Complexes of Cellulose and Trypsin

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Summary: Adsorption of trypsin to microcrystalline cellulose has been determined as functions of protein concentration and pH of the aqueous medium. The study of adsorption at several pH values indicates that interaction of trypsin to the microcrystalline cellulose interface is controlled by the electrostatic effect. The FTIR, desorption, and SEM data reveal that a part of trypsin is strongly bound to the microcrystalline cellulose matrix. Resulting complexes consist of microcrystalline cellulose, trypsin, and water.

Keywords: biopolymers; proteins

Introduction

The adsorption of proteins to solid interfaces and interactions between surfactants and proteins at liquid/solid interfaces are processes of significance to our daily life and in such fields as medicine, food processing, biotechnology, etc. Protein adsorption is also scientifically intriguing. The use of cellulose, a natural polymer, as a carrier for biologically active compounds is widely known [1]. However, pure cellulose has limited application as a matrix for proteins in spite of the fact that the combination of this natural polymer and some proteins can be promising for various medical applications. Powder microcrystalline cellulose (MCC) is used for the manufacture of drug forms of many preparations due to its valuable sorption properties [2].

It is known that protein adsorption from solutions to solid interfaces is a complicated process and adsorption value depends on many factors. The most important of them are surface properties of adsorbent, such as, its chemical composition, hydrophilicity or hydrophobicity, its charge, specific area and porosity.

The goal of this paper is to obtain multicomponent complexes on the basis of diffusion-adsorption interaction between MCC (matrix) and proteolytic ferment trypsin. Our results on adsorption of trypsin from aqueous solutions to the MCC interface are presented at various values of pH and concentration of initial ferment solutions. The possibility of trypsin to release from the interface appears to be also an important result of this study.

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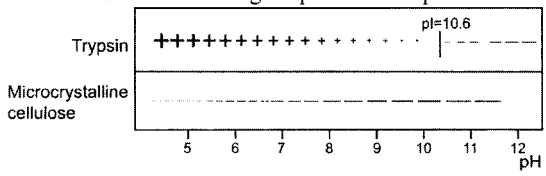
Experimental

Microcrystalline cotton cellulose had particle size < 0.05 mm and was bone-dried up to moisture content not exceeding 1.0 wt %. The DP_v of MCC was 190. Pore volume, pore radius, specific area determined by method of water vapour sorption were 2.16 cm^3/g, 20 mcm, and 230 m^2/g, respectively. Content of the end carbonyl groups in the MCC chains is $3.2 \cdot 10^{-5}$ mol/g, of the carboxyl groups - $5.8 \cdot 10^{-6}$ mol/g. MCC is negatively charged at pH range 3.0-10.0 due to its carboxyl groups. Its isoelectric point (IP) is lower than 4.0 [3].

Crystallized bovine trypsin (SPOFA) was analytical grade and its MM is 23.8 kDa. Its IP is 10.6. Trypsin molecules are positively charged in solutions at pH<IP, and negatively charged at pH>IP (see Scheme 1). The relative activity of the initial trypsin sample determined by Erlanger method [4] is 76 %.

Scheme 1. Charge dependence on pH



Adsorption interaction of MCC with trypsin in aqueous solutions and its desorption were carried out at pH range 4.2-12.0 as described elsewhere [5]. The initial ferment concentration (C_0) before adsorption as well as equilibrium trypsin concentration (C_{eq}) were estimated by Lowry method [6]. Adsorption values were calculated from difference between C_0 and C_{eq}. The quantity of desorbed trypsin was calculated in mass. % to the content of adsorbed ferment. The properties of the MCC-trypsin adsorbates in solid state were studied by FTIR and WAXS [1]. SEM study was performed to characterise changes on the MCC surface after adsorption of trypsin and its desorption. Samples were observed using raster electron microscope (Jeols JSM-35 CF).

Results and Discussion

Effect of pH of trypsin solutions on the adsorption values. Kinetics study

Dependence of adsorption values (AV, mg/g) on pH exhibits a hyperbola shape over all concentration range with a maximum at pH 8.0 - 8.5 (AV reaches 21.0 mg/g and 33.0 mg/g at C_0 2.3 mg/ml and 4.0 mg/ml, respectively). AV is markedly lower at pH 4.0 (AV=4.0 mg/g and 16.0 mg/g at C_0 2.3 mg/ml and 4.0 mg/ml, respectively) and at pH range 11.0-12.0 (AV= 3-4 mg/g and 13-16 mg/g at C_0 2.3 mg/ml and 4.0 mg/ml, respectively) (Fig. 1).

