements carried out by means of a Biochrom 4060 spectrophotometer made by Pharmacia. A New Brunswick Scientific G24 incubator shaker was used for the stability measurements. Malvern Zeta-sizer was applied for detection of size distribution of the enzyme nanoparticles.

Preparation of single enzyme nanoparticles

The preparation process of single enzyme nanoparticles (SEN) has three steps (*Fig. 5*). The detailed description of the procedure was given earlier [1, 17, 19]. The first step is a modification of CK enzyme complex and its solubilization in a hydrophobic medium. The second step is the polymerization of the vinyl group in hexane, and the final (third) step is hydrolysis and condensation of the trimethoxysilyl functional group (TMS). For the polymerization step, the enzyme should be dissolved in a hydrophobic medium (n-hexane). Polymerization on the surface of the enzyme can only be achieved if there is direct contact between the enzyme surface and the hexane medium. For this reason, the specific solubilization method of hydrophobic ion pairing may be used (*Fig. 6*). In this process, vinyl groups on the enzyme surface (synthesized in the first step) are well exposed to the organic solvent (and reagents).
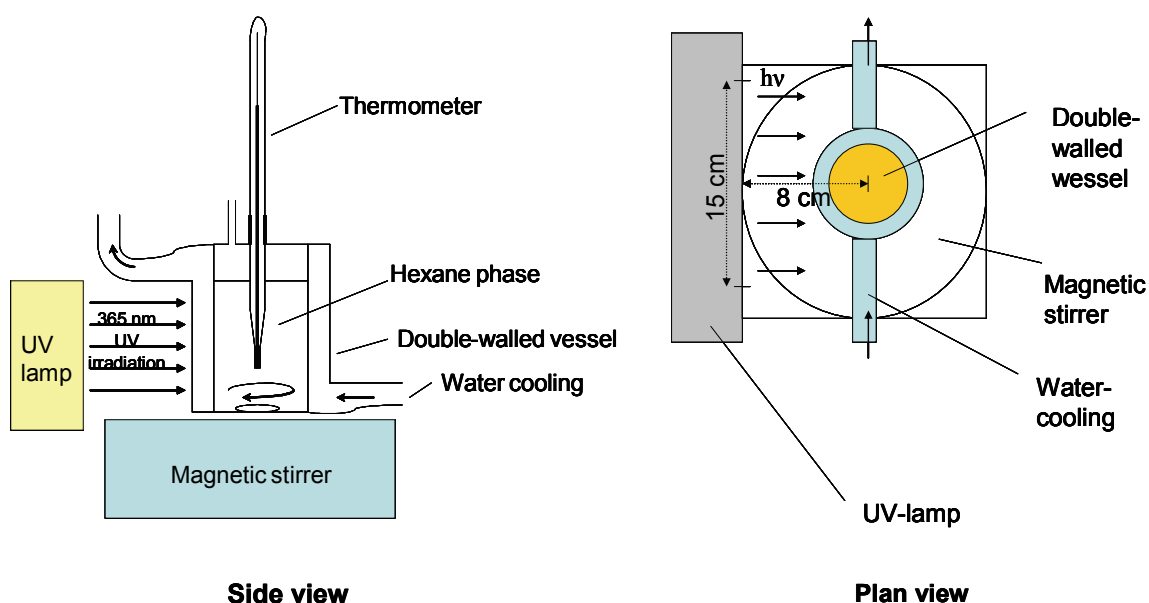

Side view   **Plan view**

Figure 4: UV-irradiation for the polymerization on the surface of the single enzyme molecules
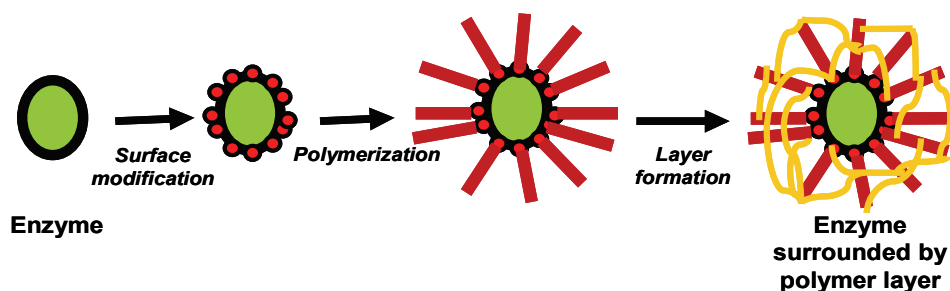

