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Characterization of a novel modified alternan

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Abstract

A novel modified alternan, produced by using newly isolated strains of *Penicillium* sp., was physically characterized. High molecular weight native alternan was progressively modified to lower molecular weight heterodisperse forms, associated with a reduction in absorbance at 225 nm, light scattering, and opalescence. Methylation analysis indicated that modified alternan has a linkage pattern similar to that of native alternan. The solution viscosity properties of modified alternan resemble those of ultrasonicated alternan and commercial gum arabic. However, alternan lacks the emulsification capacity of gum arabic. Alternan solutions are stable for at least 7 days under all conditions tested, from 4 to 70 °C and from pH 3–9. Dry preparations of alternan are bright white powders that are not highly hygroscopic. Thus, modified alternan is promising for further development as a gum arabic substitute, particularly in food applications requiring a low-viscosity bulking agent.

Keywords: Alternan; Emulsification; Gum arabic; Modified alternan; Viscosity

1. Introduction

Alternan is a branched glucan with a unique backbone structure of alternating α-(1 → 6) and α-(1 → 3)-D-glucosidic linkages (Cote & Robyt, 1982; Jeanes et al., 1954; Misaki, Torii, Sawai, & Goldstein, 1980; Seymour & Knapp, 1980; Seymour, Knapp, Bishop, & Jeanes, 1979). Alternan is branched through 3,6-di-substituted D-glucosyl residues, with approximately 7–11% branching (Seymour, Slodki, Plattner, & Jeanes, 1977). This structure imparts properties of high solubility and low-viscosity as well as resistance to most known microbial and mammalian enzymes (Cote, 1992; Cote, 2002; Cote & Ahlgren, 1995; Cote et al., 1997). Although naturally occurring strains of *Leuconostoc mesenteroides* that produce alternan also produce dextran as a troublesome contaminant, genetically improved strains for production of alternan without dextran have been isolated in recent years (Kim & Robyt, 1994; Leathers, Ahlgren, & Cote, 1997a; Leathers, Hayman, & Cote, 1995; Leathers, Hayman, & Cote, 1997b; Leathers, Hayman, & Cote, 1998; Smith, Zahnley, & Goodman, 1994). The gene that specifies alternansucrase, the enzyme that converts sucrose to alternan and fructose, also has been cloned and sequenced (Arguello-Morales et al., 2000; Kossman, Welsh, Quanz, & Knuth, 2000).

Native alternan has been estimated to have an apparent weight average molecular weight ($\bar{M}_w$) of 10^6–10^7 (Cote, 1992; Leathers, Nunnally, & Cote, 2002a). Native alternan has been modified by ultrasonication, reducing the apparent molecular weight average to $<10^6$ and altering the rheological properties of the polymer so that they more closely resemble gum arabic (Cote, 1992). Gum arabic is used in the preparation of confectionaries, beverages, encapsulated flavors, and pharmaceuticals (Whistler, 1993; Williams & Phillips, 2000). Approximately 40,000–50,000 metric tons of gum arabic are produced annually from *Acacia* trees in Nigeria, Chad, and the Sudan (Williams & Phillips, 2000). The price, quality, and availability of gum arabic vary considerably, and it would be desirable to have a domestic substitute of consistent quality. Although ultrasonicated alternan is promising for this application, ultrasonication is a relatively expensive process that would be difficult to carry out on an industrial scale. Recently, novel strains of *Penicillium* sp. were

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isolated that modify alternan to lower apparent molecular weight forms (Leathers et al. 2002a; Leathers, Nunnally, & Cote, 2002b). Physical characterizations of this novel modified alternan were carried out to evaluate its potential for commercial applications, particularly as a substitute for gum arabic.

2. Materials and methods

2.1. Carbohydrate polymers and standards

Native alternan was produced as previously described from *L. mesenteroides* strain NRRL B-21297 (Leathers et al., 1997a; Leathers et al., 1997b). Ultrasonicated alternan was produced as previously described (Cote, 1992). Briefly, an 8.0% (w/v) aqueous solution of native alternan was sonicated at 5 °C for up to 6 h using a 20 kHz, 600 W ultrasonic disruptor (Tekmar model TM600) at 80% power with an amplitude booster and a 0.75 in. probe. Modified alternan was produced using novel strains of *Penicillium* sp. as previously described (Leathers et al., 2002a; Leathers et al., 2002b). Basal liquid medium containing 1.0% (w/v) native alternan was inoculated to 10^5 spores/ml and incubated at 28 °C and 200 rpm for up to 17 days. Gum arabic (Acacia gum) was from Sigma Chemical Company, St Louis, MO. Soy protein concentrate (Profine F) was from Central Soya Company, Inc., Fort Wayne, IN.

