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Effect of cellulase on the pore structure of bead cellulose

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An enzymatic treatment with cellulases from *Trichoderma viride* was investigated in its effect on the pore structure of different types of bead cellulose. One objective of this study was to establish a suitable procedure for combined enzymatic treatment and solvent exchange that would restore the original pore structure which the beads had before drying without causing major losses in mechanical stability. Another aim was to further increase the accessible pore space and internal surface area for separation of large molecular weight compounds with regard to chromatographic applications. Finally, an attempt was made to extend the findings for unsubstituted beads to the derivatives carboxymethyl (CM) and diethylaminoethyl (DEAE) cellulose beads. The enzymatically treated samples were characterized by microscopic methods and porosity measurements such as mercury porosimetry, nitrogen sorption and size exclusion chromatography. It was found that under controlled conditions the low-porosity surface layer of dried beads could be removed making the internal pore space accessible without reducing the resistance to deformation of the beads. Additionally, a shift in pore size distribution towards larger pores was observed. Supplementary swelling treatments in solvents of high swelling power could substantially restore the former porosity of the dried beads but did not enhance the accessibility to the cellulases to a considerable extent. Internal pore volume and surface area of the derivatives were dramatically increased in the case of DEAE upon enzymatic hydrolysis, however, at the expense of mechanical stability, whereas CM was found to be less affected.

KEYWORDS: Enzymatic hydrolysis, bead cellulose, pore structure, porosity measurements

INTRODUCTION

Bead cellulose as a nontoxic and biocompatible material plays an important role in various pharmaceutical and biotechnological applications. Due to its easy availability at low cost and favourable properties it rapidly gained significance when first synthesized in the late 1960s (Determann *et al.*, 1968). At present the major preparation method for spherical bead cellulose for industrial purposes is based on the dispersion of cellulose solutions in non-miscible liquid phases followed by regeneration of the cellulose. Suitable starting materials are native cellulosic fibers, pulps, or cellulose derivatives, such as cellulose acetate. The choice of the starting material and the physical-chemical aspects of the preparation method have a decisive effect upon the pore structure and the mechanical properties of the resulting product (Schleicher *et al.*, 1989). It is obvious that the procedure allows for many variations as documented in

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numerous patents (e.g., Asahi, 1973; Chisso, 1981; Kanebo, 1988). Spherical, regular shaped particles with large pores and high mechanical stability are especially useful for applications in industrial filters, chromatographic sorption and separation processes (Štamberg *et al.*, 1982; Loth and Fanter, 1993). Other successful applications include bead cellulose in powder form for wound healing (Dautzenberg *et al.*, 1985) or as additives to cosmetics (Hatao *et al.*, 1986).

Derivatives of bead cellulose with specific sorption properties have been developed and gained importance as ion-exchangers (Peška *et al.*, 1976; Matsumoto *et al.*, 1981; Motozato and Hirayama, 1984). Useful products can be obtained, for instance, by either cross-linking (Loth and Philipp, 1989) and/or by functionalizing the bead skeleton to the desired derivative under carefully controlled conditions in order not to affect the porous structure of the particles. Depending on the degree of substitution, cross-linking of the products might become necessary to assure insolubility in aqueous media.

It was found that solvent exchange methods to replace water in the structure can help to maintain the porosity of bead cellulose which would otherwise be considerably reduced when directly dried from water (Peška *et al.*, 1978). Although it is possible at present to produce bead cellulose and its derivatives in more or less custom-made particle sizes and with a broad range of porosity (e.g., by mixing the viscose solution with a copolymer (Kajmakovic *et al.*, 1987)), research is focused on an easy method to further increase and to actually tailor pore sizes for particular end-uses.

A novel approach is the application of enzymes to bead cellulose, possibly combined with solvent treatments, which will be presented in this work. The enzymatic hydrolysis of cellulosic materials has attracted major attention lately, especially when focused on the improvement of surface properties of cotton fabrics (Olson, 1988; Pedersen *et al.*, 1992; Clarkson *et al.*, 1993). The effect of enzymes has been compared to acid hydrolysis of different cellulosic materials by different research groups (Lenz *et al.*, 1990; Buschle-Diller and Zeronian, 1994). However, little research is documented on the practical aspects of the applicability of enzymes to regenerated cellulosics. The major objective of this work was to develop a procedure involving cellulases which would specifically alter the pore structure of bead cellulose towards more and larger pores. Another aspect was whether cellulases could also be applied to the bead derivatives to improve separation of large molecular weight compounds. For the characterization of the enzymatically modified products microscopic methods and porosity measurements were used, such as nitrogen sorption, mercury porosimetry, and size exclusion chromatography. It was found that under mild conditions the enzymatic treatment led to a shift in pore size distribution and to a larger accessible surface area without affecting the mechanical strength of the beads.

MATERIALS AND METHODS

Materials

Three types of bead cellulose were purchased at SCHZ Lovosice, Czech Republic: Perloza ST (dried, low-porosity product; particle size 30–50 μm), Perloza MT50 (never dried Perloza ST) and Perloza MT200 (never dried, high-porosity product; particle size 80–100 μm).

Preparation of cellulose derivatives in bead form

Carboxymethyl cellulose (CM). As starting material for carboxymethylation any type of bead cellulose could be used: 0.1 mol of bead cellulose was soaked in water, filtered and mixed with 0.16 mol of monochloroacetic acid and 0.16 mol of sodium hydroxide. The water content of the mixture was adjusted to 80%, based on total cellulose, at 60 °C under vacuum. Then 0.21 mol of sodium hydroxide dissolved in as much distilled water as necessary for 10 mol total water in the reaction mixture was added. The reaction mixture was heated to 85 °C for 1 h, then cooled and filtered. Fractions of desired particle sizes were separated from the bulk through screen filters with distilled water. After addition of a small amount of sodium azide, the CM:water suspensions are stable up to 2 years.

Diethyl aminoethyl cellulose (DEAE). First 0.1 mol of any type of bead cellulose with known water content was added to 0.11 mol of 2-chloroethyl diethylamine hydrochloride. The water content of the mixture was adjusted to 70–75%, based on total solids, at 60 °C under vacuum. Then 0.23 mol of sodium hydroxide dissolved in 10 mol water (total) was added and the mixture was stirred vigorously at 60 °C for 10 min. The reaction mixture was then heated to 85 °C for 30 min. Within 5 min, 110 ml of 1 N HCl was slowly added under stirring. The pH should then be below 2. The mixture was allowed to stand for 30 min with occassional stirring. It was neutralized by repeated suspension with distilled water and decantation. Fractions of desired particle sizes were separated from the bulk through screen filters with distilled water. After addition of a small amount of sodium azide, DEAE:water suspensions are stable up to 2 years.

The starting material for the cellulose derivatives investigated in this work was Perloza ST in both cases. The degree of substitution was determined to 0.35 and 0.3 for CM and DEAE, respectively.

For the enzymatic hydrolysis, cellulases (EC 3.2.1.4) from *Trichoderma viride* and from *Aspergillus niger*, purchased at Sigma Chemicals, were used without further purification. Glucose, cellobiose and model dextrans of various molecular weights for gel chromatography were obtained from Serva Fine Biochemica. All other chemicals (Merck) were of reagent grade.

Swelling treatments and enzymatic hydrolysis

The enzymatic hydrolysis of bead cellulose was carried out at the pH and temperature optimum of the cellulases (pH 5, 0.05 M sodium acetate buffer; 37 °C). The following procedure uses 2.19 g of cellulose beads based on the dry weight, which in all cases was determined separately. Perloza MT50 and MT200 samples were washed with distilled water, filtered by suction and transferred to polyethylene bottles. Perloza ST was directly weighed into polyethylene bottles, 300 ml of buffer solution was added, the mixture was vigorously shaken and was adjusted to 37 °C in an incubator. Then 2500 units of cellulase in 40 ml buffer were then added to the samples which were then left in the incubator for varied lengths of time with shaking. The enzymatic reaction was terminated by washing the samples with distilled water and deactivating the enzyme with acetone (marked by 'A') or with hot distilled water (< 80 °C; marked by 'W'). The samples were slowly dried by stepwise solvent exchange using a rotary evaporator. In the case of acetone inactivation, the solvent exchange sequence was acetone, ethanol,

cyclohexane; in the case of water inactivation it was ethanol:water (50:50, 75:25, 90:10), 100% ethanol, cyclohexane.

Some of the ST samples were preswollen in ethanolamine prior to the enzymatic treatment, carefully washed with distilled water and used directly for the hydrolysis without intermediate drying.

Characterization of the products

Determination of the sedimentation volume

Cellulose suspension (8–10 ml) was placed into a 10-ml measuring cylinder and allowed to stand at room temperature for 12–24 h until equilibrium had been achieved. The sedimentation volume was read off and the suspension filtered. After drying at 105 °C the sedimentation volume was calculated based on the weight of the dry material. Three parallel measurements were averaged.

Characterization by porosity measurements

Nitrogen sorption

For the nitrogen sorption measurements a Sorptomatic 1900 (Fisons Instruments) was used. First, 100–200 mg of the samples were weighed, placed into the sample tubes and dried under vacuum at 60 °C for 24 h. Measurements were carried out in adsorption and desorption in a liquid nitrogen atmosphere and with liquid nitrogen flushing. The linear part of the BET-equation (Brunauer, Emmett, Teller) at pressures $0.05 < p/p_0 < 0.30$ was used to calculate the specific surface area for monomolecular covering (Weatherwax, 1974):

$$\frac{p}{V_a(p_0 - p)} = \frac{1}{V_m C} + \frac{(C-1)}{V_m C} \times \frac{p}{p_0}$$

with p = pressure at equilibrium, p_0 = saturation pressure, V_a = adsorption volume, V_m = volume for monolayer adsorption, C = constant.

Pore size distribution and specific pore volume were obtained using the Kelvin equation (Gregg, 1958) for cylindrical pores.

The porosity of a sample can be calculated as the fraction of pores based on the total volume of the sample.

All measurements were carried out in duplicate.

Mercury porosimetry

From 100–200 mg of the sample material were weighed accurately and placed into a glass dilatometer. Vacuum was applied for 60 min in a Macropores Unit 120 (Carlo Erba Instruments); the dilatometer was filled with mercury to the 10 mm mark and then transferred to the high pressure unit of a Porosimeter 2000 (Carlo Erba Instruments). Pressure was slowly increased to 2000 bar while recording the drop of the mercury column. Using the Washburn equation for cylindrical pores the pore radius can be calculated (Paul and Bartsch, 1972):

$$p \cdot r = -2\sigma \cos\theta$$

where p = absolute external pressure, r = pore radius, σ = surface tension of mercury and θ = contact angle.

