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Kinetic Studies on Enzymatic Hydrolysis of Cellulose(Ⅰ)
- Effect of Structural Features of Cellulose on Enzyme Adsorption -

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섬유소 가수분해반응에 관한 연구(Ⅰ)
- 효소흡착에 대한 섬유소의 구조적 특성-

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ABSTRACT

The structural properties of cellulose are significantly changed with the progress of hydrolysis reaction. The effects of changes on such properties of cellulosic substrate as crystallinity, accessibility of enzyme to the active site of cellulose surface, and particle size on the kinetics of enzymatic hydrolysis have been studied. Among those physical studies, the apparent surface active site of cellulose particle was found to have the most significant effect on the hydrolysis kinetics. Based on the experimental results, the adsorption affinity of enzyme and hydrolysis rate were mainly influenced by the surface roughness of cellulose particle. The extent of accessible active site may be expressed as the change of particle diameter. The Langmuir isotherm was proposed in terms of enzyme activity to explain the actual action of enzyme protein.

INTRODUCTION

The nature of the cellulolytic enzyme system employed determines the mode of action of cellulase, activity of each enzyme component, synergistic action among the enzyme components, and inhibitory effect on the enzyme action by the products(9, 13). The structural features of cellulose can be characterized by the crystallinity and the magnitudes of overall and reactive surface area of cellulose particles (2, 3). The interaction between the cellulase and cellulose involves the transfer of enzyme molecules from the bulk aqueous phase to the cellulose particles, enzyme-substrate complex formation by adsorption of enzyme onto cellulose, surface reaction promoted by the adsorbed enzyme, and aqueous phase reaction promoted by β-glucosidase(7).

Among these steps, the adsorption of cellulase on cellulosic substrate is very important. The adsorption of enz yme molecules on susceptible sites of cellulose surface is prerequisite step for sequent enzymatic hydrolysis reaction. For the hydrolysis reaction by cellulolytic enzyme direct physical contact between cellulase and its substrate is required (3, 6).

In this study the effect of structural features of cellulose such as crystallinity, particle size or surface area and other structural charcteristics(water retention volume and accessible active site) on enzymatic cellulose hydrolysis and enz yme adsorption was examined. The objective of this study is to develop a comprehensive kinetic model based on our

experimental observations.

MATERIALS AND METHODS

Enzyme

The cellulase enzyme used in this study was the commercial cellulase(ONOZUKA R-10, Yakult HONSHA Co.) derived from the culture filtrate of *Trichoderma viride* strain and contained appreciable activity of hemicellulase. The freeze-dried cellulase enzyme powder was diluted with buffer solution(pH 4.8, 0.05M citrate buffer) to desired concentration. Filter-paper activity was determined according to the method of Mandels, Andreotti and Roche(11).

Cellulosic Substrate

The pure cellulose material, Avicel PH 101, Avicel PH 102(FMC, Newark DE), Solka Floc BW 40(a ball milled pulp), and Solka Floc SW 40(a hammer milled pulp, Brown Co., Berlin NH) were used. The main substrates used were Solka Floc BW 40 and Avicel PH 101(microcrystalline cellulose). They were apportioned into several particle-size fractions by means of a Ro-Tap test sieve shaker. The different size fractions were tested to examine the differences in the hydrolysis reaction.

Determination of Structural Parameters

The crystallinity was measured by the powder method of x-ray diffraction using a Norelco diffractometer after the cellulose was solvent dried(6, 15).

The amount of enzyme adsorbed is a function of the number of available adsorption sites and, in turn, the amount of accessible surface area. A distinction must be made between accessible surface area and total surface area. Direct measurement of the accessible surface area is difficult. Therefore, a basic assumption in this study is that cellulose can be represented by a spherical particles(4, 16). The surface area of each swelled sample was calculated by means of the spherical particle diameter with water

Table 1. Major Structural Features of Various Fractionated Cellulose Substrates

Cellulose	wt %	WRV (g/g)	CrI %	Apparent* density g/cm³	Mean† particle size(μm)
Solka Floc BW 40					
control		4.45	77.24	1.030	133.81
60−120	66.5	5.02	78.70	1.053	187.50
120−170	13.1	4.25	78.41	1.050	106.50
170−200	16.6	4.18	76.98	1.050	81.00
200−270	2.8	3.95	74.08	1.100	63.50
270−325	1.0	3.52	71.86	1.110	48.50
Avicel PH 101					
control		3.02	84.00	1.428	54.97
120−170	17.7	2.90	81.62	1.254	106.50
170−200	15.4	2.98	83.11	1.308	81.00
200−270	13.1	3.09	83.27	1.383	63.50
270−325	17.6	3.14	85.28	1.420	48.50
325−400	36.2	3.28	86.43	1.513	40.50
Solka Floc SW 40			76.23		

*density in water

†vacuum dried cellulose

retention volume(WRV).

