Cellulose **6:** 265–282, 1999.

Effect of cellulase adsorption on the surface and interfacial properties of cellulose

F. DOURADO, M. MOTA*, H. PALA and F. M. GAMA
Centro de Engenharia Biológica - IBQF - Universidade do Minho, Largo do Paço, 4719 Braga Codex, Portugal

Abstract. The surface properties of several purified cellulose (Sigmacell 101, Sigmacell 20, Avicel pH 101, and Whatman CF 11) were characterised, before and after cellulase adsorption. The following techniques were used: thin-layer wicking (except for the cellulose Whatman), thermogravimetry, and differential scanning calorimetry (for all of the above celluloses). The results obtained from the calorimetric assays were consistent with those obtained from thin-layer wicking – Sigmacell 101, a more amorphous cellulose, was the least hydrophobic of the analysed celluloses, and had the highest specific heat of dehydration. The other celluloses showed less affinity for water molecules, as assessed by the two independent techniques. The adsorption of protein did not affect the amount of water adsorbed by Sigmacell 101. However, this water was more strongly adsorbed, since it had a higher specific heat of dehydration. The more crystalline celluloses adsorbed a greater amount of water, which was also more strongly bound after the treatment with cellulases. This effect was more significant for Whatman CF-11. Also, the more crystalline celluloses became slightly hydrophilic, following protein adsorption, as assessed by thin-layer wicking. However, this technique is not reliable when used with cellulase treated celluloses.

Key words: cellulase, cellulose, drainability, protein adsorption

Introduction

The rapid development of industrial production and utilization of technical enzymes coincides with a generally growing interest in biotechnology. The utilization of enzymatic processes in several areas of both food and non-food industries has become feasible by the development of highly specialized microorganisms, which can be employed directly or through the isolation of enzymes they produce (Miletzky, 1996). The importance of enzymes relies on their ability to provide alternative processes and products due to the specific nature of their reactions and to their compatibility with the environment.

A number of cell wall degrading enzymes have been studied in food processing as an alternative to enhance the extractability of oil from oil seeds

* Author for correspondence

(Domínguez *et al*., 1994; Domínguez *et al*., 1995; Rosenthal *et al*., 1996). In the textile industry, enzymes have been used to achieve the stone-washed look of denim garments, and on the cleaning and renewing of damaged fabric surfaces (Cavaco-Paulo *et al*., 1997). In the paper industry (Pommier *et al*., 1989; Daniels, 1992; Jackson *et al*., 1993; Grant, 1995; Stork *et al*., 1995), it has been suggested that an appropriate enzymatic treatment applied to wastepaper pulps, could overcome the limitations of a poor drainability.

The above references serve as examples to illustrate the extensive research on the potential and beneficial utilization of the enzymes, in the several areas of both food and non-food industries. However, this research is based mainly on the hydrolytic capabilities of the enzymes. It is our opinion that the effect of the adsorbed protein (enzymes) on the surface properties of the substrates should also be pondered. Since cellulose is a major source for the production of energy, food and chemicals, we attempt to understand the effect of cellulase adsorption on the surface and interfacial properties of cellulose.

Chemically, cellulose is a polymer of glucose (β-1,4-glycan) in which individual glucose units are joined by β-1,4-glycosidic linkages, forming straight chains of varied degree of polymerisation (D.P.). In general, the natural cellulose materials consist of a mixture of amorphous cellulose areas, in addition to well ordered crystalline regions.

The interactions of purified celluloses with cellulases (a group of enzymes possessing the ability to synergistically hydrolyse insoluble cellulose all the way to its monomer-glucose) has been the subject of extensive study. In fact (Walker and Wilson, 1991), over the past 15 years there has been considerable progress in understanding the basic mechanisms by which cellulases adsorb and hydrolyse cellulose. In aqueous media (Walker and Wilson, 1991; Henrissat, 1994), the adsorption of cellulases onto cellulose (the first step of the hydrolytic process) corresponds to a phase transfer of the free enzyme (in solution) to the insoluble substrate (solid phase).

The behaviour of cellulose particles in aqueous media, to what concerns the formation, stability and sedimentation of the particles agglomerates, and the stabilisation of these particles in the presence of adsorbed protein, is related to a variable extent with the kind and magnitude of the surface and with the interfacial properties of both cellulose and enzymes.

Surface interactions

In the field of surface thermodynamics, there has been considerable research to develop a theory explaining the manner by which two solid surfaces interact with each other, in a condensed medium. However, the problem of

experimental determination and theoretical calculations of solid surface free energy is still open. The available methods for this purpose contain some uncertainties as far as the interpretation of the measured quantities in terms of the free energy components is concerned. In addition, the theoretical approach to the nature of the interfacial interactions seems to be still not fully solved (Chibowski and Holysz, 1992). The approach of van Oss *et al*. (van Oss and Good, 1989), which considers the surface free energy as a sum of the apolar Lifshitz-van der Waals, γ_s^{LW}, and polar, γ_s^{AB}, interactions, is very likely to be the most popular at present (Lee, 1996). However, its validity is still a matter of controversy, other models describing these systems as well (harmonic mean, acid-base Bronsted, acid-base via Fowkes).

