Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Biosynthesis of emulsan biopolymers from agro-based feedstocks

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Keywords
Acinetobacter, adjuvant, agricultural oils, biopolymer, emulsan.

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2005/0957: received 24 August 2005, revised 31 March 2006 and accepted 3 May 2006

doi:10.1111/j.1365-2672.2006.03078.x

Abstract

Aims: The need for biocompatible, biodegradable, and versatile biopolymers permeates many fields including environmental and food technology. The goal of the study presented here is to establish the utility of agricultural oils as an inexpensive carbon source to produce materials useful for biomedical materials and offer positive attributes in terms of green chemistry.

Methods and Results: Structural variants of the complex acylated polysaccharide, emulsan, secreted from *Acinetobacter venetianus* RAG-1, were biosynthesized in cultures supplemented with agricultural feedstocks to examine the feasibility of conversion of these substrates into value-added biopolymers. *Acinetobacter venetianus* produced chemically and biologically distinct emulsan variants in culture on soy molasses and tallow oil. These variants possess significant biological function, including macrophage activation and adjuvant activity, in similar range to that observed for the standard emulsan formed on ethanol-fed *A. venetianus*.

Conclusions: The results indicate that this novel family of biopolymers can be produced in significant quantities from the readily available renewable agricultural feedstocks and the resulting structures and functions can be correlated to the chemistry of these feedstocks.

Significance and Impact of the Study: The significant quantities of agricultural oils produced annually represent an untapped source for bioconversion to valuable products. The results of this study confirm that the important polymer emulsan can be synthesized from this inexpensive carbon source.

Introduction

Emulsan is a complex extracellular acylated polysaccharide produced by the gram-negative bacterium, *Acinetobacter venetianus* RAG-1, composed of a polysaccharide backbone with *O*-acyl and *N*-acyl bound fatty acid side chains. The polysaccharide backbone consists of three amino sugars in the ratio 1 : 1 : 1 (Belsky *et al.* 1979). The fatty acid side chains range in length from 10 to 20 carbons, and represent 5–23% (w/w) of the polymer. The emulsan amino groups are either acetylated or covalently linked by an amide bond to 3-hydroxybutyric acid (Gorkovenko *et al.* 1997). The combination of hydrophilic anionic sugar main chain repeat units, along with the hydrophobic side groups leads to the amphipathic behaviour of emulsan and, therefore, its ability to form stable oil-in-water emulsions.

Our previous studies have demonstrated that the composition of the fatty acid side chains decorating the polysaccharide backbone of emulsan can be altered by changing the culture conditions of *A. venetianus* RAG-1 (Gorkovenko *et al.* 1995, 1997, 1999; Zhang *et al.* 1997). In addition, we have utilized genetic engineering to generate emulsan variants with regard to fatty acid composition (Johri *et al.* 2002). Furthermore, these structural variants result in differences in solution properties, such as emulsification and surface tension (Zhang *et al.* 1997; Johri *et al.* 2002). We have also demonstrated that emulsan-related biological activity can be related to the

structural features of the polymer. For example, depending on the structure, emulsan induces tumour necrosis factor (TNF) release from macrophages and a significant humoral immune response in a hapten-carrier model (Panilaitis *et al.* 2002).

Biopolymers are becoming increasingly attractive in many technological applications because of their versatility of functions via control of structure, biological compatibility, degradability and ease of production in green chemistry compatible formats, among many other factors. Underlying the field is the goal to tailor biopolymer structure, chemistry and morphology to the intended function. To this end, studies in many laboratories are actively bioengineering biopolymers, particularly protein polymers and bacterial polyesters, for specific functions. The biological functions summarized for emulsans, combined with the versatile control of structure and solution properties, suggested that this family of complex polysaccharides could provide a novel set of new biopolymers for many technological needs wherein emulsification is important, such as for consumer products, drug delivery (Castro *et al.* 2005, 2006), environmental remediation and oil recovery, among others.