The water retention volume which is defined as a ratio of the weight of water retained per unit weight of the cellulose, after centrifugation, was also measured(8). These structural features of various cellulose fractions are listed in Table 1.

Adsorption Experiment

Cellulose samples were suspended 5ml of 0.05M citrate buffer(pH 4.8) and preincubated in a low-temperature incubator for 1 hr at 4℃. After preincubation 5ml of cellulase enzyme solution was added. The adsorption experiment was carried out in low-temperature incubator for 1 hr at 4℃ with vigorous skaking because the hydrolysis reaction does not take place at this low temperature. After centrifugation, the protein concentration and enzyme activity in the supernatant solution were measured.

Particle Size Distribution Analysis

Cellulose dried overnight at 80℃ was fractioned with different mesh size sieves(60−120, 120−170, 170−200, 200−270, 270−325, and 325−400 mesh). They were used as the substrate for this specific hydrolysis experiment. Particle diameter was determined by means of Tyler standard sieve scale.

Analytical Methods

The reducing sugar was measured as glucose by the dinitrosalicylic acid(DNS) method(12). Soluble protein in the aqueous phase was measured by the modified Lowry method using bovine serum albumin(Sigma chem. Co.) as a standard(10). To prevent the interference by reducing sugar, each sample was precipitated by acetone[1:3(v / v)] and redissolved in citrate buffer before analysis.

RESULTS AND DISCUSSION

Relationship between Adsorbed Soluble Protein and Enzyme Activity

Since the complex commercial cellulase powder derived from *Trichoderma reesei* was used in this work, some portion might be nonenzymatic soluble protein. Lee *et al.*(8) and Ryu *et al.*(14) stated that there is a difference

Table 2. Change of Enzyme Activity after Adsorption Process

BW 40		P_{free} mg p / ml	E_{cal} FPU / ml	E_{exp} FPU / ml	θ_A E_e / E_c	P_{ads} mg p / ml	$E_{a.cal}$ FPU / ml	$E_{a.pred}$ FPU / ml
	C_o							
P_o=0.20685	40	0.04737	0.02129	0.01346	0.6322	0.15848	0.04832	0.05584
mg pr / ml	20	0.09737	0.03514	0.02568	0.7303	0.10948	0.03775	0.04829
	10	0.11832	0.03966	0.02859	0.7208	0.08853	0.03323	0.04513
	5	0.12435	0.04312	0.03295	0.7641	0.07250	0.02977	0.04271
	P_o							
C_o=10.0	0.41	0.25367	0.06887	0.04561	0.6623	0.15633	0.04786	0.06852
mg / ml	0.1034	0.05045	0.02502	0.02430	0.5717	0.05297	0.02556	0.03306
Acicel PH 101								
	C_o							
P_o=0.20685	40	0.04821	0.02454	0.01577	0.6428	0.15860	0.04835	0.05572
mg pr / ml	20	0.08301	0.03204	0.02200	0.6864	0.12380	0.04084	0.05046
	10	0.10668	0.03715	0.02496	0.6720	0.10017	0.03574	0.04689
	5	0.12583	0.04128	0.02858	0.6922	0.08102	0.03161	0.04400
	P_o							
C_o=10.0	0.41	0.15689	0.06875	0.0454	0.6604	0.15690	0.04798	0.06860
mg / ml	0.1034	0.04067	0.02291	0.01308	0.5710	0.06275	0.02767	0.03454

