RETENTION AND PAPER-STRENGTH CHARACTERISTICS OF ANIONIC POLYACRYLAMIDES CONJUGATED WITH CARBOHYDRATE-BINDING MODULES

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The retention behavior of polymers having the specific affinities of glyco-hydrolases for pulp fibers was investigated with regard to paper-strength enhancement in contaminated papermaking systems. Carbohydrate-binding modules (CBMs) of cellulases derived from *Trichoderma viride* and *T. reesei*, and of xylanase from *Thermomyces lanuginosus,* were obtained by site-directed digestion with papain, then introduced into anionic polyacrylamide (A-PAM) via a peptide condensation reaction. Three types of CBM-conjugated A-PAMs (CBM-A-PAMs) displayed different retention behavior, depending on the kind of pulp substrates, i.e. hardwood and softwood fibers. The CBM-A-PAM from *T. viride* demonstrated good additive retention for hardwood pulp fibers, resulting in high tensile strength of paper sheets, even under contaminated conditions in the presence of Ca^{2+} ions and ligninsulfonate. The CBM-A-PAM from *T. reesei* showed better performance for softwood than for hardwood sheets. The xylanase CBM-A-PAM was preferentially retained on hardwood fibers in which hemicelluloses might be present. Such an additive retention system, with inherent affinities of enzymes for pulp fibers, is expected to expand the application range of CBM-polymers in practical wet-end processes.

Keywords: Carbohydrate-binding module; Cellulase; Xylanase; Papermaking additive; Retention; Pulp fiber; Interfering substances

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INTRODUCTION

Paper products composed of cellulosic fibers have long been used in a wide variety of applications as an essential material for daily life. Functional design of paper materials is in general achieved by addition of papermaking chemicals to an aqueous fiber suspension at the wet end of a paper machine. However, this process often is considerably contaminated by recycling of both pulp fibers and process water, resulting in an increase in charged contaminants (Pelton et al. 1981). Such contaminants have interfered with the performance of most papermaking additives, because the conventional wet-end systems strongly depend on electrostatic interactions (Zhang 1999). Polyacrylamide (PAM), cationic starch, and polyamideamine-epichlorohydrin (PAE) are typical papermaking polyelectrolytes, but they are very sensitive to anionic trash and inorganic ions that have accumulated in the paper furnish. Other systems, using nonionic polymers such as polyethylene oxides and phenol formaldehyde resins, have been investigated under industrial conditions (Pelton et al. 1980; Lindström and Glad-Nordmark 1984). However, these systems have not provided a fundamental solution. For that reason there has been an upsurge in demand for a novel additive retention system that is not affected by electrostatic interactions, and is effective in the contaminated wet end.

Cellulase is an *O*-glycosyl-hydrolase (GH) that can selectively hydrolyze a β-1,4 glycosidic linkage of cellulose, and is one of the most promising candidates for solving the problems mentioned above. Most cellulases have a modular structure composed of a catalytic domain (CD) linked to a carbohydrate-binding module (CBM) (Davies and Henrissat 1995). The strong interest in utilizing CBM proteins in the design of cellulose materials results from their specific, high affinity for solid cellulose fibers without catalytic activity (Shoseyov et al. 2006; Wang et al. 2008). In previous studies we have proposed that the CBM function is effective for improving retention and performance of papermaking additives in the contaminated wet end (Kitaoka and Tanaka 2001; Yokota et al. 2008). Anionic PAM conjugated with CBM protein (CBM-A-PAM) had a nanoNewton-level specific interaction between CBM and pulp fibers, leading to good retention and high tensile strength at high conductivity and cationic demand, while commercial PAE became ineffective.

In the present study, CBM-A-PAMs were synthesized with three types of CBM proteins obtained from different GHs, and their retention behavior was compared. Cellulase is found in all microbial kingdoms, predominantly Prokaryote and Fungi. GHs other than cellulase (e.g. xylanases) also have CBM proteins with the ability to bind to carbohydrate surfaces (Hildén and Johansson 2004). To date, a variety of CBMs have been found, and they have been classified into more than 50 different families based on amino acid sequence, binding specificity, and structure (Shoseyov et al. 2006). Here, different types of CBM-A-PAMs were added to a pulp suspension of either hardwood or softwood fibers having different surface compositions, i.e. proportions of cellulose and hemicelluloses (Scott 1996). The functional diversity of CBM-conjugated polymers is discussed with regard to retention behavior and paper-strength enhancement under contaminated wet-end conditions.