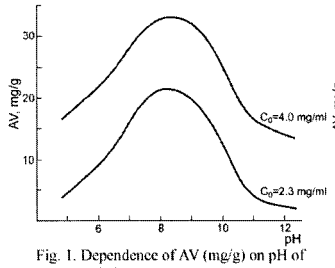


Fig. 1. Dependence of AV (mg/g) on pH of trypsin solutions

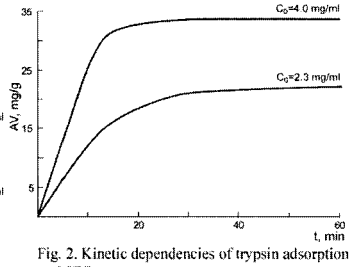


Fig. 2. Kinetic dependencies of trypsin adsorption to MCC

Kinetic dependencies of adsorption values of trypsin on MCC at different concentrations of trypsin initial solutions at pH 8.25 have a typical shape for adsorption processes taking place from solutions on porous matrices and consist of two main stages: the first one proceeds at a high rate and the second one proceeds at a slow rate (Fig. 2). The adsorption values depend on trypsin concentration. The maximum AV is 21 mg/g (at C_0=2.3 mg/ml) and 33 mg/g (at C_0 4.0 mg/ml), i.e. when C_0 increases 1.7 times AV increases 1.6 times.

Semilogarithmic adsorption anamorphoses confirm that this is two-stage process. Each stage is described by an equation of the pseudo-first-order reaction. The apparent rate constants of the first stage (K_1) are listed in Table 1. It can be seen that K_1 increases 1.1 times when C_0 of trypsin solutions increases 1.7 times. The K_1 value is lower than in the case of human serum albumin (HSA) adsorption to MCC [5].

Table 1. Maximum adsorption values, apparent rate constants, and Freundlich's constants of trypsin adsorption onto MCC

C_0, mg/ml	AV, mg/g	$K_1 \cdot 10_3$, min^{-1}	Freundlich's constants	
			1/n	$K_f 10_2$
2.3	21	7.5	1.24	6.53
4.0	33	8.5		

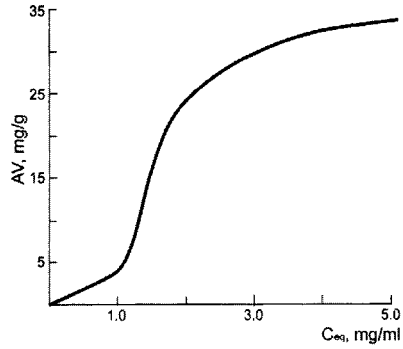


Fig. 3. Isotherm of trypsin adsorption to MCC

Isotherm of trypsin adsorption at pH 8.25 is given in Fig. 3. The AV is plotted against protein equilibrium concentration C_{eq}. Adsorption time was 24 hours. The adsorption isotherm does not exhibit a Langmuir's shape. In agreement with adsorption of other polymers and their complexes onto MCC [1, 5], it exhibits a pronounced S-shape, which is known to indicate the stepwise character of adsorption. The isotherm can be assigned to type IV, which characterises the combination of physical adsorption and chemisorption on the matrix and can not be described by Langmuir's equation but can be satisfactorily described by Freundlich's equation [1]. Freundlich's constants are listed in Table 1. Compare these constants with those obtained under adsorption of high molecular mass PVP [1] and HSA [5] from their solutions to the MCC, it can be seen that trypsin affinity (constant 1/n) to MCC is similar to that for PVP and higher than that for HSA. The relative ability of MCC to adsorb trypsin (constant K_f) is much higher than those for PVP and HSA.

Thus, complexes of MCC and trypsin under their adsorption interaction have been prepared.

Desorption of trypsin from the complexes

The ability of the complexes to emit trypsin into the solutions determines their properties as biologically active samples. It is important for their practical application.

The release values (RV) of trypsin under desorption at pH 2.0 and 8.25 depend on its concentration in the adsorbate: the higher the concentration, the higher the RV (Table 2). The maximum

RV is 69 mass. %. It is of interest that the retention of trypsin in cellulose matrix does not depend on pH solutions under desorption. Moreover, the content of ferment retained in cellulose matrix after desorption is a constant value (~10 mg/g, i.e. ~1 mass. %) and does not also depend on AV. It means that adsorption is partly reversible, and a part of adsorbed trypsin is strongly bound to the MCC matrix.

