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Short communication

Preparation of starch–sodium lignosulfonate graft copolymers via laccase catalysis and characterization of antioxidant activity☆

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ABSTRACT

Graft copolymers of waxy maize starch and sodium lignosulfonate (SLS) were prepared by *Trametes versicolor* laccase catalysis in aqueous solution. Amount of SLS grafted based on phenol analysis was 0.5% and 1.0% in the absence and presence of 1-hydroxybenzotriazole (HBT), respectively. Starch–SLS graft copolymers were effective antioxidants as judged by 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The presence of laccase caused a reduction in starch molecular weight although a cross-linked gel fraction was also observed when HBT was present. This new method for preparing starch chemically modified with phenolic compounds is simple and the resulting antioxidant polymers have potential in food, cosmetic and packaging applications.

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1. Introduction

There has been increasing interest recently in the chemical modification of starch using enzymatic catalysis since enzymes are non-toxic, renewable and are active near room temperature (require little energy) (Akkara, Ayyagari, & Bruno, 1999; Cheng & Gross, 2002; Gotlieb & Capelle, 2005). Many of the reagents currently used to make derivatized starches such as ethylene oxide, propylene oxide, cationic glycidyl ethers, acyl chlorides, and sodium hypochlorite are rather toxic so safer alternatives would be desirable. Various fatty acid esters of starch have been prepared by lipase and protease catalyzed reactions of starch and fatty acids or fatty esters (Charkraborty, Sahoo, Teroka, Miller, & Gross, 2005; Qiao, Gu, & Cheng, 2006; Rajan & Abraham, 2006) but these require organic solvents to limit water activity. Oxidized starches containing carboxylic and carbonyl groups have been prepared by laccase catalyzed oxidation of TEMPO and subsequent oxidation of starch (Mathew & Adlercreutz, 2009). Starch-graft-polyacrylamides have been prepared by horseradish peroxidase/H_2O_2 catalyzed oxidation of a mediator (pentanedione, PDO) to free radicals and subsequent chain transfer to starch (Shogren, Willett, & Biswas, 2009).

Substitution of starch with aromatic or phenolic compounds is rather difficult by conventional chemical means since functional groups directly reactive with starch hydroxyls are not present. Yet, modification of polysaccharides with natural phenolic compounds could confer some useful properties such as hydrophobicity, surface activity and antioxidant activity. Modification of cellulose fibers with a variety of phenolic compounds via laccase catalysis has been reported (Aracri et al., 2010; Elegir, Kindl, Sadocco, & Orlandi, 2008; Garcia-Ubasart et al., 2011; Liu, Shi, Gao, & Qin, 2009). Laccases oxidize phenols to radicals using oxygen as electron acceptor (Arora & Sharma, 2010; Dwivedi, Singh, Pandey, & Kumar, 2011; Kunamneni et al., 2008). Presumably, trace amounts of lignin in the cellulose are also oxidized to form phenoxy radicals and these can combine with phenolic radicals to form covalent bonds. Conjugates of water soluble polysaccharides and antioxidants have been prepared using the ascorbic acid/H_2O_2 redox system (Spizzirri et al., 2010) which can oxidize both polysaccharide and phenolics to radicals. Preparation of graft copolymers of starch with 4-phenolsuflonate using HRP/H_2O_2/PDO catalysis has been reported recently (Lv, Ma, Gong, Yan, & Hou, 2010).

Except for a study of surface cross-linking of starch and lignin films using electron beam irradiation (Lepifre et al., 2004), there have been no studies of formation of starch–lignin graft copolymers. Lignin has substantial phenolic content, is natural, abundant and inexpensive so it is a good prototype phenolic compound to investigate potential reactivity with starch. Lignosulfonates were chosen for the present study since they are water soluble and hence

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☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and use of the name by USDA implies no approval of the product to the exclusion of other that may also be suitable.

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should be miscible with starch. In addition, they contain sulfate so this represents a new way to prepare anionic starch sulfates.