EXPERIMENTAL

Materials

Commercial bleached kraft pulps of hardwood (HBKP) and softwood (SBKP) were used for handsheet-making; the Canadian Standard Freeness values were adjusted to 450 mL for HBKP, and 550 mL for SBKP, with a Hollander beater. Two cellulases (EC 3.2.1.4) from *Trichoderma viride* (Wako Co. Ltd.) and *T. reesei* (Sigma Co. Ltd.), and one xylanase (EC 3.2.1.8) from *Thermomyces lanuginosus* (Sigma Co. Ltd.) were utilized as CBM sources. Papain (EC 3.4.22.2; Sigma Co. Ltd) was used as protease for isolation of CBMs. A CBM collection kit using $\mathrm{CB_{IN}D^{TM}}$ 100 resin was purchased from Novagen Co. Ltd. Anionic polyacrylamide (A-PAM; HH-351, with molecular weight ca. 4×10^6 g mol^{-1} and charge density 0.83 meq. g^{-1}) was kindly provided by Kurita Co. Ltd. Other chemicals were pure reagent grade (Aldrich Co. Ltd.) and were used as purchased.

Methods

Separation of CBM proteins

CBM proteins were obtained according to the following methods. Papain and each GH were individually pre-incubated at 37°C for 30 minutes in 50 mM phosphate buffer (pH 6.5). A portion of the activated papain solution (1 mg mL^{-1}) was poured into 150 mL of each GH solution (10 mg mL^{-1}) with a GH/papain ratio of 30:1 (wt/wt), and the mixtures were incubated at 37°C for 30 minutes (Van Tilbeurgh et al. 1986). The CBMs were separated by affinity purification using $CB_{IN}D^{TM}$ 100 resin according to the reported protocol (Yokota et al. 2008).

Synthesis of CBM-A-PAMs

CBM/ethylene glycol solution (210 mg per 70 mL) and 3 mL of an aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) solution (10 wt%, pH 4.75) were gently mixed with 150 mL of aqueous A-PAM solution (0.1 wt%, pH 4.75) at room temperature for 3 h, keeping the initial pH constant (Kitaoka and Tanaka 2001; Yokota et al. 2008). The resultant CBM-A-PAMs were purified by three centrifugations using 2-propanol, prior to handsheet-making.

Preparation and characterization of paper sheets

The CBM-A-PAMs were added to a pulp suspension (0.15 %w/v) of either HBKP or SBKP at 0.4 % level based on dry weight of pulp. The handsheets (with basis weight 60 g m^{-2}) were prepared with tap water according to TAPPI test method (T205 sp-95). The conductivity of the pulp suspension was adjusted to 0.5 (tap water) or 4 mS cm^{-1} by addition of calcium chloride ($CaCl_2$), before addition of CBM-polymers. The cationic demand of the suspension was adjusted to 20 (tap water) or 80 μeq. L^{-1} by adding a sodium ligninsulfonate as a model for anionic trash. After conditioning at 23°C and 50 % relative humidity for 24 h, the handsheets were subjected to dry tensile strength testing (n=5) according to TAPPI test method (T494 om-88). Additive retention was determined by combustion analysis for total nitrogen on solid-phase samples (n=3).

RESULTS AND DISCUSSION

Retention and Paper-strength Characteristics of CBM-A-PAMs

Three types of CBM-A-PAMs were synthesized according to our previously reported protocol (Yokota et al. 2008). The successful collection of CBMs by removal of CDs and small peptide fragments was roughly confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), and the molecular weights of the separated CBMs (8-10 kDa) were determined (Fig. 1). The CBMs derived from *T. viride*, *T. reesei*, and *T. lanuginosus* are referred to hereafter as *Tv*CBM, *Tr*CBM, and *Tl*CBM, respectively. The N-termini of the isolated CBMs were covalently linked to the carboxyl groups of A-PAM. Undesirable self-condensation of the CBMs by EDC-mediated reaction occurred to a negligible extent, because of the much greater amount of carboxyl groups of A-PAM compared to the amount of CBM proteins.