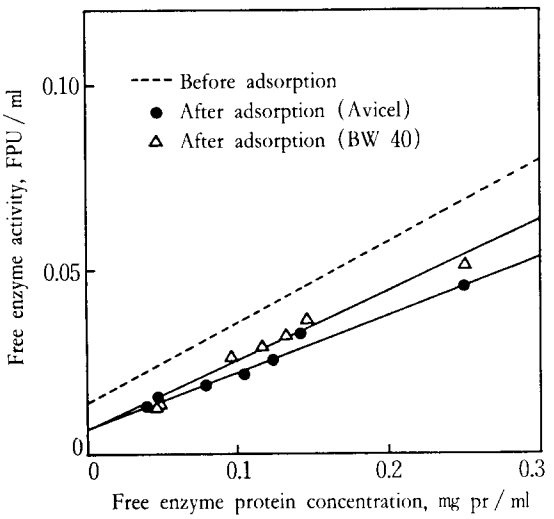

Fig. 1. Changes in enzyme activity of free enzyme protein at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml.

in adsorbabilities among the different portions of filtrate. Kim proposed that the concentration ratio of enzyme-protein and nonenzyme-protein might be proportional to total protein concentration(5). To estimate this difference, they measured the changes in the concentration of soluble protein and enzyme activity of the supernatant of cellulose suspension.

Table 2 and Fig. 1 illustrate the changes in the concentration of soluble protein and enzyme activity(FPA) of supernatant for various cellulose and enzyme concentrations on the initial adsorption. The filter-paper activity for soluble protein of supernatant decreased to 63−76% of the original level of supernatant soluble protein after initial adsorption. For example, the initial value of 0.03966 FPU / ml(0.3352 FPU / mg pr) of soluble protein reduced to 0.02859 FPU / ml(0.2416 FPU / mg pr) immediately after enzyme was added to the flasks containing cellulose. Therefore, the amount of soluble protein adsorbed cannot represent the actual enzyme activity.

According to the results, the actual adsorbed enzyme activity could be estimated by the fraction of relative adsorbability of enzyme protein. Enzyme activity of free enzyme protein decreased with the fraction of relative adsorbability θ_A because the enzymatic portion of soluble protein could be more readily adsorbed than the nonenzymatic portion.

Adsorption of Celluase Enzyme on Cellulose Effect of Structural Parameters on the Adsorption

A. Constant Enzyme Concentration

In order to investigate the effect of structural parameters on the adsorption further, the adsorption isotherms of fractionated celluloses were obtained(see Fig. 2 and 3). The following Langmuir type adsorption isotherm(6, 9) was used to relate the amount of adsorbed protein, P_{ads}, and the substrate concentration, So:

$$\theta_S=\frac{P_{ads}}{P_o}=\alpha_D\frac{S_o}{K_{ps}+S_o} \quad \text{or} \quad P_{ads}=\frac{V_{ps}\,S_o}{K_{ps}+S_o} \qquad (1)$$

Adsorption parameters, V_{ps} and K_{ps}, were determined from the nonlinear regression of experimental data since the double reciprocal plot of eq.(1) did not yield straight line. It is expected that Langmuir isotherm cannot exactly describe this system due to enzyme multiplicity, water

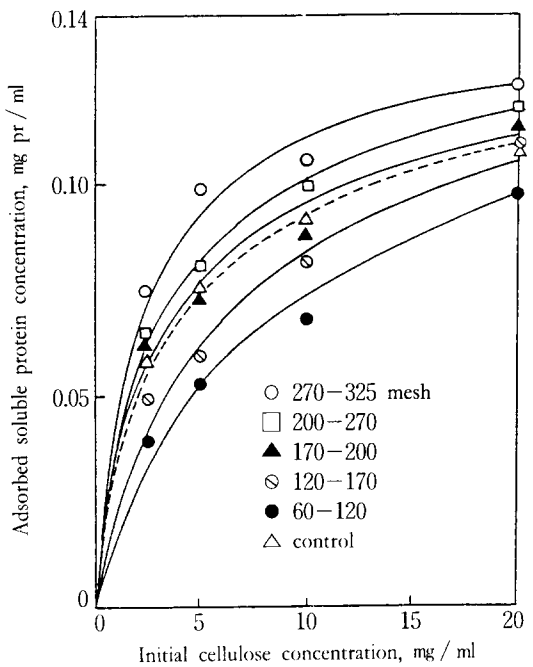

Fig. 2. Effect of particle size of cellulose(BW 40) on the adsorption at E_o=0.20685 mg pr / ml.