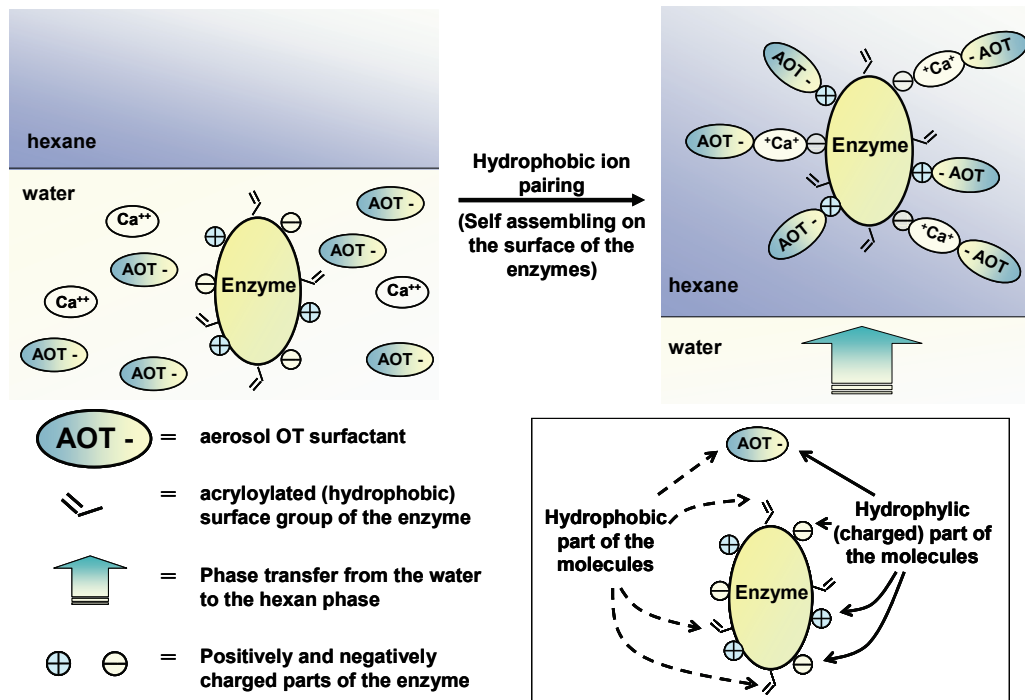*Figure 5:* Synthesis steps of single enzyme nanoparticles

Figure 6: Mechanism of hydrophobic ion pairing

Characterization of single enzyme nanoparticles

The morphology and the size of resulting SENs were examined by a JEOL-1200X transmission electron microscopy (TEM) at an accelerated voltage 80 kV. The procedure of the imaging was rather simple, the sample did not need specific preparation for their measurement. One drop of the SEN-CT solution was enough to get detectable image by TEM. The drops of SEN-CT samples were dried and after the drying the SEN-particles were detected.

Measurement and calculation of enzyme concentration

The concentration of native CK was determined by taking calibration measurements of the enzyme absorption at 280 nm. There was also no difference between the absorption properties of modified and native enzymes. The concentration of SEN-CK could not be obtained from measurement of the UV-light absorption, because the polymer layer prepared around the enzymes also has high absorption at wavelength 280 nm. The SEN-CK concentration in water was therefore calculated from the initial concentration of modified CT in hexane (in this case the absorption was measurable), assuming that after the polymerization step the full amount of SEN-CK was transferred from the hexane into the water phase, and the amount of SEN-CK precipitated during the phase transfer process was negligible.

Measurement of enzyme activity

Whatman filter paper was used as substrate (filter paper unit, FPU) and total cellulase activity was measured using DNS-probe according the instructions of Ghose [20].

Measurement of enzyme stability

The heat stability of native and prepared enzymes was measured at the following method: 1 ml 0.05 M citrat buffer (pH = 6,00) and 0.5 ml of the enzyme stick solution was mixed and filter paper sample was added into the mixture (1 cm x 6 cm Whatman No. 1. Standard filter paper) and the mixture was incubated for 60 min at the desired temperature (from 50 °C to 80 °C) without shaking. After the incubation, the enzyme-substrate mixture was cooled and the next steps were the standard activity measurement [20]. The relative activity was calculated as the ratio of residual activity to initial activity.

