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Food Chemistry **50** (1994) 191–196

0308-8146/94/$7.00

Calcium binding by cellulose and lignin

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(Received 21 July 1993; accepted 19 August 1993)

Binding of calcium ions by 0·20% suspensions of purified fibers, microcrystalline cellulose (MCC), α-cellulose (CEL), acid-swollen cellulose (ASC), alkali lignin and kraft pine lignin were investigated. More endogenous calcium was released under acidic conditions (pH < 6) and more exogenous Ca^{2+} was bound at higher pH levels for all fibers. From Scatchard analysis, MCC and CEL had little affinity for exogenous Ca^{2+}, ASC had one type of nonspecific binding site, and both lignins had two types of specific binding sites. MCC and CEL tended to bind more Ca^{2+} after enzymatic digestion, whereas ASC and both lignins tended to bind less Ca^{2+}.

INTRODUCTION

Plant cell walls consist of cellulosic microfibrils embedded in a matrix of noncellulosic polysaccharides and lignins. This structural feature makes cellulose and lignin the most abundant organic compounds found in nature (Bellamy, 1974; Cowling, 1975; Ander & Eriksson, 1978). Since cellulose and cellulose derivatives are used as bulking agents and replacements for fats and oils (McCormick, 1985; Miller *et al.*, 1989), interactions between these materials and minerals become important in terms of functional and nutritional properties of food materials. These interactions may be characterized by defining binding affinity and types of binding sites with reciprocal plots, such as Scatchard plots (Scatchard, 1949). For example, lignin has two specific binding sites for iron, and cellulose has only nonspecific binding sites for iron (Platt & Clydesdale, 1987).

James *et al.* (1978) found that calcium binding by the noncellulosic fraction of fiber reduced the availability of calcium for small intestinal absorption, but colonic microbial digestion liberated the calcium. Similarly, enzyme treatment of rice hemicellulose resulted in the release of differing amounts of bound calcium, magnesium, and manganese (Mod *et al.*, 1982). The present study was conducted to determine the release of endogenous calcium by purified cellulose and lignins, the calcium binding ability of these purified fibers, the effect of pH on the calcium binding ability of these fibers, and the effect of enzymatic hydrolysis on the binding of calcium by these fibers.

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MATERIALS AND METHODS

Sources of materials

Microcrystalline cellulose (MCC) (Sigma cell type 50) and alkali lignin (AL) (Indulin AT) were purchased from Sigma Chemical Co. (St. Louis, MO); α-cellulose (CEL) (Celufil®) was obtained from US Biochemical Corporation (Cleveland, OH); kraft pine lignin (KPL) was donated by Westvaco Research (North Charleston, SC).

Acid swollen cellulose (ASC) was prepared by a modification of the method of Hsu and Penner (1989). A 40 g sample of MCC was swollen in 600 ml of 78% (w/v) phosphoric acid at 4°C for 30 min; suspended in 3·6 litres of distilled, deionized water; filtered; and washed twice in 2·4 litres of water. This suspension of the cellulose in water was then adjusted to pH 6·2–6·4, allowed to equilibrate for 1 h, and washed three times. Following the final wash, the cellulose was lyophilized and milled until 100% of the product passed a 40 mesh sieve.

Cellulase from *Aspergillus niger* was purchased from Sigma Chemical Co. (St. Louis, MO). According to the manufacturer, 1 unit of the enzyme will liberate 1·0 μM of glucose from cellulose in 1 h at pH 5·0 at 37°C.

Lignin peroxidase was prepared and assayed as described by Kirk *et al.* (1990). Starter culture, *Phanerochaete chrysosporium* BKM-F-1767, was the gracious gift of Forest Products Laboratory (Madison, WI). The assay used for determination of activity is based on the oxidation of veratryl alcohol to veratraldehyde in the presence of hydrogen peroxide. The reaction mixture contained 1·7 ml crude enzyme, 0·6 ml 10 mM veratryl

alcohol, 0·6 ml 0·25 M sodium tartrate (pH 3·0), and 0·12 ml 10 mM H_2O_2. Change in absorbance at 310 nm was monitored; the crude enzyme gave an absorbance change of about 0·003/min.

