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Cellulose derivatives: An enzymatic approach to their modification

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Carboxymethyl cellulose (CMC) with different degrees of substitution (DS) and molecular weights (MW) have been successfully hydrolyzed by cellulases sourced from different microorganisms. The extent of enzymatic hydrolysis of CMC was shown to decrease with increasing DS. According to chromatographic analyses, the best enzymatic degradation by the crude enzymic preparations employed was 47% when cellulase T from *Trichoderma* species acted on a CMC of DS = 0·7. However, the complete hydrolysis, required for a quantitative analysis, was reached when CMCs with DS up to 0·7 were degraded by cellulase P, a purified form of celluclast from *Trichoderma reesei*.

INTRODUCTION

The importance of cellulosic materials as a renewable resource can be clearly seen from the growing number of conferences held to consider the utilization of this valuable material as well as the many aspects of biomass conversion and utilization available in the literature (Shimichi *et al.*, 1986; Martin *et al.*, 1988; Donnison *et al.*, 1989; Kennedy & Melo, 1989; Kennedy & Patterson, 1989; Kennedy & Hossain, 1992).

Enzymatic hydrolysis of cellulose results in the breakdown of cellulose to D-glucose. There are many microorganisms, such as bacteria and fungi, that produce extracellular cellulase enzymes (Beldman *et al.*, 1988; Chernoglazov *et al.*, 1988; Bhat & Wood, 1989; Szczodrak, 1989; Gogilasshcvili & Khvedelidze, 1992). Cellulose is resistant to degradation since a cellulose molecule serves largely as a structural molecule rather than as a molecule to store energy (Ahlgren & Ericksson, 1969). The susceptibility of cellulose to enzymatic hydrolysis depends on its physical-chemical and structural parameters.

Cellulose may have the hydrogen atoms of the primary and secondary hydroxyl groups replaced by reactive groups such as methyl, ethyl, carboxymethyl, etc., forming cellulose derivatives. The addition of these groups makes cellulose less crystalline and more soluble in water. The solubility is proportional to the degree of substitution (DS) and the solvating capacity of the substituent groups (Fan *et al.*, 1981, 1987; Nicholson & Merritt, 1985). The susceptibility of cellulose derivatives to enzymic hydrolysis increases as the derivatives becomes more water soluble and less crystalline up to the complete solubility point. After this point, the susceptibility decreases with increasing DS. Substituent groups with large molecular dimensions are more effective in resisting enzymatic degradation than small groups (Reese, 1957).

In this paper enzymatic hydrolysis of pure carboxymethyl (CMC) with different DS and different molecular weights (MW) has been carried out in order to obtain 100% hydrolysis and to analyze the chromatographic behaviour of their hydrolyzates.

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EXPERIMENTAL

Materials

Samples of CMC with different DS, ranging from 0·63 to 2·10, and different MW, ranging from $0{\cdot}55 \times 10^6$ to $8{\cdot}71 \times 10^6$ were obtained from Courtaulds Research, plc, Coventry, UK. Pure CMC (described as 98% pure with DS = 0·7) was also obtained from Courtaulds Research, plc. CMC BDH (a laboratory grade CMC from BDH Laboratory reagents, DS = 0·7–0·8 and 99 pure) was used as standard material.

Celluclast, made by submerged fermentation from a strain of *Trichoderma reesei* purified with isopropanol, is characterized by its contents of exoactivities, namely cellobiohydrolase (EC 3.2.1.91), and an exo-1,4-D-glucanase (EC 3.2.1.71), as well as an endo-activity, endo-1,4-D-glucanase (EC 3.2.1.4), was obtained from Novo Laboratory. Its activity is (2·206 IU/g protein). Celluclast (aged), stored at 4°C for 18 months, was also used. Cellulase A–F (1 644 IU/g protein) and cellulase T (1 290 IU/g protein) were extracted from *Aspergillus furnigatus* and *Trichoderma* species, respectively. Cellulase P (5 902 IU/g protein, — a purified form of celluclast obtained from *Trichodema* species — was a gift from Dr Jeurgen Heitsch.

