Isolation and Characterisation of Bacterial Exopolysaccharides Produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 and *Sphingomonas elodea* ATCC 31461

Massey University

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Food Technology

at Massey University, Palmerston North,

New Zealand.

Kelvin Kim Tha Goh

2004

Massey University
COLLEGE OF SCIENCES

INSTITUTE OF FOOD, NUTRITION
AND HUMAN HEALTH
Te Kura Hangarau o
Kai-oranga-ā-tāngata
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 4336
F 64 6 350 5657
www.massey.ac.nz

CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled

Isolation and Characterisation of Bacterial Exopolysaccharides Produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 and *Sphingomonas elodea* ATCC 31461
in the Institute of Food, Nutrition and Human Health at Massey University, New Zealand:

(a) is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements;

(b) that the text, excluding appendices/annexes, does not exceed 100,000 words;

(c) all the ethical requirements applicable to this study have been complied with as required by Massey University, other organisations and/or committees N.A which had a particular association with this study, and relevant legislation.

Please insert Ethical Authorisation code(s) here: (if applicable) _____

Candidate's Name:
Kelvin Kim Tha Goh

Signature: Goh

Date: 30 July 2004

Supervisor's Name:
Professor Harjinder Singh

Signature: Harjinder Singh

Date: 30 July 2004

Massey University
COLLEGE OF SCIENCES

INSTITUTE OF FOOD, NUTRITION
AND HUMAN HEALTH
Te Kura Hangarau o
Kai-oranga-ā-tāngata
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 4336
F 64 6 350 5657
www.massey.ac.nz

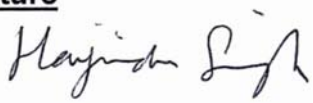
SUPERVISOR'S DECLARATION

This is to certify that the research carried out for the Doctoral thesis entitled "Isolation and Characterisation of Bacterial Exopolysaccharides Produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 and *Sphingomonas elodea* ATCC 31461" was done by Kelvin Kim Tha Goh in the Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand. The thesis material has not been used in part or in whole for any other qualification, and I confirm that the candidate has pursued the course of study in accordance with the requirements of the Massey University regulations.

Supervisor's Name

Professor Harjinder Singh

Signature



Date

30 July 2004

Massey University
COLLEGE OF SCIENCES

INSTITUTE OF FOOD, NUTRITION
AND HUMAN HEALTH
Te Kura Hangarau o
Kai-oranga-ā-tāngata
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 4336
F 64 6 350 5657
www.massey.ac.nz

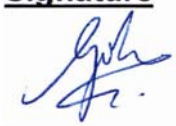
CANDIDATE'S DECLARATION

This is to certify that the research carried out for my Doctoral thesis entitled "Isolation and Characterisation of Bacterial Exopolysaccharides Produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 and *Sphingomonas elodea* ATCC 31461" in the Institute of Food, Nutrition and Human Health, Massey University, Turitea campus, Palmerston North, New Zealand is my own work and that the thesis material has not been used in part or in whole for any other qualification.

Candidate's Name

Kelvin Kim Tha Goh

Signature



Date

30 July 2004

ABSTRACT

The aim of this study was to explore the characteristics of a non-gelling exopolysaccharide (EPS) obtained from *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 and a gelling EPS obtained from *Sphingomonas elodea* ATCC 31461 (31461). The EPSs were isolated from the two bacterial strains grown in milk permeate-based media. They were purified and then characterised using light scattering and viscometric techniques. A greater emphasis of this research was placed on 2483 EPS since its physical characteristics have not been reported to date. In the case of 31461 EPS, a model for gelation of the sodium gellan was proposed based on rheological and light scattering measurements. The rheological properties of the two EPSs were also compared with several commercial polysaccharides.