The purpose of this study was to determine the feasibility of preparing starch–lignosulfonate graft copolymers using oxidoreductases as catalysts. Reactions were conducted with and without a common mediator (HBT). Lignin content in graft copolymers was assessed by UV spectroscopy and phenol analysis using Folin–Ciocalteu reaction. Antioxidant activity was measured by reaction with diphenyl-1-picrylhydrazyl.

2. Experimental

2.1. Materials

Waxy 7350 corn starch was purchased from A.E. Staley (Decatur, IL) and contained 10.4% moisture. Sodium lignosulfonate (SLS, 6.4% water), 1-hydroxybenzotriazole hydrate (HBT, 20% water), *T. versicolor* laccase (23 U/mg), Folin–Ciocalteu phenol reagent, 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and Sephacryl S500HR were from Sigma–Aldrich.

2.2. Preparation of waxy maize starch/sodium lignosulfonate graft copolymers

Starch (5.0 g dry basis) and 90 g nanopure water were heated in a sealed teflon cell equipped with magnetic stirrer and thermocouple using a Milestone Ethos 1600 Labstation microwave reactor (Milestone Inc., Shelton, CT). Heating profile was a linear ramp of 25–140 °C over 5 min. After cooling, 10 ml of 0.5 M sodium acetate buffer, pH 5.0, were added to the viscous starch solution with stirring. Into 125 ml flasks were added 25 ml starch solution (1.25 g starch), 0.8 g 31% sodium lignosulfonate in water (0.25 g SLS), 60 mg HBT (some experiments, see Table 1) and 10 mg laccase predissolved in 0.2 ml water. Flasks were sealed with rubber septa and two strands of teflon tubing were pushed through septa. Reactions were conducted in a heated shaker at 30 °C and 150 rpm for 4 h with continuous oxygen purging of the flasks. Oxygen flow was approximately 50 ml/min. After reaction, 30 ml ethanol was added to each flask to precipitate starch graft copolymer. To remove unbound SLS, crude ethanol swollen starch graft copolymer was dissolved in 20 ml water with stirring and reprecipitated with 25 ml ethanol. The precipitate was collected by centrifugation at 9000 rpm for 30 min. Dissolution in water and precipitation in ethanol were repeated 3–4× until supernatant was free of SLS. The starch graft SLS was then air dried, ground in a mortar and finally dried at 60 °C in vacuo. Above reactions as well as a control without laccase were conducted in triplicate.

2.3. Experiment to confirm that SLS is indeed grafted onto starch

As a control, 0.31 g of sample #3 and 0.0625 g of #5 were dissolved in 5 ml of water. To precipitate the sample, 6.25 ml of ethanol were added. (These ratios are consistent with what was done in Randy's paper.) The precipitate was collected by centrifugation at 9000 rpm for 30 min. Re-dissolving in water and precipitating in ethanol were repeated 3–4 times until supernatant was free of SLS.

The sample was air dried, ground in a mortar and dried at 60 °C in a vacuum oven. Presence of SLS was examined by UV spectroscopy, dissolving the sample in water (0.5% solution), using the absorption maximum at 280 nm. No SLS was seen.

2.4. Assay for SLS

Total phenol content of starch–SLS graft copolymers was determined using the Folin–Ciocalteu method of Singleton, Orthofer, and Lamuela-Raventos (1999) but at 1/100 scale. A calibration curve was constructed using SLS solutions of different concentrations (0.001–0.01%). SLS was also estimated by UV spectroscopy, specifically using the absorption maximum at 280 nm.