Given values are the average of two measurements each.

Size exclusion chromatography

Chromatographic determinations were carried out using a Kontron 322 system and an ERC 7512 RI detector. The sample material was preswollen in 0.02 M phosphate buffer and placed into normal pressure Biorad columns of length 100 mm and diameter 10 mm. At a flow rate of 0.25 ml min^{-1}, 20 μl 0.5–1.6% solute solutions of known molecular weight were applied, with glucose being the standard. As probes, cellobiose and dextrans of molecular weights from 10^3 to 5×10^6 were used. All measurements were carried out at room temperature. From the elution volumes of the probes (V_e) and the standard (V_0), which were detected by their refractive index, the specific pore volume (V_p) and pore size distributions were obtained (Rowland, 1977; Pole and Schuette, 1984):

$$V_e = V_0 + k_{av} V_p$$

with k_{av} = distribution coefficient defined as the fraction of internal water accessible to the solute. All measurements were carried out in duplicate.

Water retention values

About 0.5 g dry material was weighed into Erlenmeyer flasks and allowed to stand in 100 ml water for 1 h at room temperature. The samples were separated from excess water by sintered-glass filters without applying pressure. They were then centrifuged at 2000 g for 10 min and the material dried at 105 °C for 24 h. The total water-holding capacity of the samples was calculated from the weight difference between the wet and dry material, based on the dry weight. The data shown are the average of two to three measurements.

Characterization by microscopic methods

Light microscope

For the light micrographs a Leitz MDRB microscope with mounted camera was used. Light micrographs were used to estimate the particle volume in the water-swollen condition. About 50 beads, before and after enzymatic hydrolysis, were measured and averaged.

Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were fixed on small strips of double-sided adhesive tape and sputtered with gold-palladium to a total thickness of 8 nm. They were investigated with a Joel JSM 6300F scanning electron microscope. Electron micrographs were used to determine the particle volume in the dry state. About 50 beads, before and after various treatments, were measured and averaged.

RESULTS AND DISCUSSION

General aspects of the enzymatic hydrolysis of cellulosic materials

The degradation reaction of cellulose substrates by enzymes is very complicated and consists of a multitude of steps with the two most important being the formation of the enzyme–cellulose complex followed by the cleavage of the glycosidic bond in the cellulose molecule. Excellent reviews on the characteristics of the enzymatic degradation of cellulose are available (Finch and Roberts, 1985; Goyal *et al.*, 1991; Henrissat, 1994; Walker and Wilson, 1991). Cellulases consist of different types of depolymerases which can act in a synergistic or inhibitory manner (Woodward, 1991). These components will attack the cellulose molecule in either endo (random) or exo (terminal) mode and, combined with the action of cellobiohydrolases and cellobiases, finally yield glucose. The composition of the enzymes in the cellulase complex varies widely depending on the source and preparation method.

Cellulose as the substrate has to be regarded as a material with structural rigidity, relatively high crystallinity and large molecular weight. Provided that the enzyme–substrate complex can be formed, accessible amorphous as well as crystalline regions will be subjected to the attack of the cellulases. As a result of the heterogeneous character of the reaction, the most influential features of the cellulose are accessible surface area, crystallinity and, to some extent, degree of polymerization (Puri, 1984; Walker and Wilson, 1991). Any pretreatments that enhance the accessibility will increase the rate of attack (for example, sodium hydroxide treatment; Buschle-Diller and Zeronian, 1994). In contrast, treatments altering the chemical composition (e.g., functionalization to a high degree of substitution) and thus changing the fine structure in cellulose might have an impeding effect. In this case the enzyme and the substrate no longer 'fit'.

In general, regenerated celluloses are less ordered, have a lower degree of polymerization than native cellulose, and are usually more susceptible to enzymatic degradation. Comparative investigations on regenerated cellulose in the form of fibres and beads showed that, in both cases, partial recrystallization occurred after coagulation and separation from by-products (Schleicher *et al.*, 1989). With progressive substitution of the hydroxyl groups in the cellulose molecule the macroporous structure of the beads is replaced by a more gel-like network in the derivatives. Mechanical stability in the wet state of these materials is considerably decreased. In addition, due to the high charge density in highly substituted celluloses, the exchange efficiency becomes unfavorable. Thus, only products with a relatively low degree of substitution are interesting with regard to chromatographic applications.

Highly swollen cellulose substrates and cellulose derivatives, such as carboxymethyl or hydroxyethyl celluloses, are especially susceptible to endoglucanases which attack internal glycosidic bonds at random. However, derivatives with a degree of substitution of higher than 1.0 have been reported to be attacked only very slowly or not at all (Timmins and Lentz, 1994).

Enzymatic hydrolysis of bead cellulose as a function of time

Microscopic observations

To set the frame conditions for the enzymatic reaction, small samples of Perloza ST were treated with *Trichoderma viride*, the course of the reaction being monitored with

the help of a light microscope as well as a scanning electron microscope (SEM). With light micrographic examination, two types of beads could be distinguished. The majority had a rather smooth surface the remainder quite a rough surface. Some of the beads combine both types of surface composition as revealed by SEM (see below). The nature of the surface plays a very important role in the degradation process.

In the initial stages (up to about 1 h), no significant alteration could be observed for either type of beads. After about 2 h incubation (Fig. 1a) the surface of the coarser beads seemed to be rougher, the cavities more distinct. Figure 2 shows a micrograph of the 2 h enzyme treated sample containing both types of surface compositions with the coarse portion predominating. The detailed view of the rough part (Figs 3a, b) illustrates a very characteristic effect of the enzyme activity: compared to the untreated sample (Fig. 3a) the granularity of the surface has intensified after the treatment, the gaps look deeper. All loose material was removed leaving an 'open' structure with softened tops. In opposition, the other, smooth part of the sample appeared slightly rougher after the enzymatic attack (Figs 3c and d). The detailed view of the surface in Fig. 3d shows moderately increased granularity and unevenness of the treated sample compared to the rather self-contained surface of the untreated beads (Fig. 3c). The former round shape has flattened to some extent as visible in the SEM micrograph in Fig. 4.

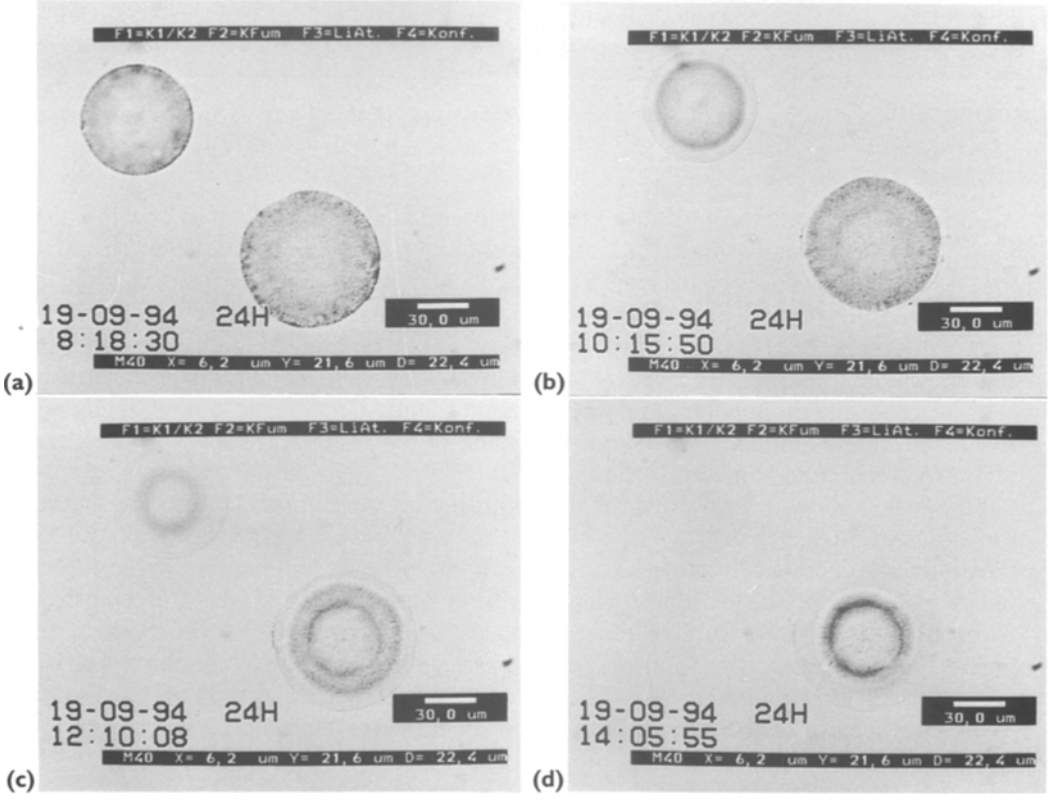


FIGURE 1. Light micrograph of Perloza ST. Course of enzymatic degradation: (a) after 2 h enzymatic hydrolysis, (b) after 4 h, (c) after 6 h, and (d) after 8 h enzymatic hydrolysis.