2.2. Analysis of modified alternan

Modified alternan samples were dissolved in 0.05 M sodium nitrate with 0.02% sodium azide. Size exclusion chromatography was performed using an 8×300 mm Shodex KB-806M column with an exclusion limit of 2×10^7 for pullulan (Showa Denko, Tokyo, Japan) equilibrated with 0.05 M sodium nitrate with 0.02% sodium azide at a flow rate of 0.5 ml/min. The column eluate was analyzed on-line by multi-angle light scattering at 690 nm (DAWN EOS; Wyatt Technology Corporation, Santa Barbara, CA) and refractive index (RI) detection at 690 nm (Optilab DSP; Wyatt Technology Corporation). A value of 0.147 ml/g was used for the dn/dc of modified alternan. Molar mass and size were calculated from the light scattering and RI signals using Astra for Windows (version 4.73; Wyatt Technology Corporation).

Methylation analysis of modified alternan was performed according to the method of Slodki, England, Plattner, and Dick (1986). Total carbohydrate was estimated using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using maltose as a standard. Solution viscosity studies were performed as previously described (Cote, 1992), using a Brookfield LVTDV-1 digital viscometer. Emulsification assays were performed by the method of Yasumatsu et al. (1972), using soy protein concentrate as a positive control. Alternan stability studies were conducted on 1.0% (w/v) solutions in defined growth medium ('WW' of Koenig and Day (1989)). The OD_{225} of appropriate dilutions of these solutions was monitored over a period of 7 days as a measure of alternan stability.

3. Results and discussion

3.1. Size and composition of modified alternan

In our previous study, novel microorganisms were isolated for their ability to degrade or modify alternan as judged by a change in the absorbance of culture supernatants at 225 nm (Leathers et al., 2002a). Alternan solutions exhibit an absorbance maximum near this wavelength, and absorbance is proportional to the concentration of alternan (Fig. 1). Kobayashi, Utsugi, & Matsuda (1986) reported similar properties for solutions of dextran. As shown in Fig. 1, medium containing 10 mg/ml native alternan is modified to produce culture supernatants with an OD_{225} of 1.0–1.5 which is equivalent to the OD_{225} of native alternan solutions of 2.4–3.2 mg/ml. Modified alternan solutions also show much less of the opalescence characteristic of native alternan solutions. However, subsequent studies revealed that the observed change in absorbance is entirely the result of a reduction in alternan molecular weight rather than concentration.

The molar mass and size of native and modified alternan were analyzed by multi-angle light scattering and RI during separation by high performance liquid size exclusion chromatography (HPLC SEC-MALS). As shown in Fig. 2A, native alternan exhibits a high degree of light scattering which is progressively reduced by modification, characteristic of polymer degradation. Concurrent detection by RI shows native alternan as a high molecular weight polymer that is progressively

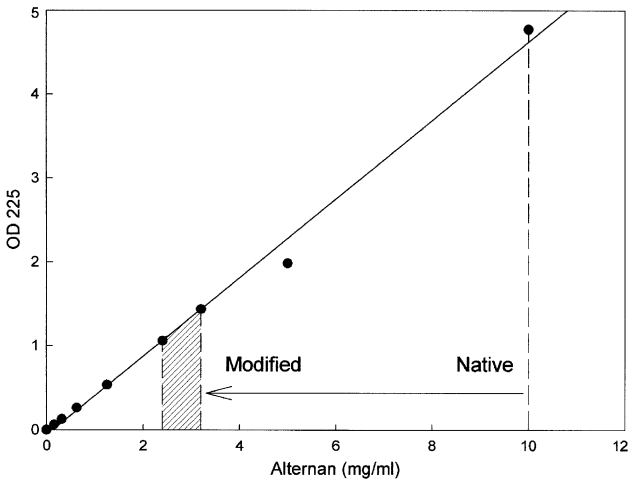

Fig. 1. Effect of alternan modification (17 h) on absorbance at 225 nm of solutions, superimposed on standard curve of native alternan concentration vs. absorbance.

