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Interaction Between Indigo and Adsorbed Protein as a Major Factor Causing Backstaining During Cellulase Treatment of Cotton Fabrics

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ABSTRACT

A model microassay system was developed to measure indigo backstaining on cotton fabrics in the presence of enzymes on a small laboratory scale. Backstaining indexes for 11 cellulase samples were measured, and the enzymes were ranked from lower to higher backstaining. Two multienzyme cellulase preparations were separated into fractions using chromatofocusing on a Mono P column. Adsorption ability and backstaining properties of purified enzyme fractions were studied. Evidence was obtained that protein adsorption on cotton fabrics is a crucial parameter causing backstaining (both for crude cellulase samples and purified enzyme components).

Index Entries: Cellulase; adsorption; textile, denim; stone washing; indigo; backstaining; microassay.

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INTRODUCTION

In the past decade, cellulases have been widely used for denim garment treatment as an alternative to the stone-washing process, in order to achieve an aged look for denim fabrics *(1–3)*. One of the problems arising during the stone washing/enzyme treatment procedure is backstaining. As a result of the treatment, indigo dye is washed out of the blue yarn in denim, and it may stain the white yarn. This diminishes the garment's look for consumers. Recently, Cavaco-Paulo et al. *(4)* have shown that the high ability of cellulase enzyme protein to bind cotton cellulose is the major issue that causes backstaining.

In this paper, the authors developed a model indigo backstaining microassay, in which the white cotton fabric was used as a model of denim's white yarn to measure the level of its staining with indigo. Using the microassay, relationships between adsorption ability of different cellulase samples and indigo backstaining were studied. In this study, the term "staining" would more precisely describe the situation because indigo dye comes to the cotton fabric from the outer suspension (not from the same garment, as in a real process of denim treatment with enzyme). However, in most cases, the term "backstaining" will be used (just to show that the microassay system is a model for a real backstaining phenomenon).

MATERIALS AND METHODS

Enzymes

Eleven laboratory and commercial cellulase preparations were used in the study. Specific activities of the enzymes are given in Table 1. Numeration of cellulase samples in the text corresponds to their numeration in Table 1.

Cellulase Activity Assays

Cellulase activity toward insoluble cellulose was analyzed with the filter-paper assay (FPA), recommended by International Union of Pure and Applied Chemistry (IUPAC) *(5)*, using Whatman No. 1 filter paper. Endoglucanase activity was determined by the viscometric method *(6)*, at pH 4.5 and 40°C, using carboxymethylcellulose (CMC, medium viscosity, Sigma, St. Louis, MO) as a substrate. CMCase activity was measured by assaying reducing sugars released after 5 min of enzyme reaction with CMC (5 g/L) at pH 5.0 and 50°C *(7)*. Avicelase activity was determined by analyzing reducing sugars released after 5 min of enzyme reaction with 5 g/L Avicel cellulose (Serva, Heidelberg, Germany) at pH 5.0 and 40°C *(7)*. Reducing sugars were analyzed by the Somogyi-Nelson method *(8)*. All

Table 1
Specific Activities of Cellulase Preparations Used in the Study

Sample no.	Source, pH region for use	Activity (U/mL or U/g)			
		FPA	Endoglucanase	CMCase	Avicelase
1	Laboratory, acid	18	321	1100	15
2	Laboratory, acid	25	382	733	30
3	Laboratory, acid	26	374	1198	27
4	Laboratory, acid	25	242	937	15
5	Commercial, acid	129	1569	4968	182
6	Commercial, acid	52	1772	21,480	52
7	Commercial, acid	68	94	904	84
8	Commercial, acid	98	532	6288	115
9	Commercial, acid	22	847	1351	3
10	Commercial, neutral	11	243	134	14
11	Commercial, neutral	16	272	1240	8

activities were expressed in international units, where 1 U of activity corresponds to the quantity of enzyme hydrolyzing 1 μmol of glucoside bonds/1 min.

Determination of Adsorption Distribution Coefficients

Adsorption distribution coefficients (K_d) for cellulase preparations were determined with Avicel cellulose at 50°C and pH 5.0 *(7)*. K_d was calculated as a slope of straight line in coordinates E_o/E_s against cellulose concentration, where E_o is initial protein concentration before adsorption, and E_s is protein concentration in solution after adsorption. Protein was analyzed by the Lowry method *(9)*, using bovine serum albumin as a standard.

Fractionation of Cellulase Multienzyme System into Components Before and After Adsorption on Cellulose

Adsorption of cellulase sample nos. 1 and 5 on Avicel cellulose (50 g/L) was carried out at 50°C and pH 5.0. Initial protein loading for the both enzyme samples, measured by the Lowry method *(9)*, was 16 mg/g cellulose. After agitation of the suspension for 15 min, a fraction of nonadsorbed proteins was separated by centrifugation.