In the present study, the goal was to determine the potential utility of readily available low cost agriculture-based (co)products as feedstocks to generate value-added bioemulsifiers termed emulsans (Ag emulsans). Further, we sought to correlate the chemistry of the feedstock to the structure and function (solution and biological) of the new emulsan variants generated on these feedstocks. Large volumes of soy molasses (or soy solubles) are generated during the manufacture of soy proteins – one of the key ingredients of soy foods that have seen explosive growth in the past several years. Similarly, millions of metric tons of vegetable and animal oils are produced annually in the United States, and exports of these products have been declining sharply in recent years (Solaiman *et al.* 2005). Therefore, an opportunity to utilize these by-products of the agricultural industry towards novel biopolymers with a wide range of technological utility was addressed. The results presented here demonstrate the feasibility of microbial conversion of such feedstocks into these bioemulsifiers and also lays the foundation for a more in-depth exploration of the relationships between feedstock composition and structure/function of the biopolymer product generated on these feedstocks.

Methods

Agricultural feedstocks

Soy molasses, also known as soy solubles (50% moisture and 30% carbohydrate), was supplied by Central Soya (Gibson City, IL, USA). The major carbohydrates in the soy solubles are sucrose (19% w/v of soy solubles), stachyose (11%) and raffinose (2%) (Montelongo *et al.* 1993). Tallow oil (acidless, low-pour grade; <1·5% free fatty acid) was supplied by Geo. Pfau's Sons Company, Inc. (Jeffersonville, IN, USA). The tallow oil has a fatty acid profile as follows (in mole per cent): C16 (23), C18 (10), C18 : 1 (46), C18 : 2 (9), C18 : 3 (<1). Unlike tallow that solidifies at room temperature, this low-pour grade feedstock remains liquified.

Experimental animals and cell lines

BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA, USA). The murine macrophage cell line, RAW 264·7 was maintained in DMEM containing 10% fetal calf serum (FCS) and 50 μg ml^{-1} of gentamycin (GIBCO, Carlsbad, CA, USA). Animal protocols were approved by the Tufts University Institutional Animal Care and Use Committee.

Emulsan preparation and structural characterization

Acinetobacter venetianus RAG-1 (ATCC 31012) was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown on minimal media [0·1 mol l^{-1} of K_2HPO_4, 0·05 mol l^{-1} of KH_2PO_4, 2 mmol l^{-1} of $MgSO_4{\cdot}7H_2O$ and 0·03 mol l^{-1} of $(NH_4)_2SO_4$], supplemented with ethanol and/or agricultural feedstocks (1% w/v) as a carbon source in 2-l baffled flasks containing 1-l media and incubated at 30°C in an orbital shaker (250 rev min^{-1}) for 6 days. Emulsan was purified as we have previously described (Gorkovenko *et al.* 1997, 1999; Johri *et al.* 2002). Emulsan variants were analysed by gas chromatography-mass spectroscopy (GC-MS) for fatty acid content as previously described (Belsky *et al.* 1979; Gorkovenko *et al.* 1999; Johri *et al.* 2002).

Emulsification assay

Emulsification activity was determined with 1·0 mg of pure emulsan dissolved in 7·5 ml of Tris-HCl (10 mmol l^{-1}, pH = 7·2) containing 20 mmol l^{-1} of $MgSO_4$ (or in 7·5-ml aliquots of cell-free microbial supernatants). Samples were placed in 125-ml Erlenmeyer baffled flasks, containing 0·1 ml of hexadecane. The flasks were incubated in a rotatory shaker for 1 h at 30°C at 200 rev min^{-1} . The turbidity was assayed at 600 nm after 10 min. The calibration curve was performed using pure emulsan from *A. venetianus* RAG-1 under the same experimental conditions.