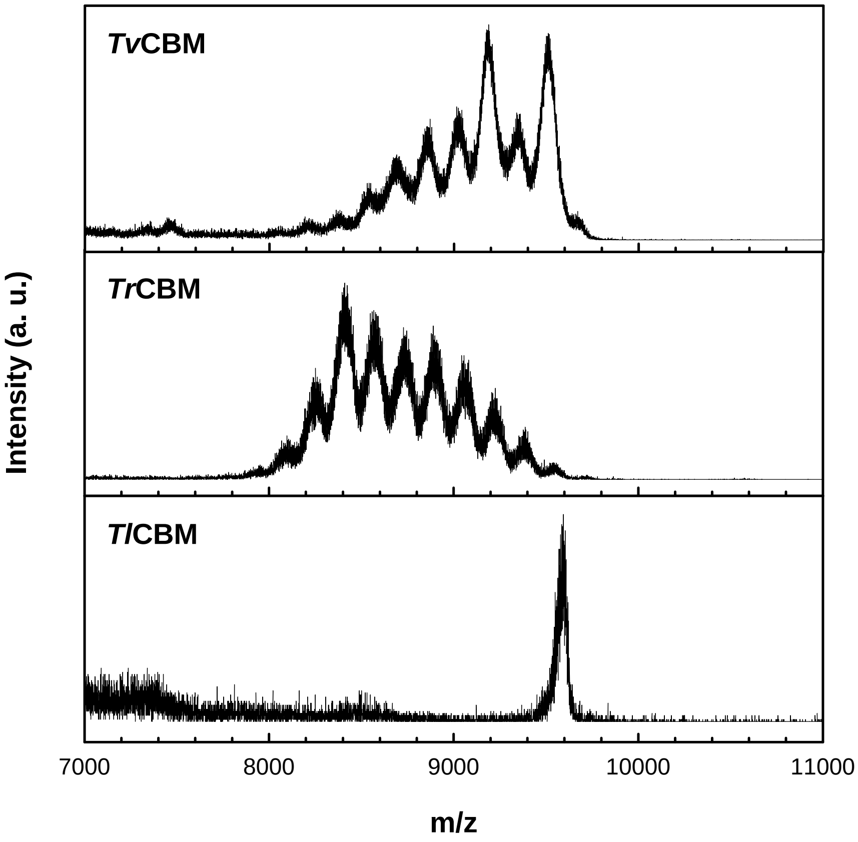


Fig. 1. MALDI-TOF mass spectra of CBMs separated by site-digestion with papain.

Figure 2 compares the relative tensile strength and additive retention efficiency of the handsheets prepared with each CBM-A-PAM and different pulp fibers. In the case of HBKP fibers, the relative tensile strength of the handsheets prepared with CBM-A-PAMs was significantly higher than for additive-free (blank) handsheets (Fig. 2a). The retention ratios of *Tv*CBM- and *Tl*CBM-A-PAMs were approximately 40 %, while that of *Tr*CBM-A-PAM reached more than 80 %. *T. reesei* cellulase is a well-known powerful GH for hydrolyzing solid cellulose, and strong affinity of its CBM for pulp fibers was presumed. The CBM-free A-PAMs have no affinity for pulp fiber, and accordingly neither retention of the additive nor paper-strength enhancement were found (Yokota et al. 2008). The CBM moieties of CBM-A-PAM molecules interacted with the surfaces of pulp fibers, and hydrogen bonding between AM units and cellulose chains may have improved the paper strength. However, additive retention did not always correspond to enhancement of tensile strength. A negative effect, such as non-hydrolytic disruption of cellulose fibers with CBMs (Wang et al. 2008), might have occurred in the case of *Tr*CBM-A-PAM.