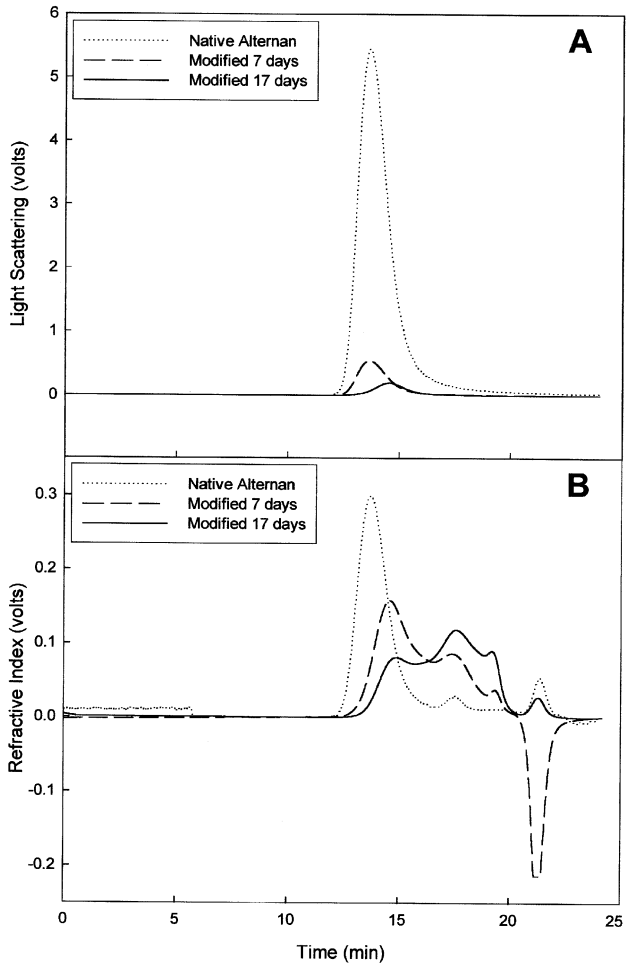

Fig. 2. Concurrent light scattering (Panel A) and refractive index (Panel B) detection of native alternan and modified alternan during separation by HPLC size exclusion chromatography (HPLC SEC-MALS). Lines: dotted, native alternan; dashed, alternan modified for 7 days; solid, alternan modified for 17 days.

modified to lower molecular weight, heterodisperse forms (Fig. 2B). As shown, areas under the RI curves for native and modified alternan are approximately equal. Total carbohydrate analysis by the phenol–sulfuric acid method indicated that alternan suffers no loss of glucose equivalents on modification, confirming that alternan is modified without substantial loss of glucose to fungal metabolism.

Modified alternan sizes were calculated by analysis of the light scattering and RI data. The molar mass weight average for native alternan is approximately 3.5×10^7 g/mol (Fig. 3A). As shown in Fig. 4A, the molar mass weight averages for the largest two peak regions of modified alternan (17 days) are 4.9×10^6 and 4.1×10^5 g/mol, respectively. These molecular weight values are somewhat higher than those previously obtained by HPSEC separations using dextran and pullulan molecular weight standards (Cote, 1992; Leathers et al., 2002a). Although light scattering calculations are independent of elution position, it is possible that opalescence in alternan solutions contributes to light scattering and inflates molecular weight values. A root mean square average radius of approximately 30 nm was calculated for native alternan (Fig. 3B). Unexpectedly, as the molar mass weight averages for modified alternan decreased, the root mean square average radii initially fell below to 10 nm limit of resolution and then increased to a radius of 80–85 nm (Fig. 4B). These radii are consistent with a model in which native alternan exists as a tightly coiled, extended rod that is further tightened and then relaxed somewhat by modification. More detailed conformational studies on alternan are underway.

Linkage analysis carried out by the methylation procedure of Slodki et al. (1986) indicated that modified alternan is fundamentally similar to native alternan (Table 1). This confirms that modified alternan retains the basic linkage pattern of alternan but does not necessarily mean that alternan is modified by random cleavage, since the relatively high molecular weight of the product would make it difficult to detect minor changes in composition.

3.2. Solution viscosity properties of modified alternan

Native alternan is highly soluble, although solutions of greater than 12–15% (w/v) are difficult to attain due to relatively high viscosity. Ultrasonicated alternan can be dissolved in water to give solutions of at least 50% (w/v) (Cote, 1992). Similarly, solutions of modified alternan were prepared at 50% (w/v). Gum arabic forms gel-like dispersions at 50% (w/v) (Whistler, 1993).

Fig. 5 summarizes the relative viscosity of gum arabic, modified alternan, native alternan, and ultrasonicated alternan as a function of concentration. As shown, modified alternan is far more similar in rheological behavior to gum arabic and ultrasonicated alternan than to native alternan. Native alternan is significantly more viscous than modified alternan, and its viscosity increases exponentially with concentration.