Measurement of endogenous calcium

Endogenous calcium was determined after wet ashing the samples following a modification of the method of Neidermeir *et al.* (1971). Endogenous calcium content of the ashed samples was analyzed with a Hitachi Model 170–50 atomic absorption spectrophotometer with an air–acetylene flame and hollow cathode lamp at 422·7 nm.

Potentiometric measurement of free calcium

The free calcium content of the samples was determined by potentiometric titration as described by Ha *et al.* (1989). Fiber materials were suspended in distilled, deionized water at a concentration of 0·20% (w/v) and stirred for 1 h. At this time, a known amount of calcium was added to 10 ml of the suspensions and free calcium measured with a Radiometer Ca^{2+}-selective electrode, type K401, using a PHM 80 research pH meter, TTA 80 titration assembly, REC 80 servograph and REA 166 tritrigraph from Radiometer Copenhagen (Copenhagen, Denmark). Immediately before each titration, a semilogarithmic standard curve was plotted based on a series of standard solutions (0–700 μM) which were prepared by diluting the 100 mM calcium standard from Radiometer Copenhagen.

Calculation of binding parameters

From the known concentrations of added calcium ions (A) and the measurements of endogenous calcium concentration (E) and free Ca^{2+} concentration (F), data for Scatchard analysis were calculated as follows:

$$\begin{cases} \text{bound calcium} = E + A - F \\ \text{unbound calcium} = F \\ \text{bound calcium/unbound calcium} = (E + A - F)/F \end{cases}$$

From Scatchard plots, k values and n values were determined. The intrinsic association constant, k, is equal to the negative slope. The intercept represents the number (n) of binding sites per g of macromolecule (Scatchard, 1949). A more complete explanation of Scatchard analysis was given by Ha *et al.* (1989).

Enzyme treatments

For each of the three types of cellulose and two types of lignin, 2·0 g of fiber was suspended in 496 ml distilled, deionized water and incubated at 37°C for 1 h with constant stirring. After this equilibration time, 4 ml of 0·4% cellulase or 3·7 ml of concentrated crude lignin peroxidase was added. Addition of 0·3 ml of 10 mM H_2O_2 started the latter reaction. After 0, 1, 3, 4, 5, 6, 12, 24, 48, and 72 h, duplicate 10 ml aliquots were removed and each was added to 10 ml distilled, deionized water. One of the aliquots was filtered, placed in a boiling water bath for 10 min, cooled on ice, and used for reducing sugar (RS1) or phenolic compound (PC1) analysis. The other aliquot was divided into two 10-ml portions. One of the portions was used for potentiometric analysis after the addition of 100–700 μM calcium standard. After the potentiometric measurements, the other 10 ml portion was filtered, placed in a boiling water bath for 10 min, cooled on ice, and used for reducing sugar (RS2) or phenolic compound (PC2) analysis. Enzyme activity during the titrations was negligible since the difference between RS1 or PC1 and RS2 or PC2 was minimal.

Reducing sugar analysis

Reducing sugar produced during cellulose hydrolysis was analyzed using the method of Nelson (1944).

RESULTS AND DISCUSSION

Endogenous calcium released by fibers

The initial pH of 0·20% suspensions and the endogenous calcium content of the fibers are given in Table 1. The endogenous calcium released by the fibers as a function of pH is given in Fig. 1. There were non-detectable levels of calcium in samples MCC and ASC regardless of the pH level, reflecting the very low endogenous levels. For samples CEL, AL, and KPL, almost 100% of the endogenous calcium was released as pH levels approached 4·0. However, little endogenous calcium was released from these three fibers when pH levels were between 7·0 and 10·0. These data compare favorably with those of Laszlo (1989) who demonstrated that little endogenous calcium was removed from corn bran and soy hull over the pH range 4·5–6·0; however, below pH 4·5, calcium was substantially extracted, with little or none remaining below pH 3·0.