It is not the aim of this work to discuss which approach would be the more adequate to the system under analysis. Even if it does not provide absolute values for the polar components of surface tension, the van Oss approach does provide relative values, a large set of data being already published and available for comparison. The essentials of van Oss approach were already described in the work of Dourado *et al*. (1998) and, in this work, the same approach was used for the characterisation of the celluloses surface properties, in the presence of adsorbed protein. The necessary equations for the calculations of the surface tension components were already presented in a previous work (see Dourado *et al*. (1998)).

Thermal interactions of water with solid surfaces

The term thermal analysis (TA) is frequently used to describe analytical experimental techniques, which investigate the behaviour of a sample as a function of temperature (Hatakeyama and Quinn, 1995).

The use of TA offers several advantages over other analytical methods. Among them, are the use of a wide range of temperatures (using various temperature programs) when studying a sample; the accommodation of a sample of almost any physical form, using a variety of sample vessels; the use of a small amount of sample for study (0.1 μg to 10 mg); the possibility of standardisation of the atmosphere in the vicinity of the sample.

Despite the above advantages, TA is not a passive experimental method, as the high-order structure of a sample, (for example crystallinity, morphology) may change during the course of measurement. In addition, a sample studied using a TA instrument is measured under non-equilibrium conditions, and the observed transition temperature is not the equilibrium transition temperature. Finally, the sensitivity and precision of TA instrument to the physico-chemical changes occurring in the sample are relatively low, compared with spectroscopic techniques. Therefore, in order to elucidate the mo-

lecular mechanisms responsible for any observed behaviour, TA data should be confronted with results from spectroscopic techniques (for example NMR, FTIR, and X-ray diffractometry) (Hatakeyama and Quinn, 1995).

Owing to the effect of water on the performance of commercial polymers and to the crucial role played by water-polymer interactions in biological processes, hydrated polymer systems are widely investigated. Several thermal studies on the interaction between water and microcrystalline cellulose have been published, such as those reported by Fielden *et al*. (1988) and Angberg *et al*. (1991).

The use of TA as an additional tool for the investigation of a solid's surface properties, can be found in several published works, such as in Staszczuk, (1984) and Chibowski and Staszczuk (1988), where the adsorption of water vapour on the surface of mineral clays was found to reflect the solid's surface properties.

The surface properties of several purified celluloses were already characterised by Dourado *et al*. (1998), with the help of the thin-layer wicking technique and the influence of cellulase adsorption on the cellulose's surface properties was studied in terms of the DLVO theory by Gama *et al*., 1996. The aim of the present work is to characterise the surface and interfacial properties of several purified cellulosic materials, before and following protein adsorption, in order to assess the changes occurring in those properties after protein adsorption. To accomplish this purpose the following techniques were used: particle size characterisation, thin-layer wicking, thermogravimetry (TGA) and differential scanning calorimetry (DSC).

Experimental

Materials

Three celluloses, Sigmacell 101, Sigmacell 20, both from Sigma, Avicel pH 101 (Fluka) and Whatman CF-11 (Whatman Chemical Separation, Ltd. England) were used for the study.

A commercial cellulase from *Trichoderma reesei*, Celluclast 1,5 L, kindly supplied by Novo, Denmark, was used for the enzymatic treatments.

Particle characterisation

The celluloses were characterised as to their size distribution and crystallinity. The experimental procedure used for this purpose is described in Gama *et al*. (1996).

Enzymatic treatment for particle size characterisation

For each type of cellulose, the experimental procedure was as follows: the reaction mixture contained 50 mg of cellulose, 3.0 ml of 50 mM acetate buffer pH 5.0 and 1.0 ml of diluted enzyme (1:100). This mixture was placed in a 50-ml flask and incubated at 50°C, for 30 min, with constant stirring, after which it was heated to 100°C, to denaturate the protein. Then, the reaction medium was centrifuged (5.000 rpm for 3 min). The fibers were washed twice with distilled water, and resuspended in 3.0 ml of distilled water (Gama *et al.*, 1996).

Determination of the celluloses surface tension components

The thin-layer wicking technique was used to determine the celluloses surface tension components. These experiments were carried out by starting with a preparation of a 5% (w/v) cellulose suspension, in ultra pure water. Aliquots of 4 ml were withdrawn and evenly distributed over a clean surface of strictly horizontal glass plates (slides sizing 25×100 mm), and left at room temperature to let the water evaporate. The slides were then dried in an oven at 110°C for 2 h, and kept in a desiccator for 5 days. The oven drying is necessary to eliminate any residual water which might interfere with the wicking measurements by diluting some of the wicking liquids (thus changing their surface tensions and viscosities) and/or changing the surface tension properties of the celluloses surface (van Oss *et al.*, 1992). Afterwards, some plates were allowed to equilibrate for 12 h in a closed vessel with the saturated vapour of the probe liquids – *n*-hexane, *n*-decane, water and formamide (Chibowski, 1992; Chibowski and Holysz, 1992; Holysz and Chibowski, 1992; Chibowski and González-Caballero, 1993). The remaining plates were kept inside the desiccator.

The thin-layer wicking experiments were carried out in a closed sandwich chamber, where the plates were placed in a horizontal position upside down. A flannel wick about 2 cm long transported the probe liquids. In the sandwich chamber, the surface of the used liquid was at the same level as that of the tested plates, to avoid any hydrostatic effects (Chibowski and González-Cabalhero, 1993). The wicking times, corresponding to each centimetre section of the cellulose thin layer, were recorded with the help of a ruler placed next to the plates. The surface tensions of the probe liquids, used for the wicking experiments were taken from van Oss and Good, (1989).

