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Effect of storage of apple on the enzymatic hydrolysis of cell wall polysaccharides

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Cell wall materials in the form of water-insoluble solids (WIS) and water-soluble fractions (WSF) were prepared from apples stored at 4°C for 30 weeks. During storage, the WIS content decreased whereas the WSF content remained unchanged. The total amount of polysaccharides decreased, in particular the pectic polymers which decreased by 10%. In contrast, the soluble pectic fraction increased by 40% whilst its degree of methoxylation remained constant. The arabinose and galactose content progressively declined. The enzymatic treatment of the apple tissues was more effective the longer the storage; yields correlated well with the enzyme hydrolysis of WIS. The accessibility of pectin to polygalacturonases in apple tissues is discussed since it was higher at the end of storage, whereas the solubilisation of pectins from WIS by polygalacturonases remained constant. On the other hand, with liquefying enzymes, the yield of pectin solubilisation from apple tissues or WIS were well correlated and increased with storage time. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

During apple processing, fruit may be treated after storage at low temperature, especially cull fruit. In some cases, cell wall polysaccharide-degrading enzymes are used as aids to improve the yield of apple juice (liquefaction) or to transform the pulp into nectar (maceration). In this industry, it is observed that the efficiency of these enzymes increases with storage time; but no investigation was carried out to assess the cell wall modifications responsible for this observation. During storage, the ripening of fruit involves the softening of fruit tissue (Knee & Bartley, 1981). This is an important mechanism which affects the edible quality of fruit as well as its processing in juices or nectars. This textural change depends on the cellular anatomy of the tissue, the water content of the cells and the composition of the cell walls (Bartley & Knee, 1982). Regarding the last point, most studies about the cell wall polysaccharides metabolism in ripening apples reported: (1) a degradation of pectin from the middle lamella (Knee, 1973; Ben-Arie & Kisley, 1979), (2) an increase in soluble pectins (Ben & Gaweda, 1985) whose molecular weight does not change (Bartley & Knee, 1982), (3) a loss of non-cellulosic neutral sugars, especially galactose and arabinose (de Vries *et al.*, 1981; Gross & Sams, 1984; Fischer & Amado, 1994), (4) a constant degree of methoxylation of pectic substances (de Vries *et al.*, 1984) and (5) a *de novo* synthesis of highly methylated esterified pectin (Knee, 1978a, b; Fischer *et al.*, 1994).

In the present investigation, we will examine whether the above changes during apple storage are capable of modifying the hydrolysis of cell wall polysaccharides with exogenous enzymes, thus improving the enzyme process. We report on isolation and enzymatic hydrolysis of the cell wall polysaccharides from apple fruits stored for 30 weeks at a low temperature.

EXPERIMENTAL

Preparation of the cell wall material

Apples of the Judeline variety were stored at 4°C and 95% relative humidity from the end of November 1992 (week 0) to June 1993 (week 30). At each sampling time, one kg of starch-free apples (negative iodine test) were randomly chosen out of a calibrated batch (ϕ 55–60 mm); fruits were peeled and cored.

The water-insoluble solids (WIS) were prepared by immersing the apple slices in distilled water and homogenizing at 4°C in a Waring blender for 20 s. The mixture was filtered through a G3 sintered-glass filter. The WIS were washed with distilled cold water until the filtrate showed a negative reaction in the phenol-sulphuric acid test (Dubois *et al.*, 1956). The residues

were freeze-dried and stored at 4°C. The filtrates and the washing water containing the water-soluble fractions (WSF) were pooled, concentrated under reduced pressure at 40°C, treated with 4 volumes of aqueous 1% HCL in 96% ethanol, and kept overnight at 4°C. After centrifugation (4500 *g*, 20 min), the pellets were dispersed in aqueous 80% ethanol and centrifuged again (3 times). Precipitates were dissolved in distilled water and freeze-dried.

Enzymes

UM10, a polygalacturonase-rich preparation (from *Aspergillus niger*), free of contaminating side chain activities in our experimental conditions, and Celluclast, a cellulase-rich preparation (from *Trichoderma reesei*), were provided by Novo Nordisk A/S (Bagsvaerd, Denmark). CPE, a pectin methylesterase-rich preparation (from *Aspergillus niger*) was provided by Gistbrocades (Seclin, France).