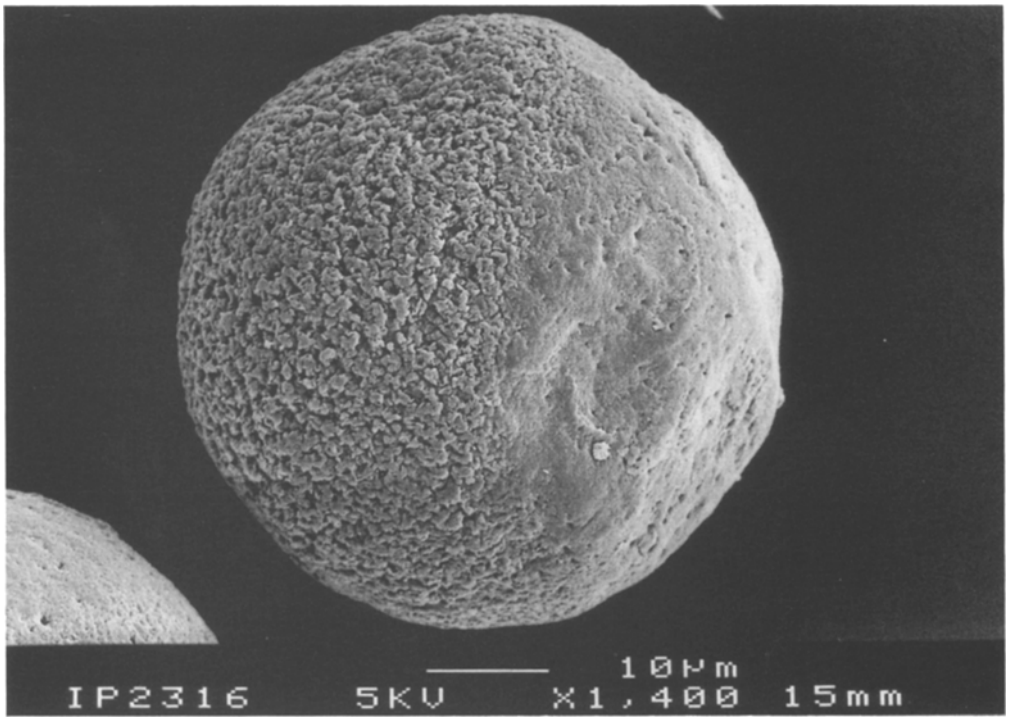


FIGURE 2. SEM micrograph of a 2 h treated ST sample combining both smooth and rough surface texture.

Continued enzymatic action caused the beads with smoother surface to develop a gel ring which became increasingly evident after about 4 h incubation time (Fig. 1b). With further enzymolysis these beads were rapidly reduced in size. Smaller beads aggregated into groups of four to six, probably forced by the gel ring formation. In comparison, the beads with coarser surface remained relatively stable for longer hydrolysis time and gelation was considerably delayed (Figs 1c, d). After 24 h incubation both types of beads were distinctly smaller with the smoother ones being almost completely dissolved. It is interesting that even after 48 h some of the beads were still visible and, as previously, maintained an almost round shape.

These observations might allow for the following interpretation. Due to the method of manufacture it seems as if the majority of the beads have a surface layer of very low porosity which encloses an interior consisting of larger pores and higher porosity. On a smaller part of the beads the surface layer is less distinct. Since the enzymatic hydrolysis is primarily a surface reaction, the surrounding skin is attacked first and removed. As a result, the interior becomes accessible for the voluminous enzyme molecules only after an initial delay and the enzymatic degradation then proceeds more rapidly. In the case of the already coarser beads, however, it could be speculated that the interior of the beads is already more accessible to the large enzyme molecules by existing macroscopic channels allowing for a more internal-type of attack. Simultaneously, prolonged cellulolytic action removes small particles from the outside of the beads as shown by intensified granularity and a 'clean' surface.

From a more application-oriented point of view, only beads with sufficient resistance to deformation and without any signs of gel formation are valuable for chromatographic purposes. Hence, with each type of cellulase as well as each type of cellulosic substrate, the hydrolysis conditions have to be established and carefully controlled. In our case the maximum incubation time for Perloza ST should not exceed 3 h.

Swelling behaviour after the enzyme treatment

From the light and the electron micrographs, an average of 50 beads were measured and the particle volume was calculated so that the reduction in size could be observed as well as the swelling behaviour as a function of incubation time. In the water-swollen state the particle volume decreased to 91% after 1 h and to 84% after 2 h of hydrolysis treatment. It is interesting that an almost identical decrease in volume was also found for the dry state (91 and 84%, respectively). Further hydrolysis reduced the volume to 78 and 73% for 3 and 4 h incubations, respectively. Thus, the reduction in size followed an almost perfectly exponential course (R^2 value = 0.994). Above 4 h treatment time, the aforementioned gel formation impeded the differentiation of the remaining solid material. The exponential relationship illustrates the rapid initial attack of the enzymes on the outside layer of the beads, then slowing down with time possibly as a result of increased accessibility of the interior of the beads (see below).

Swelling in water decreased from 271% of the untreated beads to 225% upon 1 h and 190% upon 2 h exposure to enzyme action. The removal of the surface layer thus did not increase the amount of swelling, as could be expected. The beads still maintained a high level of rigidity and mechanical stability at this stage of the enzymatic degradation.

Effect of enzyme treatment on the pore structure of bead cellulose

Table 1 shows the results of porosity measurements of ST bead cellulose in the dry state as a function of incubation time. In this series the enzyme was inactivated with acetone rinsing (A) and the sample was dried through solvent exchange. Pore size diameters in the range 7 to 100 000 nm can be determined using mercury porosimetry while measurements of nitrogen sorption indicate changes in pore size diameters from 1.5 to 150 nm.

Repeated wetting with distilled water and drying of untreated Perloza ST by stepwise solvent exchange with ethanol and ether or cyclohexane revealed an almost impervious skin around the bulk of the beads. Partial recovery of residual porosity, especially in the range of small pore sizes, could be achieved by treatment with acetone and subsequent solvent exchange. Due to the capability of acetone to quickly penetrate cellulose and displace the water molecules it is possible that some of the hydrogen bonds in the cellulose structure could not re-establish resulting in a low overall porosity of 3.44%. In addition, the organic solvents used for the solvent exchange could have a supplementary effect (Peška *et al.*, 1978). However, the observed porosity is mainly found in the small pore size range: the specific pore volume, determined by nitrogen sorption, increased from almost zero to 0.17 $cm^3\ g^{-1}$. Changes in the range of larger pore sizes were insignificant by acetone treatment alone.

The treatment with enzymes affected both specific surface area and pore volume. Changes occurred most evidently in the initial stage up to 1 h, then levelled out after

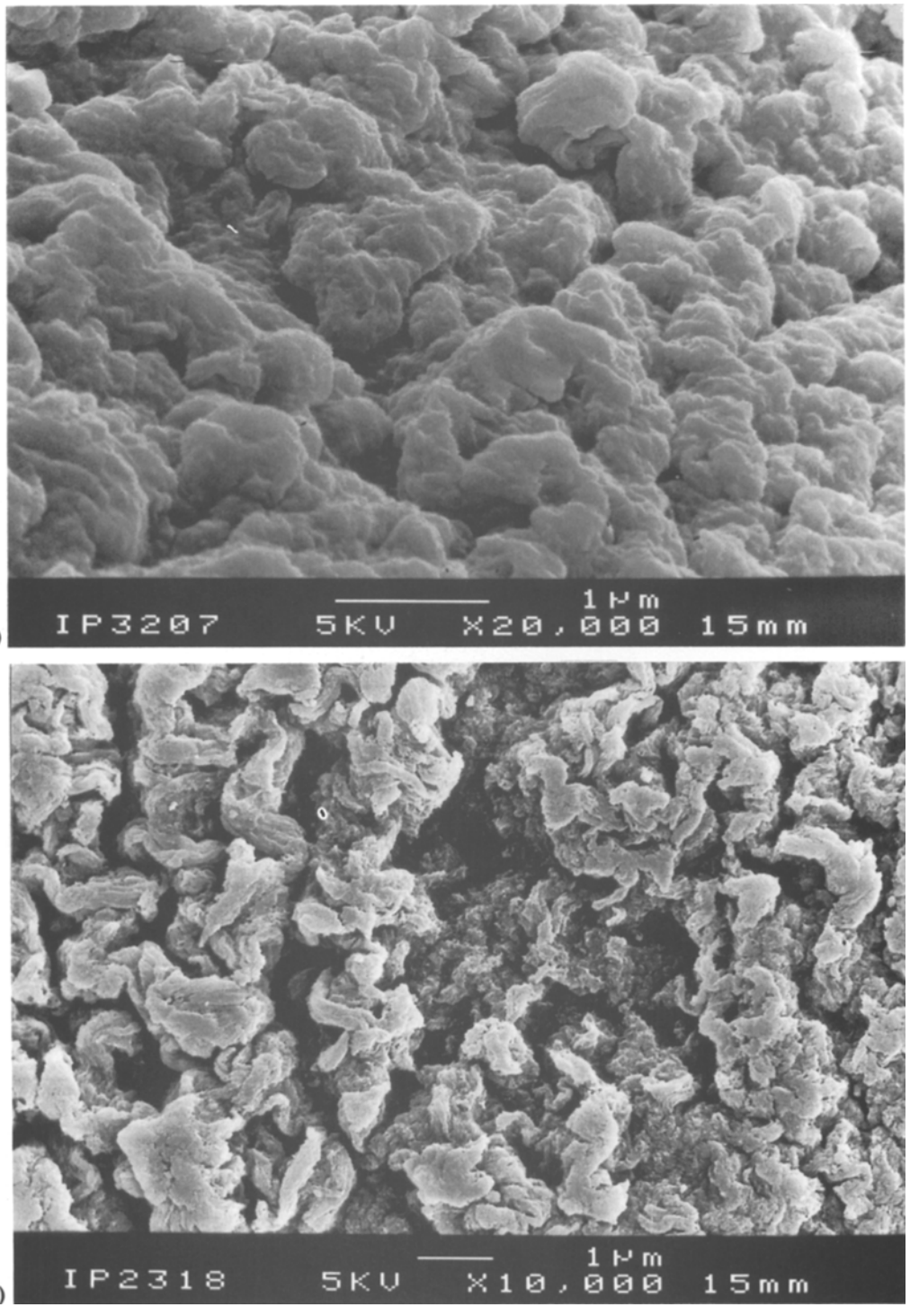
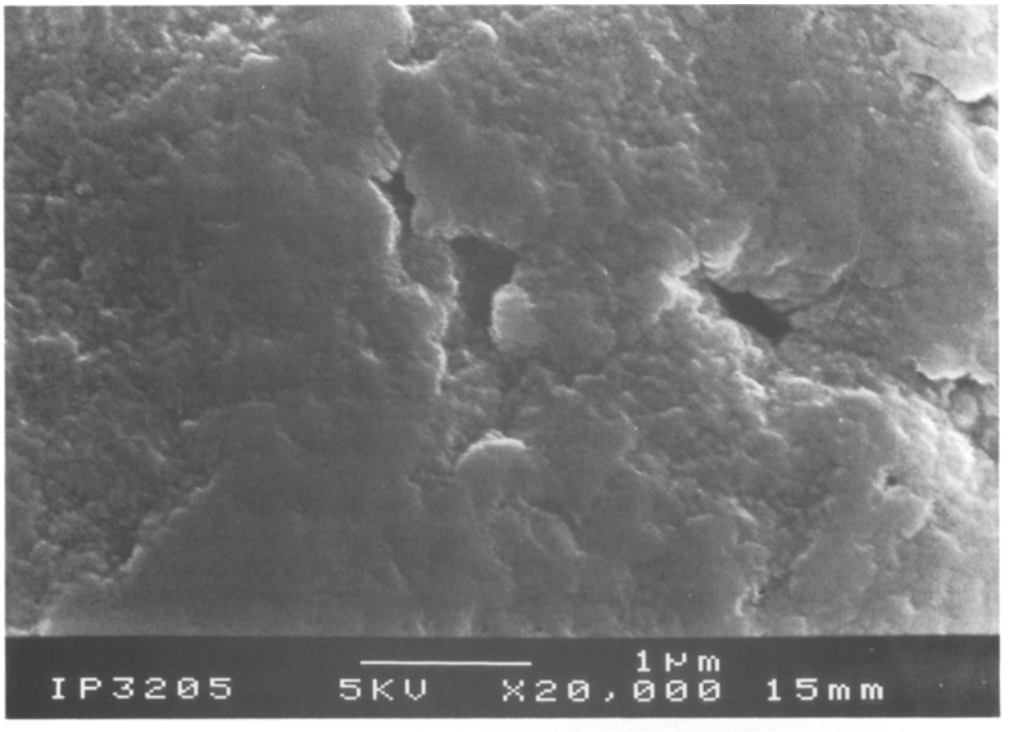
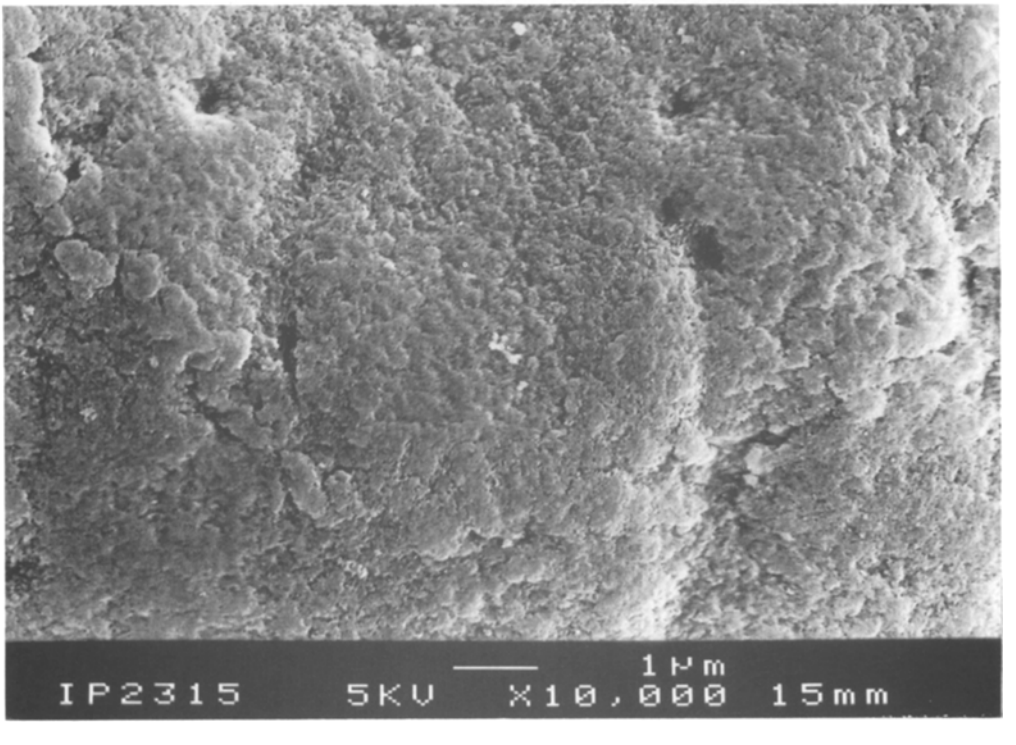