The wicking technique was also used for the enzymatically treated samples. The enzymatic treatment for this purpose is described below.

Enzymatic treatment for the thin-layer wicking technique

For each type of cellulose, a 5% (w/v) cellulose suspension was prepared, in 120 ml of ultra pure water. Then 1 ml of enzyme was added. The reaction mixture remained in constant agitation for 30 min, at 50°C, after which it was heated to 100°C, for 10 min, to inhibit the enzymatic activity. In this case, the suspension was not buffered, in order to avoid any interference from the acetate ions in the determination of the surface tension components.

Thermogravimetric and differential scanning calorimetric assay

The specific enthalpy of dehydration and the amount of adsorbed water for each type of cellulose were determined by differential scanning calorimetry and thermogravimetry. For this purpose, the cellulose samples were preconditioned in a 100% relative humidity (RH) chamber, for 7 days, at room temperature (approximately 25°C). As reported by Hollenbech *et al*. (1978), for Avicel pH 101, this period is enough to achieve the adsorption equilibrium. The same period was used for all celluloses. For each type of cellulose, 18 samples were prepared in aluminium vessels, weighing approximately 5 mg (the exact weight of the saturated cellulose was recorded). Then, these vessels were closed with perforated lids, to allow the release of evaporated water during the assays. The closed vessels were again placed in the same chamber for 5 days, to re-establish water equilibrium. The thermogravimetric assays were performed in a TGA, from Shimadzu, model 50, and the differential calorimetric in a DSC, from Mettler, model 20. Argon was used as a purge gas, with a 40 ml/min flow. In both cases, assays were performed with a heating rate of 5 °C/min, from 25 to 200°C.

These experiments were repeated following enzymatic treatment of the celluloses, in the same way as described for the wicking experiments. In this case, only 9 samples of each type of cellulose were used, owing to the good reproducibility of the experiments with the untreated samples.

XPS analysis

Cellulose pellets with 13 mm diameter were prepared with a hydraulic press by applying a pressure of 0.6 kg/m^2 for 1 min. The pellets were then analysed in an Escalab 200A (VG Scientific-Fisons) XPS analyser, equipped with a MgKα radiation source at 1253,6 eV.

Results and discussion

Several celluloses were used to evaluate the influence of particle size and crystallinity on the celluloses surface and interfacial properties, before and following protein adsorption.

For the sake of clarity, we expressed the standard deviations as a percentage of the sample mean. Thus, a dimensionless measure of the spread, the coefficient of variation (C.V.), was presented with the results.

Particle size characterisation

The results obtained for the particles size distribution and crystallinity are presented in Table 1 (data partially shown elsewhere (Dourado *et al*., 1998)). Non treated samples are presented as NT, enzymatically treated as EZ, and finally, samples sonicated after enzymatic treatment as EZ+US. Results obtained for the cellulose Whatman CF-11 are presented here, merely as a means to illustrate the separation of the agglomerates, when submitted to the different types of treatments. Since this cellulose is made up of long fibers, their actual size characterisation with the granulometer is not possible. Nevertheless, in the samples treated with the enzymes, these results allow for the detection of a significant drop in the average particle size, comparatively to the NT. For a further discussion of the results shown in Table 1, see Dourado *et al*. (1998).

From Table 1 it can also be seen that the particle size of Sigmacell 101 did not change much, irrespective of the treatment. These results indicate that this cellulose is stable in aqueous media, whereas the particles of the more crystalline celluloses agglomerate. For the more crystalline celluloses, the enzymatic treatment had a more pronounced effect than sonication in the reduction of the particles size.

Since the enzyme treatment lasted for 30 min, the fragmentation of the more crystalline celluloses could be considered the result of merely a mech-

Table 1. Cellulose crystallinity and median sizes on a volume basis (d_{50} (μm) $\pm$ C.V. (%))

	NT*	EZ	EZ + US	Icr (%)*
Sigmacell 101	15.02 $\pm$ 6.06	12.24 $\pm$ 3.10	13.00 $\pm$ 0.92	54.70
Sigmacell 20	37.20 $\pm$ 2.07	12.66 $\pm$ 3.95	12.61 $\pm$ 3.33	91.54
Avicel pH 101	49.27 $\pm$ 2.23	23.52 $\pm$ 2.00	18.04 $\pm$ 1.77	92.97
Whatman CF 11	102.14 $\pm$ 10.00	44.88 $\pm$ 20.97	43.32 $\pm$ 6.37	96.43

*Dourado *et al*. (1998).
C.V., coefficient of variation.

anical action of the enzymes. In the work of Gama and Mota (1997a), where crystalline celluloses were used as a subject of study, it can be observed that during the 30 min of enzyme reaction, a low hydrolytic activity was detected, as determined by the DNS method. A further discussion of the mechanical action of the enzymes on the cellulose particles, can be found in Klesov, (1990).

Thin-layer wicking experiments

The wicking times of untreated and enzymatically treated samples, as obtained from the wicking experiments, are presented in Tables 2 and 3. We have performed four independent assays in order to increase the statistical significance of the average wicking times. The small variations of this variable in each assay may be due to a certain degree of variability in pore size distribution and particle packing from one slide to another. From the four independent assays, we have calculated the fraction of rejected assays (R.A.), for each tested liquid according to $t_{95\%}$-Student distribution criteria. From the results obtained (Table 2), it is possible to say that the wicking experiments showed a fairly good reproducibility. The Washburn equation fitted well with the wicking process for all celluloses. Indeed, a good fit ($x^2 = f(t)$) was obtained in each wicking experiment (minimum $r = 0.996$).