Enzymatic hydrolysis

Fresh apple tissues (10 g) were immersed in 50 ml of a 0.05 M sodium acetate buffer at pH 4.5, then homogenized in a Waring blender for 5 s. After filtration through a G3 sintered-glass filter, the pulp was suspended in 50 ml of the same buffer. Enzyme preparations with 1000 nkat of polygalacturonase activity (UM10) or 500 nkat of pectin methylesterase activity (CPE) or 1000 nkat of cellulase activity (Celluclast) were added and the suspension was stirred for 6 h at 40°C. After filtration through a G3 sintered-glass filter, the residue was washed with distilled water and dried by solvent-exchange (96% ethanol and acetone) and weighed. Each hydrolysis was duplicated. The yields of liquefaction (with UM10, CPE and Celluclast) or maceration (with UM10 alone) were expressed as dry residue weight/WIS weight ratio.

The yield of solubilisation of WIS were determined as follows: the samples (150 mg) were suspended in 50 ml of 0.05 M sodium acetate buffer pH 4.5. Enzyme preparations with 150 nkat of polygalacturonase activity, 75 nkat of pectin methylesterase activity or 150 nkat of endoglucanase activity, were added and the suspensions were stirred at 40°C. After 6 h reaction, the suspensions were heated for 5 min at 95°C, then filtered through G3 sintered-glass filters. Filtrates were collected, concentrated and freeze-dried. Each experiment was duplicated and controls were carried out without enzymes. Insoluble residues were washed with distilled water, then dried by solvent-exchange (96% ethanol and acetone) and weighed.

To determine the solubilisation kinetics of galacturonic acid, WIS (10 mg) were suspended with 5 ml of a 50 mM sodium acetate buffer pH 4.5 in glass tubes fitted with Teflon-lined screw caps. Enzyme preparations with 10 nkat of polygalacturonase activity, 5 nkat of pectin methylesterase activity or 10 nkat of endoglucanase activity were added and the suspensions were stirred at 40°C. After reaction, the suspensions were heated for 5 min at 90°C, centrifuged at 450 *g* for 5 min, then filtered through 3 μm Millipore filters. Filtrates were analysed for galacturonic acid. Each kinetic experiment was duplicated and controls were carried out without enzymes.

Determination of water content

The water content of apple tissues was determined gravimetrically by drying at 70°C under reduced pression to constant weight. At each storage date, the determination was carried out three times.

Determination of galacturonic acid

The galacturonide content of the WIS was estimated photometrically at 520 nm with *m*-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973) after sulphuric acid hydrolysis (Ahmed & Labavitch, 1977). In the soluble fractions, the galacturonic acid was determined by the automated *m*-hydroxydiphenyl method (Thibault, 1979).

Determination of neutral sugars

WIS were ground (3 min) in a MM2 mixer mill (Retsch, Haan, Germany). The neutral sugar composition of WIS and WSF were determined by gas chromatography (capillary column of 30 m × 0.25 mm i.d. coated with DB225, 0.15 μm film thickness, J and W Scientific, Folsom, USA) at 215°C, using hydrogen as the carrier gas, after sulphuric acid hydrolysis (Seaman *et al.*, 1954) and derivatization to alditol acetates (Hoebler *et al.*, 1989). *Myo*-inositol was used as the internal standard.

Determination of methanol

The degree of methoxylation (DM), corresponding to the molar ratio of methanol to galacturonic acid, was calculated by determining the methanol content released after alkaline hydrolysis of the WIS. The methanol was measured by gas chromatography (Carbowax 20M capillary column of 50 m × 0.32 mm i.d., 0.2 μm film thickness, AML Chromato, Peyrelevade, France) at 70°C using hydrogen as the carrier gas, after extraction by steam distillation from a WIS suspension (2 mg/ml) in 0.2 M NaOH, for 1 h, at room temperature. Propanol was used as the internal standard.

Viscosity measurements

Specific viscosity was obtained at 30°C by measuring the flow times of solutions of freeze-dried material (2.5 g/l), corresponding to the fractions solubilised from WIS, in 155 mM NaCl and 5 mM EDTA in an auto-

matic capillary (ϕ 0.46 mm) Ubbelhode viscosimeter (Viscologic TI1, SEMATech, Nice, France).