Macrophage activation and cytokine assay

Macrophage assays were conducted as previously described (Panilaitis *et al.* 2002). Briefly, RAW 264.7 cells were plated at 8×10^4 cells per well in 200 μl in a 96-well tissue culture plate and incubated for 48 h prior to stimulation. Cells were exposed to specified concentrations of emulsan preparations overnight. Cell supernatants were collected for cytokine assays. TNF was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). All stimulations were carried out in the presence of 5 μg ml^{-1} of polymyxin B to prevent stimulation by any contaminating lipopolysaccharide (LPS).

Adjuvant activity for humoral immune response

Thirty-five 6–8-week-old female *BALB/c* mice were randomly placed in seven groups of five mice and immunized with 50 μg of KLH-DNP and 20 μg of an agricultural oil-derived emulsan. Controls included emulsan produced on our standard carbon source of 1% ethanol, as well as antigen alone. Blood was collected from the tail 3 days prior to primary immunization. Antigen and adjuvant were mixed by repeated aspiration through an 18-gauge needle, attached to a 3-cc syringe. Each mouse was immunized subcutaneously (s.c.) with 100 μl of the total volume of adjuvant and antigen. Mice were boosted s.c. after 28 days, and blood was taken every 3 days after boost until day 21 postboost, and then again at 6, 9 and 12 weeks. The total DNP-specific antibody titre was determined by ELISA as previously described (Panilaitis *et al.* 2002). The relative contributions of immunoglobulin (Ig)G2a and IgG1 isotypes to the anti-DNP response were determined by ELISA as earlier with the substitution of horseradish peroxidase (HRP)-conjugated isotype-specific antibodies (Accurate, Westbury, NY, USA) for the goat antimouse antibody. The dilution at which the absorbance was twice the baseline was determined as the total antibody titre. Antibody titres were than normalized to the standard emulsan as a per cent of control.

Statistics

Data was analysed by a paired student's *t*-test.

Results

Agricultural feedstocks and emulsan structure (fatty acid profiles)

Given the ability of *A. venetianus* RAG-1 to produce structural variants of emulsan based on the carbon source fed to the organism, it was important to examine the emulsans produced with agricultural feedstocks. GC-MS analysis of the emulsan variants derived from these conditions indicated a significant change in total fatty acid content (Fig. 1), as well as a difference in the profile of fatty acids decorating the emulsan polysaccharide backbone (Fig. 2). The addition of 1% soy to ethanol-fed *A. venetianus* RAG-1 cultures nearly doubled total fatty acid content over ethanol alone controls, while soy in the absence of ethanol resulted in less than 60% fatty acid content. Tallow-supplemented cultures produced over 30% more total fatty acid content on the backbone than control cultures, while the combination of soy and tallow produced less than 50% of this level of fatty acid substitution when compared with the control cultures ($P < 0{\cdot}001$).

When the profiles of the fatty acids were examined, subtle differences were noted (Fig. 2). Soy soluble-supplemented cultures produce emulsans with a lower number of C16 side chains and an increase in C18 side chains ($P < 0{\cdot}001$). Tallow-supplemented cultures produced emulsans that displayed similar profiles to the controls, except for the C12 side chains that were present at >10%, in contrast to the controls where this chain length was found only at trace levels. Additionally, cultures fed with combined soy solubles and tallow resulted in a significant increase in unsaturated C20 fatty acids on the backbone ($P < 0{\cdot}001$).