Calcium-binding ability of fibers

The addition of 100–700 μM of calcium standard to the fiber suspensions at their initial pH resulted in an increase in the amount of bound calcium for all fibers.

Table 1. Endogenous calcium content and initial pH of 0·20% suspensions of purified fibers

Fiber	Calcium (μg/g)[a]	pH
Microcrystalline cellulose	0·58 ± 0·01	6·0–6·2
α-Cellulose	88 ± 1·4	5·8–6·1
Acid-swollen cellulose	ND[b]	6·1–6·2
Alkali lignin	52 ± 3·1	6·7–7·0
Kraft pine lignin	80 ± 1·7	7·0–7·2

[a] Mean ± standard deviation.
[b] Not detectable.

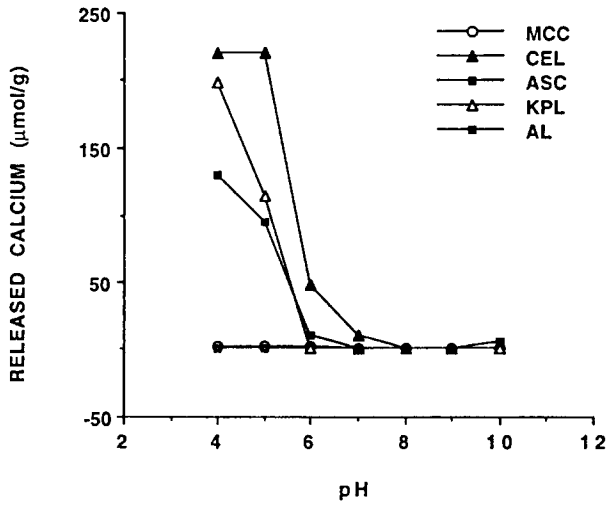

Fig. 1. Endogenous calcium released by 0·20% suspensions of fibers as a function of pH.

The addition of the standard also caused a decrease in pH; therefore, the pH was maintained by the addition of 2 mM NaOH and/or HCl. MCC and CEL had the lowest binding capacity for calcium, binding less than 10% of the added calcium for all levels of calcium addition. ASC bound 30–36% of the added calcium at all levels of calcium addition. The preparation of ASC, which involved treating MCC with phosphoric acid, may have created more accessible areas and available hydroxy groups to physically entrap or adsorb calcium (Kräsig, 1985). AL bound at least 50% of the added calcium and had the highest binding capacity for calcium among the fibers studied. KPL bound 55% of the added calcium at the 100 µM level, but this percentage gradually decreased to 22% as the level of addition reached 700 µM. Since these binding curves only provided relative affinity of calcium binding, Scatchard analysis was used to obtain quantitative binding data.

Figures 2–4 are Scatchard plots of the binding of calcium by ASC, AL and KPL at the initial pH of the suspensions. MCC and CEL had little affinity for calcium; this conclusion is based on the absence of a

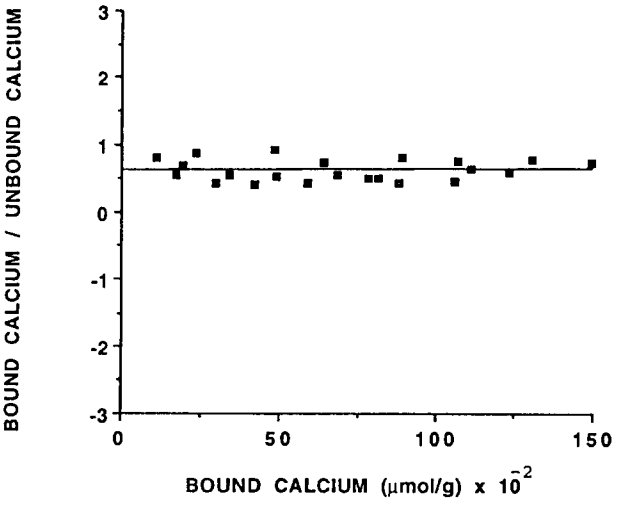

Fig. 2. Scatchard plot of the binding of calcium by 0·20% suspensions of ASC.