The shear thinning behavior of native alternan, modified alternan, ultrasonicated alternan, and gum arabic were determined at various concentrations (Fig. 6). As shown by the flatness of these curves, all tested solutions are essentially Newtonian. Only at the highest tested concentrations and very low shear rates was any evidence of non-Newtonian behavior observed. Little pseudoplasticity was observed, due to the relatively low viscosities encountered for these materials. Modified alternan and gum arabic exhibit shear thinning only at concentrations several times higher than that of native alternan.

3.3. Emulsification capacity of modified alternan

Table 2 summarizes the emulsification capacity and emulsion stability of gum arabic, soy protein, native

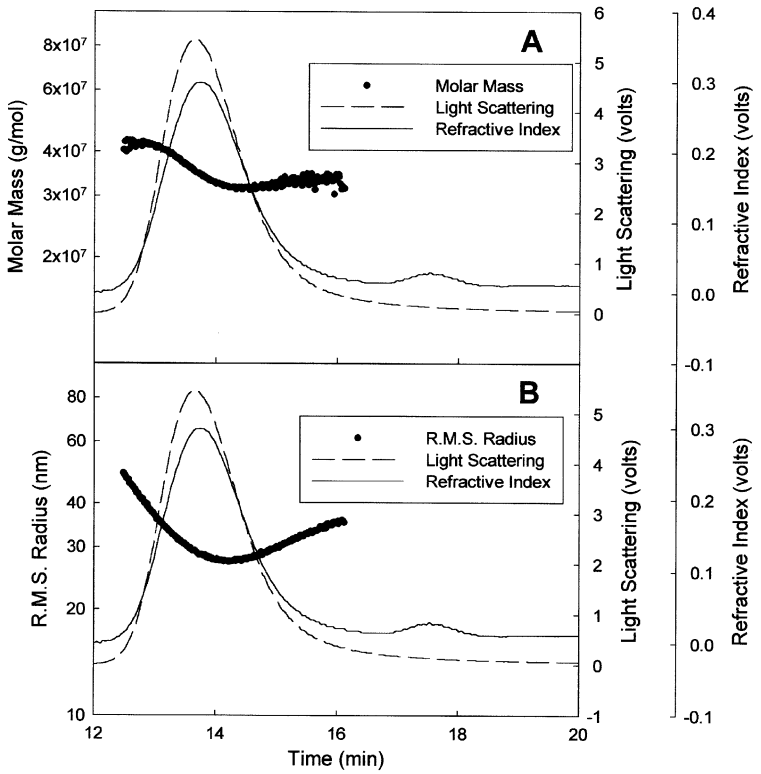

Fig. 3. Calculated molar mass weight averages (Panel A, solid circles) and root mean square average radii (Panel B, solid circles) for native alternan. Reference lines: dashed, light scattering; solid, refractive index.

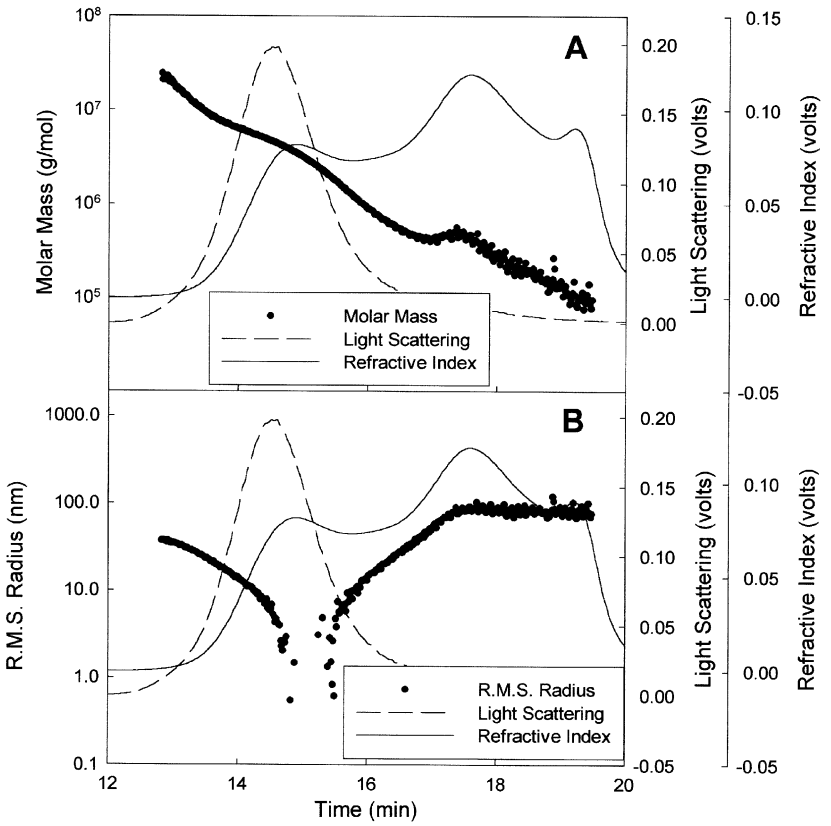

Fig. 4. Calculated molar mass weight averages (Panel A, solid circles) and root mean square average radii (Panel B, solid circles) for peak regions of modified alternan. Reference lines: dashed, light scattering; solid, refractive index.

