

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Rheo-NMR Studies of Macromolecules

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

Master of Science in Physics

at Massey University, Palmerston North, New Zealand.

Motoko Kakubayashi

2008

Abstract

In this thesis, the effects of simple shear flow on macromolecular structure and interactions are investigated in detail via a combination of Nuclear Magnetic Resonance (NMR) spectroscopy and rheology, namely Rheo-NMR. A specially designed NMR couette shear cell and benchtop shear cell, developed in-house, demonstrated that the direct measurement of the above phenomena is possible.

First, to determine whether the shear cells were creating simple shear flow, results were reproduced from literature studies of liquid crystal systems which report shear effects on: Cetyl Trimethyl Ammonium Bromide (CTAB) in deuterium oxide, and Poly(γ -benzyl-L-glutamate) (PBLG) in m-cresol.

Next, the possible conformational changes to protein structure brought about by shear were investigated by applying shear to Bovine β -lactoglobulin (β -Lg). As the protein was sheared, a small, irreversible conformational change was observed by means of one-dimensional and two-dimensional ^1H NMR with reasonable reproducibility. However, no observable change was detected by means of light scattering. A large conformational change was observed after shearing a destabilized β -Lg sample containing 10% Trifluoroethanol (TFE) (v/v). From an NMR point of view, the sheared state was similar to the structure of β -Lg containing large amounts of α -helices and, interestingly, similar to the structure of β -Lg containing β -sheet amyloid fibrils. Gel electrophoresis tests suggested that the changes were caused by hydrophobic interactions. Unfortunately, this proved to be difficult to reproduce.

The effect of shear on an inter-macromolecular interaction was investigated by applying shear during an enzyme reaction of pectin methylesterase (PME) on pectin. Experimental method and analysis developments are described in detail. It was

observed that under the conditions studied, shear does not interfere with the de-esterification of pectin with two types of PME, which have different action mechanisms at average shear rates up to 1570 s^{-1} .

Acknowledgements

I would like to thank the following people who have helped me throughout this project:

My supervisors, Dr. Bill (M.A.K.) Williams, Dr. Pat Edwards, and Dr. Robin Dykstra, for their support, enthusiasm, and constantly encouraging me to do better work.

Terry Southern, Rinni Singh, and the mechanical workshop staff for developing, improving, and constant maintenance of the rheo-device and shear cell.

Grant Platt for cutting all of my glassware.

My fellow graduate students in physics and chemistry for all of their quality advice and support.

Biomaterials group members, especially Aurelie Cucheval for teaching me everything about pectin and PME.

All the staff at the Physics department for keeping me focussed and happy during my studies.

Raymond Raman for support and technical advice during my thesis write up.

My family and friends for putting up with me, and helping to create a comforting work environment during my thesis write up.

Contents

Title page	i
Abstract	ii
Acknowledgements	iv
Contents	v
List of Figures	viii
List of Tables	xii
1 Introduction	1
1.1 Introduction	1
1.2 Rheo-NMR.....	4
1.3 Rheology of Macromolecules	6
1.3.1 Bovine β -lactoglobulin.....	6
1.3.2 Pectin.....	7
1.4 Shear.....	8
1.4.1 Shear in a Couette cell.....	9
1.5 Nuclear Magnetic Resonance (NMR).....	10
1.5.1 Basic Principles of NMR	11
1.5.1.1 NMR.....	11
1.5.1.2 Chemical Shift.....	13
1.5.1.3 Bulk Magnetization	14
1.5.1.4 Pulses	15
1.5.1.5 Relaxation	15
1.5.1.6 Water Suppression.....	16
2 Experimental Method	18

2.1	Shear Cell	18
2.1.1	NMR Couette Shear Cell	18
2.1.2	Benchtop Shear Cell.....	24
2.2	Cetyl Trimethyl Ammonium Bromide (CTAB) in D ₂ O.....	25
2.2.1	Introduction	25
2.2.2	Experimental Method.....	26
2.2.3	Results and Discussion.....	27
2.2.4	Conclusion	30
2.3	Poly(γ -benzyl-L-glutamate) (PBLG) in m-cresol	30
2.3.1	Introduction	30
2.3.2	Experimental Setup	31
2.3.3	Results and Discussion.....	32
2.3.4	Conclusion	36
3	Bovine β-lactoglobulin	37
3.1	Introduction	37
3.2	Experimental Method.....	37
3.3	Results and Discussion.....	39
3.3.1	Initial NMR Spectra	39
3.3.2	Effect of Shear on Bovine β -lactoglobulin.....	41
3.3.2.1	1D ¹ H NMR	41
3.3.2.2	2D NMR Experiments	45
3.3.2.3	Light Scattering Tests.....	48
3.3.3	Effect of Shear on Destabilized β -Lg – Addition of Trifluoroethanol	49
3.3.3.1	Gel Electrophoresis Test.....	54
3.4	Conclusions	55