FIGURE 3. (a) Surface detail of the untreated coarse beads; (b) detailed view of the coarse fraction of the treated sample shown in Fig. 2; (c) detail of the surface of an untreated smooth sample; and (d) of the treated sample shown in Fig. 4.



(c)



(d)

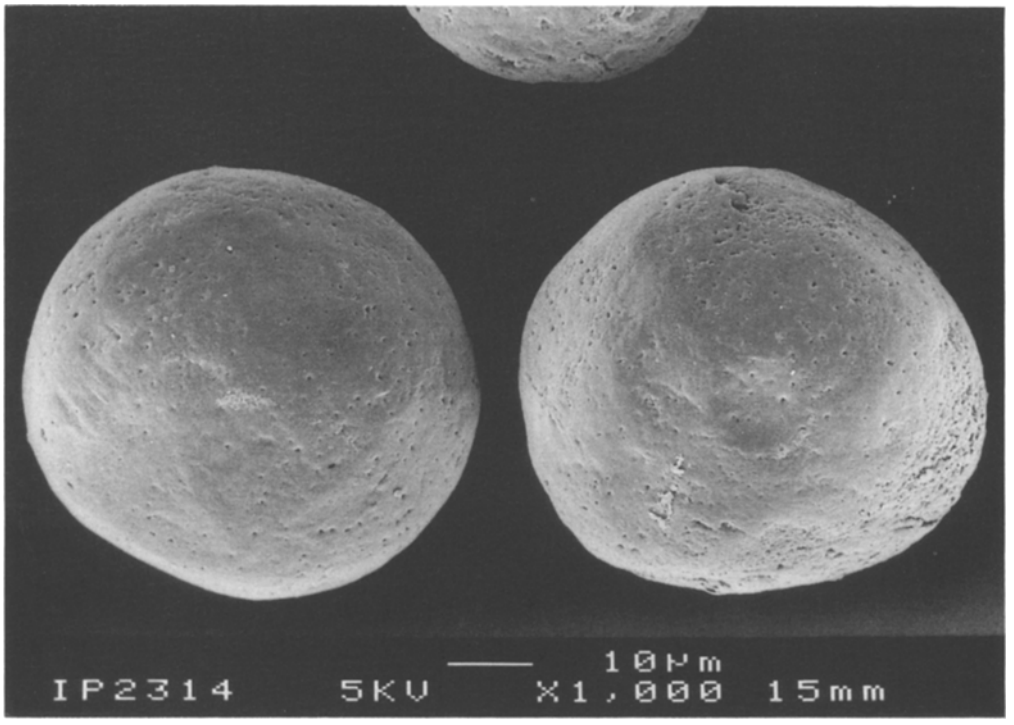


FIGURE 4. SEM micrograph of ST beads with smooth surface texture after 2 h enzymatic treatment.

TABLE 1. Porosity measurements on enzymatically hydrolysed Perloza ST as a function of incubation time

Treatment conditions	Hg-*Porosimetry*				N_2-*Sorption*	
	Specific pore volume ($cm^3\ g^{-1}$)	*Specific surface* ($m^2\ g^{-1}$)	*Average pore radius* (nm)	*Porosity* (%)	*Specific pore volume* ($cm^3\ g^{-1}$)	*Specific pore surface* ($m^2\ g^{-1}$)
untreated					0.03	0
untreated (A)[a]	0.04	7	9	3.44	0.17	68
1 h incubation (A)	0.15	25	8	8.44	0.40	183
2 h incubation (A)	0.15	24	11	10.64	0.51	258
3 h incubation (A)	0.17	26	11	14.40	0.46	202

[a]Perloza ST treated with buffer for 3 h (no enzyme); solvent exchange in the same way as with enzymatically hydrolysed samples.

longer treatment periods. Since the average pore radii remained more or less unchanged during the hydrolysis, the overall number of pores must have increased and the pore size distribution shifted. The specific surface area in the range of the larger pores increased by a factor of 3.6 from 7 to 25 $m^2\ g^{-1}$, and the surface area of pores too small to be recorded by mercury sorption almost tripled to a value of 183 $m^2\ g^{-1}$ after

1 h incubation and slightly increased further for longer treatment times. It is possible that interconnections between small pores opened during the hydrolysis, thereby forming larger pores.

The porosity values of the samples reflect the reduction in particle size of the beads during enzymatic attack by slow dismantling of the outside layer as observed by microscopy. Tiny loose particles were detached from the surface, additionally reducing the total mass of the beads. Simultaneously the number of accessible pores, channels and cavities increased due to the action of the cellulases and also contributed to the porosity.

Figure 5 exhibits the pore size distribution, obtained by mercury sorption, for the untreated ST beads in comparison to the 3 h treated sample. The graph shows a shift for very small pore sizes (radius 7–10 nm) to slightly larger sizes (about 10–19 nm) after the enzymatic treatment. The number of pores with radii between 19 and 37 nm was clearly reduced at the expense of larger pore radii in the range of 38 to about 100 nm. With the help of this graph the observed increase of both accessible surface area and pore volume at almost unchanged average pore radius can be understood.

Figure 6 graphically represents the results of size exclusion chromatography (SEC) for the same set of samples used for determination of the pore structure in the dry condition. The distribution coefficient, defined as the fraction of total internal pore water available to a solute, is plotted versus increasing molecular weights of different model solutes, and, in our case, extrapolated to 1 for the molecular weight of glucose as the standard. For reasons of clarity, values for the 2 h enzyme treatment have been omitted. This method is used to characterize the pore structure in the water swollen state which is of great significance for chromatographic problems as well as for possible further wet processes.

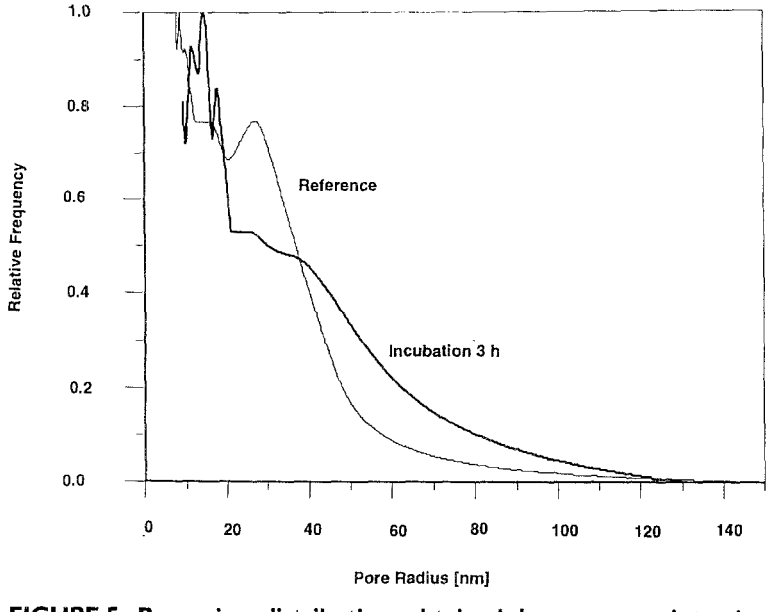


FIGURE 5. Pore size distribution obtained by mercury intrusion in ST beads, untreated and after 3 h of enzymatic hydrolysis.