Table 1
Methylation analysis of modified alternan

	Mole percentage of methylated PAAN[1] glucose derivative			
	2,3,4,6-tetra-*O*-Me	2,4,6-tri-*O*-Me	2,3,4-tri-*O*-Me	2,4-di-*O*-Me
Native alternan	10	35	45	10
Modified alternan	12	40	36	12

[a] per-*O*-acetylated aldononitrile.

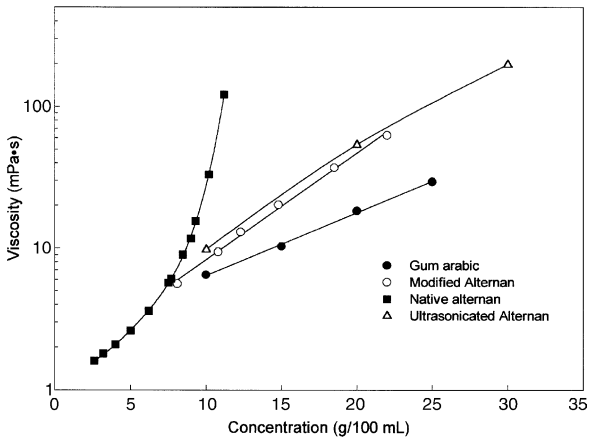

Fig. 5. Relative viscosities of solutions of gum arabic, modified alternan, native alternan, and sonicated alternan as a function of concentration. All solutions were tested at a shear rate of 39.6 s^{-1} with the exception of 30% sonicated alternan, which was at 15.8 s^{-1} (as shown in Fig. 6c, sonicated alternan behaves as a Newtonian solution at this concentration).

alternan, and modified alternan. Unfortunately, neither native alternan nor modified alternan possesses significant emulsification capacity. Gum arabic is a complex mixture of arabinogalactan oligosaccharides, polysaccharides and glycoproteins, and the protein component is believed to be necessary for its emulsification capacity (Williams & Phillips, 2000). Because some applications for gum arabic demand emulsification capacity, this is a limitation on modified alternan as a potential substitute for gum arabic.

3.4. Effect of pH and temperature on storage stability of native and modified alternan

One percent (w/v) solutions of native alternan and modified alternan were prepared in defined growth medium (pH 5.0) and held for 1 week at temperatures between 4 and 70 °C. As shown in Fig. 7A, native alternan shows little fluctuation in absorbance during this period, and modified alternan shows even less change. Solutions of native alternan and modified alternan also were adjusted to various pH levels and stored for 1 week at 4 °C. As shown in Fig. 7B, both native alternan and modified alternan exhibit excellent pH stability. Alternan solutions should have storage advantages over solutions of gum arabic, which are readily contaminated by microbial growth (Glicksman & Schachat, 1959). Due to alternan's unique structure, microorganisms that grow on alternan are rare (Wyckoff, Cote, & Biely, 1996).