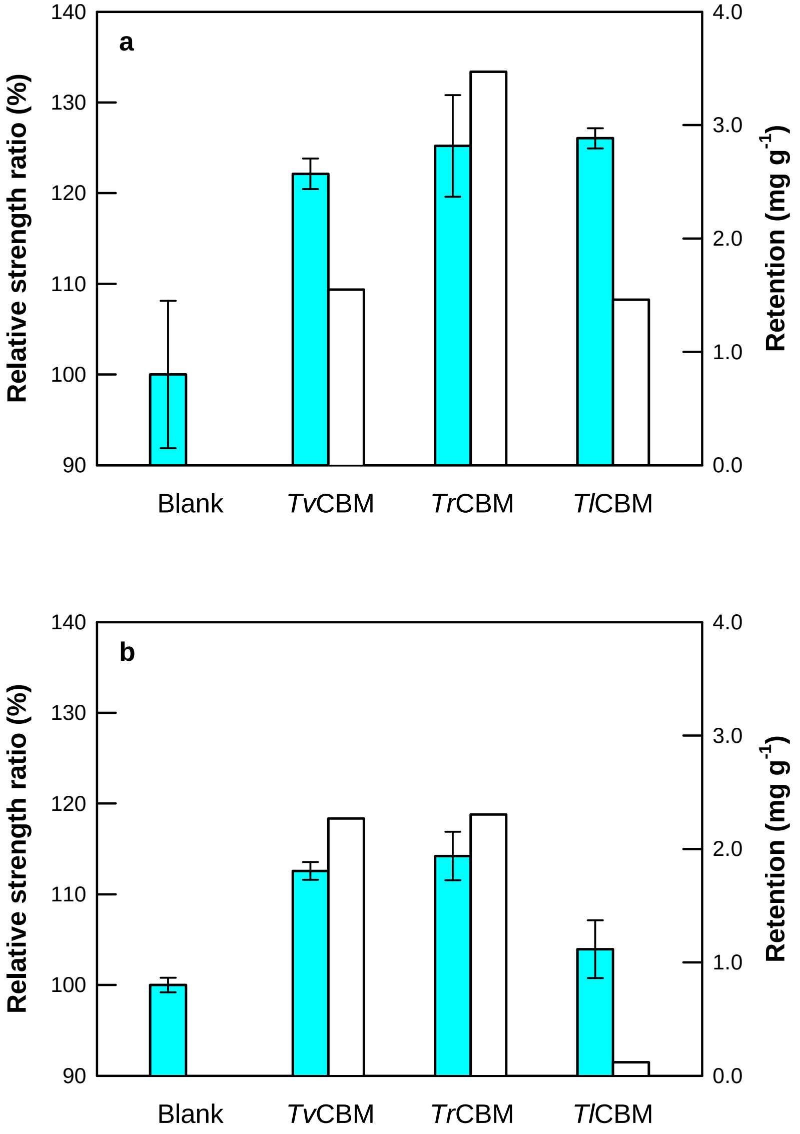
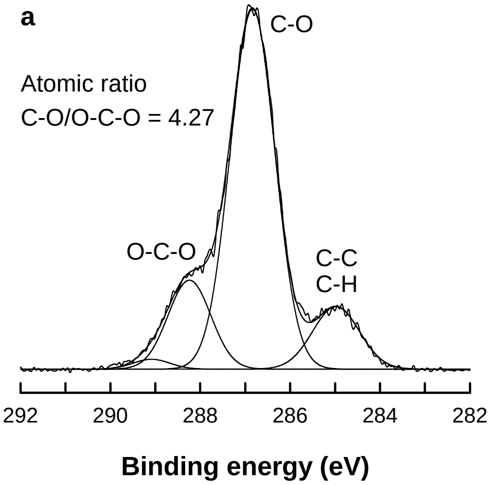


Fig. 2. Tensile strength (blue bars) and retention (open bars) of paper sheets prepared with CBM-A-PAMs. (a) hardwood handsheets; (b) softwood handsheets.

