UNIVERSITI PUTRA MALAYSIA

KESINAI (STREBLUS ASPER) PROTEASE AS A POTENTIAL MILK COAGULATING ENZYME

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FSMB 2000 6

KESINAI *(STREBLUS ASPER)* PROTEASE AS A POTENTIAL MILK COAGULATING ENZYME

By

YOUSIF MOHAMED AHMED IDRIS

Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

August 2000

This thesis is dedicated to

My father Mohamed Ahmed Idris,

My late mother Amina Ahmed Albasheer,

My wife Badria, and my children Nazim, Hala, Mawadda and Mohamed

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy.

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Chairman: **Dr. Mohd. Yazid Manap**

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Leaf extract of plant kesinai *(Streblus asper)* contains a milk coagulating protease, which could be a potential rennet substitute. However, its potential has not been investigated and the protease has not been purified and characterised. Preparation of the crude leaf extract results in an undesirable, very dark brown colour and inhibition of this browning may enhance the use of the leaf extract.

The browning inhibitors, citric acid, ascorbic acid, L-cysteine and sodium metabisulphite were used for prevention of browning and to obtain a crude extract with an acceptable colour. Metabisulphite was found to be an effective inhibitor of the enzymatic browning of the leaf extract. At 2 mM concentration it has inhibited browning and the extract obtained resulted in a white milk coagulum compared to the brown coloured coagulum of the brown extract. It is thermostable up to 85°C, with an optimum temperature at 70°C and its optimum pH is 7.2. Six mM added calcium chloride was optimum for its milk coagulation activity.

Microstructure, texture and syneresis of the milk coagulum of the crude extract were assessed by Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), the Texture Analyser, and measurement of whey volume, respectively and were compared with that of calf rennet and Fromase. Kesinai coagulum appeared as a sponge-like when examined under SEM, while calf rennet and Fromase coagulum appeared as a fibrous network. Quantification results showed that porosity of kesinai coagulum is low, and significantly different from both of calf rennet and Fromase coagulum ($P< 0.05$) and ($P< 0.01$), respectively. Kesinai coagulum was soft, and its strength is significantly lower than that of calf rennet and Fromase coagulum ($P< 0.01$). Syneresis of its coagulum was low, and the whey volume as per cent of milk volume was 34.75 % compared to 46.75% and 48.79%, for calf rennet and Fromase, respectively.

The ratio of milk coagulation activity to proteolytic activity of the extract was very low (0.02) and the protein profile of the milk coagulum and whey on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the protease was more proteolytic than calf rennet, and Fromase.

The protease was purified by ultrafiltration (UF), Fast protein Liquid Chromatography (FPLC) gel filtration with Superose 6, FPLC ion exchange using MonoQ HR 5/5 and Isoelectric Focusing (IEF) using the Rotofor system, with a puritification fold of 25, and 18% recovery. The purified protease appeared as a single band on SDS-PAGE with a molecular weight of 31.3 kDa. Characterisation of the

purified protease showed that it could be a serine protease with optimum pH of 7.2, stable in the pH range 5.0 –9.5, and its pI is 5.2. It is thermostable up to 85°C, with optimum temperature at 70°C. Zymogram analysis showed that protease activity is associated with milk coagulation activity.

It is concluded that kesinai protease could be used in the production of short ripened cheese varieties.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk Ijazah Doktor Falsafah

KESINAI (STREBLUS ASPER) PROTEASE SEBAGAI ENZIM PEGKOAGULASI SUSU YANG BERPOTENSI

Oleh

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Ekstrak dari daun pokok kesinai mengandungi protease pengkoagulasi susu yang berpotensi untuk menggantikan penggunaan rennet. Walau bagaimana pun, penggunaannya belum meluas kerana penulinan dan pencirian enzim ini belum lagi giat dijalankan. Kajian awal menunjukkan pengekstrakan enzim ini dari daun pokok kesinai memberikan ciri yang tidak digemari iaitu warnanya yang perang. Perencatan proses pemerangan diharapkan dapat meningkatkan lagi penggunann enzim ini.