Table 2. Desorption of trypsin from the adsorbates and ferment activity in solutions after desorption

AV, mg/g	pH of solutions under desorption	RV, mass. %	Content of trypsin in matrix after desorption, mg/g	Solutions of trypsin after desorption	
				Concentration of trypsin, mg/ml	Relative activity of trypsin, %
21	2.0	51	9.8	0.34	16
33	2.0	69	10.2	0.76	-
21	8.25	52	9.6	0.30	18
33	8.25	69	10.2	0.70	18

The relative activity of trypsin in solutions does not almost depend on its concentration after desorption. This unexpected result should be explained in future. The relative activity is lower than that in the original trypsin sample. This can be due to the capacity of trypsin to autolysis in solutions. However, taking into account the high maximum RV of trypsin under desorption (69 mass. %) and its independence on pH, one should propose that trypsin can principally exhibit its ferment properties in both parts of the digestive tract of animals and people.

The FTIR study of the MCC-trypsin complexes

FTIR study of complexes MCC-trypsin reveals no changes in the chemical structure of the initial MCC. The most specific absorption bands in the protein spectra are located in the absorption range of 1640-1660 cm^{-1} (the amide I) and 1530 cm^{-1} (the amide II). FTIR spectra of the MCC (1), of the complex MCC-trypsin (2), of the initial trypsin in solid state (3), and of the subtraction spectrum of trypsin in the complex (4) in the fingerprint 1500-1775 cm^{-1} are given in Fig. 4. Compare spectra 3 and 4, it can be seen that there is no shift in the position of the absorption band in the range of 1532 cm^{-1}. The shape of this band is also not changed. This means that

conformational changes of ferment molecules are not occurred. It is known that adsorption of proteins also strongly depends on the shape of molecules. Trypsin molecules have a globular shape. The existence of globular structures decreases contact capacities of macromolecules and surfaces and leads to preferable solvent adsorption. Therefore, strong competition between the MCC, trypsin, and a solvent (water) appears to be in the adsorption process. It was established that the resulting complexes are the intercalates of MCC, trypsin,and water due to high hydrophilicity of cellulose matrix. The presence of retained wa-ter (which has an absorption band in the range of 1640-1660 cm^{-1}) as in the MCC matrix as in ferment after adsorption does not allow to make any definite conclusion on the changes of ferment absorption band at 1640-1660 cm^{-1}.

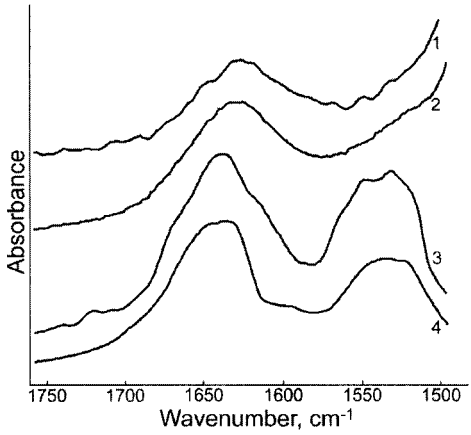


Fig. 4. FTIR spectra of the initial MCC (1), the complex MCC-trypsin (2), the initial trypsin in solid state (3), and a subtraction spectrum of trypsin in the complex (4) in the fingerprint 1500-1750 cm

Effect of the charge on the interaction of the MCC and trypsin macromolecules

The MCC The trypsin molecules are positively charged at pH range lower than its IP (pH 10.6) (see Scheme 1). The maximum AV under adsorption of trypsin to MCC is achieved at pH range 8.0-8.5. AV becomes much lower at pH range higher than IP of trypsin (pH 10.6) where its molecules are negatively charged. Thus, the adsorption of trypsin is determined by the charge of interacted molecules (at pH range lower than 8.5). This interaction can be performed via

electrostatic bonding between carboxylic groups of MCC and amine groups of ferment.

Effect of trypsin adsorption on the MCC supramolecular and morphological structure (WAXS and SEM data)

WAXS study performed on the samples of adsorbates and desorbates in solid state show that supramolecular structure of cellulose under adsorption and subsequent desorption is not changed. The structure of cellulose modification I and its crystallinity in the MCC-trypsincomplexes are similar to those of the initial MCC sample. However, one can conclude that the length of cellulose crystallites diminishes and their shape changes.

According to SEM data (Fig. 5.A-D), all complexes MCC-trypsin after trypsin adsorption to MCC reveal remarkable surface disordering compare to the initial MCC fibre. It can be concluded that trypsin deeply penetrates into the fibres. Thus, fibres of the initial MCC sample (5.A) treated with trypsin solution exhibit a loose shape with destroyed fibril structure on the surface (5.B and 5.C). This is especially noticeable in the complexes after partial desorption of adsorbed trypsin (5.D). This corresponds to the high release of trypsin under desorption. Consequently, the mutual effect of adsorption interaction between cellulose and trypsin is noticeable. Thus, some trypsin properties (for instance, its solubility) depend on adsorption process. This ferment becomes partly insoluble after adsorption onto the cellulose matrix. On the other hand, the strong effect of trypsin adsorption on the MCC morphological structure is shown by SEM study of the complexes MCC-trypsin.