4	Enzyme Activity in Pectin.....	56
4.1	Introduction	56
4.1.1	Pectin.....	56
4.1.2	Pectin Methylesterase (PME).....	57
4.1.2.1	Plant Pectin Methylesterase (pPME)	59
4.1.2.2	Fungal Pectin Methylesterase (fPME)	59
4.2	Experimental Method.....	60
4.3	Results and Discussion.....	67
4.3.1	Initial NMR spectra.....	67
4.3.2	Control Experiments	68
4.3.2.1	Michaelis-Menten Model.....	71
4.3.2.2	Simultaneous Control Experiments.....	73
4.3.3	The Effect of Shear on the Enzyme Activity of Plant PME (pPME) on Pectin	76
4.3.3.1	Shearing Experiments using the NMR Couette Shear Cell	76
4.3.3.2	Shearing Experiments using the Benchtop Couette Shear Cell	78
4.3.3.2.1	Shearing Experiments at Higher Concentrations.....	81
4.3.4	The Effect of Shear on the Enzyme Activity of Fungal PME (fPME) on Pectin.....	82
4.4	Conclusions	84
5	Conclusions	85
5.1	Summary	85
5.2	Future Directions.....	87
	Appendix A	89
	Bibliography	92

List of Figures

Figure 1.1: Shear flow in a couette cell with the inner cylinder rotating with angular velocity ω , creating a velocity gradient across the annular gap.....	9
Figure 1.2: Energy levels for nucleus with spin quantum number $\frac{1}{2}$ and positive γ	12
Figure 1.3: Hydrogen atom in a magnetic field B_0 , causing the electron to circulate and generate an extra field B'	13
Figure 1.4: Example of the bulk magnetization vector acting along the +z-axis at equilibrium, in a Cartesian reference frame.....	15
Figure 1.5: Example of a bulk magnetization vector precessing about the +z-axis.....	15
Figure 2.1(a): Scale diagram of the NMR couette shear cell used in this project.....	19
Figure 2.1(b): NMR couette shear cell set inside 500 MHz NMR spectrometer.....	19
Figure 2.2: Cross-sectional view of NMR tube setups: (a) thin-walled 5mm tube and thin-walled 3mm inner tube, (b) medium-walled 5mm tube and thin-walled 3mm inner tube.....	20
Figure 2.3: Example of the shear rate calculator: shows average and maximum shear rates, as well as the shear rate across the annular gap.	22
Figure 2.4: Collars used in shearing tests: bottom collar in (a) simple ring design and (b) cup-ring design, and (c) top collar.....	23
Figure 2.5: (a) The benchtop couette shear cell setup, and (b) a cross-sectional view. ...	24
Figure 2.6: Control experiment of CTAB system under no shear in (a) isotropic, (b) - (d) isotropic and nematic, and (e) nematic phase.	28
Figure 2.7: Shearing experiment for CTAB system at $T_{I-N} + 1$ K: (a) reference spectrum under no shear, (b) under 120 s^{-1} shear, and (c) 10 min after shear cessation.....	29
Figure 2.8: PBLG sample in NMR spectrometer, the PBLG director is in same direction	