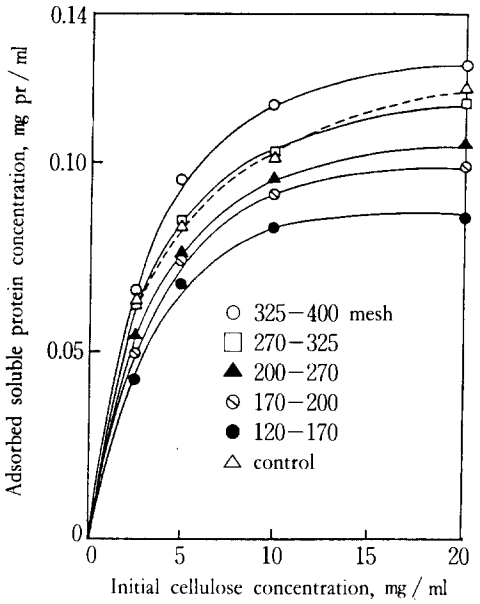

Fig. 3. Effect of particle size of cellulose(Avicel PH 101) on the adsorption at E_o=0.2 0685 mg pr / ml.

retention effect of cellulose and other effects.

Table 3 shows the values of adsorption parameters(V_{ps}, K_{ps}) and corresponding structural parameters for the standard fractionated substrate and the various cellulose. It was observed that the maximum adsorption constants for Solka Floc BW 40 were nearly constant whereas the half-saturation constants were dependent upon the crystallinity and surface area. However, the maximum adsorption constants and the half-saturation constants for Avicel PH 101 follow the inverse trends.

For Solka Floc BW 40(Fig. 2), the extent of adsorbed protein increases as particle size and crystallinity decrease. However, for Avicel PH 101(Fig. 3), the enzyme protein adsorbed reached its maximum as the substrate concentration increased due to the saturation of accessible surface sites in spite of the crystallinity increase with the particle size decrease.

Fig. 4 shown that the extent of adsorbed protein of both Solka Floc BW 40 and Avicel PH 101 increases as particle size decreases although these crystallinities follow the inverse trend. Fig. 5 also depicts that the extent of

Table 3. Adsorption Parameters and Structural Parameters for Various Fractionated Cellulosic Materials*

Cellulose adsorbents		Adsorption parameters				Structural parameters	
Cellulose	Mesh size	V_{ps} mg pr / ml	K_{ps} mg cellulose / ml	V_p mg pr / ml	K_p -1 (mg pr / ml)	CrIo %	Mean particle size, μm
Solka Floc BW 40	control	0.1257	3.2596	0.1273	20.0324	77.24	133.81
	60−20	0.1275	6.6428	0.1238	18.0058	78.70	187.50
	120−170	0.1449	6.5383	0.1267	25.6949	78.41	106.50
	170−200	0.1238	3.0368	0.1440	22.9991	76.98	81.00
	200−270	0.1318	2.8393	0.1472	31.4096	74.08	63.50
	270−325	0.1308	1.7892	0.1539	53.8508	71.86	48.50
Avicel PH 101	control	0.1339	3.3358	0.2080	10.6420	84.00	54.97
	120−170	0.1016	2.8973	0.2015	4.2801	81.62	106.50
	170−200	0.1170	3.0994	0.2077	5.5386	83.11	81.00
	200−270	0.1244	3.2590	0.1867	10.0719	83.27	63.50
	270−325	0.1319	2.7414	0.2082	10.0400	85.28	48.50
	325−400	0.1463	2.7812	0.2313	10.5933	86.43	40.50

*based on soluble protein concentration(mg pr / ml)

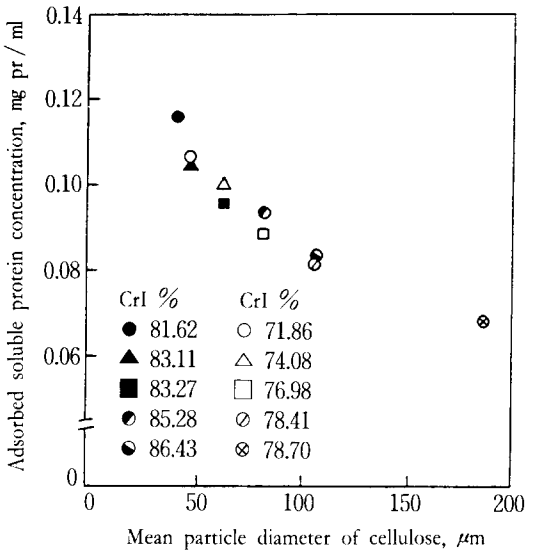

Fig. 4. Effect of particle size of cellulose on the adsorption at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml, (○△□⊘⊗) BW 40, (●▲■◐◒) Avicel PH 101.