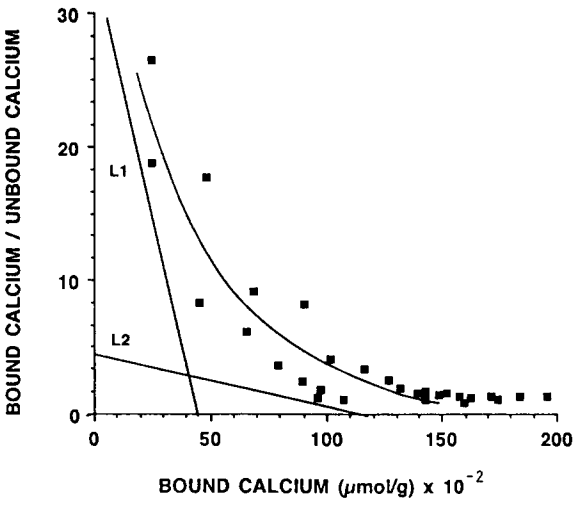

Fig. 3. Scatchard plot of the binding of calcium by 0·20% suspensions of AL. The binding curve is the best fit for Scatchard data, which is resolved into two straight lines L_1 and L_2 using the method of Ha *et al.* (1989).

specific binding line on the Scatchard plots (Figs not shown). The flat binding line for ASC (Fig. 2) indicated that ASC had a very low calcium affinity. The weak affinity of the celluloses for calcium is in agreement with an earlier report of low affinity between neutral polysaccharides and alkaline earth metal ions (Rendelman, 1978). Scatchard plots for AL (Fig. 3) and KPL (Fig. 4) indicated the presence of two types of binding sites for calcium. AL had the highest affinity for calcium and also the highest capacity to bind calcium. The two groups of binding sites bound $4{\cdot}3 \times 10^3$ µmol and $1{\cdot}15 \times 10^4$ µmol of calcium per g AL. KPL also had a comparatively high affinity and capacity for calcium. The two groups of binding sites bound $8{\cdot}00 \times 10^2$ and $5{\cdot}60 \times 10^3$ µmol calcium per g KPL. The mechanism of calcium binding was not explored in this research. However, the chemical structure of lignin is based on benzene or various polyhydroxy-phenol polymers (Nord & Schubert, 1958).

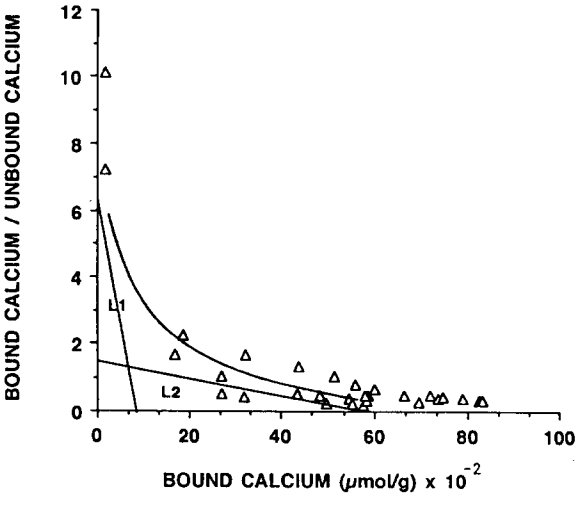

Fig. 4. Scatchard plot of the binding of calcium by 0·20% suspensions of KPL. The binding curve is the best fit for Scatchard data, which is resolved into two straight lines L_1 and L_2 using the method of Ha *et al.* (1989).

