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.

ASPECTS OF PROTEOLYSIS IN CHEESE

A Thesis presented in partial fulfilment of the requirements

for the degree

of Master of Philosophy

in Food Technology at Massey University

Christina June Coker

1994

ABSTRACT

The purpose of the present study was to elaborate methods for the detailed examination of proteolysis pathways in cheese (reviewed in Chapter 1) and to demonstrate their usefulness. Many techniques, including solvent fractionation, chromatographic separation and electrophoresis have been used previously and were revisited in this study.

Gel electrophoresis can be a powerful technique and was examined in detail. The methods investigated were: 1) a slab gel system using the apparatus of the E-C Apparatus Corporation and a polyacrylamide gel in a Tris-EDTA-borate buffer at alkaline pH and containing urea; 2) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide stacking and resolving gel with a discontinuous (Tris-chloride/Tris-EDTA-borate) buffer system that contained urea; 3) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide stacking and resolving gel and acetic acid-ammonium acetate buffers at acidic pH that contained urea; 4) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide gel with a stacking and resolving gel in Tris-HCl buffers containing sodium dodecyl sulphate (SDS) and a Tris-chloride-glycine electrode buffer.

The mini-slab alkaline urea polyacrylamide gel electrophoresis (PAGE) method was considered to be the most suitable for monitoring the loss of intact casein during cheese ripening. However, SDS-PAGE gave good resolution of para-κ-casein, β-lactoglobulin and α-lactalbumin and it could therefore be used for the analysis of cheese in which whey proteins have been incorporated or for monitoring the breakdown of para-κ-casein (Chapter 4) in cheese. Two-dimensional PAGE revealed the presence of more bands than were visible using any single method of electrophoresis. Traces of protein were found to lie beneath the α_{s1}-casein band and this explained why, even after considerable proteolysis, some α_{s1}-casein appeared to remain.

Storing cheese samples in such a way that there is a minimum of further change was examined using several different storage methods and temperatures, including storage as: freeze-dried powder at 4°C in the dark, frozen at -9, -16, -35, -75 and -100°C, and dissolved in 6 M urea solution and stored at 4 and -16°C. The trial ran for 6 months and involved the multiple sampling and detailed analysis of three Cheddar cheeses by reversed phase fast protein liquid chromatography (RP-FPLC) for the water-soluble fraction (WSF) and alkaline urea-PAGE for the protein fraction.

None of the methods used to store the cheese samples was completely satisfactory. Cheese stored at temperatures of -9 and -16°C was unstable, with proteolysis discernible after 66 days. Storage of cheese samples at these temperatures is, therefore, not recommended. Cheese stored at temperatures of -35, -75 and -100°C was unstable after 94 days, although the samples stored at -100°C were more stable. This lack of stability probably arose during thawing as well as during storage of the frozen cheese samples. Storage of freeze-dried samples at 4°C in the dark was equivalent to storing the frozen cheese at -100°C. Storage of samples in alkaline urea sample buffer was better at -16°C than at 4°C but should be for no longer than 1 month.

An indication of the differences to be expected within the normal range of Cheddar cheese was determined using three very similar Cheddar cheeses ripened at 5 and 13°C (Chapter 3, part II). Cheeses ripened at 5°C for 6 months were similar to those ripened at 13°C for 2 months and the proteolytic pathways appeared to the same at both temperatures.

The proteolytic pathways in Cheddar and Mozzarella cheeses, manufactured according to standard protocols, ripened at 13°C and sampled at regular intervals over a six month period were examined using a variety of techniques: total nitrogen (TN), non-protein nitrogen (NPN), water-soluble nitrogen (WSN), alkaline urea-PAGE, low molecular weight (LMW) SDS-PAGE, RP-FPLC and size exclusion high performance liquid chromatography (SE-HPLC). The TN and NPN analyses were done at the time of sampling whereas the other assays were done on samples that had been stored at $<$-75°C so that they could be analysed simultaneously.

The increase in WSN and NPN was greater in Cheddar cheese than in Mozzarella cheese and reflected the greater microbial enzyme activity in this cheese type.

Alkaline urea-PAGE revealed that there was more α_{s1}-casein hydrolysis (with the formation of α_{s1}-casein-I) in Cheddar cheese than in Mozzarella cheese, indicating that rennet activity was greater in Cheddar cheese. The presence of peptides believed to be β-I- (β-casein f1-189/192) and β-II-casein (β-casein f1-165) indicated that rennet may have hydrolysed β-casein. The amount of β-casein hydrolysis (and γ-casein formation) was greater in Mozzarella cheese, reflecting the greater plasmin activity in this cheese type. Both LMW SDS-PAGE and SE-HPLC of the whole cheese provided little additional information.