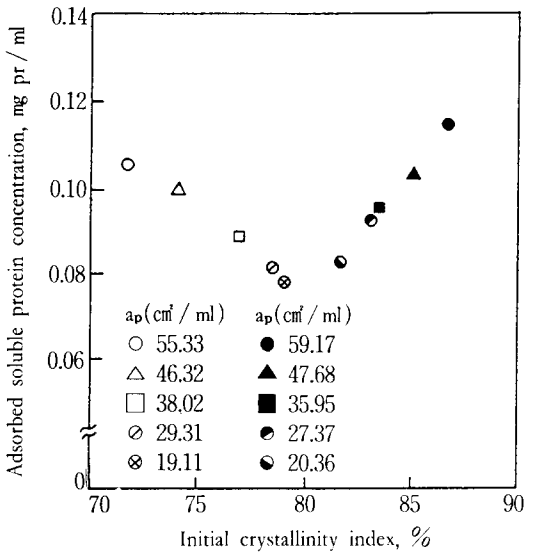

Fig. 5. Effect of initial crystallinity index on the adsorption at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml, (○△□⊘⊗) BW 40, (●▲■◐◒) Avicel PH 101.

adsorbed protein of various cellulose, which had a similar surface area with widely different crystallinity. Thus it appears that the crystallinity may not significantly affect the adsorption as reported by some workers(1).

B. Constant Cellulose Concentration

At a constant cellulose concentration(10mg / ml), adsorption isotherms for cellulase enzyme were obtained(Figs. 6 and 7). A Langmuir isotherm type equation was used to fit data. The adsorption of enzyme can be described as the following:

$$\theta_p = \frac{P_{ads}}{P_{ads\cdot m}} = \frac{K_pP}{1+K_pP}$$

$$\text{or } P_{ads} = \frac{V_pK_pP}{1+K_pP} \quad (2)$$

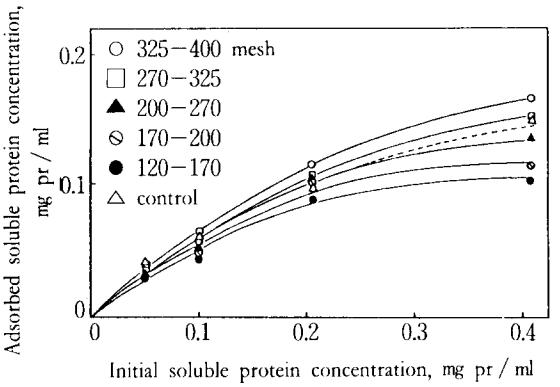

Fig. 6. Effect of particle size of Avicel PH 101 on the adsorption at C_o=10 mg / ml.

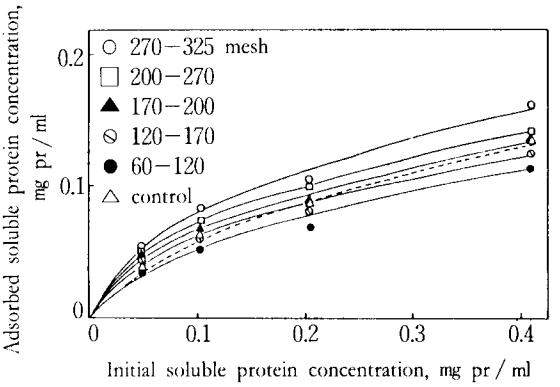

Fig. 7. Effect of particle size of Solka Floc BW 40 on the adsorption at C_o=10 mg / ml.

