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Gunnar Henriksson · Maria Christiernin
Roland Agnemo

Monocomponent endoglucanase treatment increases the reactivity of softwood sulphite dissolving pulp

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Abstract Softwood dissolving pulp was treated with a commercial monocomponent fungal endocellulase. The reactivity of the pulp for the production of rayon and cellulose derivatives as determined with the Fock method increased drastically with relatively low amounts of enzyme, and the yield loss and decrease of viscosity were moderate. The mechanism behind the increased reactivity is discussed.

Keywords Endoglucanase · Cellulase · Dissolving pulp · Viscose

Introduction

All true plants (Kingdom *Plantae*), as well as some other organisms including certain bacteria and animals, produce the technically and economically important polysaccharide cellulose. The main component of plant cell walls, cellulose, in its native form (i.e. cellulose I), is used in various textiles based on cottonseed hair [1], flax, and hemp bast fibres [18], and in various construction materials [13]. Intense chemical treatment of wood, such as kraft and sulphite pulping, removes lignin and other non-cellulose components, forming a pulp consisting of released and more flexible cell walls [17]. Chemical pulps made from wood are used mainly for paper and cardboard products, but microcrystalline cellulose, i.e. pulp, where almost all non-cellulose material has been removed and the cellulose is partially depolymerised, is also an important product, e.g. in pharmaceutical tablets and as a filler in certain polyphenolic plastics [8]. Furthermore, regenerated cellulose, i.e. cellulose that has been dissolved and re-precipitated, and cellulose derivatives, such as carboxymethyl cellulose, cellulose acetate and cellulose nitrate, are also important products, used for various purposes such as non-woven materials, explosives, textiles, optical plastics and thickeners [4]. The cellulose used for such purposes is either cotton, or special chemical wood pulps called "dissolving pulps", made either by kraft pulping of pre-hydrolysed wood chips, or by acidic sulphite pulping [10].

The main reason for the extensive use of cellulose is, in addition to its great abundance [11], the structure of the polysaccharide; cellulose is a linear, non-branched homo $\beta 1 \rightarrow 4$ glucan with a degree of polymerisation that can be as high as 8,000 in wood and perhaps even 20,000 in some algae. The unbranched glucan chains form crystalline bundles known as fibrils (also called microfibrils or elementary fibrils), which are held together by hydrogen bonds and hydrophobic interactions [19]. These vary in shape and size between different species, but typically contain from about 50 up to several hundred cellulose chains [17]. The fibrils are further organised into higher hierarchic levels in the plant cell wall [20].

The complex morphology of the plant cell wall, and the high crystallinity of cellulose are problematic for the production of cellulose derivatives and regenerated cellulose, since contamination from non-reacted cellulose particles is generally undesired. Furthermore, the chemicals used for the production of regenerated cellulose are highly toxic, e.g. the CS_2 used in the viscose process, or expensive, e.g. the solvent *N*-methylmorpholine-it N-oxide used in the Lyocell process. Thus, in the manufacture of regenerated cellulose products and cellulose derivatives, the reactivity of the pulp is important both technically and economically.

Various methods have therefore been tested for increasing the reactivity of dissolving pulps, and one of the most interesting approaches is treatment with cellulose degrading enzymes, cellulases. Treatment of cellulose with these enzymes is reported to increase the

G. Henriksson (✉) · M. Christiernin
Department of Fibre and Polymer Technology,
Royal Institute of Technology, KTH,
100 44 Stockholm, Sweden
E-mail: ghenrik@pmt.kth.se
Fax: +46-8-7906166

R. Agnemo
Domsjö Fabriker, Örnsköldsvik, Sweden

fraction of cellulose soluble in strong alkali [15, 16]. Extracellular cellulases from aerobic filamentous fungi are among the most used enzymes in biotechnology today [3]. For efficient cellulose degradation, at least four functional types of enzymes seem to be required: A-type enzymes progressively degrading cellulose chains from the reducing end in exo-mode, releasing cellobiose, i.e. enzymes that slide along the cellulose chain, hydrolysing glucosidic bonds with the release of cellobiose units; B-type enzymes attacking the cellulose in similar exo-mode, but mainly from the non-reducing end; C-type enzymes able to perform cleavage within a cellulose chain, i.e. in endo-mode; D-type small endo-enzymes that, in contrast to the other types, do not strongly adsorb to crystalline cellulose via a special cellulose binding domain (CBD) [7, 9]. The different types of enzymes cooperate in a sophisticated, yet not fully understood way [5, 14]. In purified form, all these cellulases are very inefficient in degrading cellulose individually [14], but they can, on the other hand, perform highly specific modifications of the cellulose that may be of high technical interest. For instance, it was demonstrated that C-type endoglucanases were significantly more efficient in increasing alkaline solubility than were cellobiohydrolases of A- and B-type [16]. Commercial genetically modified fungi produce large amounts of different pure, or close to pure, cellulases (of types A, B, C or D), and such monocomponent products are used, among other uses, as additives to laundry detergents.