Examination of the WSF of each cheese by PAGE analysis showed that many of the

larger peptides may have been present in both cheese types. The different concentrations of these peptides in each cheese type were consistent with different rennet and plasmin activities and suggested that they may have been products of these enzymes. RP-FPLC and SE-HPLC analysis of the WSF of Cheddar cheese revealed that, although the larger peptides continued to accumulate during ripening, there was also a large increase in the amount of small peptides and amino acids in the cheese. In the Mozzarella cheese, the larger peptides accumulated and there was little evidence of their further hydrolysis to small peptides and amino acids.

The present studies indicate that SE-HPLC using a Toyo-Soda SW 2000 column and a 36% acetonitrile/0.1% trifluoroacetic acid solvent system is a promising new technique that may be useful in determining cheese type and maturity and in relating changes in the molecular weight distribution of the peptides to changes in the textural, functional and flavour characteristics of cheese.

It was concluded that the results are consistent with the concept that differences in the manufacture of Cheddar and Mozzarella cheeses result in the formation of two cheeses, each with different amounts of similar enzymes (rennet, plasmin, and the enzymes of the starter and non-starter lactic acid bacteria), and that these differences in enzyme concentration, combined with the modifying effect of pH, temperature, moisture content and S/M, result in different enzyme activities and patterns of proteolysis in the two types of cheese and these, in turn, result in cheeses with different functional properties.

ACKNOWLEDGEMENTS

I would like to thank Drs Mike Boland (Division Manager, New Zealand Dairy Research Institute (NZDRI)), Jeremy Hill (Section Manager, Food Science Section, NZDRI) and Lawrie Creamer, my NZDRI supervisor, for the opportunity to pursue this course of study. I am particularly grateful to Dr Creamer for his guidance and countless discussions on the results of this work.

I would like to thank Dr Terry Thomas, Craig Honoré, Keith Johnston and the Cheese Division of the New Zealand Dairy Board for the funding for this study.

I am very grateful to Dr Harjinder Singh, my Massey University supervisor, for planning a very useful course of study as well as for his guidance and useful discussion.

I wish to thank Professor Ray Winger (Professor of Food Technology, Massey University) for very helpful discussion on the problems associated with frozen storage of food products and for supplying literature related to this topic.

I would also like to thank Dr Graham Pritchard (Department of Biochemistry, Massey University) for very kindly supplying literature related to proteolysis in cheese.

I wish to thank Dr Paul McSweeney for kindly supplying a copy of his Ph.D thesis and Tanoj Singh for supplying extracts from his Ph.D thesis.

I would like to thank Drs Derek Knighton, Jeremy Hill, Rose Motion, Jeff Plowman, Don Otter, Kevin Pearce and Tim Coolbear of the NZDRI for helpful discussion and advice.

Special thanks must go to Mr Richard Burr for his patient instruction on the use of the FPLC and for his assistance with mechanical problems experienced during its operation.

I must also thank Mr Steve Boleyn for providing many of the cheese samples and Dr Philip Watkinson for supplying the cheese for the cheese ripening study.

I would like to thank Ms Marlene Tsao for her assistance with the seemingly endless weighing of cheese and with some of the gel electrophoresis in the cheese storage trial.

I would like to express my thanks to Ms Jan Wilkinson for analysing my samples on the HPLC (dedicated to producing molecular weight profiles) that she operated.

Thanks must also go to Mr Paul Le Ceve for developing the photographs and for coming to the rescue with his photographic expertise when a couple of the critical photographs were overexposed.

Thanks also to Dr M Reid, Campbell Microanalytical Laboratory, Department of Chemistry, University of Otago for Nitrogen analyses and to Errol Conaghan and the Analytical Chemistry Section of the NZDRI for providing the chemical analyses of the cheese.

Lastly, a very special thankyou must go to Neil and my children, Nicholas, Michael, Justin and Timothy for their tolerance, which was sorely tested, and for their support.

TABLE OF CONTENTS

CHAPTER 2 GEL ELECTROPHORESIS: A COMPARISON OF METHODS SELECTED TO STUDY PROTEOLYSIS IN CHEESE

CHAPTER 3 I. OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES DESTINED FOR QUANTITATIVE PEPTIDE AND PROTEIN ANALYSIS and II. THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

FIGURES

TABLES