Results and discussion

In this section it will be enlight the relations among the thickness of the polymer layer on the surface of the prepared SEN-enzymes and the structure, size and stability of the resulting SEN-enzymes.
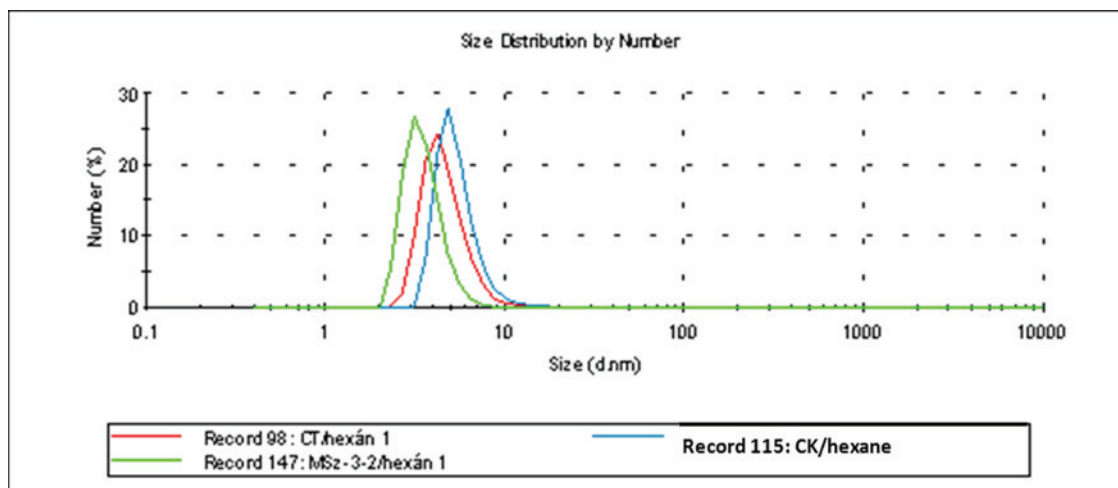
Figure 7: Size distribution of different type of single enzyme nanoparticles after the solvatation in hexane using hydrophobic ion pairing (CT =chymotrypsin, CK = celluclast enzyme complex, MSZ = mannosynthase enzyme)

Size distribution of SEN-CK during the preparation process

The size distribution of the SEN-CK enzyme complex was measured during the preparation. It was detected by Zeta Sizer. The results show that the range of the size of the enzyme nanoparticles on the hexane phase after the hydrophobic ion pairing process are between 2–10 nm (*Fig. 7*). We measured three different enzymes (CK = chymotrypsin enzyme, CK = Celluclast BG enzyme complex and MSZ = mannosynthase enzyme). In the case of CK enzyme complex during the solvatation process in hexane enzyme particles with size higher than 10 nm can not be detected.

Structural caracterization of SEN-CK enzymes

The structural and size characteristics of SEN-CT were detected by transmission electron microscopy (TEM). The size of monomeric enzyme is about 2–8 nm (Fig. 7). *Fig. 8* shows that the resulting single enzyme nano-particles have hollow spherical structures on the electron microscopic images. The surrounding silica-containing nano-gel is electrodense and results dark layer around the enzyme molecules on the picture. But these SEN nanoparticles was aggregated into greater structures. The diameter of the resulting SEN-CK molecules falls usually between 10 and 100 nm.

It can be seen a great difference of the structures of the SEN enzyme aggregates when the size of the polymer layer around the SEN particles was half (0.5x) or four times (4x) thicker than the originally described [1, 19]. The shape of the originally desribed thickness of polymer layer (Fig. 8, 1x) seems to be more spherical than that of the natural CK enzymes. But in the case of the half thickness (Fig. 8, 0.5x) we can see non-sperical objects, ellyptoid, rhomboid shapes and enzyme strings. In the case of 4x thickness (Fig. 8, 4x) there are a greater, shadowed layer around the enzyme aggregates.