As reported elsewhere (Dourado *et al.*, 1998), NT samples have a high apolar and electron donor component, while the electron acceptor component is practically null. Also, the celluloses have a slight hydrophobic character, as displayed by the small values of the work of water spreading (*Ws*) (Table 5).

It should be remarked that, as shown by Belgacem *et al.* (1995), with materials similar to those used in this work, these celluloses are not totally pure. Residual lignin may be responsible for a peak detected in the XPS spectra (Figure 1) at 284.3 eV (C-H bonds), and the peak at 289.0 is likely to be produced by carbon atoms in carboxylic groups. Of course, the level of oxidation and impurities influences the surface and interfacial properties, and should be kept in mind in the analysis of the cellulose surface properties that follows. It should also be remarked that, in the work by Belgacem *et al.* (1995), where the technique of inverse gas chromatography was used in the characterisation of cellulose surfaces, the values obtained for the dispersive characteristics of the surfaces are similar to those obtained by thin-layer wicking. However, the cellulose acid-base properties, reported by Belgacem, are not in agreement with the donor and acceptor components of the polar surface tension found in this work. The analysis of this contradiction should be the object of further work.

The predominant electron-donicity that can be observed by thin-layer wicking technique on solid surfaces is largely a consequence of the prevalence

Table 2. Average times (in seconds) and C.V. (%) for a 10 cm wicking distance for each cellulose. Untreated samples *

	Bare plates							
	Decane	R.A.	Hexane	R.A.	Water	R.A.	Formamide	R.A.
Sigmacell 101	431 ± 2.32	1/4	206 ± 2.91	0	762 ± 3.15	1/4	2905 ± 8.16	0
Sigmacell 20	438 ± 6.39	0	205 ± 6.34	0	387 ± 8.53	0	1614 ± 15.92	0
Avicel pH 101	190 ± 11.05	0	90 ± 16.67	0	189 ± 2.65	1/4	882 ± 3.63	1/4
	Precontacted							
Sigmacell 101	464 ± 2.80	0	213 ± 3.29	0	614 ± 6.03	0	3160 ± 14.49	0
Sigmacell 20	456 ± 11.18	0	206 ± 3.88	0	292 ± 2.74	0	1525 ± 21.84	0
Avicel pH 101	169 ± 4.14	0	98 ± 14.29	0	142 ± 1.41	1/4	666 ± 18.02	0

R.A., number of rejected assays/total assays, according to the $t_{95\%}$ distribution criteria.
* Dourado *et al*., 1998.

Table 3. Average times (in seconds) and C.V. (%) for a 10 cm wicking distances for each cellulose. Samples treated with enzyme

	Bare plates							
	Decane	R.A.	Hexane	R.A.	Water	R.A.	Formamide	R.A.
Sigmacell 101	779 ± 10.78	1/4	366 ± 0.55	1/4	63427 ± 27.43	1/4	8734 ± 11.27	1/4
Sigmacell 20	1283 ± 4.99	1/4	633 ± 12.95	0	70195 ± 19.39	1/4	20082 ± 21.81	0
Avicel pH 101	899 ± 10.34	1/4	396 ± 1.01	1/4	18127 ± 11.84	1/4	9408 ± 6.04	1/4
	Precontacted							
Sigmacell 101	738 ± 2.71	1/4	389 ± 2.57	1/4	67600 ± 70.41	1/4	8811 ± 14.28	1/4
Sigmacell 20	1284 ± 10.83	1/4	656 ± 11.89	0	61467 ± 4.38	1/4	31235 ± 60.47	0
Avicel pH 101	925 ± 11.78	1/4	407 ± 2.46	1/4	45000*	0	7264 ± 35.66	1/4

*Following enzymatic treatment, this cellulose adsorbed a great amount of water, making it difficult to measure the wicking distances, during the experiments. Therefore, an interval of 2 h was estimated, after which the wicking distances were measured.

Table 4. R (μm) values and C.V. (%) for the various samples

Cellulose	NT*	EZ
Sigmacell 101	1.70 ± 6.47	1.00 ± 3.00
Sigmacell 20	1.75 ± 8.00	0.58 ± 0.00
Avicel pH 101	4.18 ± 9.81	0.88 ± 9.09

*Dourado *et al.*, 1998.

Table 5. Surface tension components of the celluloses and work of water spreading, in mJ/m^2

	Cellulose	γ_s^{LW}	γ_s^+	γ_s^-	γ_s^{AB}	γ_S^-/γ_S^+	γ_s^{TOT}	W_s
NT*	Sigmacell 101	54.5	0.1	47.8	4.5	478	59.0	−3.5
	Sigmacell 20	52.9	0.1	41.7	4.2	417	57.2	−9.1
	Avicel pH 101	51.8	0.0	50.1	0.0	–	51.8	−8.2
EZ	Sigmacell 101	52.4	0.1	56.0	3.6	560	56.0	0.0
	Sigmacell 20	53.8	0.1	52.7	5.4	527	59.2	−0.1
	Avicel pH 101	52.1	0.0	61.6	0.0	–	52.1	0.7

*Dourado *et al.*, 1998.