Original solutions of cellulase sample nos. 1 and 5 (before adsorption) and supernatants, obtained after adsorption of the cellulase samples on Avicel, were desalted using a Bio-Gel P-6 column (Bio-Rad, Hercules, CA) equilibrated with 25 m*M* Imidazole-HCl buffer (pH 7.2). Desalted enzyme solutions were separated into components using chromatofocusing on a

Mono P HR 5/20 column (Pharmacia, Uppsala, Sweden) using a Pharmacia FPLC system. For no. 1 sample, elution was carried out with 40 mL diluted (1:10) polybuffer 74-HCl (gradient of pH from 7.4–4.0), and then with 15 mL of the diluted polybuffer (gradient of pH from 4.0 to 3.0). For no. 5 sample, elution was carried out with 50 mL of the diluted polybuffer 74-HCl (gradient of pH from 7.2 to 4.0), and then with 20 mL of the polybuffer (gradient of pH from 4.0 to 3.0). The flow rate was 0.9 mL/min. Protein concentration at the column outlet was monitored photometrically at 280 nm, using an UV detector. The elution profiles of enzyme solutions before and after adsorption on Avicel were used to quantify the adsorption degree of each enzyme component from sample nos. 1 and 5 (Figs. 6 and 7).

Cotton Fabric

White desized cotton mitcale swatches (10 × 10 in.) were used as a testing material. The initial swatch was cut into smaller square swatches (3.5 × 3.5 cm) for use in the staining experiments. The weight of one small swatch was 129 ± 2 mg.

Indigo

Three types of indigo were used in the experiments. The first was indigo from Sojuzchimexport (Moscow, Russia). The second was indigo from Sigma with dye content of 65%, according to Sigma certificate. The third type of indigo was produced by Buffalo Color (Parsippany, NJ) which contained 95% dye and 2% wetting agent, according to specification.

Visually, indigo samples were different. Buffalo indigo looked like a fine powder. Russian indigo had a wide distribution of particle size consisting of both small particles (looking like a powder) and relatively large crumbly aggregates (0.3–0.6 mm). Sigma indigo had a rather narrow particle-size distribution, and consisted of spherical particles (0.2–0.3 mm in diameter). During a preparation of dye suspension in water, Sigma indigo was dispersed more easily than Russian or Buffalo indigo, and gave more stable suspension. Some foaming could be observed during/after agitation of Sigma indigo suspension on a magnetic stirrer. Sigma indigo probably contained some substance that acted as a surfactant.

Preparation of Indigo Stock Suspension

Two hundred fifty mg indigo were added to 50 mL distilled water in a flask and stirred vigorously for 15–20 min on a magnetic stirrer at room temperature. Before taking aliquots for staining experiments, the suspension was placed into a Bandelin Sonorex TK52 ultrasonic bath (Berlin, Germany) for 5 min to destroy large indigo aggregates, and to make the

suspension more uniform. Two-mL aliquots were taken for staining experiments (*see* below) under magnetic stirring, in order to provide uniform indigo suspension in the reaction mixture.

Staining Procedure

A piece of white garment (3.5 × 3.5 cm) was placed into a 250-mL glass containing 18 mL enzyme solution in 0.1 *M* Na-acetate or phosphate buffer having appropriate pH, and incubated at room temperature for 10 min. Then 2 mL indigo stock suspension (5 mg/mL) were added, and the glass was placed into a water bath (50°C) on a shaker (300 rpm) and agitated for 30 min. The swatch was removed with pincers and rinsed for 5 min with 50 mL distilled water at 50°C (on a shaker at 300 rpm) 2×, then it was dried at room temperature overnight. For each enzyme sample, the assay procedure was carried out in three repetitions. Typically, 1.5 CMCase U/mL of reaction system were used when crude enzyme samples were tested. In the case of enzyme samples not possessing CMCase activity (or in the case of purified enzymes), the conditions were equilibrated by protein concentration (0.05 mg/mL).

Image Processing

Stained swatches were scanned at 300 dpi resolution on a MFS-12000SP ("Mustek," Irvine, CA) scanner. Two images of the front side and two images of the back side (one direct and one rotated 90 degrees) were obtained for each sample. Images were processed using an Adobe Photoshop 4.0 software. A histogram of color intensities was calculated for each sample, using a blue channel, and a percentile at level 120 was used as a criterion of backstaining. An average value and standard deviation were calculated for each enzyme preparation under testing. Backstaining index (BSI) was expressed in relative units (RU): The higher the value, the higher was backstaining.

Minimized Microassay Procedure

Standard indigo backstaining microassay procedure, described above, required rather large enzyme quantities (typically, 2–5 mg of protein for one assay performed with 3.5 × 3.5 cm swatches in three repetitions for statistics). When purification of individual enzyme components was performed on a laboratory scale and it was necessary to assay the BSI for purified enzyme, much lower quantities of the enzyme were available, as a rule. In that case, a modification of the method was applied, in which smaller swatches of the standard fabric (1.4 × 1.4 cm) were used in the assay; the total volume of indigo suspension was 2 mL. In the minimized

microassay, all procedures were performed as in the standard method (*see* Staining Procedure section), except that the assay was carried out in small cylindrical vials (2.2 cm diameter and 5 cm height), and all the volumes were 10× lower. The minimized microassay procedure required 10× less amounts of protein (0.2–0.5 mg).