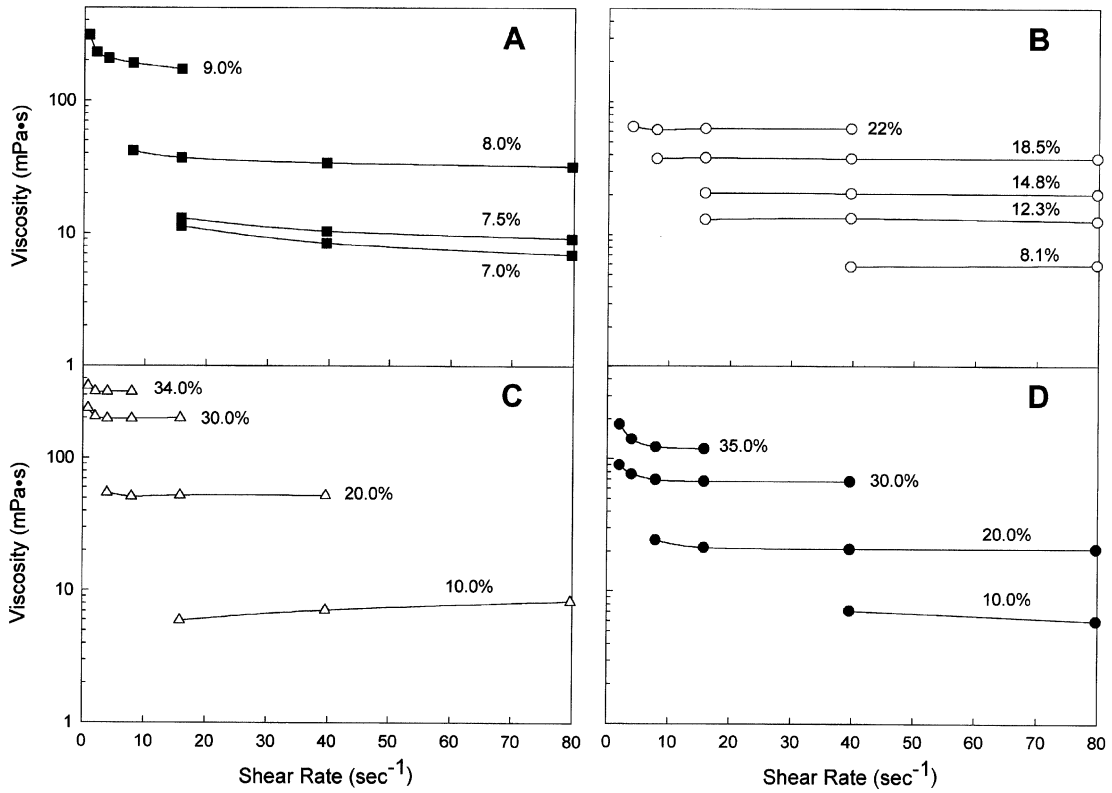

Fig. 6. Shear thinning behavior of native alternan (Panel A), modified alternan (Panel B), ultrasonicated alternan (Panel C) and gum arabic (Panel D).

Table 2
Emulsification capacity of native and modified alternan

Sample	Percent emulsification	Percent stable emulsification
Oil and water	0 ± 0	0 ± 0
Gum arabic	64 ± 2	57 ± 0
Soy protein	66 ± 0	59 ± 1
Native alternan	0 ± 0	0 ± 0
Modified alternan	0 ± 0	0 ± 0

3.5. Hygroscopicity and other properties of native and modified alternan

Dry preparations of native alternan and modified alternan were weighed, desiccated for 4 days, and re-equilibrated to room humidity for 48 h. Neither native alternan nor modified alternan is highly hygroscopic, losing only 9–12% of their initial weights on desiccation. Desiccated samples return to within 0.3% of their initial weights when re-equilibrated. By comparison, gum arabic usually has a moisture content of 13–15%, with a USP limit of 15% (Anonymous, 1983).

Gum arabic is prepared from natural plant exudates and varies greatly in color and taste. Tannins are believed to be responsible for both off-white colors and off flavors. More costly grades of gum arabic that are white and tasteless are preferred for food and pharmaceutical applications (Williams & Phillips, 2000). Both native alternan and modified alternan are bright white, amorphous powders. Although alternan has not been used as a food additive, an alternan-producing strain of *L. mesenteroides* was recently isolated from the traditional Korean fermented food, kim chi (Jung, Kim, Lee, & Jung, 1999).