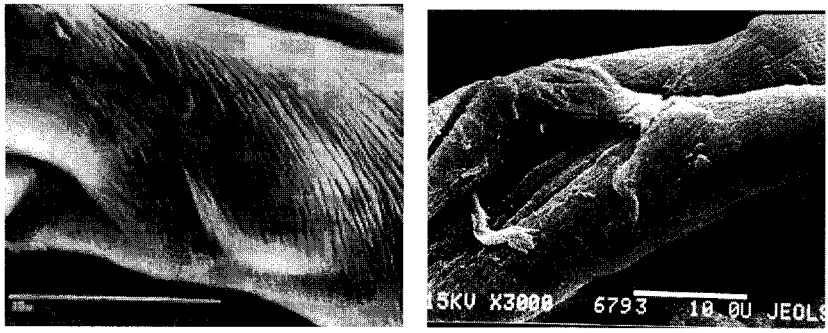


Fig. 5 (A-D). SEM micrographs of the initial MCC fibre (A) and the complexes MCC-trypsin (B, C - after trypsin adsorption to MCC; D - after trypsin desorption from the complexes)

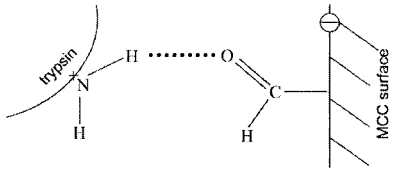
Mechanism of the trypsin interaction with cellulose matrix under adsorption

The correlation between the amount of ferment and cellulose in the complexes was estimated, taking into account AV of trypsin after adsorption to MCC matrix and subsequent desorption from the complexes (Table 3). It is seen that the amount of cellulose chains corresponding to 1mole of trypsin depends on the AV of trypsin. It has been already shown (Table 2) that the amount of trypsin retained in the MCC matrix after desorption is a constant value. This corresponds to 1 mole of ferment strongly bound to 77 moles of the MCC (Table 3, sample 5).
The MCC surface is highly hydrophilic because it is enriched with OH groups. This favours multipoint ionic interaction between the amino-and OH-groups. As a result, stable bonds (=ÑO(H)···H^+NR and =ÑO^-···H^+ NR) can be formed. Thus, three main ways for the MCC-trypsin complexes formation can be proposed. Stable bonds between MCC and trypsin are formed due to the interaction of ferment amine groups with the end aldehyde groups and surface carboxyl groups of MCC as well as with cellulose OH surface groups (Schemes 2-4).

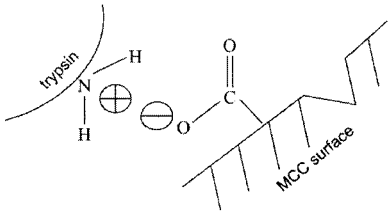
Table 3. Correlation between the amount of trypsin molecules, cellulose chains, and active groups of cellulose in adsorbates MCC-trypsin at different adsorption values

Number of sample	AV, mg/g	Amount of cellulose chains, aldehyde- and carboxyl- groups to 1 mole of trypsin		
		MCC chains	-HC=O groups	-COOH groups
1	3	256	191	45
2	13	59	59	11
3	21	36	36	7
4	33	23	23	4
5 (after desorption)	9.9	77	77	14

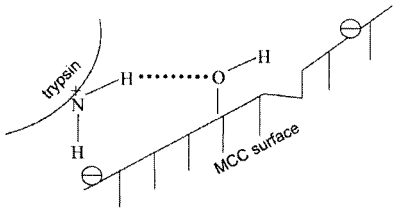
Scheme 2. Formation of aldimine bonds via interaction of trypsin amino-groups and the end aldehyde groups of MCC.



Scheme 3. Electrostatic interaction of trypsin amino- groups and surface carboxyl groups of MCC



Scheme 4. Formation of H-bonds via interaction of trypsin amino- groups and OH groups of MCC



The important conclusion of this study is that the strong competition between MCC, trypsin, and a solvent (water) occurs in the adsorption process.

Conclusion

1. Adsorption of trypsin to microcrystalline cellulose matrix has been determined as functions of ferment concentration and pH of its solutions. The maximum adsorption value is reached at the pH range 8.0-8.5, i.e. lower than the isoelectric point of trypsin. This indicates that interaction of trypsin with the MCC interface is controlled by the electrostatic effect.
2. The isotherm with respect to the adsorption of trypsin is S-shaped and described by Freundlich's equation.
3. Desorption data reveal that a part of adsorbed trypsin is strongly bound to the MCC matrix. Mechanisms of bonding of trypsin to cellulose matrix are proposed.

Acknowledgement

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