We can see white spot on the darker halo of the darker thick layer around the enzyme aggregates. It is very likely that these white spots are "enzyme footprints" of enzymes existing on the sample without polymer layer.
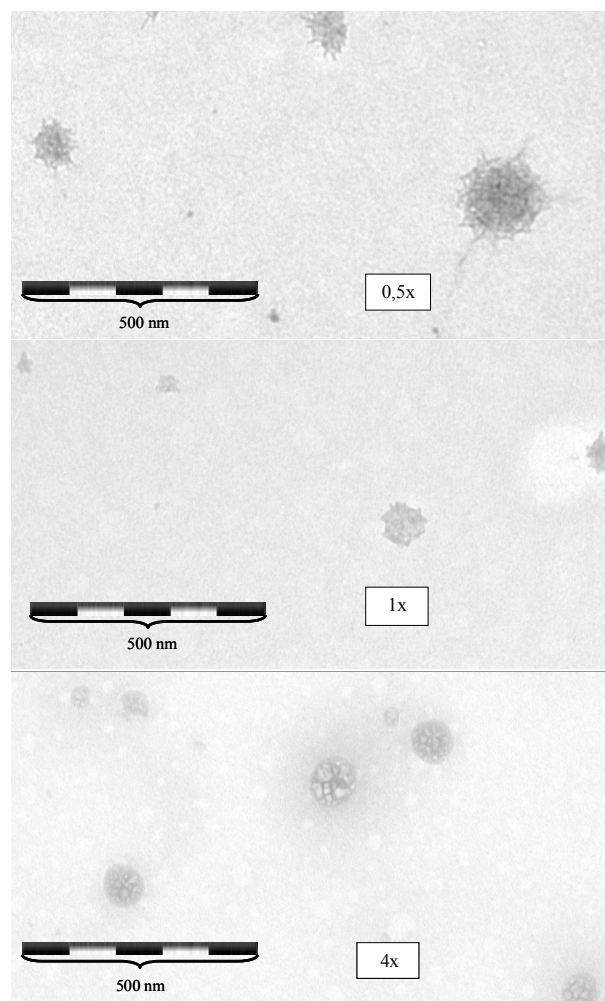

Figure 8: TEM images of the SEN-CK enzyme complexes with 0.5x, 1x and 4x quantity of monomers of the polymer layer

Results obtained confirm that this technique is suitable to realize not only single enzyme nanoparticles but also single enzyme complex nanoparticles (make polymer layer around each single enzyme molecules of the enzyme complex) which are separable (little particles in *Fig. 10*) or in some cases a few number of enzymes can be included in the nanoparticles (greater particles in Fig. 10). The filtration of the nanofluid, applying filter paper with cutoff of 10 000 D, could not be carried out during our preparation procedure. The thickness of the polymer layer can be estimated to be about 3–7 nm according to Fig. 10. The larger particles with size of 10–100 nm can not cause practically diffusion resistance against the substrate molecules diffusing to the active centre of the enzyme.

Activity change of enzyme complexes with the changes of the thickness of the polymer layer

Preliminary results show that the thickness of the cross-linked polymer layer around the enzyme complex can also reduce the activity of the single enzyme nanoparticles of Celloclast BG enzyme complexes (SEN-CK). But this reducing effect is not dramatical because when the polymer layer around the enzyme complex is 4 times thicker, the activity of the SEN-CK composite decreases to about 60%, of its original value. (We assume here, that the thickness of the polymer layer is proportional to the quantity of monomer given in *Fig. 9*).
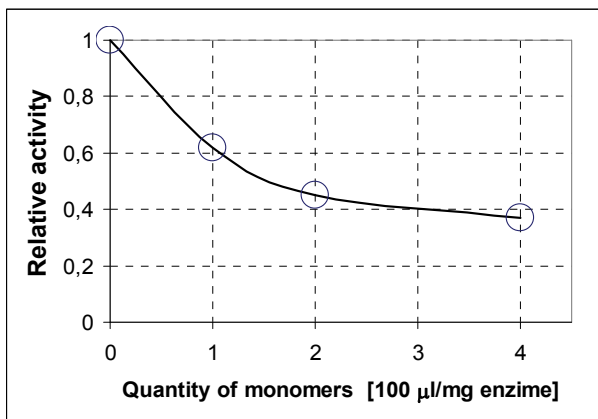