Figure 2b shows the paper strength and additive retention in the case of softwood handsheets modified with CBM-A-PAMs. Paper sheets treated with *Tv*CBM- and *Tr*CBM-A-PAMs possessed good retention efficiency and higher strength than the blank sheet. On the other hand, only a slight amount of *Tl*CBM-A-PAM was retained on the softwood sheets, resulting in negligible improvement of paper strength. The BKP fibers are composed mainly of cellulose but have residual hemicelluloses on the surface; the surface compositions depend on the wood origin. Approximately 90 % of the hemicellulose content of hardwood is 'xylan' consisting of a β-1,4-linked xylopyranose backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains (Scott 1996; Gregory et al. 1998). Glucomannan is the major constituent in softwood hemicelluloses, and most of it is removed by Kraft pulping (Scott 1996). In this study, larger amounts of pentose components on HBKP than on SBKP fibers were found from the atomic ratios of C-O and O-C-O determined by X-ray photoelectron spectroscopy (XPS, Fig. 3). Xylanase must preferentially adsorb on xylan, and thus it was presumed that *Tl*CBM-A-PAM interacted with hemicelluloses on the HBKP fibers, resulting in high retention and paper strength.



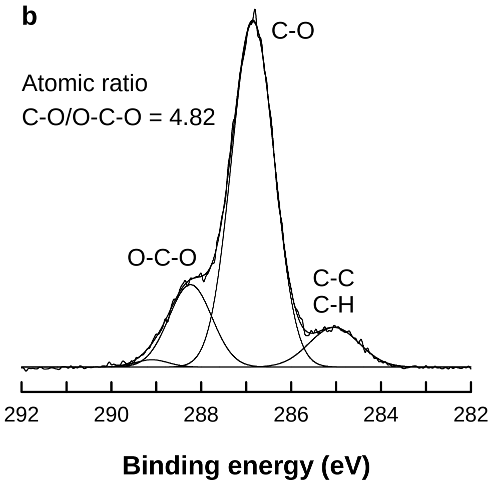


Fig. 3. XPS C1s narrow region spectra of the surfaces of pulp fibers. (a) HBKP; (b) SBKP. The binding energies for all spectra were determined with respect to the reference signal (unoxidized C-C and/or C-H band) at 285.0 eV. Theoretical values of C-O/O-C-O of pure glucose and xylose are 5 and 4, respectively.

Additive Performance of CBM-A-PAMs in the Contaminated Wet End

Hardwood and softwood handsheets were prepared with CBM-A-PAMs under wet-end conditions at high conductivity (4 mS cm^{-1}) or high cationic demand (80 μeq. L^{-1}), realized by adding $CaCl_2$ or sodium ligninsulfonate, respectively. Figure 4 shows the influence of paper stock conductivity and cationic demand on the tensile strength and additive retention of the paper sheets prepared with *Tv*CBM-A-PAM. Both tensile strength and retention for hardwood sheets remained almost unchanged, even under the contaminated conditions (Fig. 4a), similar to the behavior observed in our previous reports (Kitaoka and Tanaka 2001; Yokota et al. 2008). On the other hand, *Tv*CBM-A-PAM was less effective for SBKP fibers, and a higher concentration of contaminants reduced the additive retention and resultant paper strength (Fig. 4b). In particular, conductivity was a fatal factor for inducing ineffectiveness of *Tv*CBM-A-PAM.