In this paper we report the ability of a commercial C-type endo-cellulase to increase the reactivity of dissolving pulp by the Fock method [6], which is a more relevant method than alkaline solubility for predicting chemical consumption in the viscose process.

Materials and methods

Materials

Industrial sulphite pulp was produced from a mixture of 80% Norway spruce (*Picea abies*) and 20% Scots pine (*Pinus sylvestris*). The wood chips were cooked to a kappa number of 5, corresponding to a lignin content of 0.75% of produced pulp. Cooking was performed in two stages. In the first stage the chips were impregnated with 60 kg sodium hydrogen sulphide in solution form per ton wood (dry weight) at a wood to liquor ratio of 1:4.5, with a pH of 4–5, for 2–3 h at 142–153°C. The cooking liquor was then replaced with a sulphur dioxide solution at 6.0 bars pressure. For the second stage the temperature was 140–142°C, the duration was 12 h, and the pH was 2. The wood to liquor ratio was 1:3.5. Thereafter the pulp was bleached by an alkaline extraction stage followed by two washings, and an alkaline hydrogen peroxide stage followed by two washings.

The endoglucanase preparation Novozyme 476 produced from a genetically modified *Aspergillus* fungus was a kind gift from Novozyme, Denmark. According to the manufacturer, Novozyme 476 is a monocomponent cellulose, and SDS-PAGE showed a single dominant band with only traces of other bands (L. Hildén, Uppsala University, personal communication). The substrate specificity of the enzyme strongly indicated that Novozyme 476 contained endocellulase activity with no significant exo-cellulase activity (not shown). The product was used without any further purification. The cellulolytic activity was determined by the manufacturer following analytical standards, and is expressed in endo cellulase units (ECU). The enzyme activity of the preparation was 5,000 ECU/g. All other chemicals used were of analytical grade.

Enzymatic treatment of pulp

Before enzymatic treatment, the un-dried pulp was adjusted to pH 7 with sulphuric acid (as a 3% suspension in deionised water), and washed with deionised water until the conductivity was below 5 μS. Incubations with enzyme were carried out under optimal conditions for the enzyme according to the manufacturer (pH 7 and 50°C) in 50 mM bis-Tris buffer with a pulp concentration of 1% under stirring with RW 20 rotors with a stirring blade for 22.5 h. Thereafter, the fibre suspension was filtered through a 45 mesh nylon filter in a Buchner funnel, and freeze dried.

Reactivity measurements

The reactivity of the cellulose was determined by the method described by Fock [6] with slight modification. The Fock method is a micro-scale process simulating the industrial viscose process for making regenerated cellulose. The test was carried out in two steps.

Step 1: Preparation of viscose from dissolving pulp and collection of the regenerated cellulose

Pulp samples of 0.5 g were weighed in a 100-mL Erlenmeyer flask with a stopper, and mixed with 50 mL NaOH (90 g/L) and 1 mL CS_2. The samples were stirred with magnetic stirrer (300 L/min) for 3 h at room temperature, and were transferred quantitatively to a 150-mL test tube with a glass stopper, diluted to 100 g using deionised water, and shaken. The solution was then left for 2 h to allow undissolved cellulose to sediment. Ten millilitres of the clear solution from the upper part of the contents of the measuring cylinder was weighed in a 100 mL beaker and neutralised using 5% H_2SO_4 (the yellow solution was de-coloured) then left overnight in a fume cupboard to evaporate.

Step 2: Measurement of the residual cellulose

The dried regenerated cellulose samples were mixed with 20 mL68 % H_2SO_4 and stirred with a magnetic stirrer for

1 h. The milky solutions were then transferred to a 250 mL Erlenmeyer flask, diluted with deionised water to 50 mL and mixed with 10 mL 1 *N* (1/6 M) $K_2Cr_2O_7$. The solutions were refluxed for approximately 10 min to clear solution. When the regenerated cellulose samples were oxidised, the solutions were transferred to a 100 mL-measuring flask and diluted with de-ionised water. Aliquots (40 mL) of the solutions were transferred to 250-mL beakers containing 0.5 g KI. The solutions were stirred using a magnetic stirrer and titrated with 0.1 *N* $Na_2S_2O_3$. When the brown solutions started to change colour, half a spoonful of starch was added and the solutions turned dark-blue/violet. The titration continued until the solution turned almost green, indicating that all the I_2 was reduced. The volume of $Na_2S_2O_3$ required in each case was determined.

Determination of degree of polymerisation

The degree of polymerisation of the cellulose was determined by gel permeation chromatography (GPC) according to SCAN-CM 15:99 [2].