2.5. Evaluation of antioxidant activity

Radical scavenging by starch–SLS graft copolymers was carried out by a method similar to that of Spizzirri et al. (2009). To 0.6 ml of 0.5% copolymer solution in water was added 0.6 ml 0.2 mM DPPH in ethanol with shaking. After incubating at 25 °C for 1 h, samples were centrifuged at 12,000 rpm for 20 min and the optical absorption of the supernatant was measured at 517 nm. The scavenging activity of the starch–SLS graft copolymers was measured as the decrease in the absorbance of DPPH radical and expressed as % inhibition = $(A_0 - A_1)/A_0 \times 100$ where A_0 is absorbance without added copolymer and A_1 is absorbance with copolymer.

2.6. SEC-MALLS

Conventional SEC was conducted with a 95 cm × 1.4 cm column packed with Sephacryl S500HR and eluted with 0.01 M NaOH. Approximately 2.5 ml sample (2–3% for samples 1 and 3; 0.1% for sample 5) was applied to the column. Flow rate was 20 ml/h and fractions (2.5 ml) were collected with an Isco Foxy Junior fraction collector (Teledyne Isco Inc., Lincoln, NE). Fractions were assayed for starch and phenol using phenol-sulfuric (Chaplin, 1986) and Folin–Ciocalteau (Singleton et al., 1999) methods, respectively.

SEC-MALLS was carried out using a Waters 1525 HPLC system, a Waters 2414 refractive index detector and a Wyatt Technologies Dawn EOS light scattering detector. GPC analyses were performed using a TSK-Gel 6000PW column (300 mm × 7.5 mm), an injection volume of 20 μl of 0.25% solutions and a temperature of 40 °C. The HPLC was set to an isocratic flow rate of 0.5 ml/min of 0.01 M NaOH. A value of 0.146 determined for starch (Yoo & Jane, 2002) was assigned for the refractive index increment (dn/dc) for the copolymers and a value of 0.195 was used for SLS (Fredheim, Braaten, & Christensen, 2002). Since the copolymers contain only about 1% SLS, dn/dc for the copolymers would not be expected to be significantly different from starch. Weight and number average molecular weights were calculated using Wyatt Astra software.

2.7. Water solubility and absorption

1% aqueous solutions of starch–SLS graft copolymers were centrifuged at 10,000 rpm for 5 min. The supernatant was carefully removed, dried in an oven and weighed. The gel fraction, if any, was weighed wet and the water absorption index (WAI) calculated as WAI = gel weight/total dry weight. The water solubility was calculated as $\text{WSI} = W_{\text{sup}}/W_{\text{tot}}(V_t/V_t - V_{\text{gel}})$, where W_{sup} is dry weight of copolymer in supernatant, W_{tot} is total dry weight of copolymer, V_t is total volume, and V_{gel} is volume of the gel phase. The latter factor corrects for the soluble polymer residing within the gel fraction.

3. Results and discussion

The experimental design is shown in Table 1. Reaction 1 contains starch, SLS and laccase while reaction 2 adds the mediator HBT. Reaction 3 is a control without laccase. Reaction 4 is a control to determine if any laccase remains in starch after ethanol/water precipitation. Reaction 5 is a control containing SLS and laccase meant to determine if higher molecular weight lignosulfonate species might be formed which could co-elute with starch.

UV spectra of the 3 samples and SLS are shown in Fig. 1. A maximum at 280 nm was evident for samples 1 and 2 which corresponded to the presence of aromatic groups in SLS. There was no peak at 280 nm for the reaction 3 indicating that all unbound SLS

Table 1
Summary of reaction conditions.

Sample #	Starch (g)	SLS (g)	HBT (mg)	Laccase (mg)	Time (h)	Color after reaction
1	1.25	0.25	0	10	4	Tan
2	1.25	0.25	60	10	4	Light brown
3	1.25	0.25	0	0	4	White
4	1.25	0	0	10	4	White
5[a]	0	0.25	0	10	4	Brown

SLS = sodium lignosulfonate and HBT = 1-hydroxybenzotriazole hydrate.

[a] No ethanol/water precipitation after reaction.