RESULTS AND DISCUSSION

Backstaining for Different Cellulase Preparations with Indigo from Different Sources

Figure 1 shows BSIs obtained with Russian indigo for different cellulase preparations. The enzymes differed significantly in the BSI. In general, enzyme samples can be roughly divided into three groups characterized by low (<40 RU), moderate (40–70 RU), and high (>70 RU) BSI.

Data on backstaining levels for six cellulase samples, obtained as Datacolor measurements on a Datacolor 3890 instrument after full-scale processes of enzymatic treatment of denim garment in a washing machine, were compared to the results of the microassay. Figure 2 shows the relationship between those data and BSI data. Correlation coefficient between the two methods was found to be 0.91. Such a good correlation indicates that the model microassay can predict the backstaining levels observed on a larger scale under real conditions of enzymatic treatment of denim garment.

A comparison of indigo from different sources was then carried out using three selected cellulase samples with low, moderate, and high BSI (Fig. 3). Acetate buffer (0.09 *M*, pH 5.0) without an enzyme was used as a control in the comparison study. For all enzyme preparations, the BSI decreased in the following order: Russian indigo > Buffalo indigo > Sigma indigo. However, in the absence of enzyme (when acetate buffer was used), the situation was opposite: Russian indigo < Buffalo indigo < Sigma indigo. Extremely high BSI for the buffer with Sigma indigo can be explained by the presence of formulation wetting component(s) in this indigo sample (dye content in this preparation was only 65%, according to specification). Buffalo indigo contained 2% wetting agent, according to certificate. This may explain the moderate BSI for the buffer with Buffalo indigo (Fig. 3), which was higher than the BSI obtained with Russian indigo, which did not contain any formulation or wetting components.

Although absolute BSI values for a particular enzyme preparation were different with different types of indigo, when several enzyme samples were tested in a row, they could be ranked from lower to higher backstaining with either type of indigo (in the same order as in Fig. 1). In subsequent experiments, the authors used either Russian or Sigma indigo, or both.

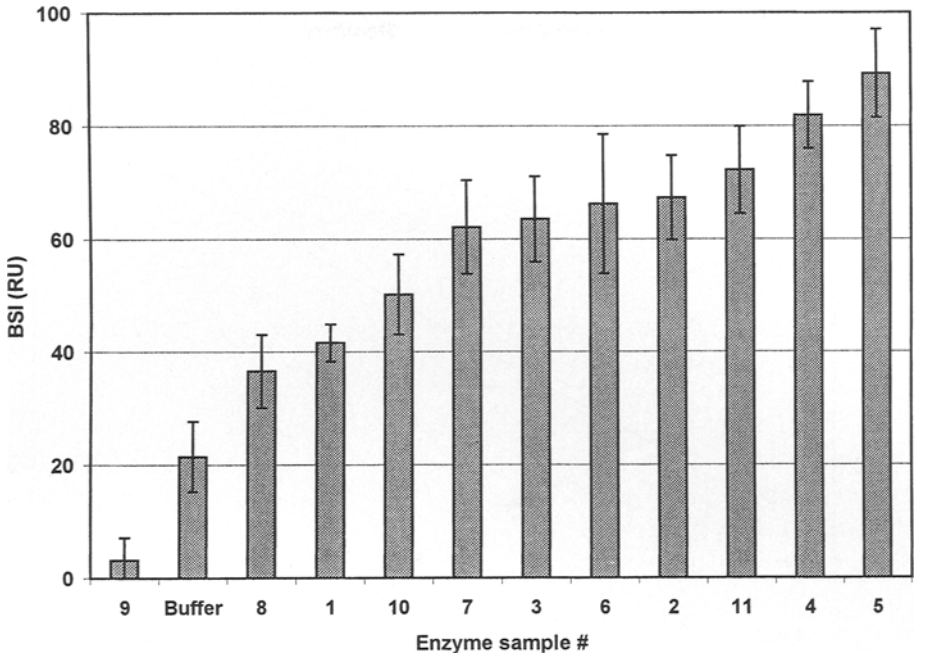

Fig. 1. Backstaining obtained with Russian indigo for different cellulase samples, pH 5.0.

Figure 4 shows the effect of pH on BSI of different cellulase preparations. Backstaining for the sample of neutral cellulase (no. 11) decreased with increase in pH. This is in good agreement with the literature data *(1,4)*. However, in the case of acid cellulase sample (no. 5), as well as in the case of buffers without an enzyme, the BSI increased on transition from acid to neutral pH region. This is in some disagreement with a point of view that backstaining is minimal in neutral pH region *(1,4)*. The disagreement may be explained by different assay conditions. In a real enzyme biowashing process, activity of acid cellulases decreases dramatically with increase in pH from 5.0 to 7.0 resulting in a significant decrease of efficiency of indigo removal (reduction of indigo concentration in the outer solution) at neutral pH *(1)*. Lower indigo concentration causes less backstaining. In the authors' model microassay system, indigo concentration in the outer suspension was always constant. This may explain unusual behavior of the acid cellulase sample no. 5 in Fig. 4.

Some bends at pH 6.0 for the cellulase no. 1 sample and for the buffers in Fig. 4 can be explained by changing the chemical nature of buffer: Acetate buffer was used for creating pH 5.0, and phosphate buffers were used for creating pH 6.0–8.0.