RESULTS AND DISCUSSION

Change of cell wall material content during storage

The water, WIS and WSF contents of apples can be seen in Table 1. The water content of the apple tissue decreased and lost a significant 2.5% during the 30 weeks storage period. This result agreed with that of Fischer and Amado (1994) and corresponded in all probability to evaporation of water during ripening. At the beginning of storage, the WIS and WSF contents were 2.06 and 0.43 g/100g of apple flesh, respectively. These amounts of cell wall material were in agreement with previous reported results on apple cell walls (Voragen *et al.*, 1983; Stevens & Selvendran, 1984; Renard *et al.*, 1990). Over 30 weeks, the WIS content decreased progressively by 14%, whereas the WSF content remained unchanged. As previously observed (Fischer & Amado, 1994), the reduction of cell wall material during storage could be explained by catabolic activity (Fischer & Bennet, 1991) of the endogenous apple enzymes such as pectin methylesterase, exopolygalacturonase, β-galactanase (Wallner, 1978; Bartley & Knee, 1982) or endo-polygalacturonase (Wu *et al.*, 1993). A WIS preparation was preferred to alcohol-insoluble solid (AIS) preparation because (1) the endogenous enzymes should act as they did in the crushed apple pulp and (2) the cell wall polysaccharides were more accessible to exogenous enzymes in the WIS than in the AIS material (Ella Missang *et al.*, 1993).

Table 1. Water content, water-insoluble solids (WIS) and water-soluble fraction (WSF) of apples during storage

Weeks	Water content (g/100g)	WIS (g/100g)	WSF (g/100g)
0	86.4(0.5)(a)	2.06	0.43
2	86.3(0.4)	2.01	0.38
4	86.4(0.5)	2.19	0.41
6	86.0(0.7)	1.97	0.39
8	85.9(0.5)	2.12	0.34
10	85.7(0.7)	1.89	0.38
12	85.8(0.8)	1.99	0.41
14	85.6(0.5)	1.91	0.35
16	85.4(0.4)	1.75	0.41
20	85.2(0.6)	1.77	0.39
25	84.9(0.6)	1.89	0.39
30	84.2(0.4)	1.78	0.42

(a) standard deviation.

Changes of composition in cell wall polysaccharides during storage

Tables 2 and 3 show the composition of WIS and WSF and the DM of the pectic substances of WIS and WSF. In the WIS, the weight of total polysaccharides decreased by 15% over 30 weeks. The molar ratio of galacturonic acid remained constant whereas the arabinose and galactose content decreased with time, by 44 and 28% over 30 weeks, respectively. In contrast, during the same period, the β-glucan content, cellulose or xyloglucans, rose by 49%, corresponding both to an increase in the glucose molar ratio and a loss of pectic neutral sugars. The total polysaccharide content in the WSF increased by 16% with storage time (Table 3). As with the WIS, the arabinose and galactose molar ratio decreased by 54 and 33%, respectively, over 30 weeks. In contrast, the galacturonic acid content rose by 24%. If we consider the sum totals of the WIS and the WSF, the polysaccharide content decreased by 10% during storage and about 20% of the total polysaccharides became water soluble after 30 weeks. The total amount of pectin, expressed as galacturonic acid, decreased by 10% during storage, whereas the soluble fraction, which was less rich in neutral sugars, increased by

Table 2. Sugar composition of the WIS of apples during storage

Weeks	Sugar composition (mol%)									Total (mg/g)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal.A	DM(a)	
0	1.6	1.5	19.0	11.0	2.9	10.4	17.0	36.7	78	769
2	1.7	1.3	18.4	12.7	3.2	9.2	17.5	36.0		774
4	1.5	1.1	18.4	11.9	3.2	9.5	17.4	36.9	73	747
6	1.6	1.3	18.4	13.1	3.0	9.2	18.3	35.1		731
8	1.7	1.5	17.0	12.9	2.7	9.2	19.4	35.5	76	723
10	1.9	1.5	16.4	12.9	3.1	9.4	19.1	35.8		697
12	1.9	1.5	15.8	14.5	3.0	9.0	20.4	33.8		717
14	1.8	1.5	12.1	13.2	3.2	8.7	21.5	38.0		700
16	1.9	1.6	10.7	13.5	3.7	8.3	22.8	37.5	69	702
20	2.2	1.8	11.2	13.5	3.4	8.1	22.8	37.1		693
25	2.0	1.8	12.4	14.0	3.4	7.7	23.4	35.4		699
30	2.0	1.7	10.7	13.0	3.1	7.5	25.3	36.7	72	655

(a) Degree of methoxylation based on the galacturonic acid content of the WIS.