Fig. 6 and 7 show that the extent of adsorbed protien increased as the particle size decreased due to surface area increase. However, the difference of the extent of adsorbed enzyme protein was not significantly increased, because the accessible surface site of substrate is not proportional to the surface area of substrate.(see Fig. 8) V_p and K_p for Solka Floc BW 40 were increased with decreasing crystallinity and particle size whereas the adsorption parameters for Avicel PH 101 were nearly constant with changing crystallinity and particle size.(See Table 3)

Evaluation of apparent adsorption affinity on the cellulose surface

Enzyme protein concentration will be expressed in activity units(FPU / ml), because enzyme action in hydrolysis reaction was represented to enzyme activity(16). According to its concept, eq.(2) is expressed as the following;

$$E_{ads} = \frac{V_p' E}{K_p' + E} \quad (3)$$

Fig. 9 and 10 show Langmuir type adsorption of the adsorbed enzyme activity with respect to the free enzyme

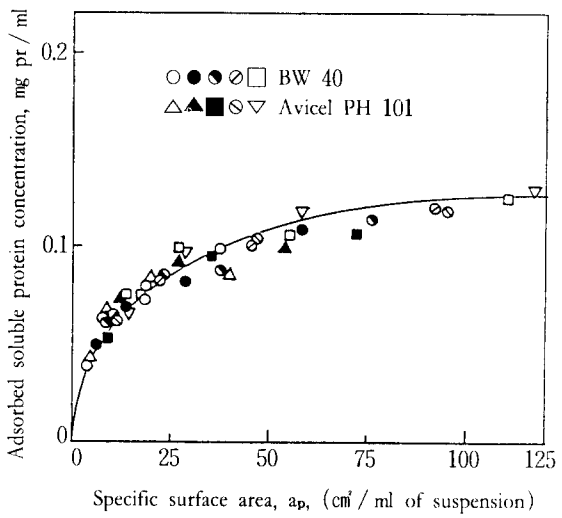

Fig. 8. Effect of surface area of various cellulose on the adsorption at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml.

Table 4. Adsorption Affinity Parameters for Various Fractionated Cellulosic Materials*

Cellulose Adsorbents	Adsorption Affinity Parameters				Structural Parameters	
	V_p' FPU / ml	K_p' FPU / ml	k_n FPU / cm² ×10²	k'' FPU / cm² ×10³	a_p (cm² / ml)	CrIo %
BW 40						
control	0.14583	0.06098	0.600	2.5087	24.31	77.24
60−120	0.10338	0.04738	0.541	2.4793	19.11	78.70
120−170	0.11335	0.03838	0.3867	1.359	29.31	78.41
170−200	0.12731	0.04052	0.3348	1.0657	38.02	76.98
200−270	0.12020	0.03286	0.2595	0.70935	46.32	74.08
270−325	0.11037	0.02077	0.1995	0.3754	55.33	71.86
Avicel PH 101						
control	0.13148	0.04023	0.3147	0.9630	41.78	84.00
120−170	0.18353	0.10867	0.9014	5.3373	20.36	81.62
170−200	0.14068	0.05000	0.5141	1.8271	27.37	83.11
200−270	0.14282	0.04127	0.3973	1.1480	35.95	83.27
270−325	0.13418	0.03128	0.2814	0.6560	47.68	85.28
325−400	0.12717	0.02376	0.2149	0.4015	59.17	86.43

*based on the enzyme activity(FPU / ml)

activity.

It was assumed that these adsorption parameters will be dependent on the specific active site of cellulose. To describe the effect of the accessible surface active sites, it was also assumed that V_p' and K_p' were a function of the accessible surface site for enzyme activity because these values were expressed as the adsorption affinity of enzyme on the cellulosic substrate.

Therefore, the maximum adsorbed enzyme concentration and the equilibrium constant can be expressed as the following: $V_p' = K_n a_p$, $K_p' = K'' a_p$ (4)

Table 4 shows that the apparent adsorption affinity

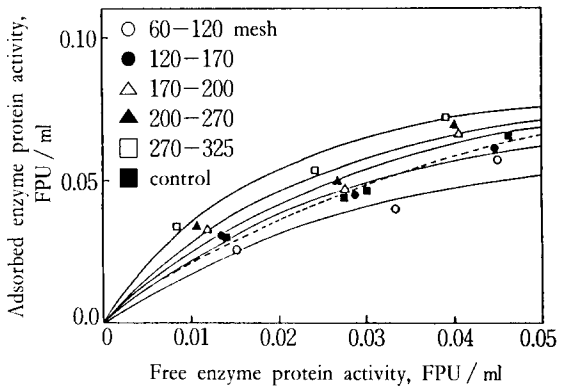

Fig. 9. Effect of particle size of cellulose(BW 40) on the adsorption at C_o=10 mg / ml.