Yield determination

The pulp yields of the treatments were measured by the determination of released reducing carbohydrates in the filtrate after the enzyme incubations. The colorimetric assay based on parahydroxy benzoic acid was used [12]. The assay solution consists of 2 g parahydroxy benzoic acid hydrazid and 294.5 mg $CaCl_2$ dissolved in 40 mL 0.5 M HCl. A working solution was made by mixing 5 mL assay solution with 20 mL 0.5 M NaOH, and was used immediately. Samples of 0.5 mL were mixed with 0.9 mL working solution, heated to 97°C for 8 min, cooled and centrifuged for 5 min at 13,000 *g*. Absorbance at 415 nm was determined and concentrations were determined from glucose standards.

Results and discussion

The dissolving pulp was treated with three different amounts of the monocomponent cellulose (Novozyme 476). The reactivities of the dissolving pulps were measured with the Fock method [6], which simulates the viscose process, i.e. swelling in alkali, transformation to cellulose xanthate with CS_2, which makes the polymer soluble, and removal of the $–CS_2$ group in diluted sulphuric acid forming re-precipitated cellulose fibres [6]. Even with the lowest amount of enzyme used, the cellulase-treated samples had highly increased reactivity. In spite of this, the decreases in degree of polymerisation expressed as viscosity were well within acceptable levels—to form rayon of good quality, the viscosity of the pulp actually needs to be lowered to 200–300 cm^3/g during the viscose process. Furthermore, yield losses during the enzymatic treatments were low (Table 1). These data compare favourably with those of other methods for increasing the reactivity of dissolving pulps. Thus, the concept of enzymatic activation of dissolving pulps appears to be an attractive alternative for enhancing the viscose process, and maybe also in the manufacture of cellulose derivatives.

What is the mechanism behind the activating effect of the endocellulase treatment? The relatively moderate decrease in viscosity suggests that a simple decrease in degree of polymerisation is not the answer. Endoglucanases, i.e. cellulases of the C- and D-type, are known to preferably degrade amorphous cellulose rather than highly crystalline cellulose [14]. Since less crystalline, or at least structurally diverse cellulose, seems to occur between, and on the surface of, cellulose fibrils [19, 20], endoglucanase activity might therefore lead to a swelling of the cell wall, leading to increased exposure to solvents and reagents of the dissolving pulp cellulose. A further possibility is that cellulose fibrils can contain short segments with less ordered cellulose; endoglucanases can attack these regions and eventually cut the fibril. This might also lead to increased reactivity of the pulp. Figure 1 presents a speculative model of cellulose-mediated activation. Degradation of cellulose II (antiparallel chains) by the enzyme might also play a role in reactivity.

The advantage of using a monocomponent endoglucanase over a "culture filtrate" type of commercial cellulase, which contains a complete set of cellulolytic enzymes, is that the reaction is better controlled, and that the yield losses will probably be lower, i.e. crystalline cellulose will be degraded to a lesser extent.

Table 1 Effects of endoglucanase treatment of dissolving pulp. *ECU* Endo cellulase units

Enzyme load (ECU/g dry pulp)	Reactivity of pulp (%)[a]	Viscosity (mL/g)[b]	Molecular weight of cellulose (Da)[c]	Yield(%)
0	77.5 ± 1.5	517	420,000	100
5	90.4 ± 1.7	489	290,000	99.5
50	90.5 ± 1.6	420	280,000	99.1
500	90.9 ± 0.8	343	280,000	98.7

[a]Determined by the Fock method [6], see Materials and methods
[b]Determined according to SCAN method [2]
[c]Determined by gel permeation chromatography as average molecular weight

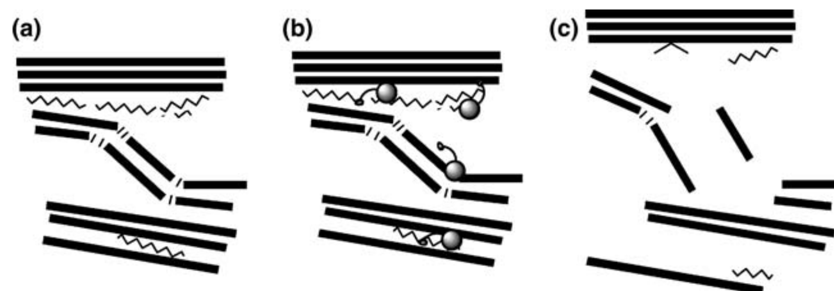

Fig. 1a–c Hypothetical mechanism of the activating effect of the monocomponent cellulase. **a** The cellulose in the pulp fibres consists mainly of crystalline fibrils (▬), but more amorphous cellulose is located on the surface and between fibrils (〰), or in shorter segments within the fibrils (ıııı). **b** The monocomponent C-type endoglucanase (⁀●) preferentially attacks the more amorphous cellulose. **c** Partial degradation and nicking of the amorphous regions in the cellulose leads to separation of the fibrils, i.e. to a swelling of the fibre that increases its reactivity. Although some fibrils have been cut at amorphous regions, the degree of polymerisation is not drastically decreased

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