Table 3. Sugar composition of the WSF of apples during storage

Weeks	Sugar composition (mol%)								Total (mg/g)
	Rha	Ara	Xyl	Man	Gal	Glc	Gal.A.	DM(a)	
0	1.8	17.1	1.0	1.0	15.3	1.6	62.2	67	647
2	1.7	14.1	1.3	1.0	13.5	3.5	64.8		640
4	1.9	13.1	1.5	0.5	12.4	2.0	68.6	70	611
6	1.9	13.1	1.2	0.7	13.3	2.2	67.5		634
8	2.2	13.9	1.2	0.5	14.6	2.3	65.3	75	656
10	2.0	14.6	1.1	1.1	14.0	2.0	65.3		677
12	2.0	13.4	0.8	0.6	12.3	1.8	69.1		708
14	1.9	9.2	0.8	0.5	8.9	1.6	77.1		749
16	2.0	9.4	0.7	0.0	10.8	1.5	75.7	81	786
20	1.6	8.6	0.8	0.9	11.5	2.0	74.6		803
25	2.1	9.2	0.7	0.8	11.6	1.4	74.2		691
30	1.8	7.8	0.7	0.7	10.2	1.9	76.9	86	750

(a) Degree of methoxylation based on the galacturonic acid content of the WSF.

about 40%. The DM based on the galacturonic acid content of the WIS was high (78–72) and agrees with that reported by de Vries *et al.* (1981) and Renard *et al.* (1990). No significant change of DM was observed during storage, in agreement with de Vries *et al.* (1984). On the other hand, the DM of pectins solubilised in WSF increased from 67% to 86% over 30 weeks. These results which confirmed previous observations (Bartley & Knee, 1982; Yoshioka *et al.*, 1992; Fischer & Amado, 1994) about the increase of pectin solubility during apple ripening were in accord with the hypothesis that during ripening, (1) highly methylated pectin could be synthesized (Knee, 1978a, b) and (2) the water-soluble pectin content increased while a degradation of arabinan and galactan side chains took place confering less protection to polygalacturonase action (Gross & Sams, 1984).

Changes in enzyme hydrolysis of cell wall polysaccharides during storage

When the apple tissues were treated with a polygalacturonase preparation for maceration (Fig. 1), the solubilisation yield of the cell wall material was 61% for the first week and rose to 80% after 30 weeks of storage. The increase was greatest between week 4 (end of December) and 8 (end of January). The liquefaction yield was higher and also increased from 85 to 93%. These results which agree with previous research (Massiot *et al.*, 1994) dealing with apple parenchyma liquefaction (85–95%) confirmed that a fraction (5–7%) of the cell wall material remained insoluble.

The solubilisation of polysaccharides from the WIS with a mixture of pectin methylesterases, polygalacturonases and cellulases is shown in Table 4. At

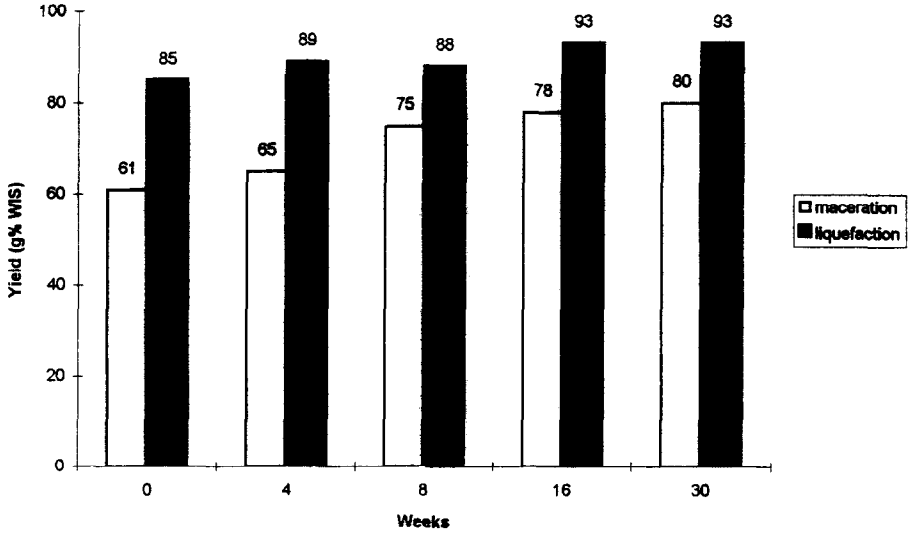

Fig. 1. Solubilisation of cell wall material (as % of WIS) when apple tissues were treated with liquefying or macerating enzyme preparation (see Experimental).