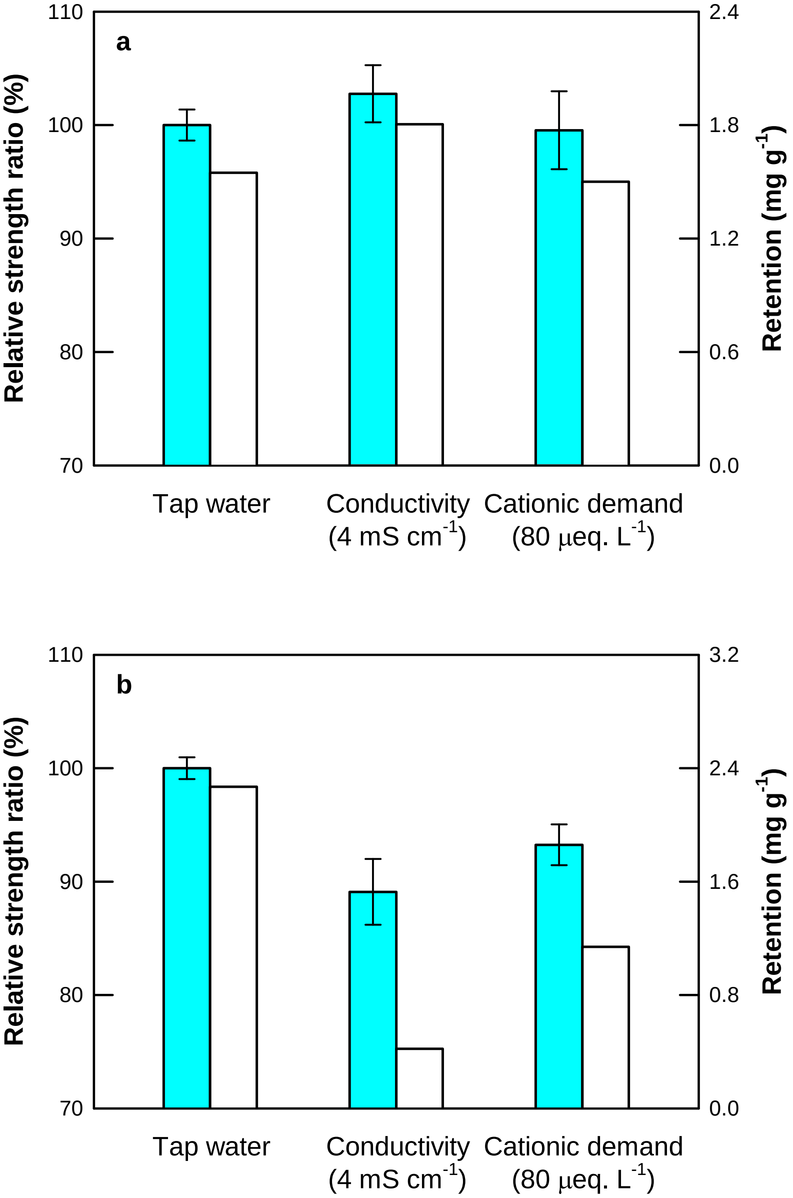


Fig. 4. The influence of paper stock conductivity and cationic demand on tensile strength (blue bars) and retention (open bars), of paper sheets prepared with *Tv*CBM-A-PAM. (a) hardwood handsheets; (b) softwood handsheets.

Figure 5 displays the influence of conductivity and cationic demand on *Tr*CBM-A-PAM performance. Under the contaminated wet-end conditions, both additive retention and tensile strength of HBKP sheets prepared with *Tr*CBM-A-PAM became relatively low (Fig. 5a). In the case of SBKP fibers both additive retention and tensile strength remained almost unchanged (Fig. 5b). Thus *Tr*CBM-A-PAM appeared to be effective for

SBKP fibers, in contrast to *Tv*CBM-A-PAM. The difference in retention behavior of A-PAMs conjugated with CBMs from two *Trichoderma* species was interesting, and showed a striking contrast for the contaminated system. The different cellulose substrates were relevant to the apparent activity of cellulase for cellulose degradation (Ortega et al. 2001). Consequently, the unique characteristics of CBM-A-PAMs possibly resulted from the inherent functions of CBMs from different GHs.