Because, in this paper, the authors worked mostly with acid cellulases, pH 5.0 was used in further experiments.

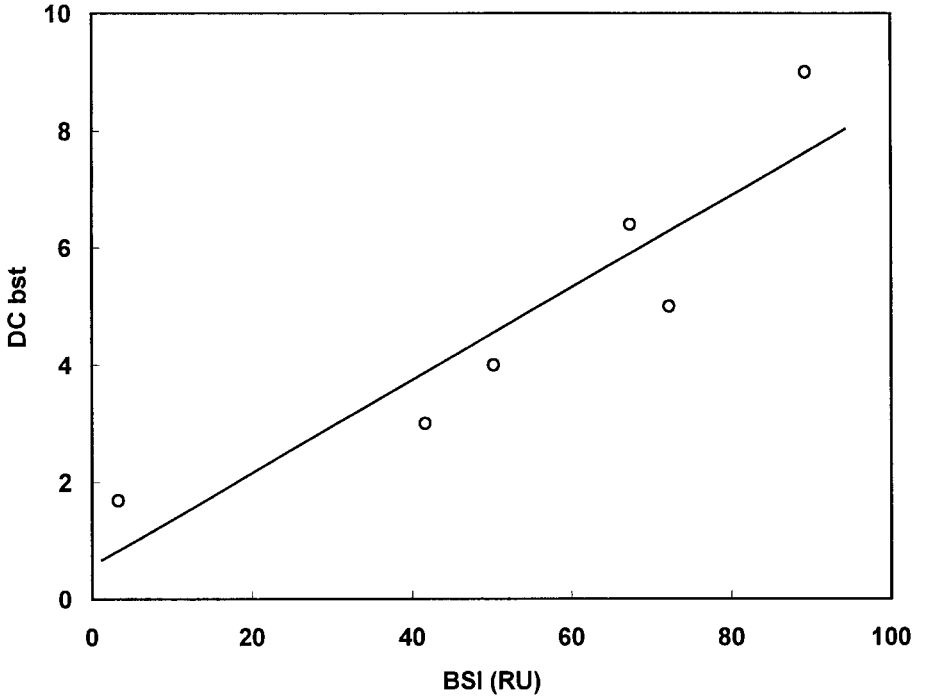

Fig. 2. Relationship between the BSI determined with the model microassay developed in this article and backstaining measured on a Datacolor 3890 Instrument (DC bst) after enzyme treatment of denim garment in a washing machine.

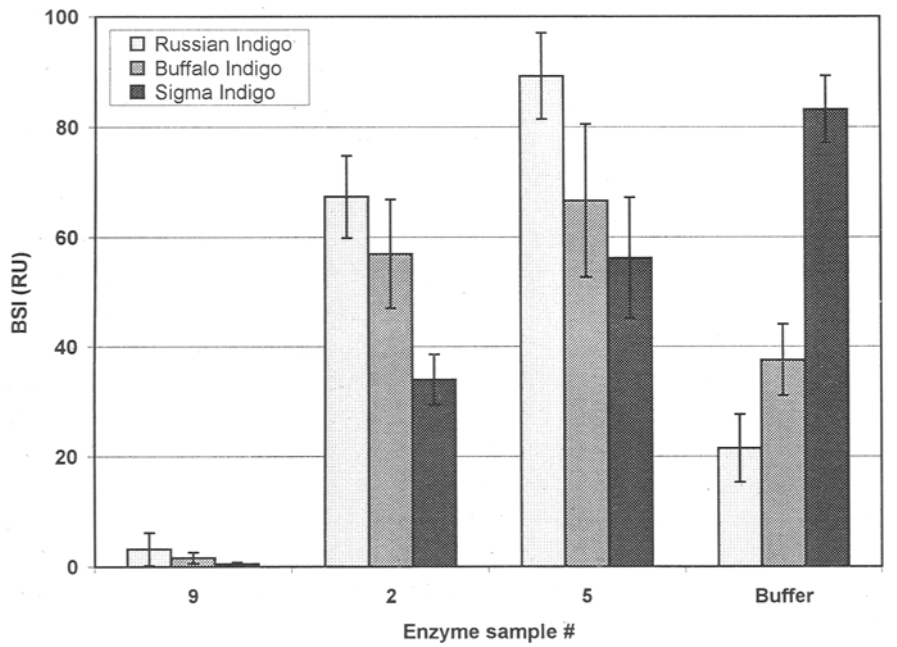

Fig. 3. Comparison of different types of indigo in the BSI microassay at pH 5.0.