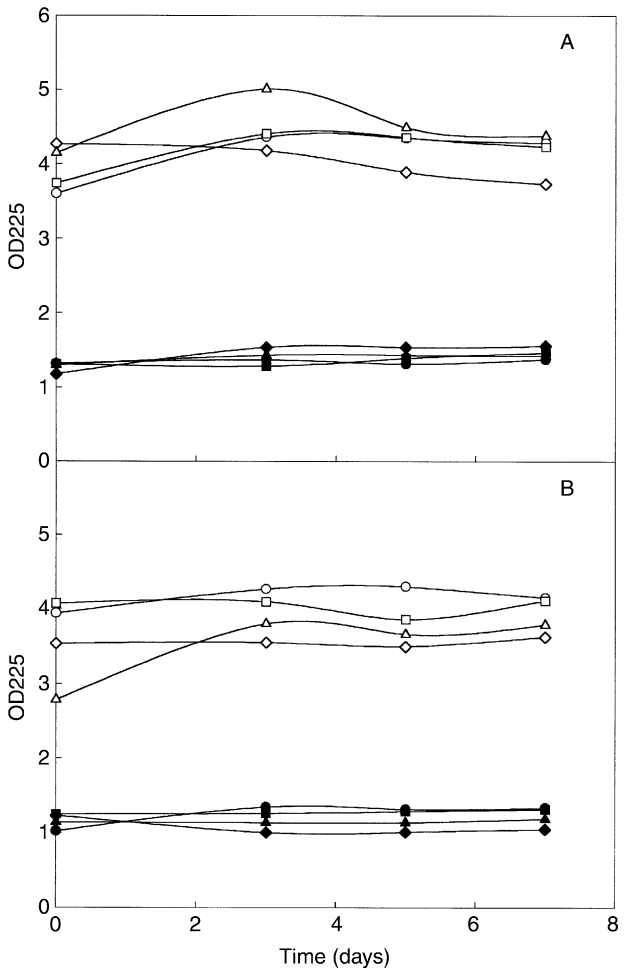

Fig. 7. Effect of temperature (Panel A) and pH (Panel B) on the storage stability of native alternan and modified alternan solutions. Symbols: open, native alternan; closed, modified alternan; circles, 4 °C or pH 3.0; squares, 25 °C or pH 5.0; triangles, 37 °C or pH 7.0; diamonds, 70 °C or pH 9.0.

4. Conclusions

A novel modified alternan produced by a bioconversion process was characterized as a reduced molecular weight, heterodisperse form of alternan. Modified alternan exhibits solution viscosity properties that resemble those of commercial gum arabic, suggesting that it might be developed as a substitute for this product. Alternan solutions show excellent stability but lack the emulsification capacity of gum arabic that is important for certain applications. Efforts are underway in our laboratory to develop methods to impart emulsification capacity to modified alternan.

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References

Anonymous (1983). Acacia. In M. Windholz, S. Budavari, R. F. Blumetti, & E. S. Otterbein (Eds.), *The Merck index* (pp. 2–3). Rahway: Merck & Co.

Arguello-Morales, M. A., Remaud-Simeon, M., Pizzut, S., Sarcabal, P., Willemot, R.-M., & Monsan, P. (2000). *Leuconostoc mesenteroides* NRRL B-1355 *dsrC* gene for dextransucrase. *GenBank Accession No. AJ250172*.

Cote, G. L. (1992). Low-viscosity α-D-glucan fractions derived from sucrose which are resistant to enzymatic digestion. *Carbohydrate Polymers*, *19*, 249–252.

Cote, G. L. (2002). Alternan. In E. J. Vandamme, S. De Baets, & A. Steinbuchel (Eds.), *Biopolymers. Vol. 5. Polysaccharides I: polysaccharides from prokaryotes, Chap. 13* (pp. 323–350). Weinheim: Wiley.

Cote, G. L., & Ahlgren, J. A. (1995). Microbial polysaccharides. In J. I. Kroschwitz, & M. Howe-Grant (Eds.), (4th ed.) (*Vol. 16*) (pp. 578–612). *Kirk-Othmer encyclopedia of chemical technology*, New York: Wiley.

Cote, G. L., Leathers, T. D., Ahlgren, J. A., Wyckoff, H. A., Hayman, G. T., & Biely, P. (1997). Alternan and highly branched limit dextrans: Low-viscosity polysaccharides as potential new food ingredients. In A. M. Spanier, M. Tamura, H. Okai, & O. Mills (Eds.), *Chemistry of novel foods* (pp. 95–110). Carol Stream, IL: Allured Publishing Corp.

Cote, G. L., & Robyt, J. F. (1982). Isolation and partial characterization of an extracellular glucansucrase from *L. mesenteroides* NRRL B1355 that synthesizes an alternating (1 → 6), (1 → 3) α-D-glucan. *Carbohydrate Research*, *101*, 57–74.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Glicksman, M., & Schachat, R. E. (1959). Gum arabic. In R. L. Whistler, & J. N. BeMiller (Eds.), *Industrial gums: Polysaccharides and their derivatives* (pp. 213–298). New York/London: Academic Press.

Jeanes, A., Haynes, W. C., Wilham, C. A., Rankin, J. C., Melvin, E. H., Austin, M. J., Cluskey, J. E., Fisher, B. E., Tsuchiya, J. H., & Rist, C. E. (1954). Characterization and classification of dextrans from ninety-six strains of bacteria. *Journal of the American Chemical Society*, *76*, 5041–5052.

Jung, H.-K., Kim, K.-N., Lee, H.-S., & Jung, S.-H. (1999). Production of alternan by *Leuconostoc mesenteroides* CBI-110. *Korean Journal of Applied Microbiology and Biotechnolgy*, *27*, 35–40.

