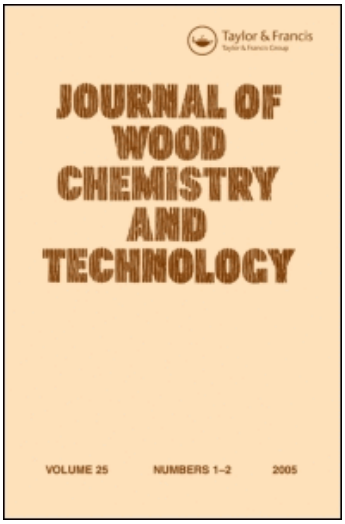

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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To cite this Article Roy, S. , Desrochers, M. and Jurasek, L.(1989) 'Proteins as Potential Retention Aids in the Pulp and Paper Industry', Journal of Wood Chemistry and Technology, 9: 3, 407 — 420
To link to this Article: DOI: 10.1080/02773818908050307
URL: http://dx.doi.org/10.1080/02773818908050307

JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 9(3), 407-420 (1989)

PROTEINS AS POTENTIAL RETENTION AIDS IN THE PULP AND PAPER INDUSTRY

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ABSTRACT

Retention of fines and additives by retention aids is important for pulp yield and paper quality in the pulp and paper industry. Measurement of the adsorption of various proteins on different cellulosic substrates was used to evaluate their potential as retention aids. Commercially obtained proteins and bacterial proteins extracted from a Bacillus subtilis strain showed substantial adsorption on three of the five cellulosic substrates tested. Proteins with high pI values were better adsorbed on cellulosic substrates.

INTRODUCTION

Pulp fines retention is of great interest to the pulp and paper industry, especially in the thermomechanical and chemi-thermomechanical pulping processes. These processes produce a high percentage of fine cellulosic particles in suspension [1]. Retention of the fines is important for pulp yield and paper quality.

Pulp washing through successive dilution and filtration is crucial in paper manufacturing. Filtering is difficult because particles finer

than the screen are not retained in the pulp [2,3]. Loss of these cellulosic particles and/or of other suspended additives causes changes in the paper properties and raises costs [4,5]. The addition of various retention aids to the pulp allows fine particles and fibres to adhere or aggregate, thus promoting their retention [6], which is especially important for the filtration that occurs on a paper machine. Organic polymers having high molecular weights and positive, negative or neutral net charges [7-11] are the most common retention aids in use in the industry. Fines retention occurs via two mechanisms: mechanical filtration and colloidal forces [12]. Fines retention dependent on colloidal forces is of special interest since these forces are chiefly electrostatic attraction and other adsorption forces between the fines and fibre surfaces: If these are suitably exploited they can lead to much better retention of fines in the paper [2,3,9,13,14].

As cellulosic particles naturally bear negative charges in suspension [2], many positively charged (cationic) polymers offer good binding possibilities. By their structure and charge, many proteins are good candidates. Proteins, i.e. polypeptides of high molecular weight, are polymerized amino acid residues, some of which bear either carboxylic, amino, or guanidinium groups that ionize and confer different charges according to the pH of the solution [15,16]. Therefore, the net charge of the proteins may vary as a function of the pH of the solution and he pI (pH at which the protein has no net charge) of the proteins.

We report here the evaluation of certain proteins and other peptides as potential retention aids in the paper industry.

MATERIALS AND METHODS

Protein Sources

The proteins used in this study were obtained from commercial sources and a bacterial culture. Lysozyme, trypsinogen, myoglobin, carbonic

anhydrase and protease from Bacillus subtilis (Type VIII, subtilopeptidase A) were purchased from Sigma Chemical Co. A protein mixture purified from a Bacillus subtilis culture supernatant was also studied. The medium used for the production of the B. subtilis proteins was nutrient broth (Difco) and contained (g/L): beef extract, 3.0 and peptone, 5.0. A 1% inoculum of an overnight culture of the Bacillus subtilis strain (ATCC 21228) (American Type Culture Collection, Maryland, U.S.A.) grown at 30°C was used to inoculate a fermentor (Biostat, E.B. Braun) containing 15 L of the same medium. The culture was grown overnight at 30°C and 200 rpm agitation, with an aeration rate of 8 L/min. The cells were harvested by centrifugation at 17,000 x g for 20 min and the supernatant recovered. The extracellular proteins were concentrated from the supernatant about 3 fold with a Pellicon ultrafiltraton system (Millipore, Massachusetts, U.S.A.). The concentrated fraction was then dialysed for 24 hours in dialysis tubing (MW cutoff = 10,000 d) against 20 mM Na-acetate, pH 5.0 buffer. The negatively charged proteins were then extracted by mixing with DEAE-Sephadex A-25 ion exchanger (Pharmacia fine chemicals, Uppsala, Sweden) for approximately 1 hour at 4°C (1 g gel/g protein). The ion exchanger was removed using a glass fiber filter (Millipore) and the filtrate was frozen for further experiments. After each step, a sample was subjected to isolectric focusing (IEF). For IEF, polyacrylamide gels containing ampholytes covering pH 3-10 (Phast system, Pharmacia) were used [17]. Proteins were visualized by silver-nitrate staining [18].