Figure 9: The change of the enzyme activity in the function of the quantity of monomers (MAPS)

Heat stability of CK and SEN-CK enzyme compexes

According to our previous investigations carried out by chymotrypsin enzyme, the prepared chymotrypsin enzyme (SEN-CT) is more stable than the natural one [17, 19]. Similarly to it, the SEN-CK particles are really more stable than natural ones (CK) at room temperature as well as under extreme conditions (50–80 °C, Fig. 10). The native enzyme complex loses about 90% of its activity at 80 °C after 1 h incubation, but in the case of

SEN-CK particles using an originally described amount of monomer in the polymerization step (Fig. 10, 1x layer) there is no significant change in its thermal stability between 50–80 °C (Fig. 10).
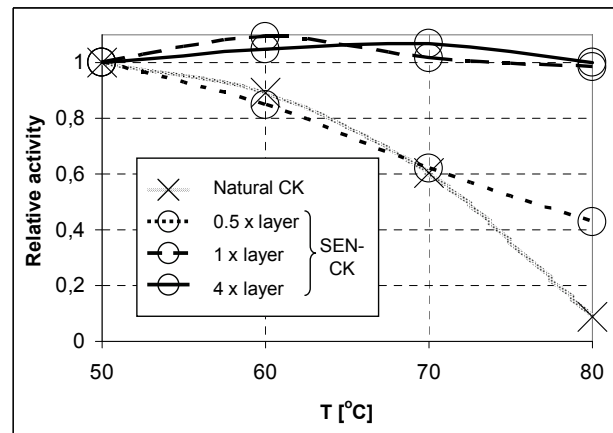

Figure 10: Heat stability of the CK enzyme complex using different amount of monomer during the polymerization of the layer on the surfaces of the enzymes

There is no significant difference among the stability of SEN-CK enzymes, when the amount of the monomer is four times higher (4x layer, Fig. 10) But if the thickness of the polymer layer is lower than the originally described (0.5x, Fig. 10), then the heat stability of the SEN-CK enzyme complex converges to the heat stability of the naurtal ones (CK) without polymer layer.

Conclusions

Single enzyme complex nanoparticles was synthesized following the method of Kim et al [1]. The enzyme complex was cellulosome that it is a large spatial association of enzymes. Single enzyme nanoparticle of celluclast BG cellulose enzyme complex (SEN-CK) can degrade substrates with large molecules as natural cellulose. It means that four different types of cellulolytic enzymes are located at the well defined place in the enzyme complex during the degradation of cellulose substrate.

The diameter of cellulosome in the case of *C. thermocellum* is about 18 nm [21]. During the solvatation process of CK enzyme complex in hexane enzyme particles with size higher than 10 nm are not detectable. It means that the hydrophobic ion pairing process can solve the different enzymes of CK enzyme comlex separately and the ionic interactions between the different enzymes and the detergent molecules on the surface of the enzymes seemed stronger than the interactions between the different functional parts of the enzyme complex. The results suggest, that self-arrangement mechanism takes place in the third step of the preparation of SEN-CK enzyme complexes.

After the polymerization step and the cross linkage between the polymer fibers on the surface of the

enzymes the resulting SEN-CK enzyme complexes have at least one order of magnitude higher average size (Fig. 7). It means that during the third step of the preparation of SEN enzyme complexes, the separated enzyme nanoparticles, containing polymer fibers on the surfaces of the enzyme molecules, can aggregate into larger structures. It was assumed that the altering size distribution of the resulting SEN-CK enzyme complex was caused by the higher concentration of monomer at the third step of the synthesis of SEN-CK. (cross-linkage between the polymer chains). The probability of the intermolecular binding is higher and the size of the resulting partiles will be longer due to the higher concentration of enzymes.

It was proved that the stability of SEN-CK enzyme complex is significantly better than that of native enzyme complex (enzyme without pretreatment). Activity of SEN-CK does not decrease practically at 80 °C for 1 hour incubation time.

ABBREVATIONS

AOT: sodium bis(2-ethylhexyl) sulfosuccinate or aerosol OT
CT: α-chymotrypsin enzyme
CK: Celloclast BG enzyme complex (Novozymes)
SEN: single enzyme nanoparticles
MAPS: methacryloxypropyltrimethoxysilane
TEM: transmission electron microscope

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