General preparation of CMC samples

CMC (10 mg) was added to 0·1 M citrate buffer pH 4·8 (1·0 ml) and stirred until completely dissolved. The CMC sample was then incubated in a water bath to reach the desired temperature required by the enzyme to be used.

Enzymatic assay of CMC samples with different degrees of substitution and molecular weights

CMC samples (10 mg) with different DS was prepared as described above and then incubated at 50°C with celluclast, aged celluclast, cellulase T and cellulase P, or at 60°C with the thermostable cellulase A–F. After the substrate solutions had reached the desired temperature, enzyme (0·1 ml, 1·64 IU/ml) was added to them. Aliquots (100 μl) were collected at intervals of 0, 5, 15 and 30 min, and 1, 2, 4, 8 and 24 h. These were added to 3,5-dinitrosalicylic acid (DNS) reagent (1·0 ml) and heated in a boiling-water bath for 10 min. After cooling to 25°C, absorbance was measured spectrophotometrically at 570 nm.

Chromatographic analysis of enzymatic hydrolyzates of CMC

CMC hydrolyzate (20 μl) was loaded on to a chromatographic column (50 cm × 1·0 cm i.d.) packed with Biogel® P2 (400 mesh). The column was connected to an automated carbohydrate detecting system, where an eluent was mixed with L-cysteine–sulphuric acid reagent, heated to 98°C and analyzed spectrophotometrically at 415 nm.

RESULTS AND DISCUSSION

The rate of enzymatic degradation of cellulose fibres is affected by the degree of crystallinity of the cellulose. An indication of an inverse linear relationship between crystallinity and degradibility was observed by Fan *et al.* (1987).

Celluclast aged, cellulase A–F (thermostable) and cellulase T enzyme preparations were used to degrade CMC. A decrease in enzymatic hydrolysis with increasing DS was observed for all enzyme activities (Fig. 1). This could be explained by the difficult access of the enzyme to the 1,4-β-D-glycosidic linkage due to steric hindrance from the carboxymethyl groups, located mainly at C-2. The distribution of these carboxymethyl groups in the cellulose molecule may also be important as there is an increase in resistance of the cellulose ether to enzyme attack as the number of contiguous substituted D-glucose units increases (Nicholson & Merritt, 1985).

CMC is produced with a wide range of degrees of substitution and molecular weights. The physical-chemical properties of cellulose ethers, for instance CMC, are dependent on the degree of polymerization, the degree of substitution and the substituent distribution pattern. The action of cellulase enzyme preparations on seven CMC samples with DS = 0·7 and MW ranging from 0·55 to $8{\cdot}71 \times 10^6$ Da was not affected by the large difference of the molecular size (Table 1). It has been reported that the degree of polymerization does not influence the efficiency of cellulose hydrolysis (Knappert *et al.*, 1980). Cellulase components, existing

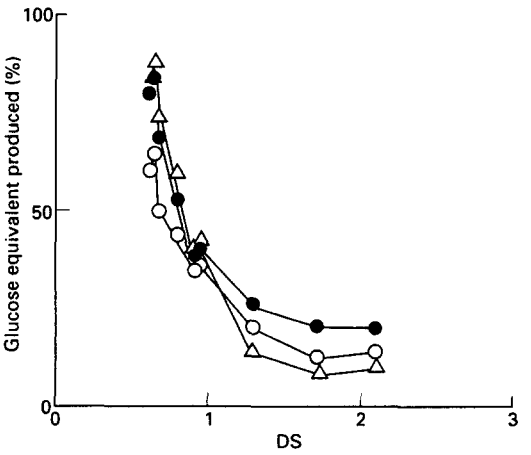

Fig. 1. Percentages of glucose equivalent produced from CMC with different DS measured by the DNS reducing sugar assay. Aged celluclast (●), cellulase A–F (○) and cellulase T (△).