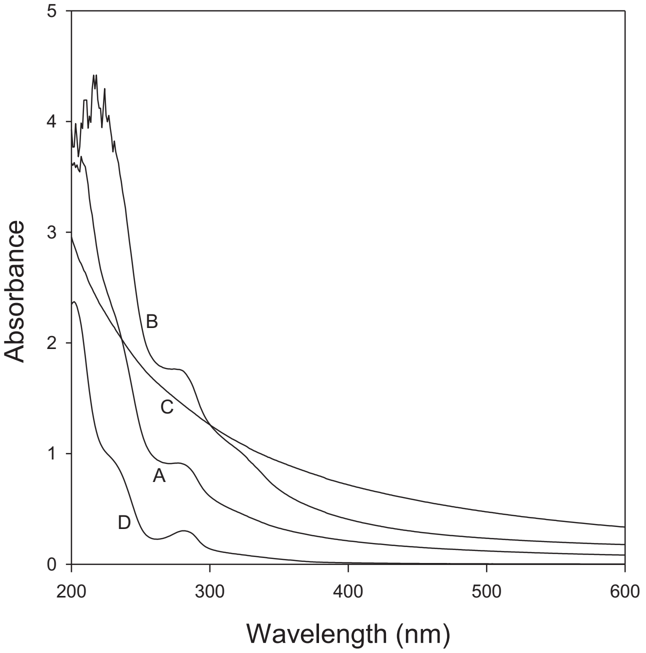

Fig. 1. UV/vis spectra of samples in water: 0.5% #1 (A), 0.5% #2 (B), 0.5% #3 (C), and 0.0025% SLS (D).

was washed out of the sample by the ethanol/water precipitations. SLS is known to be soluble in a wide range of ethanol/water mixtures (Luh & Frederick, 1992) while starch is insoluble at ethanol concentrations greater than about 25%. Therefore, these data suggest that laccase catalyzed covalent coupling of the SLS to starch occurred in reactions 1 and 2.

Quantitative estimates of the amount of SLS bound to starch as measured by the Folin–Ciocalteu phenol assay are given in Table 2. Weight percentages of SLS in starch–SLS graft copolymer samples 1 and 2 were 0.55% and 0.97%, respectively. SLS contains about 1.4 mmol phenol per g SLS (Areskogh, Li, Gellerstedt, & Henriksson, 2010). There was no phenol detected in sample 3 again suggesting that all SLS was washed out of the sample. There was also no phenol detected in sample 4, indicating that residual laccase was probably not a contributor toward phenol content in samples 1 and 2.

Table 2
Antioxidant activity of starch–SLS graft copolymers.

Sample #	Total phenolic content (%)[a]	Inhibition of DPPH radicals (%)[b]
1	0.55 ± 0.03	15 ± 3
2	0.97 ± 0.04	31 ± 4
3	0.003 ± 0.003	6 ± 1
4	0	–

[a] g SLS/100 g starch–SLS graft copolymer.

[b] $(A_0 - A_1)/A_0 \times 100$ for 0.5% copolymer solutions.