the beginning of storage, the buffer and the enzymes solubilised 13.4 and 75.0% of the cell wall material, respectively. The buffer-soluble fraction increased with storage time to 25.1% after 30 weeks whereas the enzyme-soluble fraction declined to 66.1%. As a whole, during storage, it was the increase of water-soluble polysaccharides as reported by Yoshioka *et al.* (1992), and not the more effective action of exogenous enzymes which explained why the enzyme treatment involved a higher solubilisation of the cell wall material. The correlation between the yield of tissue liquefaction (Fig. 1) and the solubilisation of WIS was good

Table 4. Yield of solubilisation of WIS after 6 h of reaction with pectin methylesterases, polygalacturonases and cellulases

Weeks	Fractions (g/100g)		
	Insoluble	Enzyme soluble	Buffer soluble
0	11.6	75.0	13.4
4	12.0	74.4	13.6
8	10.6	74.5	14.9
12	10.1	72.9	17.0
16	9.9	70.9	19.2
20	9.1	68.7	22.2
30	8.8	66.1	25.1

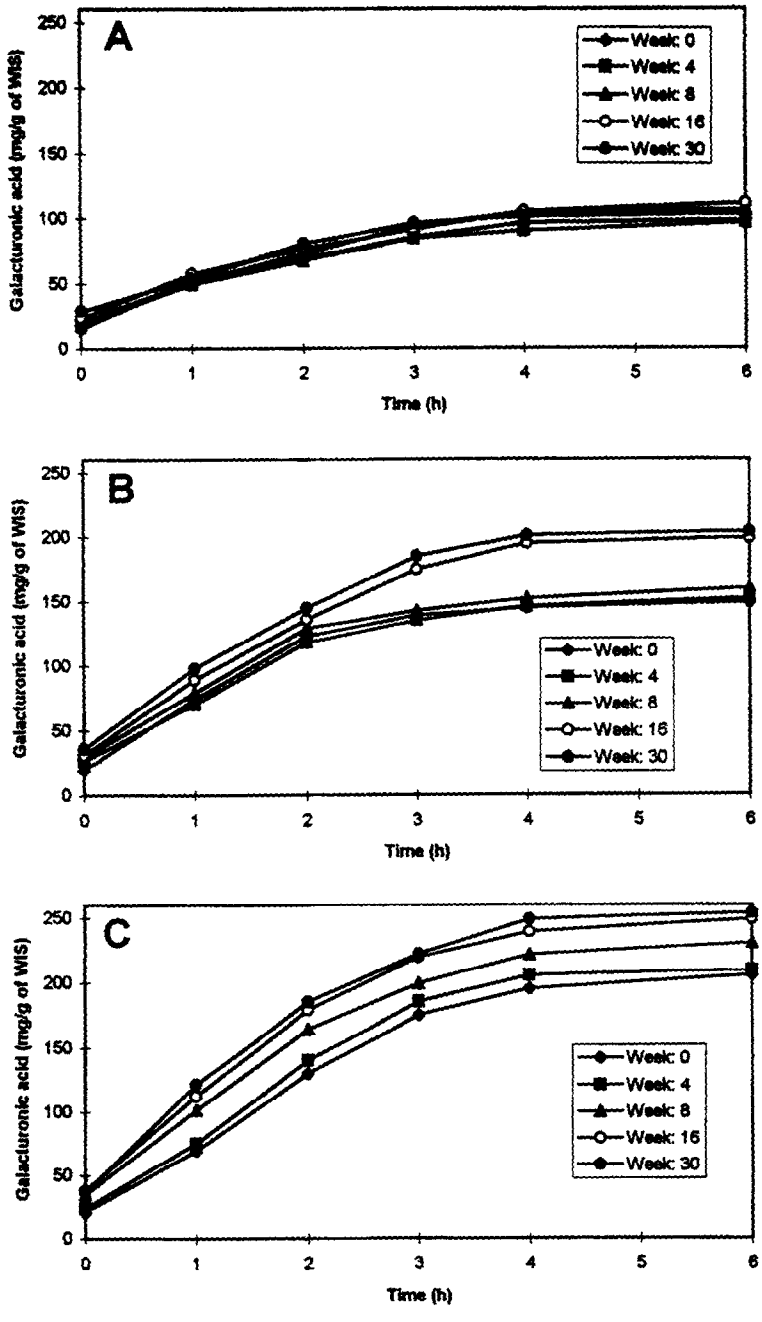

Fig. 2. Content of galacturonic acid in the soluble fraction after hydrolysis of the WIS with polygalacturonases [A], polygalacturonases and pectin methylesterase [B], pectinases and cellulases [C].

(Table 4). Table 5 shows the specific viscosity of the fractions solubilised with the buffer or the enzymes and standardised at the same concentration. Specific viscosity of the buffer-soluble fraction rose with time indicating that the hydrodynamic volume of the soluble cell wall polysaccharides from WIS increased during ripening, in agreement with Fischer *et al.* (1994) who observed that pectins shifted towards higher molecular weights. After enzyme hydrolysis of the WIS, the specific viscosity reached to 0.024 (week 0) and increased after 8 weeks of storage. These results indicated that the longer the apple was stored, the more hydrolysis residues from the polysaccharides contributed to the increase in viscosity of the soluble phase.

The solubilisation of pectins, expressed as galacturonic acid, from the WIS with a mixture of enzymes is presented in Fig. 2. With the polygalacturonase activity alone (A), the kinetics were close to each other, with limits of around 40% of galacturonic acid content in the WIS. The combined action of polygalacturonase and pectin methylesterase activities (B) increased this value to 60% for the WIS (weeks 0, 4 and 8) and to 75% for the WIS (weeks 16 and 