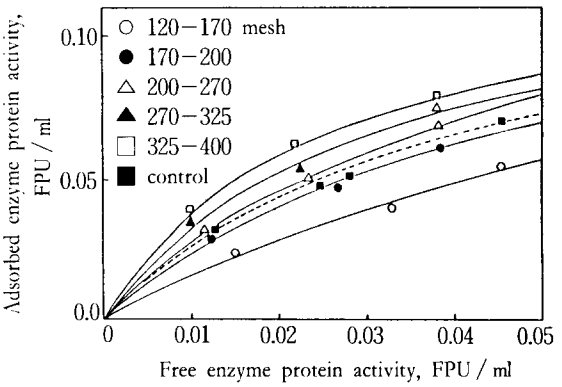

Fig. 10. Effect of particle size of cellulose(Avicel PH 101) on the adsorption at C_o=10 mg / ml.

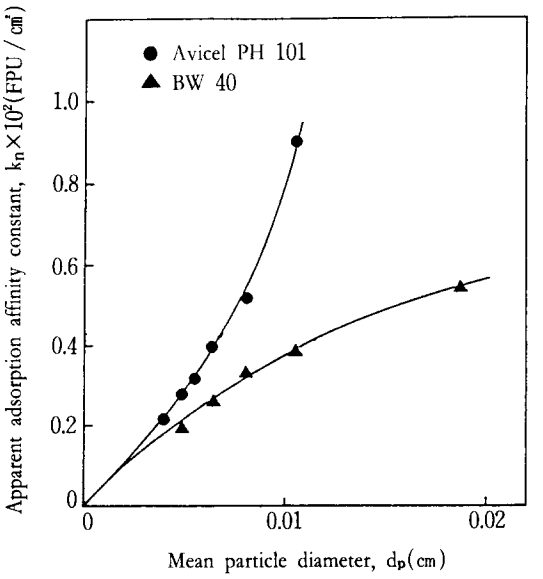

Fig. 11. Changes in maximum adsorption affinity for mean particle size of cellulose.

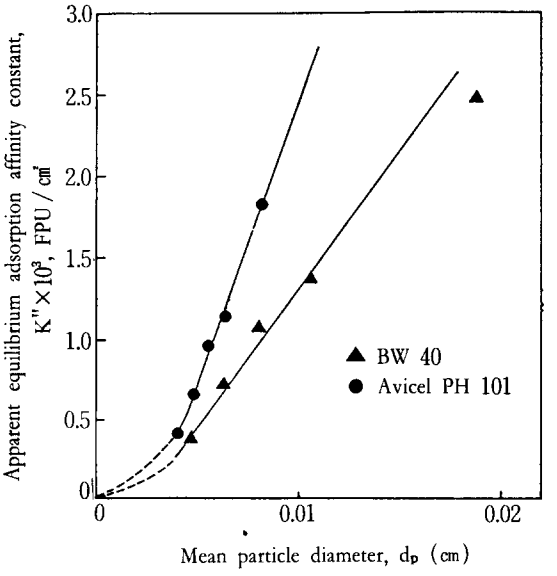

Fig. 12. Change in equilibrium adsorption affinity for mean particle size of cellulose.

parameters decrease as the particle diameter decreases. Fig. 11 and 12 show that both Solka Floc BW 40 and Avicel PH 101 represent the distinct pattern, The profile of K_n of Avicel PH 101 is more sharply increased than Solka Floc BW 40. Otherwise, K" is represented by a simple linear plot with particle size in this particle diameter regions. From the curves of Fig. 11 and 12, for BW 40

$K_n=0.47894\ d_{po}-10.118\ d_{po}^2$, $K''=0.1693\ d_{po}-0.00039$, (5)

and for Avicel PH 101,

$K_n=0.27646\ d_{po}+50.878\ d_{po}^2$, $K''=0.3536\ d_{po}-0.00106$ (6)

It will be described that the large particles of cellulose are more accessible and rougher than the small ones. The small difference of adsorption of the different sized substrate particle will be explained by the fact that there are the interaction among the structural features, fragmentation of large particles and probably manufacturing method. Since the adsorption is a surface phenomenon, the bulk adsorbent concentration cannot describe the effective adsorbent concentration or the adsorption site of cellulose adsorbent.