of oxygen in Earth's lower atmosphere and in the Earth's surface. Thus, all the biopolymers and common mineral particles are oxides and/or hydroxides, which makes them preponderantly electron-donors. However, many of these materials tend to show a small but not negligible γ_s^+, in addition to a (usually more sizeable) γ_s^-. Thus, if, on a solid surface, a non negligible value of

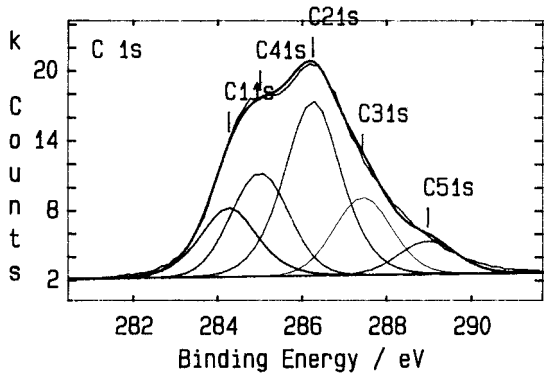

Figure 1. Carbon C1 XPS spectrum obtained with Avicel pH 101.

Table 6. Surface tension components of several polymers (in mj/m2).

	γ_s^{LW}	γ_s^+	γ_s^-
Lysozyme (dry)*	41.2	0.07	23.4
Lysozyme (hydrated)*	31.5	≈4.5	≈56.2
H.S.A. (dry)**	41.0	0.13	17.2
H.S.A. (2 layers of hydration)**	26.8	6.0	51.5

*van Oss, 1994.
**van Oss and Good, 1989.

Table 7. Amount of adsorbed water, enthalpy of dehydration and respective C.V (%)

	NT		EZ	
	mg H_2O / mg dry solid	ΔH (j/g H_2O)	mg H_2O / mg dry solid	ΔH (j/g H_2O)
Sigmacell 101	0.365 ± 8.49	1577.48 ± 7.29	0.364 ± 5.22	1886.13 ± 3.09
Sigmacell 20	0.243 ± 5.76	1444.95 ± 6.58	0.357 ± 5.88	1795.20 ± 6.20
Avicel pH 101	0.233 ± 5.58	1464.30 ± 5.74	0.418 ± 2.15	1707.60 ± 2.36
Whatman CF 11	0.214 ± 7.48	1360.56 ± 5.11	0.283 ± 1.41	1933.96 ± 12.76

γ_s^+ is found, one may assume the presence of a residual polar liquid on that surface. This liquid could be simply hydration water, due to imperfect drying of the surface. In this case, the value of γ_s^{AB} can be a measure of the degree of residual hydration and the ratio γ_S^-/γ_S^+ indicates the degree of orientation of the residual water of hydration (van Oss *et al*., 1997). According to this hypothesis and despite the small values of γ_s^+ (Table 5), as compared to γ_s^-, the highest values of γ_s^{AB} and especially γ_S^-/γ_S^+ from Sigmacell 101 suggest that this cellulose has the strongest affinity for water.

It is interesting to notice that the small values of γ_s^{AB} from Sigmacell 101 and Sigmacell 20 (Table 5) are similar. Knowing that these celluloses have similar particle sizes, as shown in Table 1, it is possible that there is a similar and small amount of residual water of hydration at the surface of these cellulose particles. However, and since these celluloses have different structural properties (Table 1, *Icr*) the highest values of γ_S^-/γ_S^+ from Sigmacell 101 indicate that, at the surface of this cellulose particles, the adsorbed water molecules display a higher degree of orientation. Hence, this cellulose has a stronger affinity for water molecules. These observations agree with the results obtained from the calorimetric assays (Table 7) – a more amorphous cellulose, like Sigmacell 101, not only adsorbs a greater amount of water, but also more strongly.

If the observations for Sigmacell 101 and Sigmacell 20 were based on the celluloses structural properties (on account of their similar sizes), that is not the case of Sigmacell 20 and Avicel, since, despite their similar crystallinities, these last two have different particle sizes. Therefore, a straightforward comparison of the γ_s^{AB} and γ_S^-/γ_S values is not possible. Based only on the similar structural properties of these two celluloses, similar γ_s^{AB} and γ_S^-/γ_S^+ values were expected. For Avicel, the null value of the electron donor component, γ_s^+ (Table 5), suggest that there is virtually no residual water of hydration.

It is interesting to observe that, after the enzymatic treatment, the wicking times increased greatly (Table 3), when compared to those of the untreated samples (Table 2). The presence of new ionic and polar residues from the adsorbed protein (and since proteins have an hydrophilic character) should have strengthened the interactions with the probe polar liquids, by increasing the work of adhesion of the liquids on the treated surfaces. Thus, smaller wicking times would be expected.

These results may be partially be justified as follows: human serum albumin (HSA), and lyzosyme, like other serum proteins, are initially insoluble in water when dry, but become very soluble upon hydration (van Oss and Good, 1989). When these proteins are added to water as dry powders, the particles first have to swell, before they undergo dissolution as a second step.

Likewise, and regarding EZ samples in bare plates, it is very much likely that the adsorbed protein is first undergoing a continuous rehydration process, as it gets in contact with the 'liquid front' of the polar probe liquids (like water and formamide). Hence, the rehydration process may slow down the liquids migration velocity (higher wicking times), and therefore could be considered a limiting step in the wicking process.