Table 1. Percentages of glucose produced after 24 h of enzymatic hydrolysis of CMC with different MW. Glucose measured by the DNS reducing sugar assay

Molecular weight (weight average) (× 10^6, by GPC[a])	Glucose produced (%)		
	Aged celluclast	Cellulase A–F	Cellulase T
1·74	21·1 ± 0·7	17·0 ± 0·5	24·3 ± 0·5
0·55	24·1 ± 0·5	18·5 ± 0·5	27·7 ± 0·5
1·11	20·0 ± 0·7	13·3 ± 0·7	23·0 ± 0·5
3·16	21·0 ± 0·2	14·0 ± 0·2	24·2 ± 0·7
2·51	25·0 ± 0·5	16·7 ± 0·5	28·8 ± 0·7
1·43	20·1 ± 0·5	13·4 ± 0·5	23·1 ± 1·4
8·71	22·2 ± 1·4	14·8 ± 0·5	25·5 ± 1·4

[a]GPC = Gel permeation chromatography.

in a single microorganism, were found to have different modes of attack on CMC (Fan *et al.*, 1987; Wood & Bhat, 1988; Bhat & Wood, 1989). In this case, CMC samples with different molecular weights did not respond differently to the cellulase action, because they were probably hydrolyzed by both components of the enzyme preparations (endo-1,4-β-D-glucanases and exo-cellobiohydrolases) which led to a more uniform enzymic breakdown of the CMC samples.

Figure 2 shows that the hydrolysis of pure standard CMC BDH (DS = 0·7) by cellulase T was higher than when aged celluclast or cellulase A–F was used. (The enzymatic hydrolyzates had their reducing power measured by the 3,5-dinitrosalicylic acid.) A similar behaviour was observed for the same enzyme preparations acting on pure CMC sample 21 (DS = 0·7, Fig. 3). However, after 24 h of hydrolysis of CMC BDH and CMC sample 21 by the three cellulase preparations, higher percentages of D-glucose were produced from the degradation of CMC sample 21 for each of the three enzyme preparations compared to the hydrolysis of CMC BDH by the corresponding enzyme preparation (Table 2). This could be due to a more efficient contact of the active site of the enzyme preparation with the 1,4-β-D-glycosidic linkages of the CMC sample 21 compared with CMC DBH, leading to more effective catalysis. However, the different rates of hydrolysis found for each of the three enzyme preparations acting on the same substrate could be due to the different mode of attack of the components present in each enzyme preparation, resulting in different types of breakdown of the substrate with liberation of products with different molecular size which could respond differently to the 3,5-dinitrosalicylic acid assay.

Despite a large number of studies on the mechanism of action of cellulases, the exact mechanism of the biodegradation of cellulose is poorly understood. Many questions still remain unanswered and their resolution seems to rely on the development of either purification (Okada, 1975; Berghem *et al.*, 1984; Nisizawa *et al.*, 1978; Hong *et al.*, 1986; Yamanobe *et al.*, 1988) or genetic engineering (Gilkes *et al.*, 1984; Owolabi *et al.*, 1988; Gilkes *et al.*, 1988) techniques. At present, there is no publication reporting that CMC can be completely hydrolyzed to its monomers by cellulase enzymes. In this work, several crude cellulase preparations obtained from different sources were used in order to hydrolyze

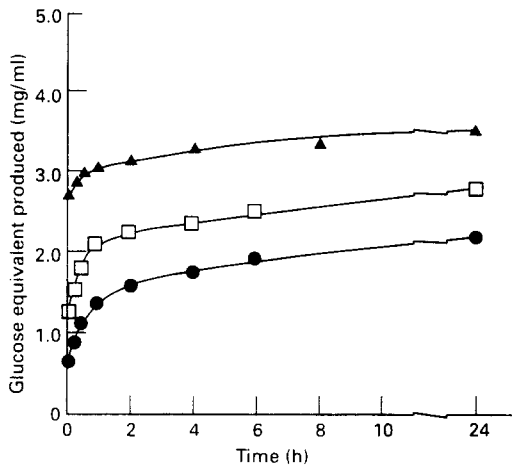

Fig. 2. Kinetics of enzymatic hydrolysis of CMC BDH by cellulase A–F (●), aged celluclast (□) and cellulase T (▲).