Therefore from the adsorption experimental data of fractionated cellulose, the actual adsorbed enzyme protein was estimated by means of relative adsorbability of enzymatic portion of soluble protein and the apparent adsorption affinity parameters suggested in this work.

According to the result obtained, the active site of substrate is not proportional to the surface area of substrate and probably depends on the surface roughness(edges, projections, etc). In this work, the adsorption affinity parameters(K_n and K") will probably determine the level of the surface roughness or accessiblity of active site.

요 약

본 연구에서는 섬유소물질의 구조변화, 섬유소물질의 결정성, 표면적 및 입자의 크기와 그에 따른 섬유소의 accessible active site 변화등 물리적 특성이 효소 흡착에 미치는 영향을 연구하였다. 정제하지 않은 섬유소 분해 효소에는 흡착성 protein과 비흡착성 protein이 포함되어 있음을 실험적으로 알 수 있었으며, 따라서 실제 흡착효소의 활성도를 추정할 수 있었고, 섬유소에 대한 흡착된 실제효소의 활성을 나타내기 위하여 효소의 활성도로 Langmuir isotherm을 표현할 수 있었다. 섬유소의 구조변화에 따른 효소의 흡착현상은 섬유소물질의 결정성, 표면적, 입자크기등에 상호 연관적으로 영향을 받으며 특히, 섬유소의 표면적과 입자크기의 영향이 크므로 이들 구조적 특성을 섬유소의 accessible active site로 고려하여 섬유소 입자크기 변화에 따라 효소 활성도의 섬유소에 대한 흡착 친화도를 결정하였다. 섬유소의 accessible active site는 섬유소 표면의 roughness에 큰 영향을 받는 것으로 보여진다.

NOMENCLATURE

a_p: Equivalent spherical interfacial surface area (cm^2/ml)
C_o: Initial cellulose concentration (mg / ml)
CrI_o: Initial crystallinity index (%)
d_{po}: Initial surface mean particle diameter (μm)
E_{free}, E: Free enzyme activity (FPU / ml)
E_{free}': Actual free enzyme activity (FPU / ml)
$E_{a,\ cal}$: Adsorbed enzyme activity obtained from Fig. 1 (FPU / ml)
E_{ads}, $E_{a,pred}$: Actual adsorbed enzyme activity (FPU / ml)
E_{cal}, E_o: Free enzyme activity obtained from Fig. 1 (FPU / ml)
E_{exp}, E_e: Free enzyme activity experimentally measured (FPU / ml)
E_o: Initial enzyme activity (FPU / ml)
K": Apparent equilibrium adsorption affinity constant (FPU / cm^2)
K_n: Apparent maximum adsorption affinity constant (FPU / cm^2)
K_p: Half-saturation constant for protein $(mg\ pr / ml)^{-1}$
K_p': Equilibrium constant for enzyme activity (FPU / ml)
K_{ps}: Half-saturation constant for substrate (mg of substrate / ml)
P: Soluble protein concentration (mg pr / ml)
P_{ads}: Adsorbed soluble protein concentration (mg pr / ml)
$P_{ads,\ m}$: Maximum adsorbed soluble protein concentration (mg pr / ml)
P_{free}: Free soluble protein concentration (mg pr / ml)
P_o: Initial soluble protein concentration (mg pr / ml)
S_o: Initial substrate concentration (mg pr / ml)
V_p: Maximum amount of protein for protein (mg pr / ml)
V_p': Maximum adsorbed enzyme concentration (FPU / ml)
V_{ps}: Maximum protein adsorption constant for substrate (mg pr / ml)
WRV: Water retention volume (g / g)

t: Hydrolysis reaction time, or incubation time (hr^{-1})

GREEK LETTERS

α_D: Cellulase fraction that is adsorbed

θ_A: Fraction of relative adsorbability of free enzyme protein

θ_S: Fraction of the surface coverage of substrate

θ_P: Portion of the surface substrate occupied by enzyme

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