However, this hypothesis does not justify the also higher wicking times of the precontacted plates, in EZ samples. This is especially surprising in the case of water, since the adsorption of water vapour should have rehydrated the adsorbed protein, thus yielding smaller wicking migration times, than the ones obtained (Table 3).

Moreover, even if the period of 12 h was not sufficient to reach the adsorption equilibrium with the vapours of the polar liquids, that would not be the case of the plates precontacted with the vapours of the apolar ones, since these have a high vapour pressure. Hence, the wicking velocities of either bare or precontacted plates with the apolar liquids (Table 3), should not differ much from those of the untreated. This would especially be the case of the precontacted plates, since, according to the Washburn equation, the wicking velocity should depend only on the liquids properties.

This observation brings us to the validity of the calculated *R* parameter. It can be observed in the work of Dourado *et al*. (1998) that, although sonication

contributed to a great decrease in the particles median size, the *R* parameter did not change much. From Table 4, the *R* values for EZ have greatly decreased, when compared to those of the NT. Even though the enzymatic treatment had a more pronounced effect than sonication, in the reduction of the particles size of the more crystalline celluloses (Table 1), the reduction of the *R* parameter (as compared to that of the NT) should have not been so significant, since the average size of proteins is around 60–80 Å.

Bearing in mind that, aside from active enzyme protein, almost every commercial enzyme preparations contains sugars, carbohydrates and inorganic salts, as stabilising agents of the final crude extract, the possibility of these additives influencing the wicking process was questioned. Therefore, additional experiments were performed with Sigmacell 101. In these experiments, we reduced the period of enzymatic treatment to 5 min, EZ5, to exclude the possibility of any hydrolytic effect from the enzymes.

We also used a smaller protein concentration (1/4 of the initial concentration used in the experiments), but maintained the 5 min of enzyme treatment. The reason for using a smaller protein concentration is associated with the need to avoid excess, hence unbound, protein. In this situation, the following operations were performed:

- the enzyme extract was ultrafiltered (1/4 EZ5U) prior to the enzymatic reaction, to remove the additives,
- the enzyme extract was ultrafiltered and following the enzymatic reaction, the treated cellulose suspension was centrifuged (1/4 EZ5UC) to remove any unbound protein.

A final experiment was conducted with BSA (bovine serum albumin), as a protein control without any hydrolytic activity. The reaction mixture of cellulose and BSA was held in suspension also for 5 min (BSA5).

The data gathered from these experiments were not presented here, since, for the various experiments performed with the enzyme celluclast, the obtained values for the *R* values, surface and interfacial properties, did not change significantly, when compared to the original experiments presented in Tables 4 and 5 (EZ). As to the experiments performed with BSA, the obtained values of the above parameters, were very similar to the ones of NT (Tables 4 and 5).

As a consequence of the above observations, it appears that the thin-layer wicking technique should not be applied in the case of celluloses with adsorbed protein.

Despite the observations presented here, following protein adsorption the apolar and electron donor component of the celluloses remained high, whereas the electron acceptor component remained practically null. The surface ten-

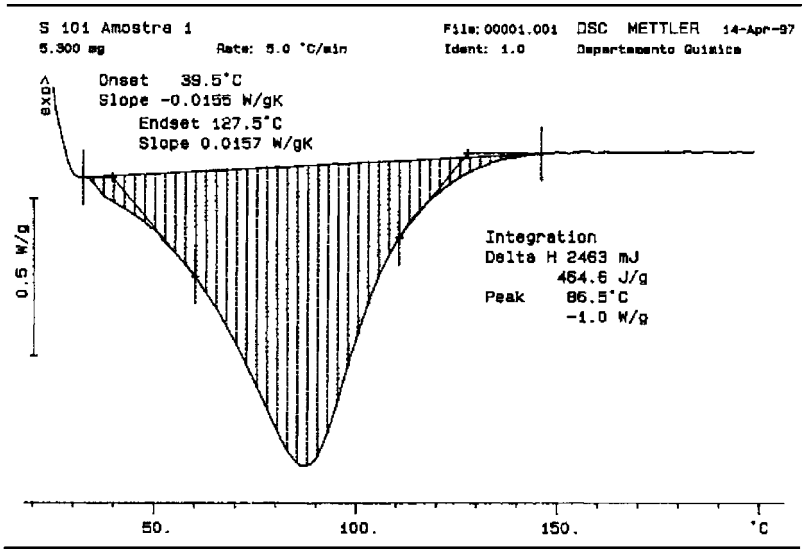

Figure 2. Example of a DSC curve, for Sigmacell 101, (NT).

sion components of EZ samples (Table 5) were expected to be closer to the ones of dry lysozyme and dry human serum albumin (HSA) (Table 6).

According to the small positive values of the work of water spreading, W*s*, (Table 5), the celluloses became slightly hydrophilic. However, only the surface character of the more crystalline samples varied significantly. These results agree well with those obtained from particle size characterisation (Table 1), where it was seen that the enzyme adsorption increased the stability of the more crystalline particles. For Sigmacell 101, this effect was, however, insignificant. Therefore, the adsorption of enzyme increased the stability of the more crystalline particles, by breaking the agglomerates and keeping the individual particles apart. The same effect was reported in Gama *et al*. (1996), for Avicel pH 101, by analysing the optical density attenuation of cellulose suspensions.