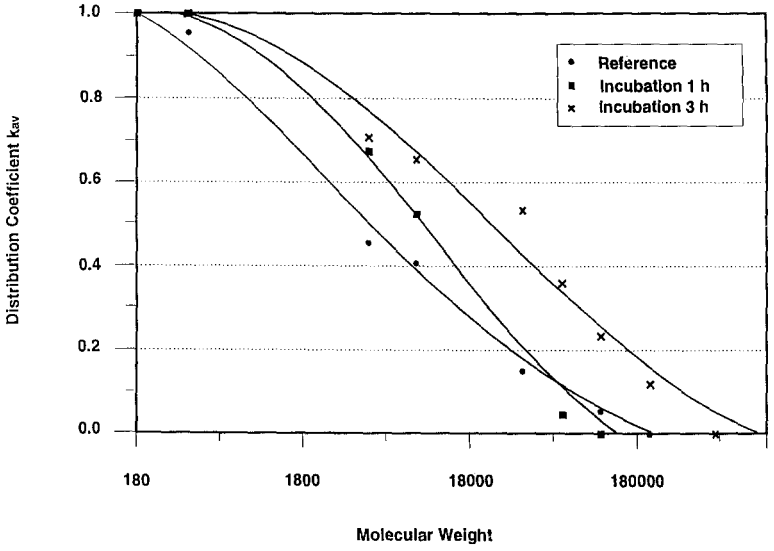


FIGURE 6. SEC distribution coefficient k_{av} as a function of the molecular weight of various solutes in solution. The graph shows a comparison of an untreated ST sample and ST samples treated for 1 and 3 h.

The curves clearly indicate a shift to increased accessibility of the wet structure for larger molecules after the enzymatic treatment. The accessibility for a dextran with an average molecular weight around 20 000, for example, increased from about 20% in the case of the untreated material to approximately 35 and 50% after 1 and 3 h enzymatic hydrolysis, respectively. Especially after 3 h incubation larger pores were made available for solutes in the molecular weight range of 50 000 and 1 250 000 while the untreated as well as the sample after 1 h enzyme treatment did not show any discernible accessibility in this range. It is interesting to note that in the molecular weight range of the cellulase components (approximately between 26 000 to 80 000; Hurst *et al.*, 1977; Finch and Roberts, 1985) the accessibility of the cellulose structure dramatically increased within 2–3 h of hydrolysis. This effect could be assigned to the likely absence of the impeding surface layer making an internal attack of the enzyme molecules possible, combined with simultaneous development of internal pores. As observed by microscopic investigations the deterioration of the beads proceeded more and more rapidly from 3 h incubation onwards.

If the internal volume V_i is plotted against the molecular diameter of the probe molecules, information about the total pore volume can be obtained by extrapolation to the size of the water molecule (2.6 Å). Table 2 shows the results for the total water holding capacity of the beads measured by SEC in comparison with values determined by the method of water retention (WRV) for the untreated ST reference as well as for samples after 2 and 3 h of enzymatic hydrolysis. Although based on two very different determination methods the data agree astonishingly well. They show a significant initial increase in total pore volume due to the enzymatic attack but no further increase within 3 h treatment time. The higher WRV in the case of the 2 h sample probably does not reflect the true internal pore water but might be attributed to the surface having not yet

TABLE 2. Total pore volume of enzymatically treated ST samples, determined by water retention (WRV) and size exclusion chromatography (SEC)

Treatment conditions	WRV (ml g^{-1})	SEC (ml g^{-1})
untreated (A)[a]	1.290	1.184
2 h incubation (A)	1.586	1.482
3 h incubation (A)	1.485	1.485

[a]see Table 1.

been completely cleaned from the attached small particles. This, in combination with an already more open structure, might suggest a higher internal volume.

Effect of intermediate drying during fabrication on the course of the enzyme treatment

For easier handling, bead cellulose is often sold in dried form. However, drying can have disadvantageous effects on the pore structure of the beads as when drying occurs directly from water, the majority of the pores will collapse. These pores will then remain inaccessible to water swelling due to the formation of strong hydrogen bonds.

Solvent exchange methods with organic solvents can help prevent the irreversible collapse of the swollen structure. If solvent exchange was used for drying, the re-wetted beads will show a similar swelling behaviour in water as shown in their respective solvents (Peška *et al.*, 1978). Another possibility for improving swelling after drying is to use protic solvents of high swelling power that are capable of more or less restoring the original swollen structure. As liquid retention experiments showed (Philipp *et al.*, 1973), ethanolamine proved especially suitable and led to very high swelling values with different types of regenerated cellulosic materials.

Dry Perloza ST was exposed to ethanolamine swelling followed by solvent exchange in order to see whether a porous structure similar to Perloza MT50, the never-dried ST product, could be obtained. Table 3 presents a comparison of pore structure data of ST and MT50 as well as of ethanolamine swollen ST samples and the effect of the

TABLE 3. Effect of intermediate drying after fabrication on the course of enzymatic treatments

Sample/treatment	*Particle size* (μm)	*Specific surface area* ($m^2\ g^{-1}$)[a]	*Specific pore volume* ($cm^3\ g^{-1}$)[a]	*Water retention value* (%)	*Sedimentation volume* (ml g^{-1})
ST reference, dry	30–50	0	0.03	139	3.4
ST, ethanolamine swelling					
untreated		195	0.40	211	3.9
1 h incubation (A)		188	0.56	170	3.9
MT50 reference, wet	30–50	182	0.55	158	3.8
MT50, 1 h incubation (A)		233	0.64	152	3.3
MT200 reference, wet	80–100	190	0.64	499	13.4
MT200, 1 h incubation (A)		269	1.11		

[a]Values determined by nitrogen sorption.

enzymatic hydrolysis on the samples. Data on Perloza MT200 are included. This also consists of never dried beads, but has larger particle sizes and larger initial pore volume.

The reference samples of MT50, MT200 and the ethanolamine swollen ST sample all have values for the accessible surface area which are of the same order of magnitude, while ethanolamine swelling reproduced only about 73% of the specific pore volume of the MT50 sample. It is interesting that the ethanolamine pretreatment created approximately the same pore structure situation in the range of small pore diameters as the 1 h enzyme treatment on the unswollen ST sample (cf. Table 1). It was expected that the enzymatic attack would proceed much faster on the preswollen sample. However, this was not the case. While MT50 and MT200 showed a further increase in accessible surface area, swollen ST samples did not. The pore volume increased in all cases to some extent. While the MT50 samples were still rather stable, MT200 samples with the highest increases in both surface area and pore volume upon enzymatic attack suffered considerable losses in mechanical stability after only 1 h incubation. Light microscopic investigations showed the collapse of the macroscopic structure and successive breaking of the beads into small pieces. This is also documented in the high values for water retention and sedimentation volume.

The water retention value of the ethanolamine swollen sample was distinctly higher than that of the untreated MT50 (158%) and decreased upon enzyme treatment from 211% to 170%. An explanation for this behaviour might be found in the sponge-like structure as indicated by SEM micrographs. Figure 7 shows the surface detail of a

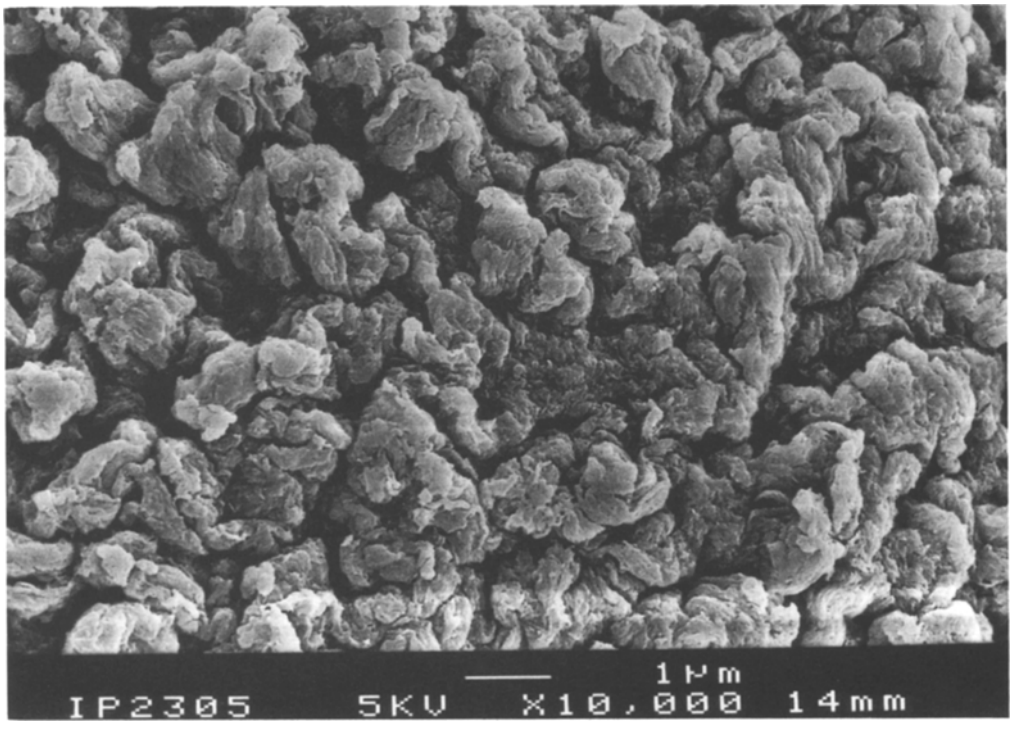


FIGURE 7. SEM micrograph of a detail of the surface texture of ethanolamine swollen ST beads.

sample after ethanolamine treatment and subsequent solvent exchange. The texture is very similar to that shown in Fig. 3b, but less open. The granularity of this structure should be capable of taking up large amounts of water as well as reagent solution. It is surprising, though, that the porosity of the sample did not change significantly after enzymatic hydrolysis. Obviously the attack of the enzymes is more concentrated on the surface of these samples removing some unevenness and reducing the possibility of water entrapment as expressed by the decreased WRV.