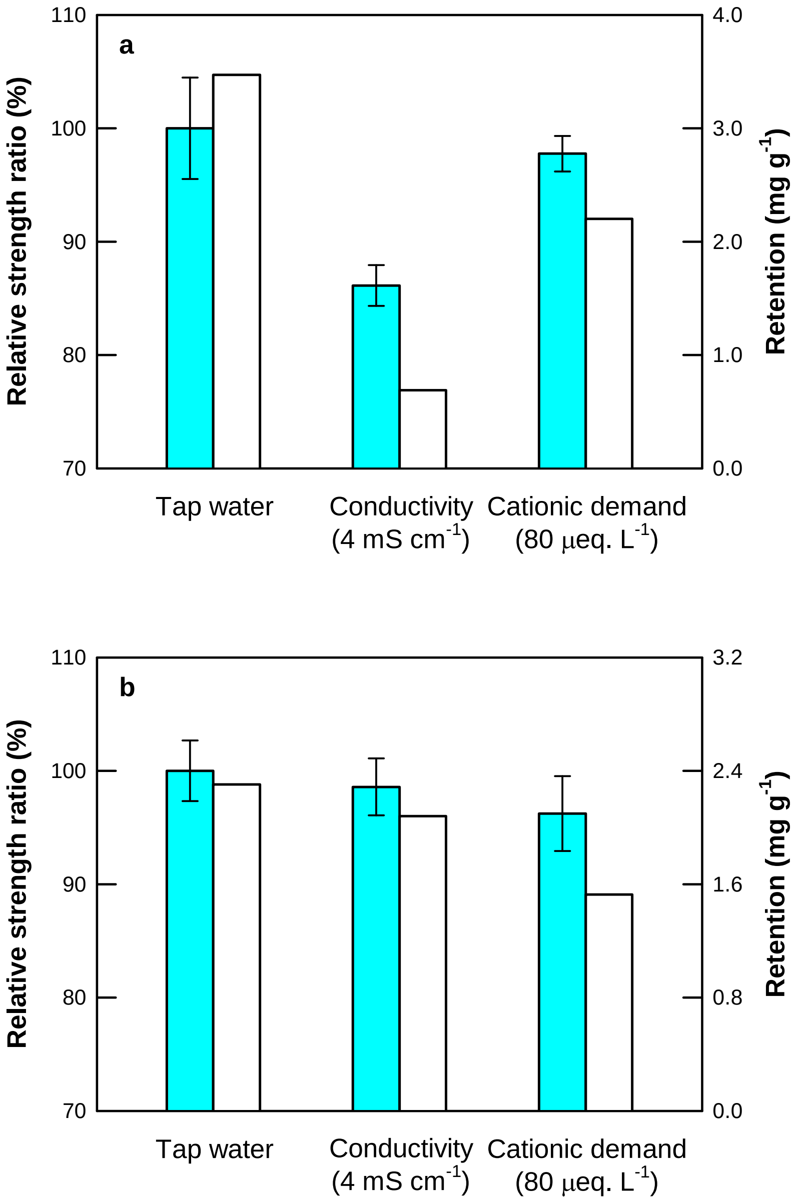


Fig. 5. The influence of paper stock conductivity and cationic demand on the tensile strength (blue bars), and retention (open bars), of paper sheets prepared with *Tr*CBM-A-PAM.
(a) hardwood handsheets; (b) softwood handsheets.

Subsequently, the influence of wet-end contaminants on the additive performance of *Tl*CBM-A-PAM was investigated. The tensile strength and the additive retention of HBKP sheets were negatively affected by the addition of inorganic ion and anionic trash (Fig. 6).