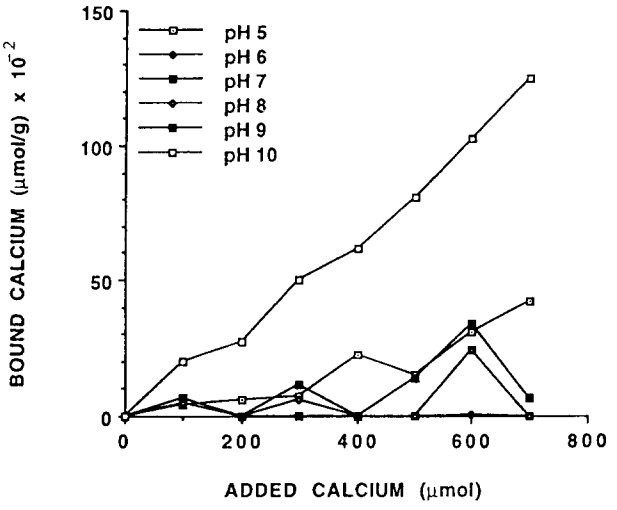

Fig. 5. Effect of pH on the binding of calcium by 0·20% suspensions of MCC.

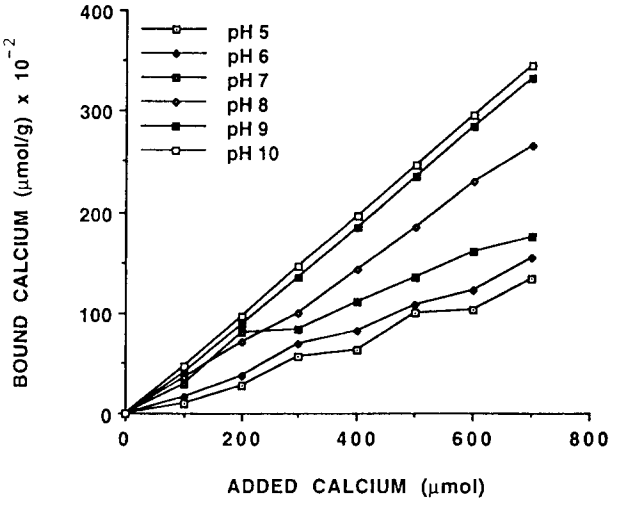

Fig. 7. Effect of pH on the binding of calcium by 0·20% suspensions of ASC.

The hydroxyl and methoxy groups of lignin are similar to those of catechol, which react with iron to form hexadentate complexes of high stability (Fieser & Fieser, 1965; Fernandez & Phillips, 1982).

Effect of pH on calcium binding

Calcium binding for MCC and CEL did not change as pH was increased from 5.0 to 9.0; however, at pH 10.0, calcium binding increased significantly ($P < 0·05$) for these two celluloses (Figs 5 and 6). These results are consistent with those of Rendleman (1982) who demonstrated very little interaction between cellulose and calcium in the pH range of 5–8. The use of sodium hydroxide to increase the pH of the suspensions in this study may have loosened the structure of cellulose, resulting in creation of accessible areas for entrapping or adsorbing calcium as the pH was raised to 10 (Whitaker *et al.*, 1974; Kräsig, 1985). Scatchard analysis of the binding of calcium by MCC and CEL at all levels of pH indicated that these celluloses had only nonspecific binding for calcium and that the amount of bound calcium increased more than three times at pH 10 (Figs not shown). For ASC, calcium binding capacity increased significantly ($P < 0·05$) with calcium addition as pH increased to 10 (Fig. 7). These results are in agreement with earlier reports that the ionization of polyhydroxy compounds in the presence of a base facilitates formation of a metal–alcoholate complex (Rendleman, 1973, 1978). There were no significant increases in bound calcium at higher pH levels for either AL or KPL (Figs not shown).

Effect of enzymatic hydrolysis on calcium binding

In order to investigate the effect of enzymatic hydrolysis on the binding of calcium ions at the initial pH of the fiber suspensions, potentiometric titrations for free calcium were performed after addition of 100–700 μM calcium standard to enzyme treated suspensions of each fiber.