Microscopy examination of 2483 EPS was carried out using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). In CLSM, the lectin SBA (from *Glycine max*) Alexa Fluor 488 conjugate was used to stain the EPS since it has affinity for galactopyranosyl residues present in 2483 EPS. The CLSM micrographs showed a random distribution of EPS aggregates in the culture medium. At high magnification, the SEM micrographs showed web-like EPS structures. These structures were formed during the critical point drying process, when the EPS, which filled the interstices and channels of the protein aggregates, dehydrated. The 2483 EPS aggregates were found to be stable at neutral or low pH (~3.9) but were disrupted at high pH (pH 8-10).

Procedures commonly used to quantify EPS from culture medium were found to be unreliable. In the development of an improved EPS assay, each of the processing steps was examined. Key improvements included the use of Flavourzyme for protein hydrolysis; optimising ethanol concentration to prevent lactose crystallisation yet allowing complete EPS precipitation; and a suitable centrifugation regime to minimise EPS loss. The improved EPS assay gave reproducible results (5% coefficient of variation).

The isolation of 2483 EPS from milk media proved to be a difficult task because of interference from non-EPS components. An effective and simple approach allowing maximum EPS recovery involved the use of a hydrolysed milk medium which was ultrafiltered (UF) to remove molecular species larger than 2.5 x 10^5 Da. The UF permeate was suitable for the growth of 2483 with an EPS yield of ~400mg/L. Two EPS fractions (namely a soluble and an insoluble fraction) were isolated by ethanol precipitation and the soluble 'ropy' fraction was further purified to achieve ~98% purity. The elemental analysis of the purified fraction revealed the presence of nitrogen (~2.7% w/w). This could be due to the interaction of some peptides (from the growth medium) with the EPS. The polysaccharide composition of the soluble EPS fraction comprised of galactose, glucose, rhamnose and mannose residues (5:1:0.6:0.5). Traces of glucosamine were also found in the fraction.

The purified fraction of 2483 EPS was characterised. Using a capillary viscometer, an intrinsic viscosity of ~2013mL/g was determined. The flow curves of the 2483 EPS solutions obtained using a rotational viscometer showed shear-thinning behaviour and an exponent value of ~0.76 (based on the Cross-type model) is typical of random coil polymers. The concentration dependence of the viscosity plot produced gradients of ~1.1 in the dilute domain and ~3.3 in the semi-dilute to concentrated domain. The coil overlap parameters at three concentration domains ($c^*[\eta], c_{cr}[\eta]$ and $c^{**}[\eta]$) were 0.55, 2.86 and 5.67 respectively. The molecular parameters of the 2483 EPS were found via static light scattering measurements to have a weight-average molar mass (M_w) of ~2 x 10^6 Da, a z-average root-mean-square radius ($\left(r_g^2\right)_z^{1/2}$) of ~165nm and a low polydispersity index (M_w/M_n ~1.15). The plot of M_w versus $\left(r_g^2\right)_z^{1/2}$ gave a gradient of approximately 0.5, which also suggested that the EPS polymer adopted a random coil conformation.

The second part of the research involved gellan gum. Two gellan samples were studied. The first gellan sample was obtained from the fermentation of *Sphingomonas elodea* ATCC 31461 using milk permeate-based medium (31461). The second sample was a commercial high acyl gellan (LT100). Both gellan samples were converted to their