Figure 8 demonstrates the close similarity of ethanolamine swollen ST and untreated MT50 in the wet state. Almost identical in the accessibility range for low molecular weight molecules, slight differences were found for the exclusion limit of higher molecular weight substances. A further small shift towards better accessibility was observed with the enzyme treated MT50 while the ethanolamine treated ST sample did not change considerably (not shown in Fig. 8).

The sedimentation volume of highly swollen compounds is first and foremost of practical significance. The values (Table 3) varied from 3.3 ml g^{-1} to 3.9 ml g^{-1} for the different ST and MT50 samples. In that range, aqueous suspensions are easily manageable and easy to separate by centrifugation or similar methods. MT200 already reached a rather high value for the sedimentation volume but was still easy to handle in unmodified condition. After 1 h enzymatic treatment, however, MT200–water suspensions showed minimal settlement indicating high amounts of deteriorated beads and advanced gelation. The determination of the sedimentation volume could thus serve to decide quickly about the suitability of enzyme treatment for a particular type of bead cellulose. In the case of MT200 the reaction time was reduced to a maximum of 20 min.

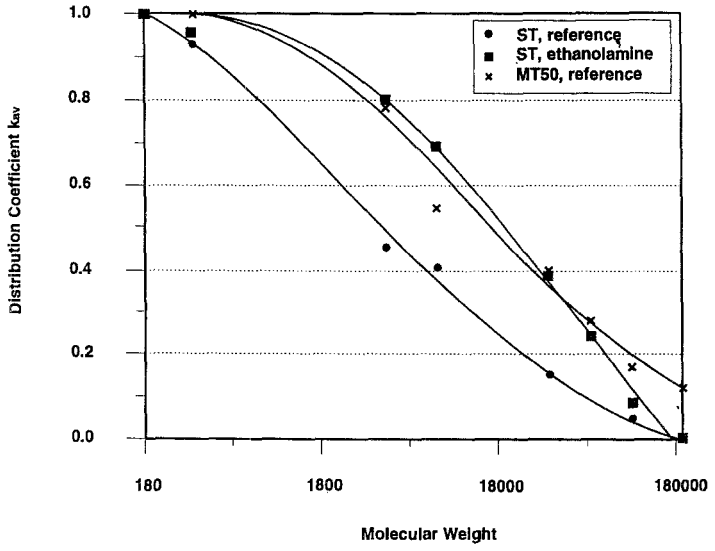


FIGURE 8. SEC distribution coefficient k_{av} as a function of the molecular weight of various solutes in solution. The graph shows the comparison of untreated ST, ethanolamine swollen ST and untreated MT50 samples.

Influence of the enzyme deactivation method on the pore structure

Since acetone rinsing and subsequent solvent exchange were already sufficient to open up the structure of Perloza ST to some extent, a study was made of whether the method of enzyme deactivation would influence the structure of the product. Thus, in a series of experiments the enzymes were inactivated by hot water ('W') followed by a stepwise solvent exchange with ethanol:water and finally cyclohexane. Average data from porosity measurements are summarized in Table 4. For reasons of comparison, one sample was first treated with acetone, followed by intermediate water rinsing, and then solvent exchange-dried as in the case of the acetone deactivation. The only major difference to acetone inactivation was found for the 1 h treatment. In this case it seemed as if the hot water had a certain levelling influence on the effects of enzyme treatment (cf. Table 1). Only about half to two thirds of the values could be obtained compared to the equivalent treatment followed by acetone inactivation. It could be speculated that in the initial phase the enzyme modified structure is less stable and hot water could effect some structural rearrangement. At longer periods of treatment time the differences in pore structure caused by the alternative enzyme inactivation more or less levelled out (compare 3 h treatment, Table 1 and 3). The deactivation method thus seems to be critical for the structure of the substrate only at the initial stage of the enzymatic treatment.

Enzymatic treatment of CM and DEAE bead celluloses

Ion-exchange materials based on cellulose such as CM and DEAE bead cellulose show excellent flow properties and good resistance to changes in ionic strength of the fluid phase. Their separation efficiency has proved to be superior to comparable materials (based on dextran). In CM, the hydroxyl groups of cellulose have been partially substituted with carboxymethyl groups, in DEAE with diethyl aminoethyl groups. Substitution occurred after the regeneration of cellulose on accessible surfaces of the beads. Using this mode of modification, the ratio of ordered regions is higher than for celluloses which have been derivatized prior to regeneration (Pharmacia, 1972).

TABLE 4. Influence of the use of hot water for deactivation of the enzymes and effect of preswelling in ethanolamine on the pore structure of Perloza ST

Treatment conditions	Hg-*Porosimetry*				N_2-*Sorption*	
	Specific pore volume ($cm^3\ g^{-1}$)	*Specific surface* ($m^2\ g^{-1}$)	*Average pore radius* (nm)	*Porosity* (%)	*Specific pore volume* ($cm^3\ g^{-1}$)	*Specific pore surface* ($m^2\ g^{-1}$)
untreated (A)[a]	0.04	7	9	3.44	0.17	68
untreated (W)[a]	0.06	11	9	6.80	0.24	125
untreated (A, W)[b]	0.06	11	9	4.35	0.22	102
1 h incubation (W)	0.08	14	8	6.03	0.25	124
3 h incubation (W)	0.17	29	10	11.28	0.54	239

[a]See Table 1.

[b]This sample was first rinsed with acetone, followed by washing with water. The solvent exchange was then carried out as in the case of enzymatically treated samples (inactivation with hot water).

With the help of enzyme treatment the aim was to further increase pore sizes for specific separation of high molecular weight substances. Both derivatives, CM and DEAE, behaved differently upon enzymatic attack. DEAE was far more susceptible to enzymatic hydrolysis than CM or the unsubstituted materials. Although both derivatives had similar degrees of substitution (0.3 and 0.35 in DEAE and CM, respectively) and the derivatization process was comparable, the diethyl aminoethyl groups in DEAE are more bulky than the substituent groups in CM. Hence they might result in a generally looser structure and thus ease enzyme penetration. Due to their bulky nature it might also be speculated that their distribution along the cellulose chain is less balanced than in the case of CM. During the enzymatic reaction DEAE turned yellowish indicating that some decomposition processes had occurred.

Figure 9a presents a light micrograph of DEAE in the enzymatic reaction solution after 10 min incubation with cellulases from *Trichoderma viride*. Due to the relatively large average pore radius (11 nm) and high porosity (13.6%) of the starting DEAE as depicted in Table 5, it is most probable that deterioration predominantly occurred from the interior of the beads causing them to collapse and to disintegrate. Parallel to the observed decomposition, the surface of some of the beads seemed to have burst. After separation from the resulting debris, the remaining beads were subjected to porosity measurements. As shown in Table 5, after 10 min incubation a slightly smaller average pore radius was found along with an almost linear increase in accessible surface area and pore volume, indicating increasing amounts of smaller pores. The number of pores in the overlapping range recorded by both nitrogen sorption and mercury sorption decreased at the expense of larger ones. The overall porosity of the samples remained more or less constant indicating that the internal enzymatic attack must have dominated in this case. Inactivation of the enzyme with hot water ('W') here resulted in approximately the same pore structure situation as after inactivation with acetone.

A cellulase complex from *Aspergillus niger* proved to be far less aggressive in terms of the DEAE substrate and the degradation turned out to be far less pronounced. Figure 9b shows a light micrograph of DEAE in reaction solution after 10 min incubation. The number of disintegrated beads was distinctly smaller. However, the effect of the enzyme treatment on the pore structure was also less prominent. In this case the formation of very small pores dominated while all other pore parameters showed less desired effects.

After 20 min treatment time (*Trichoderma viride*) the tendency towards further increase in pore volume and average pore size but decreasing accessible surface area evidently indicates that fewer but larger pores developed at the expense of the smaller ones. The average pore radius increased to 15 nm and the porosity reached 24.3%. In addition, the amount of very small pores also increased. However, the product was rather fragile after 20 min incubation and mechanically too unstable to be used for chromatographic applications without successive cross-linking.

Carboxymethyl cellulose (CM) proved to be less susceptible to the enzymatic attack than DEAE cellulose. Untreated CM consisted of spherical particles with greater differences in particle size distribution than in the case of unsubstituted ST. Smaller beads frequently stuck to larger ones or formed accumulations of four to six. The surface of the untreated CM showed a relatively fine-grained texture (Fig. 10a). The higher magnification micrograph in Fig. 10b reveals fine cross-links on the surface. Pore structure determinations (Table 5) showed that the untreated CM must already contain