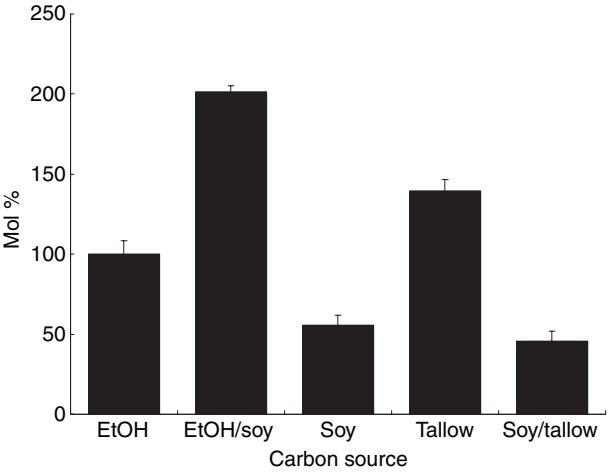

Figure 1 Total fatty acid content of emulsans produced in cultures supplemented with or without ethanol and/or agricultural feedstocks. EtOH, 1% ethanol supplemented; EtOH/Soy, 1% ethanol and 1% soy solubles supplemented; soy, 1% soy solubles supplemented; tallow, 1% tallow supplemented; soy/tallow, 1% soy solubles and 1% tallow supplemented. Fatty acid content was determined in quadruplicate, and error bars represent SD.

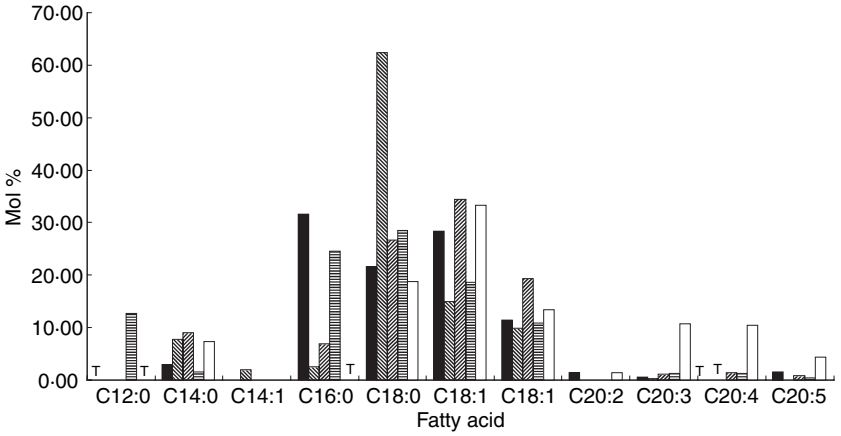

Figure 2 Fatty acid profiles of agricultural feedstock-produced emulsans. The individual identity of fatty acid side chains are expressed as mole per cent. Fatty acid content was determined in quadruplicate, and error bars represent SD. T, trace levels of the identified fatty acid. ■, EtOH; ▧, EtOH/soy; ▨, soy; ▤, Tallow; □, soy/Tallow.

Emulsification properties

As the fatty acid side chains are essential for emulsification properties of emulsan, it was important to determine the impact of agricultural oil as a carbon source on structure and then the emulsification behaviour. Emulsification behaviour was similar for the emulsan generated on the soy molasses to the standard ethanol-fed control in soy molasses-supplemented cultures; however, tallow-supplemented cultures produced emulsan with approximately 30% higher emulsification behaviour than the controls ($P < 0{\cdot}05$; Fig. 3).

Macrophage activation

To examine the effect of these alternative carbon sources on biological activity of the emulsan polymers, the agricultural feedstock-produced variants of emulsan were studied for the macrophage response they induced. The Ag emulsans were used at a range of concentrations to stimulate RAW 264·7 cells to determine the level of macrophage activation based on release of TNF. All the emulsan preparations activated the macrophages; however, there were noticeable differences in the response induced by each preparation. The standard ethanol/minimal medium-produced emulsan and the tallow-produced emulsan induced a TNF response that was at the upper limit of the assay for the higher two concentrations examined. Therefore, the macrophage activation of each preparation was compared at the 50 ng ml^{-1} concentration. The emulsan from the soy molasses-supplemented cultures displayed significantly ($P < 0{\cdot}05$) lower activation potential (Fig. 4), while the other three preparations examined were not significantly different. The emulsan from tallow-supplemented cultures induced a similar level of macrophage activation as with the controls (ethanol as the carbon source).