as magnetic field B	32
Figure 2.9: PBLG 14.02% shearing test: sample (a) under no shear, (b) under shear, and (c) after shear stopped for a few minutes.....	33
Figure 2.10: Theoretical plot of $\left 3\cos^2\theta - 1\right $ vs. angle made between PBLG director and magnetic field in the NMR spectrometer.	34
Figure 2.11: PBLG 12.54% shearing test: sample (a) under no shear, (b) under shear, and (c) after shear stopped for a few minutes.....	35
Figure 3.1: The β -Lg backbone structure, and a standard spectrum of β -Lg under no shear, recorded in a 500 MHz NMR spectrometer showing backbone amide protons, backbone α protons, and side chain protons $H_{side\ chain}$	40
Figure 3.2(a): Shearing experiment carried out on a β -Lg sample subjected to an average shear rates of 120 s^{-1} overnight, and compared with a spectrum of the sample prior to shear application.	41
Figure 3.2(b): Shearing experiment carried out on a β -Lg sample subjected to an average shear rate of 120 s^{-1} over 2 days, and compared with a spectrum of the sample prior to shear application.....	42
Figure 3.2(c): Shearing experiment carried out on a β -Lg sample subjected to an average shear rate of 120 s^{-1} over 4 days, and compared with a spectrum of the sample prior to shear application.....	43
Figure 3.3: Rate of conformational change in a β -Lg shearing experiment.	44
Figure 3.4(a): 2D TOCSY spectrum of 6 mM/g β -Lg.	46
Figure 3.4(b): TOCSY β -Lg structure assignment established by Uhrinova et al. [1]...	47
Figure 3.5: Fingerprint region overlay of 2D TOCSY spectra before and after shear application.	48
Figure 3.6: β -Lg containing various amounts of TFE (v/v), under no shear, at 298 K...	50

Figure 3.7: Shearing experiment on a β -Lg sample containing 10% TFE (v/v) under an average shear rate of 1570 s^{-1} over 2 days.	51
Figure 3.8(a): Comparison of the sheared β -Lg sample (containing 10% TFE (v/v) and sheared at 1570 s^{-1} for 2 days) with a control sample (containing 10% TFE (v/v)), and a reference β -Lg sample containing 20% TFE.....	52
Figure 3.8(b): Comparison of the sheared β -Lg sample (containing 10% TFE (v/v) and sheared at 1570 s^{-1} for 2 days) with a β -Lg sample known to contain amyloid fibrils.....	53
Figure 4.1: Linear chain of the smooth homogalacturonan region in pectin	57
Figure 4.2: The de-esterification of pectin, catalyzed by pectin methylesterase.	58
Figure 4.3: An example of pPME acting on a pectin chain over time.	59
Figure 4.4: An example of fPME acting on a pectin chain over time.....	60
Figure 4.5: Setup of sample preparation: syringe used to de-gas NMR sample, and a sonicator used on a low sonicating setting for 1 min to remove air bubbles.	64
Figure 4.6: NMR tube set up of control sample.....	65
Figure 4.7: NMR spectrum showing peaks from the methyl ester group COOCH_3 , methanol CH_3OH , and DSS, before and after pPME was added.....	68
Figure 4.8(a): Control experiment showing the rate of methanol liberated over time....	70
Figure 4.8(b): Rate of de-esterification (DM) over time corresponding to figure 4.8 (a).....	70
Figure 4.9(a): $1/V$ vs. $1/[S]$ plot of experimental data of pPME acting on pectin.	72
Figure 4.9(b): Theoretical Michaelis-Menten model derived using K_m and V_{max} from figure 4.9 (a), compared with experimental data for pPME acting on pectin.....	73
Figure 4.10: Amount of methanol liberated over time, with experiments conducted on different days.....	74
Figure 4.11: Control experiments showing the rate of methanol liberated over time,	

carried out at different temperatures: 303.2 K and 305.3 K.....	75
Figure 4.12(a): Control experiment of pPME and pectin under optimized protocol, with a control sample, and a sample in the NMR couette shear cell with no shear applied.	77
Figure 4.12(b): Shearing experiment with pectin and pPME sample sheared at 240 s^{-1} , and compared with a control sample that showed no change in kinetics.....	78
Figure 4.13: (a) Benchtop couette shear cell setup, and (b) close up of the sample waterbath being heated by water from a variable temperature waterbath.	79
Figure 4.14: Shearing experiment with pectin and pPME sample sheared at 1570 s^{-1} , and compared with a control sample that showed no change in kinetics.	81
Figure 4.15: Shearing experiment on a 5% pectin sample (5 times usual concentration) with an average shear rate of 1540 s^{-1} , compared with control sample that showed no change in kinetics.	82
Figure 4.16: Shearing experiment with an average shear rate of 240 s^{-1} applied to a pectin and fPME sample, and compared with control sample that showed no change in kinetics.	83
Figure A.1: Diagram of sample loaded in the annular gap between two cylinders, with the inner cylinder rotating with angular velocity ω	89

List of Tables

Table 2.1: Summary of possible NMR tube setups and shear rates using couette

shear cell.....	22
-----------------	----