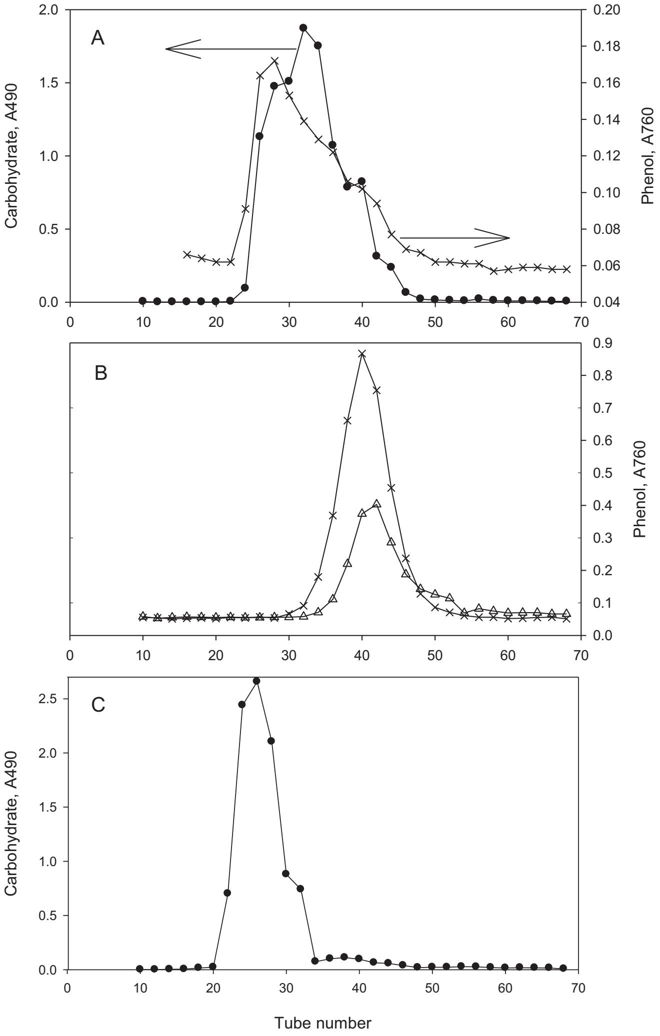

Fig. 2. Sephacryl S500HR size exclusion chromatography (SEC) of sample #1 (A), SLS (x) and sample #5 (Δ) (B) and sample #3 (C).

Sephacryl S500HR size exclusion chromatographs for samples 1, 3, and 5 and SLS are shown in Fig. 2. SEC of sample 1 (Fig. 2A) shows that starch and SLS coelute at low elution volumes (high molecular weights). In contrast, SLS elutes at higher volumes (lower molecular weights) as shown in Fig. 2B. This data suggests that grafting of SLS to starch has taken place since molecules much larger than SLS were produced which contain both starch and phenolic moieties. Lignins can, under some conditions, be polymerized to higher molecular weights by laccase treatment so a control reaction (#5 in Table 1) was conducted to test this possibility. There was, however, little change in the elution volume of SLS after laccase treatment (Fig. 2B), indicating that homopolymerization of SLS by laccase was not responsible for the large decrease in elution volume of phenolic groups in Fig. 1A. The phenol content of SLS after laccase

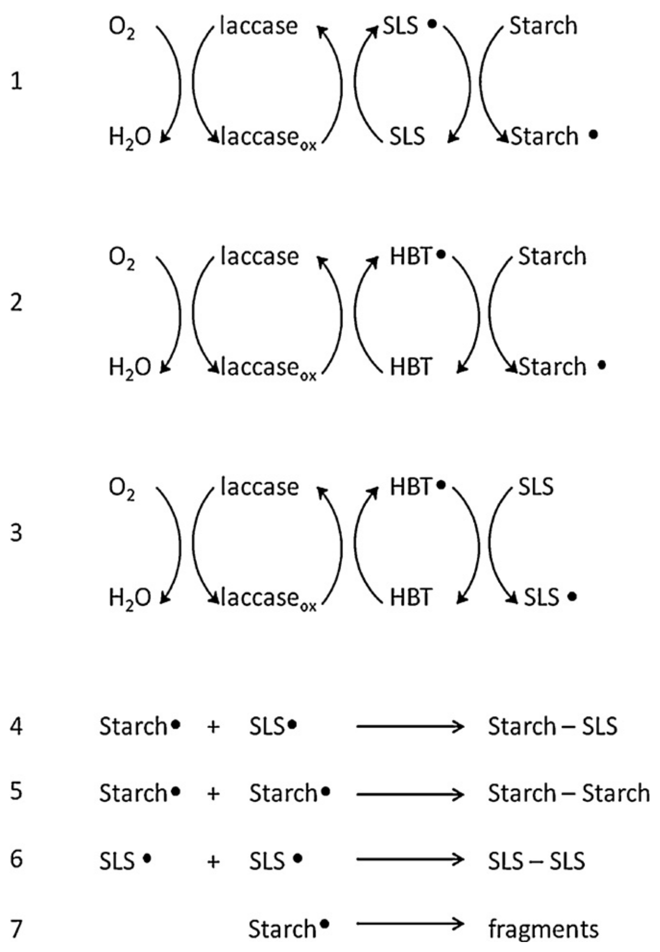

Fig. 3. Schematic diagram of proposed reactions.