The amount of bound calcium increased after 24 and 48 h hydrolysis for MCC (Fig. 8). Although the differ-

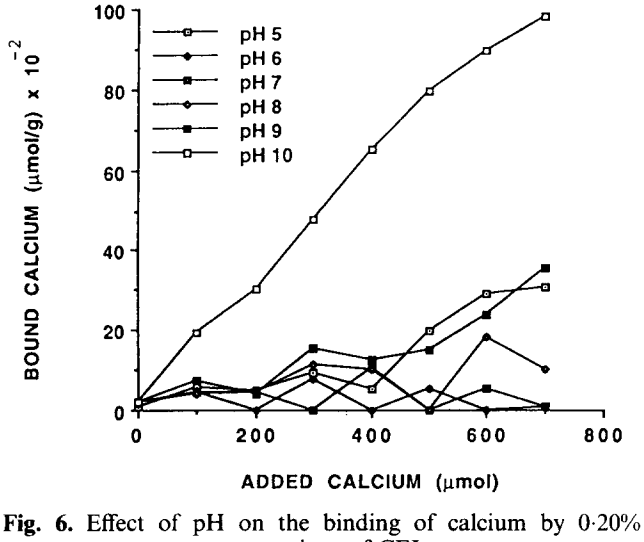

Fig. 6. Effect of pH on the binding of calcium by 0·20% suspensions of CEL.

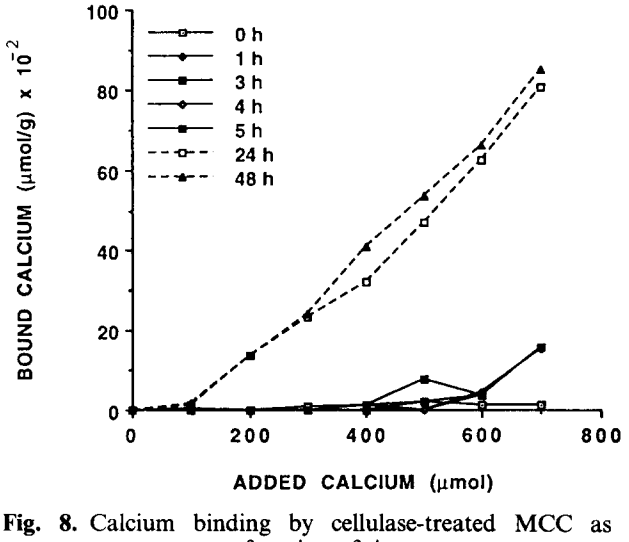

Fig. 8. Calcium binding by cellulase-treated MCC as a function of time.

Table 2. The amount of reducing sugar after cellulase hydrolysis[a]

Cellulase treatment (h)	MCC	CEL	ASC
0	0	0	0
1	26·0	45·0	86·7
3	49·7	78·8	187
4	57·8	48·3	217
5	67·9	54·4	279
24	164	43·6	845
48	187	54·9	1 032

[a] MCC, microcrystalline cellulose; CEL, α-cellulose; ASC, acid-swollen cellulose.

ences were not significant, calcium binding by CEL tended to increase and that by ASC tended to decrease after cellulase hydrolysis (Figs not shown). The results of the reducing sugar analysis (Table 2) suggested the partial hydrolysis of MCC and ASC by cellulase. However, the results of the reducing sugar analysis did not necessarily correspond with those of the changes in calcium binding accompanying hydrolysis. MCC and CEL contain mostly crystalline microfibrillar structures, which have been found to adsorb metal ions on the surface. Therefore, cellulase hydrolysis of MCC and CEL may have increased the amount of surface area and, subsequently, increased the amount of bound calcium. The tendency of ASC to bind less calcium after cellulase treatment may reflect a mild destruction of the swelling structure during cellulase hydrolysis.

KPL bound significantly less ($P < 0.05$) calcium after 24 and 48 h of enzyme treatment. Results of Scatchard analysis were a shift of the binding curves from L1 to L3 (Fig. 9), suggesting that the number of binding sites was decreased by enzymatic hydrolysis. AL also bound significantly less ($P < 0.05$) calcium after enzymatic hydrolysis, especially at 72 h (Fig. 10). These results suggest that the calcium binding sites for AL and KPL were gradually destroyed by lignin peroxidase.