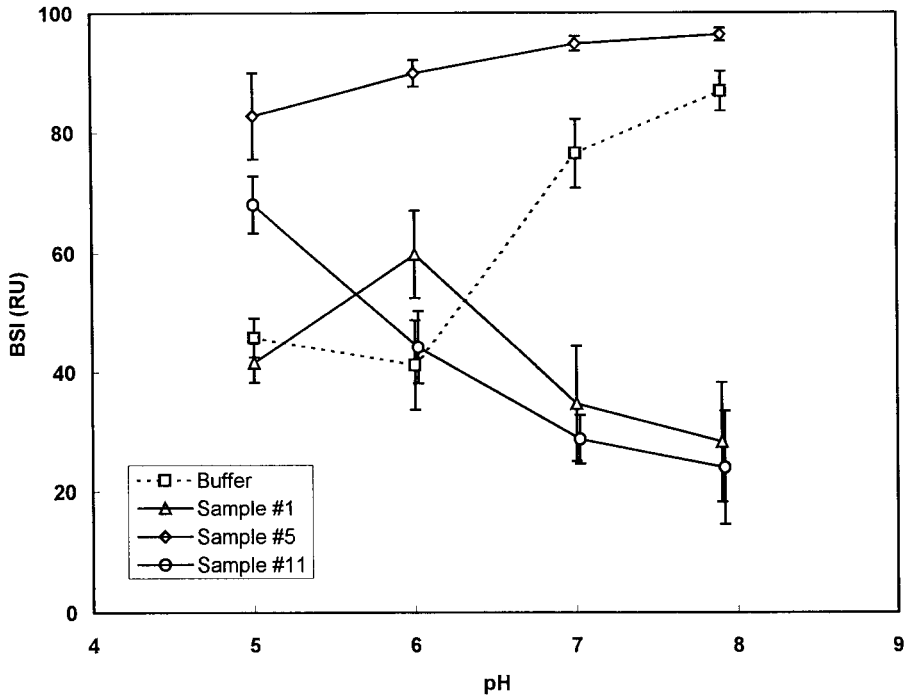

Fig. 4. Effect of pH on backstaining for different enzyme samples with Russian indigo.

Relationship Between Protein Adsorption Ability and Backstaining

An attempt was made to find a relationship between the adsorption ability of enzymes and their BSI. It is well known that in the presence of cellulosic material (fiber, powder, or fabric), part of cellulase protein adsorbs on cellulose surface, but another part remains in solution *(6,7)*. Distribution coefficient K_d is often used as a criterion of adsorption ability of cellulases on cellulose. K_d is calculated as a ratio between quantities of adsorbed enzyme and enzyme in solution, and it is typically expressed in L/g units *(7)*. The greater is the K_d, the higher is the adsorption ability of the enzyme (more enzyme is adsorbed on cellulose surface under the same conditions of assay).

Distribution coefficients for a variety of cellulase preparations, based on protein assay were determined using microcrystalline cellulose (Avicel) as a substrate, and the BSIs for the same preparations were obtained using indigo from two different sources. Figures 5A and B show a relationship between the adsorption ability of protein from the samples tested and the BSI. Figure 5A demonstrates a relationship in coordinates BSI against K_d. In Figure 5B, the percentage of protein adsorption (calculated as a difference

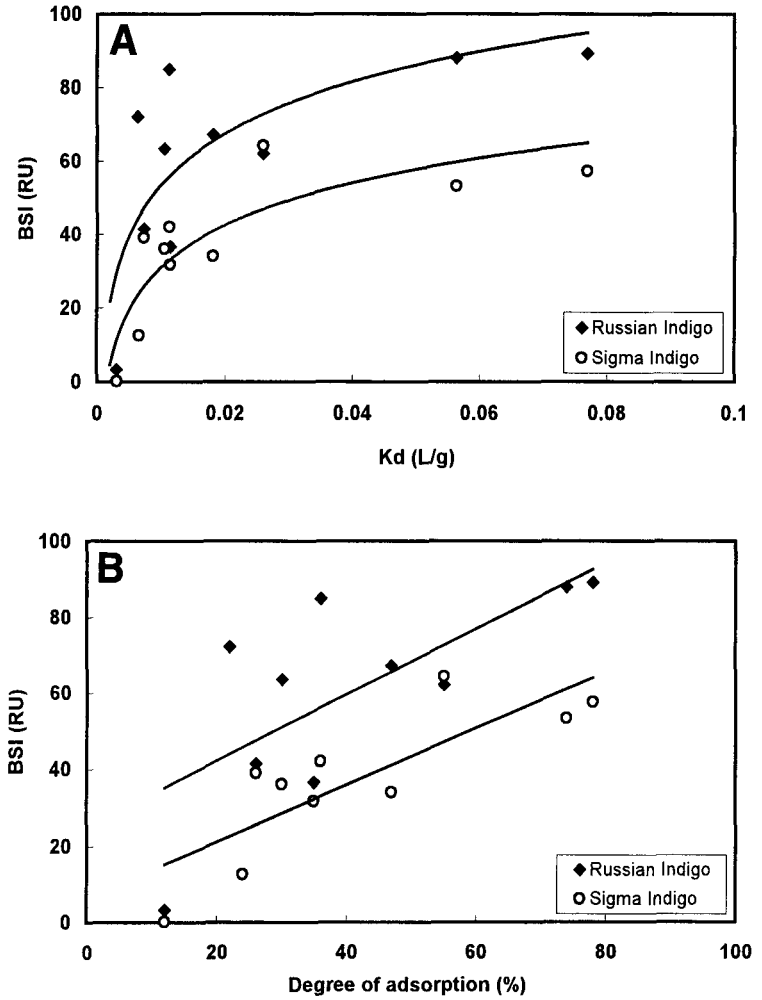

Fig. 5. Relationship between cellulase adsorption ability and backstaining at pH 5.0.

of protein concentration in solution before and after adsorption on Avicel) is used as the abscissa axis. As can be seen from the figures, the higher was the adsorption ability, the higher was BSI. In the first case (Fig. 5A), a nonlinear dependence can be observed. In the second case (Fig. 5B), the results were fitted to a straight line using linear regression. Correlation coefficients for Russian and Sigma indigo were found to be 0.70 and 0.82, respectively, demonstrating a rather satisfactory correlation between the enzyme adsorption ability and backstaining.