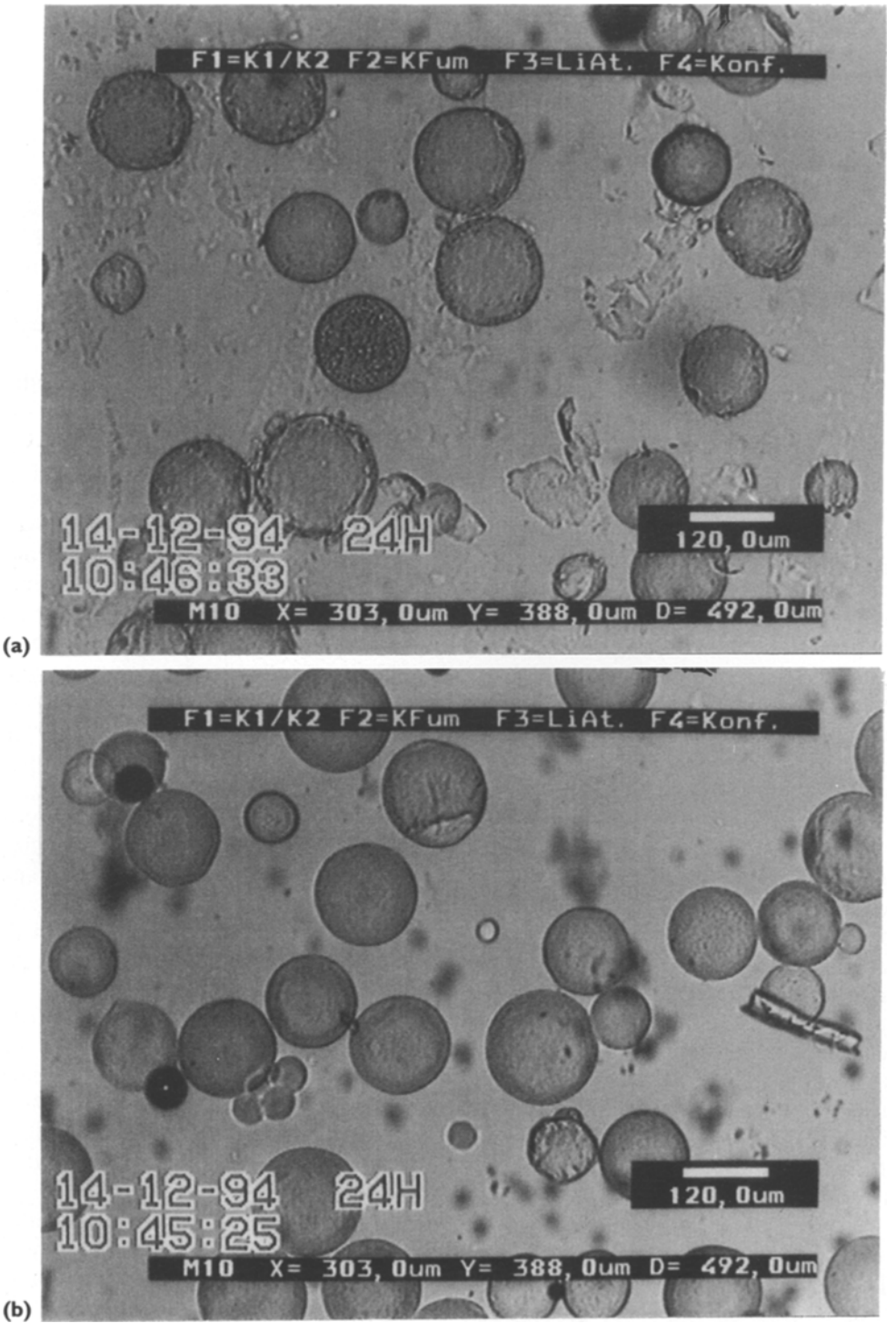


FIGURE 9. (a) Light micrograph of DEAE beads after 10 min reaction with cellulase from *Trichoderma viride*; (b) light micrograph of DEAE beads after 10 min reaction with cellulase from *Aspergillus niger*.

TABLE 5. Effect of enzyme treatment on cellulose derivatives

Treatment conditions	*Hg-Porosimetry*				*N_2-Sorption*	
	Specific pore volume ($cm^3\ g^{-1}$)	*Specific surface* ($m^2\ g^{-1}$)	*Average pore radius* (nm)	*Porosity* (%)	*Specific pore volume* ($cm^3\ g^{-1}$)	*Specific pore surface* ($m^2\ g^{-1}$)
DEAE:						
untreated (A)[a]	0.23	30	11	13.60	0.45	122
10 min incubation (A)	0.43	70	9	13.00	0.27	94
10 min incubation (A)[b]	0.28	70	7	6.33	0.32	178
10 min incubation (W)	0.46	65	12	14.72		
20 min incubation (A)	0.50	43	15	24.30	0.70	177
CM:						
untreated (A)[a]	0.40	44	23	24.53	0.80	201
1 h incubation (A)	0.47	50	22	26.82	0.96	203
2 h incubation (A)	0.21	37	10	12.13	0.37	159

[a]See Table 1.

[b]Instead of cellulase from *Trichoderma viride* a less reactive enzyme complex from *Aspergillus niger* was used.

a high number of large pores and channels as indicated by high overall porosity (24.53%) and large average pore radius (23 nm). It could be expected that the enzyme would easily penetrate the CM beads and possibly lead to a further enhanced porous structure by internal attack.

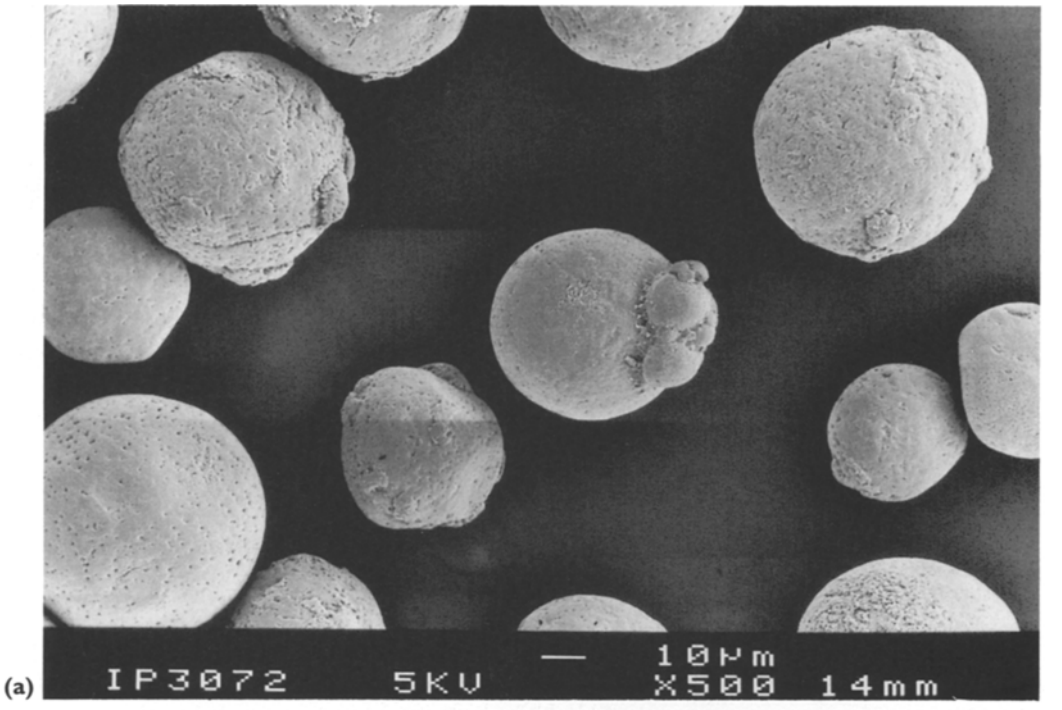
However in contrast to DEAE and the unsubstituted products, enzymatic hydrolysis of 1 h duration did not affect the CM beads. The pore structure data (Table 5) remained almost unchanged and the pore size distribution of CM before and after 1 h treatment turned out to be almost identical.

Similar results were obtained for the wet surface. Before and after the enzyme treatment (1 h incubation) the water retention value amounted to 1.68 ml g^{-1}. With SEC a total pore volume of 2.32 ml g^{-1} for the untreated sample was determined which slightly decreased to 2.12 ml g^{-1} after 1 h enzymatic hydrolysis.

SEM investigation of the treated CM beads showed that the surface had apparently lost most of its cross-links. The only salient difference to the untreated sample in this case was the accumulation of small particles on the outside of the beads giving them an almost 'hairy' appearance (Fig. 10c). This observation is in striking contrast to the usual effect of the enzymes to clean the surface.

If the treatment time was extended to 2 h the properties of the CM beads quickly declined. SEM micrographs showed a flattening of the bead surface (Fig. 10d) as was also observed in the case of enzymatically treated ST samples. Pore volume, accessible surface area and porosity decreased in some cases even below the values before the enzyme treatment, indicating the break-down of the former structure. Although much smaller in individual particle size, only small amounts of collapsed or completely deteriorated beads were found as was the case for DEAE.

Although the degree of substitution was relatively low in CM, it is quite possible that the resulting structural changes strongly interfered with the enzymatic attack. It could also be speculated that the heterogeneous character of the substitution reaction brought about a structural situation unfavorable to the steric requirements of the cellulases.