30). On the other hand, the DM of the WIS were similar (Table 2). This indicates that pectin methylesterases were more active on methylated galacturonans from WIS (weeks 16 and 30) and so enhanced the action of the polygalacturonases. We suggest that the repartition of esterified galacturonic acid units was different, probably due to the action of endogenous pectin methylesterase (John & Dey, 1986, Fischer & Bennet, 1991). A mixture of pectinases and cellulases (C) increased the limit of solubilisation; the kinetic pattern was the same (as B) with the WIS (week 8) half way between the two. These results (1) suggested that endogenous pectinases could facilitate the action of cellulases on and after week 8, and (2) confirmed that the hydrolysis of cellulose increased the solubilisation of pectins with exogenous pectinases (Voragen *et al.*, 1980; Massiot *et al.*, 1992).

The degree of maceration of cell wall material in the tissues (Fig. 1) increased with the length of storage time whereas the solubilisation of pectins from the WIS with the polygalacturonases (Fig. 2A) was similar. We suggest that accessibility of galacturonans in the tissue depends on many factors like turgor pressure, cell size and cell wall hydration which influence enzyme diffusion in the tissue. Under our conditions, the access of polygalacturonases to pectins is influenced by the period of apple storage but not their solubilisation. In contrast, there was a good correlation between the degree of liquefaction of cell wall material in the tissue (Fig. 1) and the solubilisation limits of pectins from the WIS (Fig. 2C), indicating that the cellulose hydrolysis and the breaking of cells improved access to the pectins. Hence, under liquefying conditions, as apple storage time lengthened, the pectic polysaccharides became more accessible to the enzymes.

Table 5. Specific viscosity of soluble fractions (a) after treatment of the WIS with pectin methylesterases, polygalaturonases and cellulases

	Storage time (in weeks)				
	0	4	8	16	30
Controls (b)	$46\ 10^{-3}$	$48\ 10^{-3}$	$51\ 10^{-3}$	$64\ 10^{-3}$	$70\ 10^{-3}$
Assays (c)	$24\ 10^{-3}$	$24\ 10^{-3}$	$27\ 10^{-3}$	$34\ 10^{-3}$	$38\ 10^{-3}$

(a) with a fixed concentration of 2.5 g of freeze-dried material per l.
(b) Fraction solubilised with buffer only.
(c) Fraction solubilised with enzymes.

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