Cellulosic Substrates

Commercial cellulose (Solka floc BW 200, Brown Company, N.H., U.S.A.) wood meal (Hurricane pulverized spruce wood, HPW), holocellulose (cellulose obtained from spruce HPW by acid chlorite treatment), thermomechanical pulp (TMP) (spruce, 1st stage) and chemithermomechanical pulp (CTMP) (unbleached

CTMP; 75% aspen + 25% spruce) were used as cellulosic substrates. The pulps came from eastern Canadian mills.

Holocellulose Preparation

HPW wood meal (5 g) was mixed with 50 mL of a 10% solution of $NaClO_2$ (100 g of sodium chlorite + 1,000 mL H_2O) and 33 mL of a 10% buffer (50 g of sodium acetate + 100 mL of acetic acid in 1,000 mL of H_2O) in a 250 mL beaker and left overnight in a fume hood. The resulting holocellulose was then collected by centrifugation (17,000 x g, 30 min.). The pellet was twice resuspended in water and recovered by centrifugation. The holocellulose was then dried overnight at room temperature.

Adsorption Test

A 5% (dry wt./vol.) cellulosic substrate suspension (2.5 mL) and 0.5 mL of a protein solution of known concentration were mixed in a 10 mL beaker for 1 h at room temperature. The mixture was then transferred to tubes and centrifuged at 3,500 x g for 10 min. The supernatant was filtered using a 0.45 μm pore size filter (Millipore) and kept for protein assay. Three different protein determination techniques were used, depending on the sensitivity needed: optical density at 280 nm, the Bio-Rad phosphoric acid protein assay (Bio-Rad, California, U.S.A.), and Folin-Ciocalteu alkaline method [19].

FPLC and SDS-PAGE

The protein concentration was also monitored on 500 μL samples with a fast protein liquid chromatograph (FPLC; Pharmacia) equipped with a Mono-S column (a monidisperse cation exchanger; Pharmacia). Proteins were eluted using linear gradients ranging from 0-85% NaCl (or 25% in the case of protease) in 20 mM Na-acetate pH 5.0 buffer at a flow rate of 1 mL/min. Proteins were detected by UV absorption at 260 nm. The same samples were

also subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in an SDS buffer system (Phast system, Pharmacia) and proteins were stained with silver nitrate [18].

Physical Testing

The physical properties of papers containing various protein concentrations were tested. Three lysozyme concentrations (1.2%, 2.4% and 10%; based on oven-dried pulp) plus a control (no protein added) were used with two pulp suspensions: TMP and CTMP in phosphate buffer, 20 mM, pH 7.0. Physical testing was done using standard (CPPA) methods. Freeness, basis weight, burst index, tear index, breaking length, stretch and Z-span breaking length were measured [20,21].

RESULTS

Preliminary work consisted of screening of a few commercially available cationic (positively charged) proteins for their adsorption on commercial cellulose. The best adsorption was recorded with lysozyme on Solka floc cellulose. The influence of the concentration of the protein on the degree of adsorption was studied. According to the results obtained in the preliminary work, and according to polymer concentrations cited in the literature [10,11,22], various lysozyme concentrations from 0 to 0.167% (based on oven-dried pulp in phosphate buffer, 20 mM, pH 7.0) were assayed at a Solka floc cellulose at a consistency of 5%. The results (Figure 1) showed strong binding at low protein concentrations with gradual saturation of cellulose protein binding sites, whereas, at higher protein concentrations, as shown in Figure 2, there seemed to be an equilibrium between free and cellulose-bound proteins, with about 20% of the protein being bound.