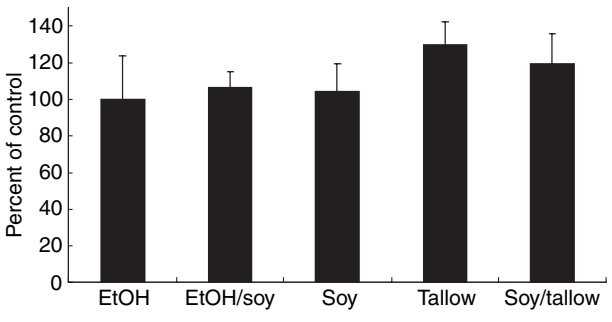

Figure 3 Emulsification activity of agriculture-based (Ag) emulsans. Results were normalized to the ethanol-fed controls. Activity was determined in quadruplicate, and error bars represent SD.

Adjuvant activity

To further evaluate the biological function of the emulsan variants generated on agricultural feedstocks, a hapten-

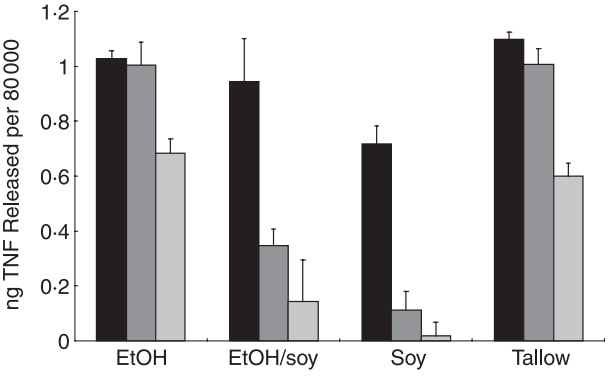

Figure 4 Macrophage activation by emulsan variants. Emulsans at the specified concentrations were used to stimulate RAW 264.7 cells. Stimulations were performed in triplicate, and error bars represent SD. The results presented are a representative example of three separate runs. ■, 5 ng ml^{-1}; ▩ 500 ng ml^{-1}; ▩, 50 ng ml^{-1}.

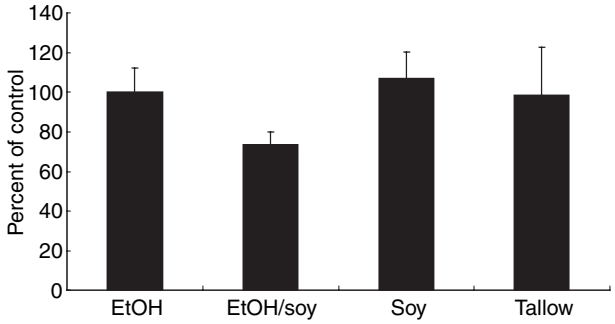

Figure 5 Adjuvant activity of agriculture-based (Ag) emulsans. Relative anti-DNP titres from day 14 postboost serum collection were normalized to ethanol-fed control cultures. Enzyme-linked immunosorbent assays (ELISA) were performed in triplicate, and error bars represent SD. Each bar represents the average antibody titre of five animals.

carrier immunization protocol was used *in vivo*. This model has been previously utilized to establish the adjuvant activity of the standard emulsan (Panilaitis *et al.* 2002). Moreover, emulsan has been demonstrated to have significant adjuvant activity in models of Lyme disease, botulinum intoxication and *Yersinia pseudotuberculosis* (unpublished results). The purpose of these experiments was to verify that the Ag emulsans maintained this property of standard emulsan. Animals immunized with antigen in the presence of emulsan exhibited significantly higher DNP-specific antibody titres over the course of the experiment than those animals immunized with antigen alone (data not shown). When compared with the response to the standard emulsan (ethanol-produced), the emulsans supplemented with tallow and soy in the absence of ethanol induced anti-DNP titres similar to controls (Fig. 5). The only significantly different titre was induced by emulsan supplemented with soy and ethanol that had titres *c.* 25% less than controls ($P < 0{\cdot}05$). Additionally, the immune response produced was long-lived, as significant titres in all groups were found more than a year after the final boost (data not shown). The relative contribution of IgG1 and IgG2a isotypes to the total serum response of the immunized animals was examined at the fourteenth day after the final boost. Emulsan-immunized animals demonstrated a switch to IgG2a as was previously reported (Panilaitis *et al.* 2002); however, no significant difference in the degree of this switch was observed (data not shown). The animals immunized showed no changes in behaviour or survival.