sodium forms (Na-31461 and Na-LT100 gellan) using cation exchange resin and purified. The Na-gellan samples were highly sensitive to changes in Na^+ concentrations. From oscillatory measurements, it was found that the complex moduli of the two Na-gellan samples superimposed closely at a specific Na^+ concentration. The model for the conformational changes of Na-gellan molecules from a solution to a gel was proposed based on rheological and light scattering data. At very low Na^+ concentrations (<19mM, in the case of Na-LT100), Na-gellan molecules were single-stranded (M_w ~2.5 x 10^5 Da) and adopted random coil conformation (exponent value based on the Cross-type model of ~0.76). At a slightly higher Na^+ concentration (~19-24mM), Na-gellan molecules formed double-helices which led to a two-fold increase in molecular weight (M_w ~5.2 x 10^5 Da). The double-stranded molecule appeared to be stiffer (exponent value of the Cross-type model ~0.82) and the mechanical spectra (G', G'') demonstrated 'weak gel' characteristics. A further increase in the Na^+ concentration (>24mM) resulted in the formation of a gel network. The study also found that at low Na^+ concentration, both single-stranded and double-stranded Na-gellan molecules had a tendency to form aggregates under zero-shear conditions. The interactions involved in these aggregates were considered weak and transient, according to the Cox-Merz plot and light scattering data.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my chief supervisor, Professor Harjinder Singh for providing excellent guidance, invaluable advice, support and encouragement throughout the course of my study. I would also like to express my appreciation to Dr Derek Haisman, who with his wealth of knowledge, zest, availability and friendliness, has served as a remarkable co-supervisor. In addition, I wish to acknowledge Assoc. Professor Ian Maddox of the Institute of Technology and Engineering for providing valuable co-supervision and encouragement throughout the period of my research. My gratitude also extends to Professor Richard Archer, formerly of Fonterra Co-operative Group Limited, who never ceased to provide stimulating suggestions during our fortnightly meetings. I would also like to acknowledge Dr Vaughn Crow of Fonterra Co-operative Group Limited for helpful suggestions during the fortnightly meetings.

I am grateful to Massey University for the opportunity to pursue my doctoral study part-time while employed as a lecturer at the Institute of Food, Nutrition and Human Health. I would like to acknowledge the funding for the research from Fonterra Co-operative Group Limited and the New Zealand Foundation for Science and Technology through the Technology for Business Growth programme.

I would like to thank Dr Stephen Podzimek, Dr Adeline Lodder and Dr Michelle Chen of Wyatt Technology, USA, for their helpful suggestions in the use of the DAWN DSP via e-mail correspondence. A colleague and a friend who deserves special mention is Dr Yacine Hemar. His helpful advice in the area of rheological measurements is deeply appreciated.

Many wonderful people have assisted me in different ways with the research and completion of my doctoral thesis, and for whom I am indebted to: Ms Suzanne Bassett, Ms Michelle Curry, Mr Debjit Dey, Mr Steven Wang for providing technical help even beyond working hours; Mr Gary Radford for assisting in the operation of the UF plant and for being ever so willing to help in any way needed; Mr Byron McKillop for constructing any apparatus or tools needed for my experiments; Ms Michelle Tamehana for her patience and help with the light scattering experiments; Mr Doug Hopcroft and

Mr Raymond Bennett for assisting with processing of samples for electron microscopy; Dr Paul McJarrow for helping with the analysis of sugar composition with the Dionix; Ms Elizabeth Nickless for providing training in the operation of the confocal laser scanning microscope; Mr Peter Jeffery and Mr Bryden Zaloum who provided competent computer and software support; Ms Christine Ramsey, Ms Kathryn Tulitt, Ms Karen Pickering and Ms Yvonne Parkes for giving efficient and friendly administrative support. Mr Warwick Johnson, Mr Chris Hall, Mr Steve Glasgow, Ms Ann-Marie Jackson, Ms Felicity Jackson, Mr Michael Sahayam; Mr John Edwards; Mr Jon Palmer, Mr John Sykes and Mr Mike Stevens, all of whom have assisted me in so many ways which I deeply appreciate.

I would like to thank Mr Wong Shee Way, Mr Tang Mun Hay, Mr Jeffrey Heng, Mr Samuel Aw, Dr Ye Aiqian, Dr Rogerio Periera, Lydia Pedley, Cliff and Mary Foote for their friendship, humour and encouragements. These are only a few names, since I cannot adequately acknowledge all of the people to whom I am indebted. I hope the rest will know who they are and that I thank them very much.

Finally, I wish to acknowledge my family: mum, dad, sisters, brothers, in-laws, nephews and nieces for their prayers, love and concern.

Last, but not least, my special gratitude to my wife, Sang Mui for her love, companionship, understanding, patience and unceasing prayers. Without her support and encouragements, this thesis would not have been completed.

Above all, praise and glory to my Lord Jesus Christ, whose grace and faithfulness endure forever.

TABLE OF CONTENTS