Kim, D., & Robyt, J. F. (1994). Production and selection of mutants of *Leuconostoc mesenteroides* constitutive for glucansucrases. *Enzyme Microbial Technology*, *16*, 659–664.

Kobayashi, M., Utsugi, H., & Matsuda, K. (1986). Intensive UV absorption of dextrans and its application to enzyme reactions. *Agricultural and Biological Chemistry*, *50*, 1051–1053.

Koenig, D. W., & Day, D. F. (1989). Induction of *Lipomyces starkeyi* dextranase. *Applied and Environmental Microbiology*, *55*, 2079–2081.

Kossman, J., Welsh, T., Quanz, M., & Knuth, K (2000). Nucleic acid molecules encoding alternansucrase. *Patent Cooperation Treaty (PCT) International Publication No. WO00/47727*.

Leathers, T. D., Ahlgren, J. A., & Cote, G. L. (1997a). Alternansucrase mutants of *Leuconostoc mesenteroides* strain NRRL B-21138. *Journal of Industrial Microbiolgy and Biotechnology*, *18*, 278–283.

Leathers, T. D., Hayman, G. T., & Cote, G. L. (1995). Rapid screening of *Leuconostoc mesenteroides* mutants for elevated proportions of alternan to dextran. *Current Microbiology*, *31*, 19–22.

Leathers, T. D., Hayman, G. T., & Cote, G. L (1997b). Microorganism strains that produce a high proportion of alternan to dextran. *United States Patent* 5,702,942.

Leathers, T. D., Hayman, G. T., & Cote, G. L (1998). Rapid screening method to select microorganism strains that produce a high proportion of alternan to dextran. *United States Patent* 5,789,209.

Leathers, T. D., Nunnally, M. S., & Cote, G. L. (2002a). Modification of alternan by novel *Penicillium* spp. *Journal of Industrial Microbiolgy and Biotechnology*, *29*, 177–180.

Leathers, T. D., Nunnally, M. S., & Cote, G. L (2002b). *Penicillium* isolates for modifying alternan. *United States Patent* 6,479,275.

Misaki, A., Torii, M., Sawai, T., & Goldstein, I. J. (1980). Structure of the dextran of *Leuconostoc mesenteroides* B-1355. *Carbohydrate Research*, *84*, 273–285.

Seymour, F. R., & Knapp, R. D. (1980). Unusual dextrans. 13. Structural analysis of dextrans from strains of *Leuconostoc* and related genera, that contain 3-*O*-α-D-glucosylated α-D-glucopyranosyl residues at the branch points, or in consecutive linear position. *Carbohydrate Research*, *81*, 105–129.

Seymour, F. R., Knapp, R. D., Bishop, S. H., & Jeanes, A. (1979). Structural analysis of *Leuconostoc* dextrans containing 3-*O*-α-D-glucosylated α-D-glucosyl residues in both linear-chain and branch-point positions, or only in branch-point positions, by methylation and by 13C-N.M.R. spectroscopy. *Carbohydrate Research*, *74*, 41–62.

Seymour, F. R., Slodki, M. E., Plattner, R. D., & Jeanes, A. (1977). Six unusual dextrans: methylation structural analysis by combined GLC-MS of per-*O*-acetyl aldononitriles. *Carbohydrate Research*, *53*, 153–166.

Slodki, M. E., England, R. E., Plattner, R. D., & Dick, W. E., Jr (1986). Methylation analyses of NRRL dextrans by capillary gas–liquid chromatography. *Carbohydrate Research*, *156*, 199–206.

Smith, M. R., Zahnley, J., & Goodman, N. (1994). Glucosyltransferase mutants of *Leuconostoc mesenteroides* NRRL B-1355. *Applied and Environmental Microbiology*, *60*, 2723–2731.

Whistler, R. L. (1993). Exudate gums. In R. L. Whistler, & J. N. BeMiller (Eds.), *Industrial gums. Polysaccharides and their derivatives* (3rd ed.) (pp. 309–339). San Diego: Academic Press.

Williams, P. A., & Phillips, G. O. (2000). Gum arabic. In G. O. Phillips, & P. A. Williams (Eds.), *Handbook of hydrocolloids* (pp. 155–168). Cambridge: CRC Press.

Wyckoff, H. A., Cote, G. L., & Biely, P. (1996). Isolation and characterization of microorganisms with alternan hydrolytic activity. *Current Microbiology*, *32*, 343–348.

Yasumatsu, K., Sawada, K., Moritaka, S., Misaki, M., Toda, J., Wada, T., & Ishii, K. (1972). Whipping and emulsifying properties of soybean products. *Agricultural and Biological Chemistry*, *36*, 719–727.