Testing Adsorption and Backstaining for Purified Enzyme Fractions

In order to find out what components of cellulase multienzyme system are responsible for high backstaining, two cellulase preparations (nos.

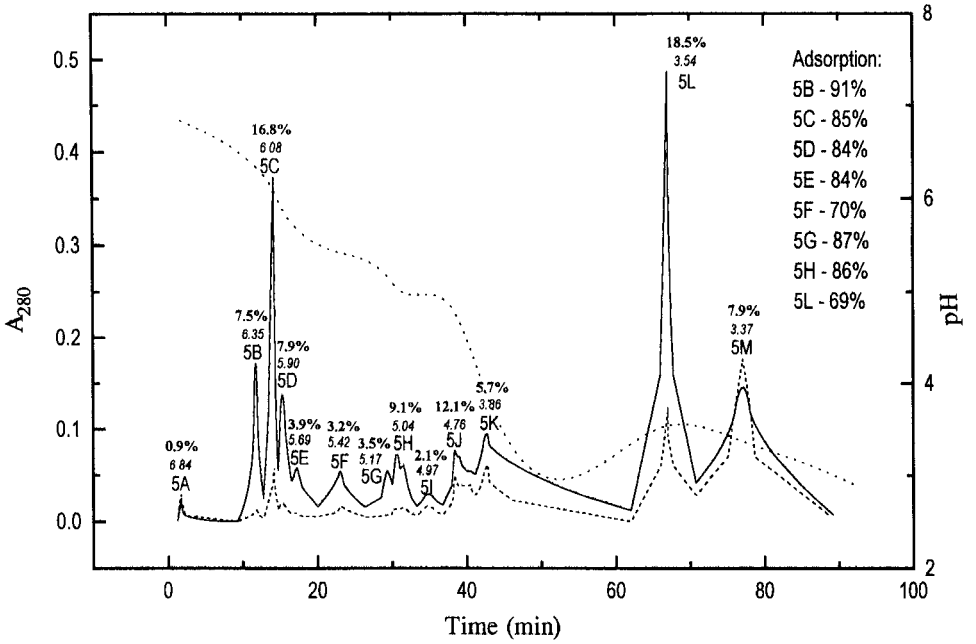

Fig. 6. Monitoring of adsorption of protein components from cellulase sample no. 5 on Avicel cellulose by chromatofocusing of the enzyme preparation on a Mono P HR 5/20 column. ——, protein elution profile of the original preparation (before adsorption); – – –, protein elution profile of supernatant after adsorption; . . ., pH profile.

1 and 5) were separated into components by chromatofocusing on a Mono P column, using Pharmacia FPLC system. For each cellulase sample, two separations were made: of original enzyme preparation and supernatant obtained after the adsorption of original preparation on microcrystalline cellulose (Avicel). This allowed quantitative monitoring of adsorption ability of individual enzyme fractions on cellulose (Figs. 6 and 7). Each component fraction of the cellulase sample nos. 1 and 5 was marked by a combination of number and letter. Numbers above peaks in Figs. 6 and 7 show a relative content (%) of the corresponding component in the crude preparation and pH, at which the component is eluted from the column (this pH relates to p*I* of proteins).

Most protein fractions from the no. 5 sample were adsorbed on cellulose to a very significant degree (Fig. 6). For each of eight components (5B, 5C, 5D, 5E, 5F, 5G, 5H, 5L), >69% of protein bound to cellulose surface (numbers in the right part of the figure show the degree of adsorption of these components on Avicel).

Enzyme components from the no. 1 sample were more loosely adsorbed on cellulose (Fig. 7). Some adsorption of protein from 1E, 1F, 1G, 1I, 1J, 1L, 1M, 1N fractions was detected, but the adsorption was not very