The next step consisted of measuring the degree of adsorption of lysozyme on five different cellulosic substrates. As in the previous experiments, the Solka floc cellulose consistency was set at 5% and, in the light

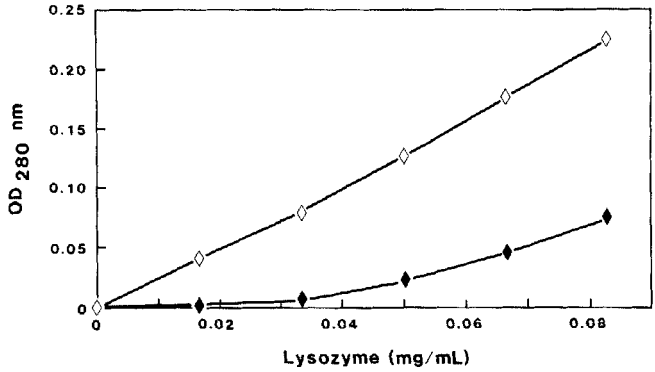

Figure 1. Adsorption of low concentrations of commercial lysozyme on Solka floc 5% in phosphate buffer, pH 7.0; lysozyme (◊), and lysozyme + Solka floc, 5% (♦).

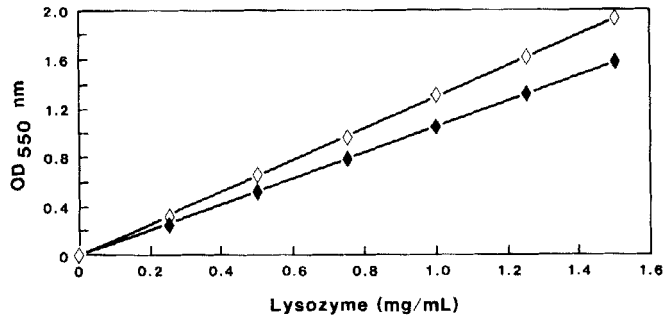

Figure 2. Adsorption of high concentrations of commercial lysozyme on Solka floc (5%) in phosphate buffer, pH 7.0. Symbols as described in Figure 1.

of the prior results, the lysozyme concentration was varied from 0.0167% to 0.167% (based on oven-dried pulp in phosphate buffer, 20 mM, pH 7.0). The five substrates assayed were: cellulose, wood meal, holocellulose, and thermomechanical and chemi-thermomechanical pulps. Each assay was performed three times and the results presented in Figure 3 are an average of these assays. Figure 3 shows that, as the lysozyme concentration is

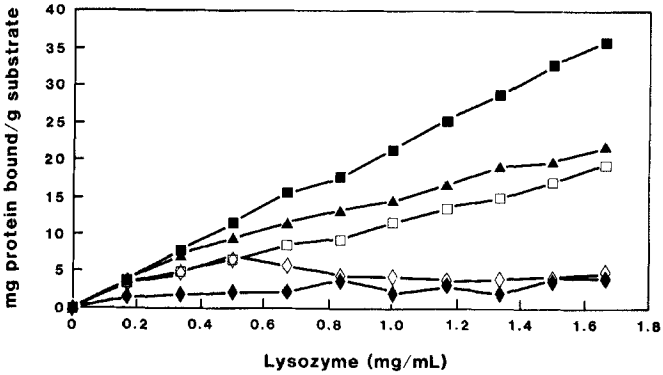

Figure 3. Adsorption of lysozyme on five different cellulosic sustrates (5%) in phosphate buffer, pH 7.0. Holocellulose (■), CTMP (▲), TMP (□), wood meal (◊), and cellulose (♦).

raised, much more lysozyme is adsorbed on holocellulose and on TMP and CTMP than on cellulose and wood meal.

Figure 4 shows the results obtained in the adsorption of B. subtilis protease on the same five substrates. Protease was chosen because of its high pI (around 8.5). The pH was fixed at 5.0 (in acetate buffer 20 mM, pH 5.0) to avoid the precipitation of the proteases, insoluble at pH 7.0. The same concentration of lysozyme as of protease was used. It appears that, as observed with lysozyme, there was much more protease adsorbed on holocellulose, TMP, and CTMP than on cellulose and wood meal.