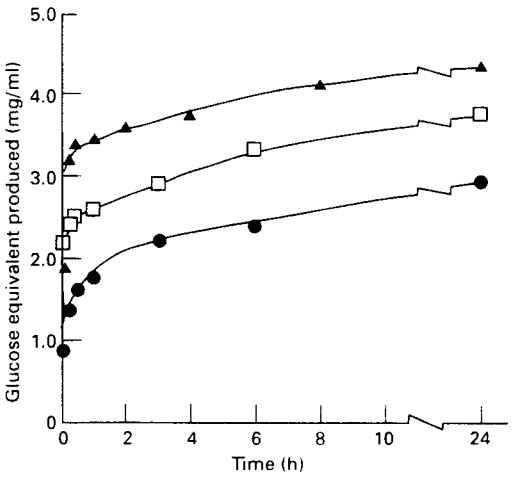

Fig. 3. Kinetics of enzymatic hydrolysis of pure CMC (sample 21) by cellulase A–F (●), aged celluclast (□) and cellulase T (▲).

industrial grade CMC samples. Forty-seven per cent hydrolysis of CMC (DS = 0·7), measured by the reducing power of its products, was the best result that could be achieved when cellulase T was used (Table 2). However, complete hydrolysis of CMC (DS = 0·7), and CMC (DS = 0·63), respectively also measured by the reducing power of their products, were found for cellulase P, a purified form of celluclast. This purified preparation indeed improved the hydrolysis of CMC with DS up to 0·92 (Table 2). However, as expected even with the purified cellulase, a decrease in enzymatic degradation was observed with increasing DS.

A chromatographic system using Fractogel® TSK-HW has been developed (Hamacher & Sahm, 1985) for separating enzymatic degradation products of CMC. A decrease in enzymatic hydrolysis of CMC with increasing DS was also observed using Fractogel® TSK-HW. However, the higher the DS, the lower the resolution obtained for the fragments formed by enzymatic breakdown. Figure 4 shows the chromatographic separation

Table 2. A comparison between enzymatic hydrolyses of CMC samples with different DS by the action of different enzyme preparations. Time of hydrolysis 24 h

DS	Enzyme preparation	Glucose produced (%)
0·63	Cellulase A–F	30·0 ± 0·7
0·69	Cellulase A–F	25·0 ± 0·7
0·78	Cellulase A–F	22·0 ± 1·4
0·92	Cellulase A–F	17·2 ± 0·5
1·73	Cellulase A–F	6·3 ± 0·5
0·7	Cellulase A–F	36·5 ± 1·4
BDH	Cellulase A–F	25·3 ± 0·7
0·63	Cellulase P	100·1 ± 1·4
0·69	Cellulase P	99·4 ± 1·4
0·78	Cellulase P	94·0 ± 0·7
0·92	Cellulase P	88·0 ± 0·7
1·73	Cellulase P	0·0 ± 0·0
0·7	Cellulase P	97·2 ± 0·5
0·63	Celluclast	42·5 ± 1·4
0·69	Celluclast	35·0 ± 0·5
0·78	Celluclast	25·5 ± 0·7
0·92	Celluclast	20·0 ± 1·4
1·73	Celluclast	8·4 ± 0·5
0·7	Celluclast	40·1 ± 0·7
0·63	Cellulase T	42·0 ± 1·4
0·69	Cellulase T	36·3 ± 1·4
0·78	Cellulase T	29·9 ± 0·7
0·92	Cellulase T	20·0 ± 0·5
1·73	Cellulase T	4·0 ± 0·5
0·7	Cellulase T	47·0 ± 0·7
BDH	Cellulase T	38·0 ± 1·4
0·63	Celluclast (aged)	40·1 ± 0·7
0·69	Celluclast (aged)	34·5 ± 0·5
0·78	Celluclast (aged)	26·5 ± 1·4
0·92	Celluclast (aged)	19·0 ± 0·7
1·73	Celluclast (aged)	6·0 ± 0·0
0·7	Celluclast (aged)	43·3 ± 1·4
BDH	Celluclast (aged)	27·5 ± 0·7