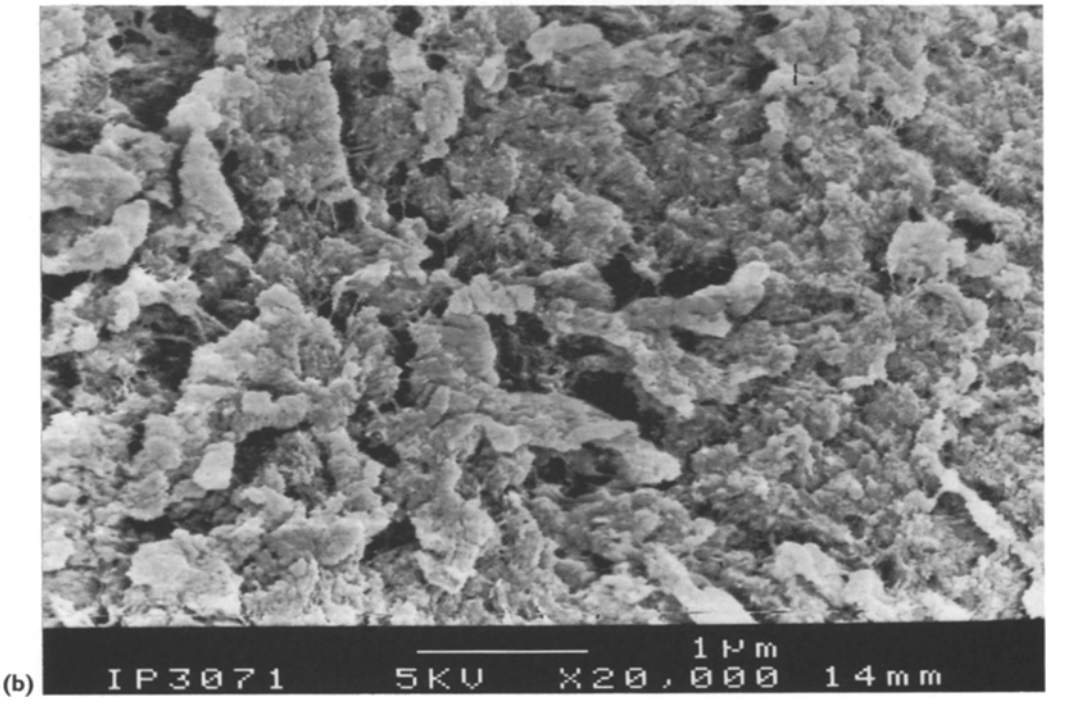
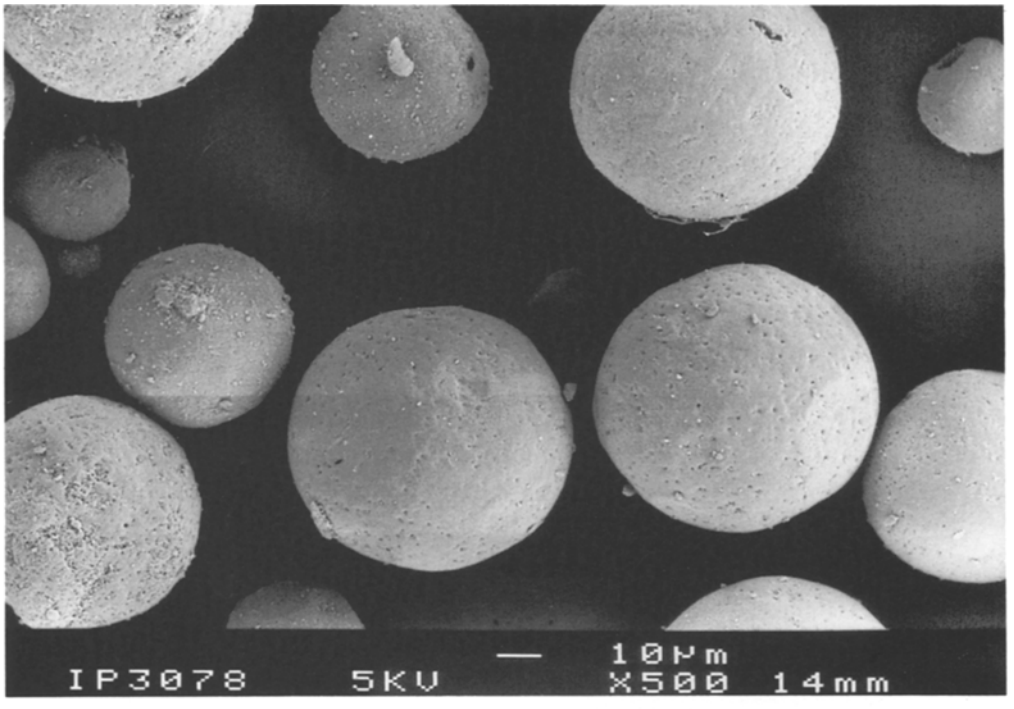
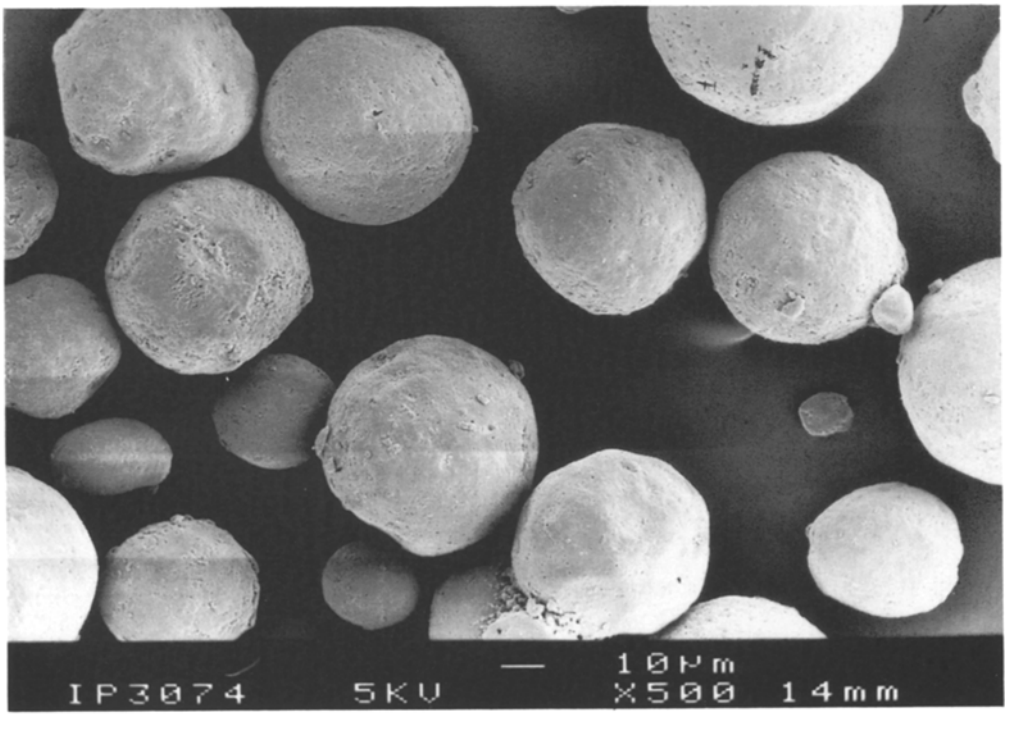


FIGURE 10. (a) SEM micrograph of the surface of untreated CM beads; (b) detailed view of the beads in Fig. 10a at higher magnification; (c) SEM micrograph of CM beads after enzymatic treatment for 1 h; (d) SEM micrograph of CM beads after 2 h treatment with enzyme showing flattened parts on the beads.

(c)



(d)



Thus, at first exposure, the CM structure might have functioned as a barrier for the enzyme, inhibiting the hydrolysis reaction. With longer incubation times, however, the situation must have dramatically changed. Further research will be required to turn enzymatic hydrolysis into a beneficial treatment for both CM and DEAE bead celluloses and to give explanations for the observations which have been discussed.

CONCLUSIONS

The results of the work clearly show that a treatment with cellulase can be used to change the pore structure of cellulosic partition support materials. For the final properties of the bead celluloses, however, treatment variations, e.g., specific after treatments or the method of enzyme deactivation, might also play a significant role. Especially in the case of cellulose derivatives further research will be necessary to improve mechanical stability by successive crosslinking reactions.

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REFERENCES

Asahi Chemical Industry Co., Ltd. (1973) Japanese Patent JP 48-60 753.
Buschle-Diller, G. and Zeronian, S. H. (1994) *Text. Chem. Color.* **26**, 17–24.
Chisso Corporation (1981) Japanese Patent JP 56-24 430.
Clarkson, K. A., Larenas, E. and Weiss, G. L. (1993) U.S. Patent 5 246 853.
Dautzenberg, H., Loth, F., Wagenknecht, W. and Philipp, B. (1985) *Das Papier* **39**, 601–607.
Determann, H., Rehner, H. and Wieland, T. (1968) *Makromol. Chem.* **114**, 263–274.
Finch, P. and Roberts, J. C. (1985) in: *Cellulose Chemistry and its Applications* (T. P. Nevell and S. H. Zeronian, eds.). New York: Wiley, pp. 313–343.
Goyal, A., Ghosh, B. and Eveleigh, D. (1991) *Bioresource Technol.* **36**, 37–50.
Gregg, S. J. (1958) *Oberflächenchemie fester Stoffe*. Berlin: VEB Verlag Technik.
Hatao, M., Takada, S., Saito, T. and Nishikawa, M. (1986) *Chem. Abstr.* **105**, P 84959s.
Henrissat, B. (1994) *Cellulose* **1**, 169–196.
Hurst, P. L., Nielson, J., Sullivan, P. A. and Shepherd, M. G. (1977) *Biochem. J.* **165**, 33–41.
Kajmakovic, M., Hace, D. and Moraca, S. (1987) *Polimeri, Zagreb* **8**, 213–217.
Kanebo, Ltd.; Kanebo Rayon K. K. (1988) European Patent EP 264 853.
Lenz, J., Esterbauer, H., Sattler, W., Schurz, J. and Wrentschur, E. (1990) *J. Appl. Polym. Sci.* **41**, 1315–1326.
Loth, F. and Fanter, C. (1993) *Das Papier* **47**, 703–710.
Loth, F. and Philipp, B. (1989) *Makromol. Chem., Macromol. Symp.* **30**, 273–287.
Matsumoto, K., Hirayama, C., Motozato, Y. (1981) *Nippon Kagaku Kaishi* **12**, 1890–1898.
Motozato, Y. and Hirayama, C. (1984) *J. Chromatogr.* **298** (3), 499–507.
Olson, L. (1988) *Am. Dyest. Rep.* **77**, 19–22.
Paul, D. and Bartsch, D. (1972) *Faserforsch. Textiltech.* **23** (5), 187–195.
Pedersen, G. L., Screws, G. A., Jr. and Cedroni, D. M. (1992) *Canadian Text. J.*, 31–35.
Peška, J., Štamberg, J. and Hradil, J. (1976) *Angew. Makromol. Chem.* **53** (1), 73–80.

Peška, J., Štamberg, J. and Pelzbauer, Z. (1978) *Cellulose Chem. Technol.* **21**, 419–428.
Pharmacia Fine Chemicals (1972) U.S. Patent 3 652 540.
Philipp, B., Schleicher, H. and Wagenknecht, W. (1973) *J. Polym. Sci., Symp.* **42**, 1531–1543.
Pole, C. F. and Schuette, S. A. (1984) *Contemporary Practice of Chromatography*. Amsterdam: Elsevier.
Puri, V. P. (1984) *Biotechnol. Bioeng.* **26**, 1219–1222.
Rowland, S. P. (1977) in: *Textile and Paper Chemistry and Technology*, (J. C. Arthur, Jr., ed.). ACS Symposium Series 49. Washington, D.C.: American Chemical Society.
Schleicher, H., Loth, F. and Lukanoff, B. (1989) *Acta Polymerica* **40** (3), 170–177.
Štamberg, J., Peška, J., Dautzenberg, H. and Philipp, B. (1982) in: *Affinity Chromatography and Related Techniques* (T. C. J. Gribnau, J. Visser and R. J. F. Nivard, eds.). Amsterdam: Elsevier, p. 131–141.
Timmins, M. R. and Lenz, R. W. (1994) *Trends Polym. Sci.* **2** (1994), 15–19.
Walker, L. P. and Wilson, D. B. (1991) *Bioresource Technol.* **36**, 3–14.
Weatherwax, R. C. (1974) *J. Colloid Interface Sci.* **49**, 40–48.
Woodward, J. (1991) *Bioresource Technol.* **36**, 67–75.