In the case of the cationic proteins extracted from B. subtilis strain, their adsorption on the five cellulosic substrates was measured in the same manner at pH 5.0 (in acetate buffer, 20 mM, pH 5.0) and protein concentration was varied from 0 to 0.05%. Lower concentrations were used because of the difficulties encountered with concentration of the proteins from the culture supernatant.

The B. subtilis cationic proteins adsorbed on the five cellulosic substrates. Comparison of the results obtained (Figure 5) with the B.

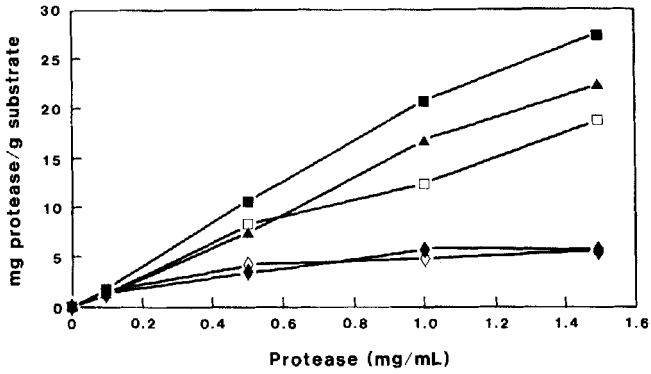

Figure 4. Adsorption of a B. subtilis protease (subtilopeptidase A) on five different cellulosic substrates (5%) in acetate buffer, pH 5.0. Symbols described in Figure 3.

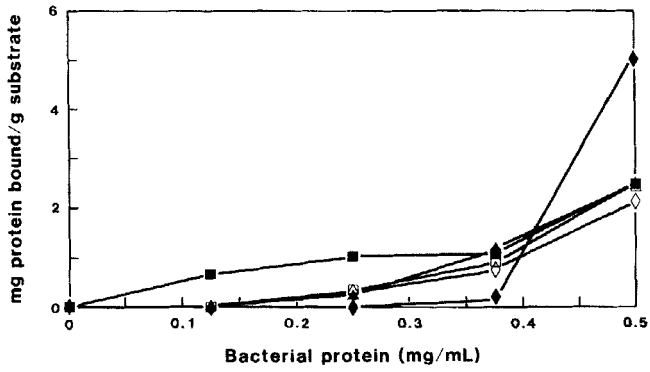

Figure 5. Adsorption of B. subtilis cationic proteins on five different cellulosic substrates (5%) in acetate buffer, pH 5.0. The proteins were partially purified from a B. subtilis culture grown in nutrient broth medium. Symbols as described in Figure 3.

Table 1. Adsorption of several proteins on 5 different cellulosic substrates[a]

Protein	Lysozyme	Trypsinogen	Myoglobin	Carbonic anhydrase	*B. subtilis* protease	*B. subtilis* basic secreted protein (crude)
Isoelectric point (pI)	11.0	9.3	7.3	5.9	8.5	8.3
Molecular weight (MW)	14,400	24,000	17,200	29,000	-	-
Cellulose	++	+	-	+	++	+
Lignocellulose	++	++	+	+	++	++
Holocellulose	++	++	++	+	++	++
TMP	++	++	+	+	++	++
CTMP	++	++	+	+	++	+

[a] The tests were performed in acetate buffer, 20 mM, pH 5.0 at room temperature. The proteins (lysozyme, 0.025 mg/mL; trypsinogen, 0.025 mg/mL; myoglobin, 0.5 mg/mL; carbonic anhydrase, 0.1 mg/mL; commercial protease, 0.1 mg/mL and bacterial protease, 0.5 mg/mL) were mixed for about 1 hour with 5% of substrate. The results were measured as adsorption of proteins at 280 nm (FPLC) or by silver nitrate staining (electrophoresis).

Legend: Adsorption (FPLC datum) +
No Adsorption (FPLC) -
Adsorption (FPLC and electrophoresis data) ++

Table 2. Modification of physical properties of TMP by the addition of different concentrations of lysozyme.

Sample No.	1	2	3	4
C.S. Freeness (mL)	649	660	656	655
Grammage (g/m²)	58.3	60.6	59.4	59.5
Burst Index (kPa.m/g)	0.39	0.41	0.44	0.48
Tear Index (millinewton.m/g)	4.18	3.80	4.18	4.99
Breaking Length (km)	1.02	0.97	0.98	1.12
Stretch (%)	1.55	1.71	1.76	1.74
Z-Span Breaking Length (km)	8.53	8.87	8.85	9.20
Tensile Index (N.m/g)	9.99	9.55	9.61	10.97

[a] Samples consisted of 5% TMP in 20 mM phosphate buffer, pH 7.0 with different lysozyme concentrations. Percent based on oven-dried pulp: 1-0%; 2-1.2%; 3-2.4%; 4-10%.