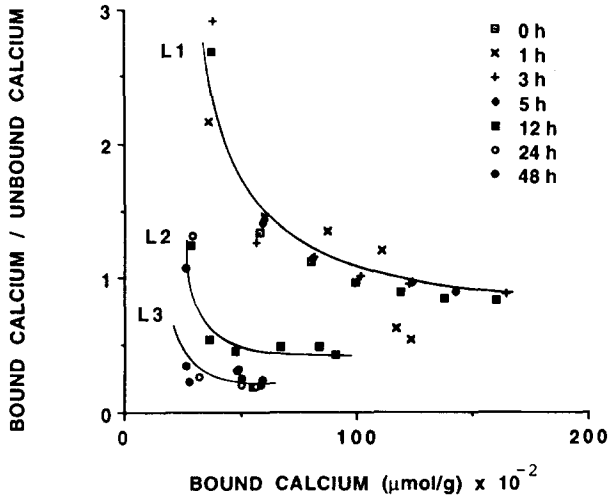

Fig. 9. Scatchard plot for calcium binding by lignin peroxidase-treated KPL as a function of time. L1 is the binding curve for 0–5 h, L2 is for 12 h and L3 is for 24 and 48 h.

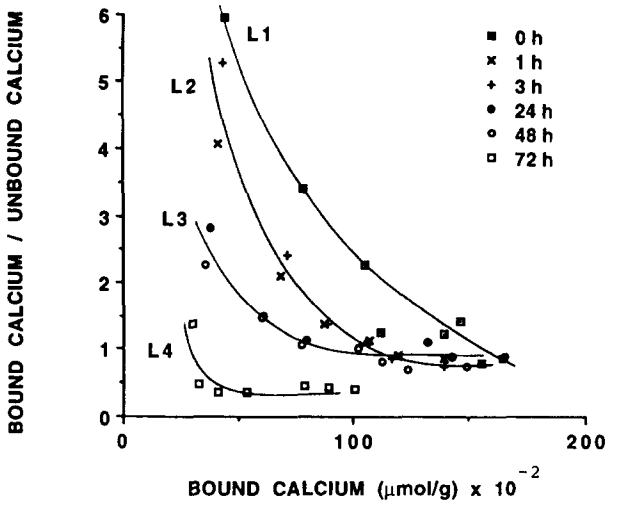

Fig. 10. Scatchard plot for calcium binding by lignin peroxidase-treated AL as a function of time. L1 is the binding curve for 0 hr, L2 is for 1 and 3 h, L3 is for 24 and 48 h, and L4 is for 72 h.

CONCLUSIONS

MCC and CEL, both highly purified celluloses, had little affinity for calcium at pH levels between 5 and 9 and greater affinity at pH 10 ($P < 0.05$). Both these celluloses tended to bind increased amounts of calcium after enzymatic hydrolysis. The structure of these celluloses may have been loosened to produce more accessible areas or exposed hydroxy groups for entrapping or adsorbing calcium as pH was increased to 10 or by cellulase hydrolysis.

ASC had a very low affinity for calcium. The amount of bound calcium increased as pH increased from 5 to 10. Less calcium was bound after treatment with cellulase. These results indicated that the calcium-entrapping structures created during acid swelling of cellulose were enhanced by increased pH but destroyed by cellulase hydrolysis.

Both AL and KPL had two types of specific binding sites for calcium. AL had 1·1–1·4 times greater affinity and 2·1–5·4 times greater capacity to bind calcium than KPL. Both lignins tended to bind more calcium at higher pH levels, which may reflect ionization of polyhydroxy groups of lignin at higher pH levels. Both lignins bound less calcium after treatment with lignin peroxidase. Based on Scatchard analysis of both lignins, many binding sites were destroyed by lignin peroxidase.

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