Discussion

The goal of these studies was to determine the feasibility of utilization of agriculture-based substrates as feedstocks to generate value-added bioemulsifiers. In addition, our goal was to characterize the chemical and biological properties of the emulsans produced from these feedstocks. This family of complex biopolymers possesses potent immunomodulatory properties that are sensitive to structural changes of the polymer. Therefore, the present studies offer a new option for the generation of these bioemulsifiers in which the structural differences attributable to the chemistry of the carbon source impacts both solution properties and biological activity. Biological functions including the activation of the innate immune response and adjuvant carrier for specific humoral responses were demonstrated with these new structural variants. Our prior work demonstrated that the fatty acid components of emulsan were critical to emulsification activity and macrophage activation potential (Zhang *et al.* 1997; Johri *et al.* 2002; Panilaitis *et al.* 2002). The fatty acid profiles of the emulsan biopolymers produced from agricultural feedstock-supplemented cultures were also different, resulting in the altered solution and biological properties.

The emulsification properties of the emulsan generated on soy soluble-supplemented cultures were similar to the controls (grown on ethanol as carbon source), while the tallow-supplemented cultures generated biopolymers that were approximately 30% more effective at emulsification of hexadecane. The emulsan from the tallow-supplemented cultures possessed approximately a third higher degree of substitution by fatty acids (fatty acids per milligram of emulsan) than the control samples (on ethanol) and our previous data demonstrated a trend that increased total fatty acid content (substitution) results in increased emulsification behaviour. However, the cultures supplemented with soy solubles and ethanol produced approximately twice the degree of substitution by fatty acids compared with controls, but no significant difference in emulsification. When the individual fatty acid profile is examined, the major differences were that the emulsan from the tallow-supplemented culture had a higher percentage of C12 side chains, and a lower percentage of C18 : 1 side chains. Given the fact that emulsifiers are dependent on the presence of hydrophobic and hydrophilic components, the presence of a short chain fatty acid may not intuitively suggest an increase in emulsification activity. However, the emulsan from the tallow-supplemented culture had a significant content of this side chain and the highest emulsification activity.

The results of macrophage activation assays with these emulsan variants demonstrated that changes in structure lead to differences in the macrophage-activating capabilities. While the exact mechanism of this activation remains elusive, our working hypothesis based on our previous work (Panilaitis *et al.* 2002) is that the identity, degree of

substitution, and perhaps clustering of particular fatty acids may be involved. The emulsan from the tallow-supplemented cultures had similar macrophage activating potential to the controls while the polymer from the soy molasses-supplemented cultures resulted in only 10–30% of the activity of the controls. An examination of fatty acid profiles indicated that the major changes in fatty acid profiles was an increase in the percentage of C18 side chains, and a corresponding decrease in the percentage of C16 side chains. Interestingly, the level of C16 side chains in the samples from the tallow-supplemented samples was close to the control, perhaps indicating a causal relationship. However, it is likely that more subtle structural properties, such as clustering of particular side chains, or the order of individual side chains, may be important in terms of the molecular recognition of these acylated polysaccharides by macrophages.