Table 3. Modification of physical properties of CTMP by the addition of different concentrations of lysozyme.

Sample No.	1	2	3	4
C.S. Freeness (mL)	196	212	198	203
Grammage (g/m²)	59.5	58.8	58.5	59.9
Burst Index (kPa.m/g)	0.80	0.76	0.77	0.76
Tear Index (millinewton.m/g)	3.34	3.31	3.28	3.18
Breaking Length (km)	2.02	2.02	1.99	1.99
Stretch (%)	1.41	1.63	1.49	1.57
Z-Span Breaking Length (km)	8.64	8.69	8.78	8.63
Tensile Index (N.m/g)	19.78	19.81	19.51	19.48

[a] Samples consisted of 5% CTMP in phosphate buffer, 20 mM, pH 7.0, with different lysozyme concentrations. Percent based on oven-dried pulp: 1-0%; 2-1.2%; 3-2.4%; 4-10%.

subtilis protease (Figure 4) is difficult because of the low concentrations of cationic protein preparation compared to the commercial protease.

Qualitative measurements were also made of the adsorption of commercial cationic proteins, commercial protease and bacterial proteases on the five cellulosic substrates by FPLC and SDS-PAGE electrophoresis. SDS-PAGE confirmed the results obtained with the FPLC system. The results are presented in terms of adsorption of the six proteins tested (Table 1). The results seem to indicate that the proteins having higher pI values (and thus the highest positive charge) are better adsorbed on cellulosic substrates.

Physical tests were performed on two pulp suspensions containing four different concentrations of lysozyme at pH 7.0. The results presented (Tables 2 and 3) show that the addition of lysozyme to TMP and CTMP had little effect on the physical properties of the pulps.

DISCUSSION

The first experiment to determine how the proteins adsorb on commercial cellulose (Figure 1 and 2) showed that, within a certain concentration range, increases in the amounts of added proteins cause an increased adsorption but only in a certain ratio (in opposition to the complete adsorption at low concentrations). These results suggest that there are two mechanisms involved in the adsorption of lysozyme to Solka floc cellulose.

As all lysozyme molecules have the same conformation in solution, it is proposed that the observed difference in adsorption among tested proteins is caused by the availability of suitable adsorption sites on the cellulose. It has been shown that the most significant binding between retention aids and cellulosic substrates is via charge-charge interactions [23]. These offer weak binding which is reversible if subjected to shearing forces or competitor ions for the binding sites. Therefore, there are

two kinds of binding sites, some that are hidden within the cellulose structure and allow lasting binding (saturation), and others that are exposed to shearing forces and do not allow strong binding.

We have observed saturation only when protein was added in low concentrations. Nevertheless, when adding high protein concentrations, the saturation also occurs.

In the case of the B. subtilis protease, we noted that the quantity adsorbed on the five cellulosic substrates was similar to lysozyme, except in the case of holocellulose, where less is bound. The neutralization of many binding sites by the numerous H^+ ions present in solution at pH 5.0 could explain this phenomenon.

Results of measurement of the adsorption of purified cationic proteins by FPLC and SDS-PAGE (Table 1) are difficult to compare because the variation in the initial concentrations of the proteins may have affected binding to the adsorption sites on the substrate. Comparing the four proteins, pI seems to have had an effect on the degree of adsorption. The B. subtilis proteases and B. subtilis seem to adsorb well on all the substrates. The degree of adsorption of bacterial proteases is as high as the degree of adsorption of commercial proteases.

Tables 2 and 3 show that an increase in the lysozyme concentration added to TMP seems to cause a slight strengthening of the pulp whereas in the case of the CTMP, there is no effect. This difference could be due to the different fines contents of the pulps. TMP's contain many more cellulosic fines and fiber fragments on which lysozyme can adsorb and enhance physical properties.

In view of the adsorption studies presented here it would be interesting to see if addition of cationic proteins could improve fines retention.

ACKNOWLEDGEMENT

We wish to thank R.W. Baine and the Physical Testing Group for expert analyses of handsheet properties.

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