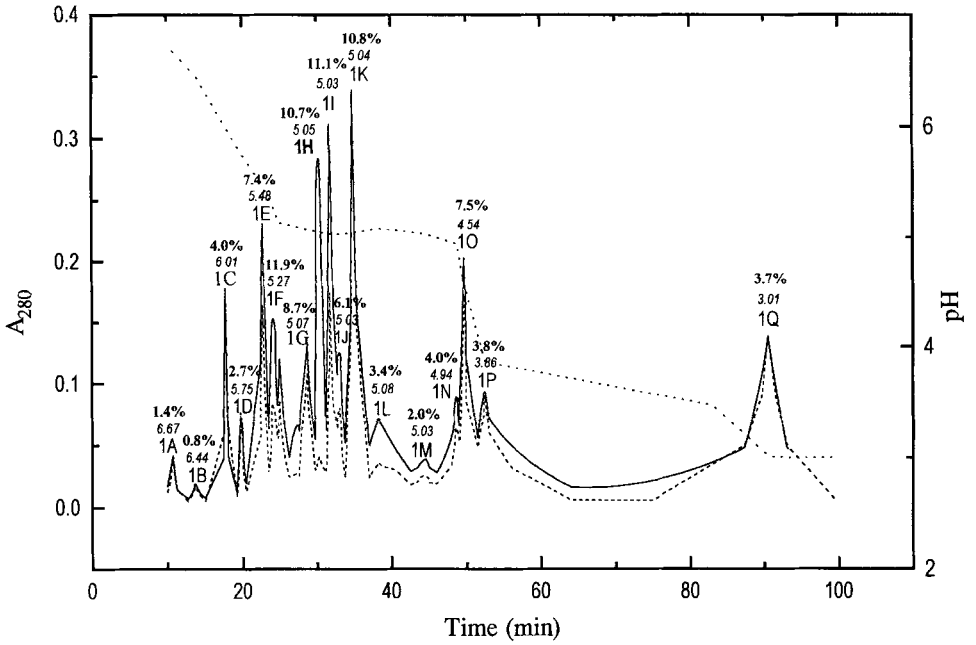

Fig. 7. Monitoring of adsorption of protein components from cellulase sample no. 1 on Avicel cellulose by chromatofocusing of the enzyme preparation on a Mono P HR 5/20 column. ——, protein elution profile of the original preparation (before adsorption); – – –, protein elution profile of supernatant after adsorption; . . ., pH profile.

strong or the components were not major (as in the case of 1L). The most strongly adsorbed and major component was that from 1H fraction, with the adsorption degree of 82%. This component represented 11% of the whole protein in the no. 1 sample. Rather surprisingly, it was not cellulase but xylanase, because it had a high activity toward xylan, and had no activity toward insoluble cellulose.

In Figure 8, the BSIs for different enzyme fractions of the separated multienzyme systems are shown. Here, the minimized BSI microassay procedure (1.4 × 1.4 cm swatches, 2 mL vol, indigo from Sigma) was used. The conditions in the assay system were equilibrated by a protein concentration, because not all the components possessed CMCase activity. The fact that the 1H fraction (which had no activity toward cellulose) showed very high BSI indicates that it was not a modification of cotton fabric with adsorbed enzyme that caused high backstaining. A similar observation can be made for the 5I fraction that was completely inactive toward insoluble cellulose (it had relatively high activity toward soluble β-glucan), but it showed high BSI. Data from Cavaco-Paulo et al. *(4)*, in which catalytically inactive mutant of endoglucanase from *Cellulomonas fimi* demonstrated high backstaining,

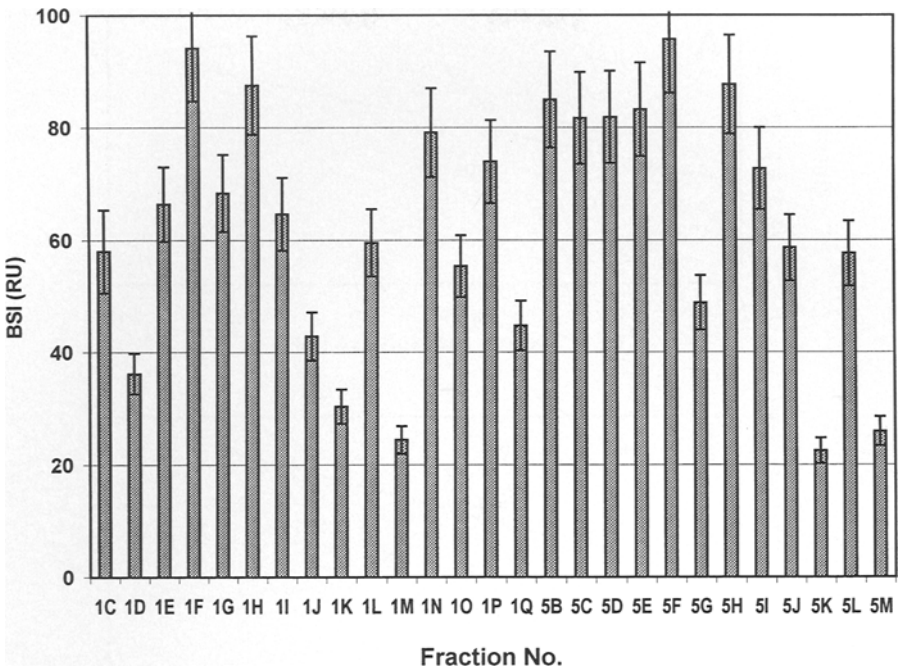

Fig. 8. BSI for component fractions of cellulase sample nos. 1 and 5 determined by the minimized microassay at pH 5.0, using indigo from Sigma. Fraction numbers correspond to peak numbers in Figs. 6 and 7.

also support the idea that indigo redeposition on cellulose fibers is not a result of enzyme hydrolytic action on cellulose surface. Additionally, it was shown in the same paper *(4)* that there was not an increase in backstaining after a modification of cellulose surface with an acid.