Calorimetric experiments

Regarding the thermogravimetric assays (Table 7, NT), it can be seen that the amorphous Sigmacell 101 adsorbed the greatest amount of water. Besides crystallinity, the specific surface area determines the amount of adsorbed water. Therefore, admitting that the water is more strongly bound to a more hydrophilic (amorphous) surface, like that of Sigmacell 101, there should be a correlation between crystallinity and specific enthalpy of dehydration, thus eliminating the effect of the surface area. As presented in Table 7 (enthalpy of

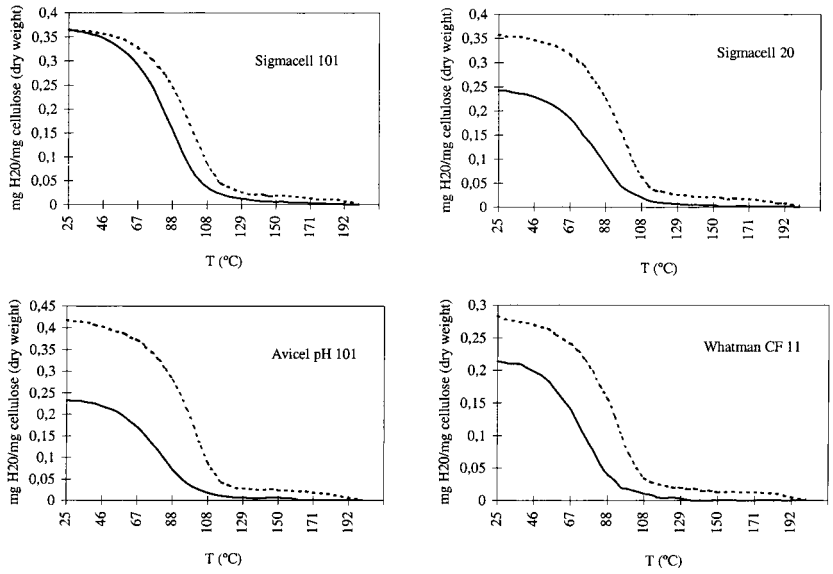

Figure 3. Dynamic thermogravimetric curves of water desorption from the saturated cellulose samples, before (NT) (—) and after enzymatic treatment (EZ) (- - -).

dehydration, NT), this hypothesis was in fact confirmed since the specific heat of dehydration seemed to correlate roughly with the cellulose crystallinity.

These results, as obtained from calorimetric assays, were consistent with those obtained from thin-layer wicking. Sigmacell 101, which was stable in aqueous media, was the least hydrophobic of the analysed celluloses (*Ws* values, Table 4), and had the highest specific heat of dehydration. The other celluloses, which agglomerated in aqueous media, showed less affinity for water molecules, as assessed by the two independent techniques.

After protein adsorption (Table 7, EZ), Sigmacell 101 adsorbed a similar amount of water. However, this water was more strongly adsorbed, since it had a higher specific heat of dehydration. The more crystalline celluloses adsorbed not only a greater amount of water, but also more strongly. This effect was more significant for Whatman CF-11, and this may be associated to the higher fraction of surface coverage by the enzymes, resulting from its lower specific surface. This suggestion was based on the observations by Gama and Mota (1997b), for Whatman CF-11, where the XPS technique was used to determine the fraction of the particles surface covered by protein.

Figure 2 shows a differential calorimetric thermogram, where the recorded peak characterises the endothermic process of water desorption. For all types of cellulose, similar thermograms were obtained. The results from the thermogravimetric assays are graphically displayed in Figure 3. It is possible to see that, following enzyme adsorption, a shift of the curves to higher temper-

atures occurred. Also, regarding EZ curves, it is possible to observe a region of strongly adsorbed residual water (between 125–200°C), which was more difficult to desorb from the surface of the treated samples. Probably, this water is adsorbed on polar groups from the proteins.

Based on the discussion here presented, it is our opinion that the effect of the enzymes on the interfacial properties of cellulosic materials should also be pondered, when studying biotechnological applications of glycanases.

The calorimetric assays proved to be a useful technique for assessing the differences in the celluloses affinity for water. New studies are being conducted with pulp fibers, with and without enzymatic treatment, in quasi-isothermal conditions so that the equilibrium equations can be used to assess the fibers surface properties.

Acknowledgement

The authors wish to acknowledge the financial support from JNICT/Portugal, through the project INTERREGII/BM/03/REGII/3/97.

References

Angberg, M., Nyström, C. and Castensoon, S. (1991) Evaluation of heat-conduction microcalorimetry in pharmaceutical stability studies. IV. The influence of microcrystalline cellulose on the hydration rate of anhydrous lactose. *Int. J. Pharmaceutics* **77**, 269–277.

Belgacem, M. N., Czeremuszin, G., Sapihea, S. and Gandini, A. (1995) Surface characterization of cellulose fibres by XPS and inverse gas chromatography. *Cellulose* **2**, 145–157.

Cavaco-Paulo, A., Cortez, J. and Almeida, L. (1997) The effect of cellulase treatment in textile washing processes. *JSDC* **113**, 218–222.

Chibowski, E. (1992) Solid surface free energy components determinations by the thin-layer wicking technique. *J. Adhesion Sci. Technol.* **6**(9), 1069–1090.

Chibowski, E. and Holysz, L. (1992) Use of the Washburn equation for surface free energy determination. *Langmuir* **8**, 710–716.