The mechanism of emulsan macrophage activation and adjuvant activity has not been determined, although this is quite common in the adjuvant field. Some attempts have been made to better understand structure–function relationships in other adjuvant systems. This is particularly true of the saponin family of adjuvants (Oda *et al.* 2003; Sun *et al.* 2005). The saponins consist of a triterpenoid aglycone with unbranched sugar side chains and, like many other adjuvants have an amphipathic structure (Lefrancier *et al.* 1978; Hunter *et al.* 1991; Chinnah *et al.* 1992; Stewart-Tull 1995). Based on a panel of soyasaponins, it was determined that increased immunopotentiation could be correlated to an increased hydrophile–lipophile balance value (HLB). In addition, the presence of an aldehyde on the triterpenoid nucleus was a critical component of T helper Type 1 (TH1)-activation by saponin adjuvants (Oda *et al.* 2003). The effect of acyl side chain structures were also examined in the context of the cellular activation induced by synthetic lipopeptides. Triacyl-lipopentapeptide compounds were examined for their ability to activate murine bone marrow-derived macrophages based on the composition of their acyl chains, demonstrating a positive effect in preparations containing saturated C10, C14 or C16 fatty acid side chains (Muller *et al.* 2004).

Our working hypothesis is that macrophage activation and emulsification properties are positive indicators of adjuvant activity for an emulsan preparation, although these correlations have not been fully examined in a mechanistic study because of their extreme complexity as described earlier. The emulsan generated on soy soluble-supplemented cultures with ethanol have differences in their fatty acid profiles, and diminished macrophage activation, when compared with controls. Subsequent analysis of the adjuvant activity of this preparation indicates a decrease of approximately 25% of anti-DNP titres in relation to controls. This supports the working hypothesis regarding the important role of macrophage activation and emulsification properties on adjuvant activity. Upon examination of similar comparisons between controls and soy or tallow alone (no ethanol), the hypothesis is not supported. Tallow-supplemented cultures had the same macrophage-activating potential, enhanced emulsification behaviour and a higher mole percentage of fatty acid substitution; however, this bioemulsifier structural variant generated antibody titres with no significant difference from the control. The titres induced by soy only-supplemented emulsan were also statistically the same as control, even with twice the fatty acid substitution. Perhaps the increase in fatty acid content was offset by a significant decrease (70–90%) in macrophage activation, which was observed with the soy-supplemented emulsans. These studies indicate the need for further study to determine which specific structural and biological properties lead to significant adjuvant activity.

Soy molasses is a co-product of the manufacture of soy-protein products, such as soy isolates and soy concentrates. Growth of the soy foods market at a rate of 10–15% in the past few years has made available a large volume of this co-product. Because of its high content of potentially fermentable carbohydrates and its low cost (2–5 cents per pound), soy molasses is an attractive feedstock for bioconversion processes. It has been studied as a substrate for the microbial production of industrially important chemicals, such as sophorolipids (Solaiman *et al.* 2004), lactic acid (Montelongo *et al.* 1993) and butanol (Qureshi *et al.* 2001). Likewise, animal fats such as tallow, lard and recycled grease are rendering products generated at a rate of about 5 million metric tons per year in the United States alone. They are also viable fermentation feedstocks that have attracted the attention of researchers (Deshpande and Daniels 1995; Cromwick *et al.* 1996; Haba *et al.* 2000). Soy solubles and tallow are thus among the readily available agriculture-based renewable resources attractive as potential fermentation feedstocks in 'green' bioprocesses to produce products with the desired structural and functional attributes.

The availability of significant quantities of renewable agricultural feedstocks as carbon source supplements, with microbial conversion to value-added bioemulsifiers, provides a new opportunity in bioconversion technology. This approach is further buoyed because of the ability to alter structural details of these complex polymers, and thus alter solution and biological behaviour. The results presented in this study lay the groundwork for the expansion of such studies to further explore agricultural oils and co-products in the context of bioconversion to this family of bioemulsifiers. Furthermore, better correlations between structure and function will provide a template

for the study of more selective applications to exploit the novel properties of these complex polymers. The green chemistry approach offered, from synthesis to degradation, combined with the novel functions of this family of biopolymers, should lead to new technological impact.

Acknowledgements

We thank the USDA for grant support (99-355504-7915) of this work.

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