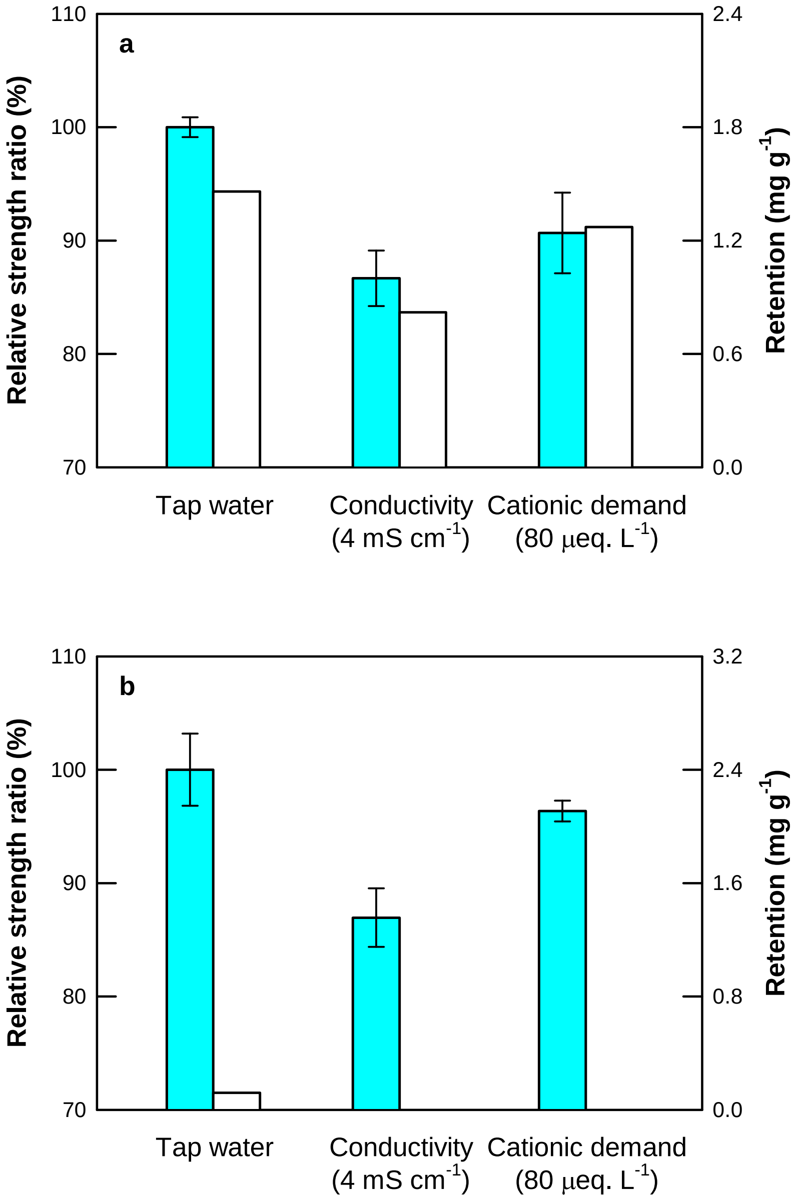


Fig. 6. The influence of paper stock conductivity and cationic demand on the tensile strength (blue bars), and retention (open bars), of paper sheets prepared with *Tl*CBM-A-PAM. (a) hardwood handsheets; (b) softwood handsheets.

Thus, *Tl*CBM-A-PAM was relatively sensitive to the wet-end conditions. In the case of SBKP fibers, virtually no additive was retained in paper sheets, regardless of the wet-end condition. It has been reported that the binding of xylanase on xylan substrate was sensitive to electrostatic conditions such as pH and ion strength (Tenkanen et al. 1995). Hence the proteinic characteristics of *Tl*CBM might be directly involved in the weak affinity of *Tl*CBM-A-PAM for pulp fibers in the contaminated wet-end system, resulting in unfavorable decrease in the physical properties of handsheets.

Although further work should be carried out both to elucidate in more detail the retention mechanism and to evaluate other paper properties (freeness, tearing strength, etc.), the unique behavior of CBM-polymers having different responses to the types of pulp fibers and the wet-end conditions was clearly shown in the sequence of handsheet investigations. Of the three CBM-A-PAMs tested in this study, *Tv*CBM-A-PAM showed the best performance as a paper-strength additive for hardwood sheets (Figs. 4-6a). *Tr*CBM-A-PAM and softwood pulp fibers were also a good match (Figs. 4-6b), whereas xylanase CBM-A-PAM appeared to be limited to uncontaminated systems. These results imply that the functional characteristics of the CBM-A-PAMs depend a great deal on the properties of the original CBMs. That dependence expands the functional design of papermaking additives by CBM-conjugation, for potential applications to advanced cellulose-based materials.

CONCLUSIONS

1. Carbohydrate-binding modules (CBMs) were successfully conjugated with A-PAM, which otherwise has no affinity for pulp fiber, by an EDC-mediated condensation reaction. The synthesized CBM-A-PAMs displayed good retention and paper-strength enhancement.
2. CBM-A-PAMs designed using different CBMs demonstrated unique retention behavior, depending on the combination of enzyme sources and pulp types, possibly because of their inherent affinities for carbohydrate-based substrates.
3. CBM-A-PAM from *T. viride* showed good additive retention on hardwood pulp fibers, resulting in high tensile strength of paper sheets, even under the contaminated conditions. On the other hand, *T. reesei* CBM-A-PAM showed better performance for softwood sheets. Xylanase CBM-A-PAM was effective for hardwood rather than softwood fibers in a pure system.

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