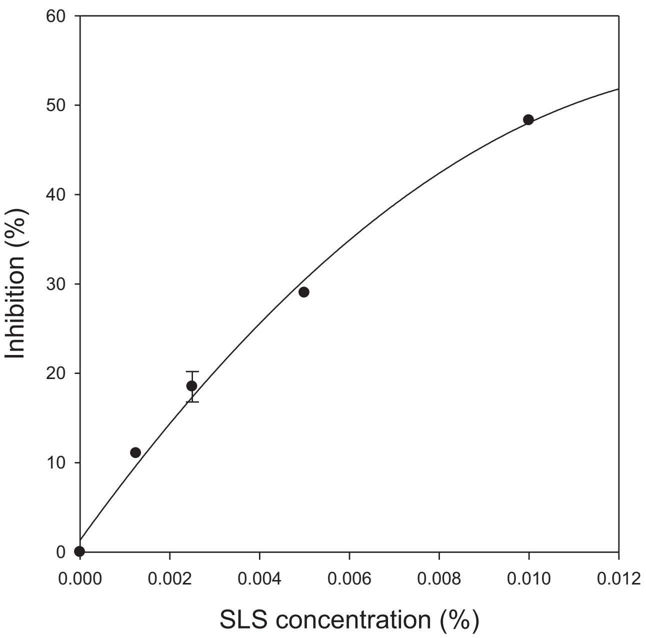

Fig. 4. Percent inhibition of DPPH radicals by sodium lignosulfonate (SLS).

treatment (sample #5 in Fig. 2) was about half that of unmodified SLS, indicating about 1/2 the phenol groups of SLS were oxidized by laccase during the 4 h treatment period. SEC of sample #3 in Fig. 3C shows that starch control (no laccase) elutes at somewhat lower volumes than sample 1 in Fig. 1A, suggesting that laccase causes some reduction in molecular weight of starch.

A proposed series of reactions is given in Fig. 3. In reaction 1, laccase is oxidized by oxygen which then catalyzes the oxidation of phenol groups in SLS to phenoxy radicals. These can then abstract hydrogen from starch forming starch radicals. SLS thus serves as a mediator since laccases cannot oxidize starch directly. In reactions 2 and 3, HBT is oxidized by laccase and serves as a mediator to oxidize starch and SLS to free radicals. Once formed the starch and SLS macroradicals can undergo a number of reactions including combination to form graft copolymers (reaction 4), starch cross-linking (reaction 5), SLS cross-linking (reaction 6) and starch depolymerization (reaction 7). The overall level of grafting is low ($\leq$1%) since enzyme and radical concentrations are low and thus it is relatively improbable for two macroradicals to meet and combine. Since HBT is readily oxidized by laccases, more radicals are formed than when it is absent and grafting level is higher. Previous work has demonstrated that laccases catalyze the polymerization of SLS both in the presence (Prasetyo et al., 2010) and absence of HBT (Areskogh et al., 2010). Depolymerization of starch via free radical catalysis has also been proposed (Bertolini, Mestres, Colonna, & Raffi, 2001).

Water solubilities and weight average molecular weights for starch–SLS graft copolymers and SLS are shown in Table 3. Water solubilities for samples 1 and 3 were nearly 100%. Water solubility for sample 2, which utilized HBT in the reaction, was 84%. Sample 2 also contained a gel fraction which was highly swollen in water, absorbing 53 g H_2O/g sample or 330 g H_2O/g dry gel. Gel formation was probably due to cross-linking by combination of starch–SLS radicals. Such water absorbencies are higher than normally observed for cross-linked neat starch gels (15–30) (Sasaki, Yasui, Matsuki, & Satake, 2002) and probably reflect the repulsion of the negatively charged sulfate groups which are attached to the starch–SLS copolymers.