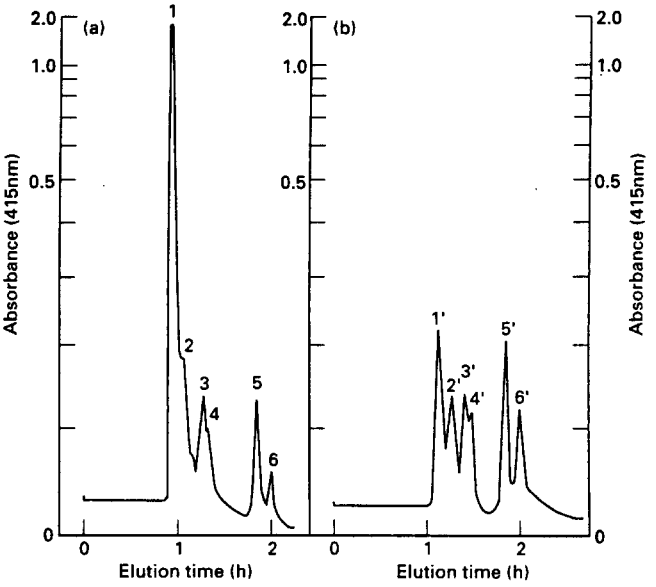

Fig. 4. Chromatographic behaviours of the hydrolyzate of pure CMC sample (DS = 0·7) obtained after enzymatic degradation by aged celluclast (a) and cellulase P (b). 1, High molecular weight CMC; 2, cellooligosacharide; 3, cellotetraose; 4, cellotriose; 5, cellobiose; 6, D-glucose. 1', Cellooligosaccharide; 2', cellotetraose; 3', cellotriose; 4', not identified; 5', cellobiose; 6', D-glucose.

of CMC sample 21 fragments obtained after enzymatic hydrolysis by aged celluclast (Fig. 4(a)) and cellulase P (Fig. 4(b)). The products obtained by celluloytic breakdown of the CMC chain with DS = 0·7 were analyzed using a chromatographic column packed with Biogel® P2 (400 mesh). The chromatographic fragment distribution of the enzymatically degraded CMC sample (DS = 0·7) confirms the improved degradation of CMC, shown in Table 2, by purified enzyme preparation since no peak corresponding to high molecular weight material was found in the sample hydrolyzed by cellulase P.

The assignment of the peaks is given in the legend to Fig. 4.

Peak 4', in Fig. 4(b), appears at an elution time that does not correspond to any peak in Fig. 4(a). The appearance of this peak could be due to liberation of a molecule with a molecular weight of a size between cellobiose and cellotriose, e.g. CM-cellobiose in a detectable amount, as a result of the enzymatic breakdown of CMC.

CONCLUSIONS

This work has shown that the extent of enzymatic hydrolysis of CMC decreases with increasing DS. The best enzymatic degradation by the crude enzyme preparation was 47% when cellulase T was used on a CMC of DS = 0·7, which was confirmed by chromatography. However, complete hydrolysis, required for a

quantitative analysis, was reached when CMCs with DS up to 0·7 were degraded by the purified cellulase P. This enzyme was very effective at hydrolyzing CMCs with DS up to and including 0·92.

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