Chibowski, E. and González-Caballero, F. (1993) Theory and practice of thin-layer wicking. *Langmuir* **9**(1), 330–340.

Chibowski, E. and Stasczczuk, P. (1988) Determination of surface free energy of kaolinite. *Clay and Clay Min.* **36**(5), 455–461.

Daniels, M. J. (1992) Using biological enzymes in papermaking. *Paper Technol.* **33**, 14–17.

Domínguez, H., Núñez, M. J. and Lema, J. M. (1994) Enzyme-assisted hexane extraction of soya bean oil. *Food Chem.* **54**, 223–231.

Domínguez, H., Núñez, M. J. and Lema, J. M. (1995) Enzymatic pretreatment to enhance oil extraction from fruits and oilseeds: a review. *Food Chem.* **49**, 271–286.

Dourado, F., Gama, F. M., Chibowski, E. and Mota, M. (1998) Characterization of cellulose surface free energy. *J. Adhesion Sci. Technol.* **12**(10), 1081–1090.

Fielden, K. E., Newton, J. M., O'Brien, P. Rowe and R. C. (1988) Thermal studies on the interaction of water and microcrystalline cellulose. *J. Pharm. Pharmacol* **40**, 674–679.

Gama, F. M, Carvalho, M. G., Figueiredo, M. M. and Mota, M. (1996) Comparative study of cellulose fragmentation by enzymes and ultrasound. *Enzyme Microb. Technol.* **20**, 12–17.

Gama, F. M. and Mota, M. (1997a) Enzymatic hydrolysis of cellulose (I): relationship between kinetics and physico-chemical parameters. *Biocatal. Biotransfor.* **15**, 221–236.

Gama, F. M. and Mota, M. (1997b) Enzymatic hydrolysis of cellulose (II): X-ray photoelectron spectroscopy studies on cellulase adsorption. Effect of the surfactant Tween 85. *Biocatal. Biotransfor.* **15**, 237–250.

Grant, R. (1995) Enzymes help to increase pulp paper production. *Tappi* **78**(8), 26–27.

Hatakeyama, T. and Quinn, F. X. (1995) *Thermal Analysis: Fundamentals and Applications to Polymer Science*. 2nd edn. Chichester: John Wiley, pp. 1–3.

Henrissat, B. (1994) Cellulases and their interaction with cellulose. *Cellulose* **1**, 169–(196.

Hollenbeck, R. G., Peck, G. E. and Kildsig, D. O. (1978) Application of immersional calorimetry to investigation of solid–liquid interaction: microcrystalline cellulose–water system. *J. Pharm. Sci.* **67**(11), 1559–1606.

Holysz, L. and Chibowski, E. (1992) Surface free energy components of α-alumina from thin-layer wicking. *Langmuir* **8**, 717–721.

Jackson, L. S., Heitmann, J. A. and Joyce, T. W. (1993) Enzymatic modifications of secondary fiber. *Tappi* **76**(3), 147–153.

Klesov, A. A. (1990) Biochemistry and enzymology of cellulose hydrolysis. *Biochem.* **10**(55), 1731–1765.

Lee, L.-H. (1996) Correlation between lewis acid-base surface interaction components and linear solvation energy relationship solvatocromic α and β parameters. *Langmuir* **12**, 1681–1687.

Miletzky, F. (1996) Biotechnology in the pulp and paper industry. Proc. 6th Inter. Conf. Biotechnology in the pulp and paper industry, Vienna, Austria, pp. 9–15.

Pommier, J.-C., Fuentes, J.-L. and Goma, G. (1989) Using enzymes to improve the process and the product quality in the recycled paper industry. Part 1: the basic laboratory work. *Tappi* **72**(6), 187–190.

Rosenthal, A., Pyle, D. L. and Niranjan, K. (1996) Aqueous and enzymatic processes for edible oil extraction. *Enzyme Microb. Technol.* **(19**(1), 402–420.

Staszczuk, P. (1984) Application of thermal analysis for determination of film pressure and film surface energy on silica gel surface. *J. Thermal Analysis* **29**, 217–225.

Stork, G., Pereira, H., Wood, T. M., Düsterhöft, E. M., Toft, A. and Puls, J. (1995) Upgrading recycled pulps using enzymatic treatment. *Tappi* **78**(2), 79–88.

van Oss, C. J., Chaudhury, M. K. and Good, R. J. (1987) Monopolar surfaces. *Adv. Colloids Interf. Sci.* **28**, 35–64.

van Oss, C. J., Giese, R. F., Murphy, Z., L. L., K., Norris, J., Chaudhury. M. K. and Good, R. J. (1992) Determination of contact angles and pore sizes of porous media column and thin layer wicking. *J. Adhesion Sci. Technol.* **6**(4), 413–428.

van Oss, C. J. (1994) *Interfacial Forces in Aqueous Media*. Marcel Dekker, New York, pp. 179.

van Oss, C. J., Giese, R. F. and Wu, W. (1997) On the predominant electron-donicity of polar solid surfaces. *J. Adhesion* **63**, 71–88.

van Oss, C, J. and Good, R. J. (1989) Surface tension and the solubility of polymers and biopolymers: the role of apolar and polar interfacial free energies. *J. Macromol. Sci. Chem.* **A26**(8), 1183–1203.

Walker, L. P. and Wilson, D. B. (1991) Enzymatic hydrolysis of cellulose: an overview. *Bio/Technol.* (**9**), 3–14.