M_w for the starch–SLS graft copolymer samples 1 and 2 were $10–12 \times 10^6$ and these were lower than the control starch in absence of laccase (sample 3, 20×10^6). Thus the presence of free radicals likely promotes depolymerization reactions of starch. These molecular weights are much lower than that of native waxy maize starch ($\sim 8 \times 10^8$) (Yoo & Jane, 2002). Heating at elevated temperatures and pressures in the microwave reactor as well as shear from stirring (Millard, Dintzis, Willett, & Klavons, 1997) and flow through the HPLC (Cave, Seabrook, Gidley, & Gilbert, 2009) are known to reduce starch molecular weight, especially highly branched amylopectin. It should be noted that recovery from the column was low for sample 3 indicating portions of the starch were

Table 3
Physical properties of starch–SLS graft copolymers.

Sample #	Water solubility (%)	Water absorption index (WAI)	M_w [a]	Recovery (%)[b]
1	98 ± 3	0	$9.8 \pm 0.5 \times 10^6$	88
2	84 ± 3	53 ± 8	$12 \pm 0.6 \times 10^6$	86
3	96 ± 3	0.7 ± 0.2	$20 \pm 1 \times 10^6$	15
SLS	100	0	$54 \pm 5 \times 10^3$	100

[a] Weight average molecular weight.

[b] Mass recovery from column eluate calculated from integration of refractive index peak.

binding to the column. This could affect the measured value of M_w if species of different molecular weight were preferentially absorbed. M_w for SLS was 54,000 and this was very similar to a literature value of 64,000 (Fredheim et al., 2002).

Radical scavenging activities of the starch–SLS graft copolymers are shown in Table 2. In the DPPH assay, antioxidants reduce the purple colored stable DPPH radical to a pale yellow colored compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen donating ability of the antioxidant (Spizzirri et al., 2010). For a 0.5% solution of sample 1, 15% of DPPH radicals were reduced compared to 31% for sample 2. The control samples consisting of starch only (#3) had minimal inhibition of DPPH (6%). Thus, it appears that antioxidant activity is proportional to phenol content. A plot of percent inhibition versus concentration for SLS alone is shown in Fig. 4. Using this plot and percent inhibitions for starch–SLS copolymer samples 1 and 2, one can back calculate % SLS of approximately 0.42% and 1.0% respectively. These are similar to SLS contents determined by Folin–Ciocalteu assay. Previous studies have also found that lignins have substantial antioxidant activity (Faustino, Gil, Baptista, & Duarte, 2010; Salanti, Zoia, Orlandi, Zanini, & Elegir, 2010; Ugartondo, Mitjans, & Vinardell, 2008).

4. Conclusions

In summary, graft copolymers of starch with polyphenolic compounds (lignosulfonates) were prepared for the first time and these had antioxidant activity consistent with their phenolic content. The laccase grafting method is also likely to be applicable to other phenolic compounds. Some possible applications for starch-antioxidant graft copolymers might include food additives and preservatives as well as cosmetics. In the latter application, the strong UV absorption of lignin could help serve as a sunscreen. Some polyphenolics have antimicrobial properties (Cruz, Dominguez, Dominguez, & Parajo, 2001; Elegir et al., 2008; Pyla, Kim, Silva, & Jung, 2010) so such starch grafts could be useful as wound dressings, coatings for fruits and vegetables or other packaging. Since SLS contains significant levels of sulfate (1.6 mmol/g; Areskogh et al., 2010), starch–SLS graft copolymers should be anionic and thus could be useful for cation binding and water absorption when cross-linked.

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