Figure 9 shows a relationship between the adsorption ability (taken as a degree of adsorption of components on Avicel, based on data from Fig. 6 and 7) and BSI for purified enzyme components. In Fig. 9A, data for all fractions studied from both sample nos. 1 and 5) are shown. In Fig. 9B and 9C, the data for nos. 1 and 5 enzyme samples are presented separately. Satisfactory correlations were observed in all cases, correlation coefficients being varied in the range of 0.61–0.68. As a rule, components having high adsorption ability also demonstrated high backstaining.

CONCLUSIONS

A microassay method was developed for measuring indigo staining levels on white cotton fabric in the presence of enzymes, in which the mitcale fabric was used as a model of white yarn presented in denim. The

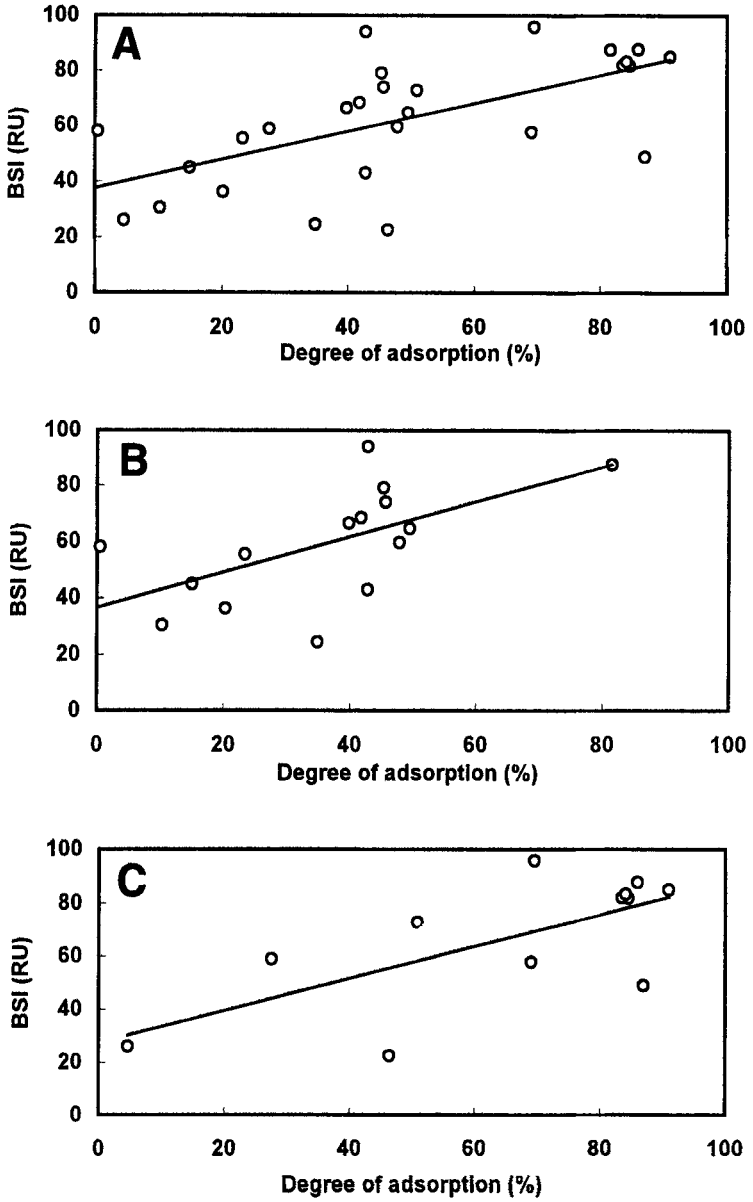

Fig. 9. Relationships between the adsorption ability of protein components of cellulase samples no. 1 and no. 5 and their BSI. **(A)** Data for all protein components are given together; **(B)** data for purified components from sample no. 1; **(C)** data for purified components from sample no. 5.

method can be used for testing enzyme samples on a small laboratory scale, and can predict the backstaining levels on a larger scale under real conditions of enzymatic treatment of denim garment.

Different cellulase enzyme preparations were tested using indigo samples from three different sources. The absolute BSIs for a particular enzyme were different with different types of indigo, indicating that formulation components contained in the dye samples notably influence the results of staining. When several enzyme samples were tested in a row, they could be ranked from lower to higher backstaining with either type of indigo.

A highly efficient method for one-stage separation of cellulase multienzyme system into protein fractions before and after adsorption on Avicel was developed, using chromatofocusing on a Mono P (Pharmacia) column. It allowed obtaining all major enzyme components in purified form in quantities sufficient for testing their backstaining properties. The method also allowed monitoring of adsorption ability of individual enzyme components.

Satisfactory correlations were found between the adsorption ability of enzymes and their BSIs (both for crude cellulase samples and purified enzyme fractions), providing evidence that protein adsorption on cotton garment is a crucial parameter causing backstaining.

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