Agen perencat pemerangan seperti asid sitrik, asid askorbik, L-cystein dan sodium metabisulphite telah digunakan untuk mencegah pemerangan keatas ekstrak mentah, seterusnya menghasilkan warna yang boleh diterima. Metabisulphite telah didapati berkesan jika dibandingkan dengan bahan kimia lain. Ia telah dapat merencat proses pemerangan pada kepekatan 2 mM dan menghasilkan susu terkoagulasi yang berwarna putih. Ekstrak mentah ternyahwarna kaya dengan bahan phenolic dan aktiviti pengkoagulasinya meningkat dengan penambahan $CaCl_2$ sehingga 6 mM. Ianya tahan haba sehingga 85°C dengan suhu dan pH optimumnya 70°C dan 7.2, masing-masing.

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Mikrostruktur, tekstur dan sineresis koagulum susu dengan ekstrak mentah telah ditentukan dengan menggunakan SEM, TEM, penganalisis tekstur, dan isipadu whey dengan susu telah dibandingkan dengan calf rennet dan Fromase. Koagulum susu yang dihasilkan menggunakan ekstrak kesinai mempunyai struktur seperti span apabila dilihat dibawah SEM, manakala koagulum susu yang dihasilkan menggunakan calf rennet dan Fromase mempunyai struktur jaringan berfilamen. Keputusan pengiraan menunjukkan bahawa keporositian adalah rendah, dan menunjukkan perbezaan yang ketara dengan calf rennet ($p<0.05$) dan Fromase ($p<0.01$). Koagulum dengan kesinai lembut dan kekenyalannya lebih rendah dari koagulum dengan calf rennert dan Fromase ($p<0.01$). Aktiviti sineresisnya juga rendah dan peratusan isipadu whey kepada isipadu susu adalah 34.75% berbanding dengan 46.75% dan 48.79% oleh calf rennet dan Fromase, masing-masing.

Profil protin koagulum dan whey yang dihasilkan dengan ekstrak kesinai telah dikaji menggunakan kaedah SDS-PAGE. Nisbah diantara aktiviti koagulasi kepada aktiviti proteolitik keatas susu adalah sangat rendah (0.02) dan profil protin koagulum dan whey menunjukkan ekstrak kesinai adalah lebih proteolitik berbanding calf rennet dan Fromase.

Protease telah ditulinkan menggunakan ultrafiltration (UF), Fast Protein Liquid Chromatogaphy (FPLC), gel filtration dengan Superose 6, FPLC ion exchange

menggunakan MonoQ HR 5/5 dan isoelectric focussing (IEF) menggunakan sistem Rotofor dengan peringkat penulinan 25 dan hasil 18%. Ekstrak kesinai yang tel;ah ditulenkan hanya memberikan satu jalur sahaja diatas SDS-PAGE dengan berat molekul 31.3 kDa. Pencirian keatas ekstrak kesinai yang telah ditulinkan menunjukkan bahawa ia mungkin jenis serine protease dengan pH optimum 7.2, stabil pada julat pH antara 5.0 – 9.5 dan pI nya 5.2. Ianya tahan haba sehingga 85°C dengan suhu optimum 70°C. Analisis zymogram menunjukkan bahawa aktiviti protease dan activiti coagulasi adalah berkaitan.

Sebagai kesimpulan, enzim protease dari ekstrak pokok kesinai berpotensi untuk digunakan sebagai pemangkin proses penghasilan variasi keju pematangan singkat.

ACKNOWLEDGEMENTS

I am indebted to my supervisors Dr. Mohd. Yazid Manap and Associate Professor Dr. Abdullah Sipat for guidance, advice, valuable discussions, encouragement, assistance and support throughout the period of my study at Universiti Putra Malaysia. I am also indebted to my supervisors Associate Professor Dr. Salmah Yusof and Dr. Mohd. Suria Affandi for their valuable comments and advice.

I am appreciative of the valuable assistance of Dr Menon K. Vidyadaran, Miss Azilah, Mr. Ho, Miss Suleka and Miss Faridah on Electron microscopy. I wish to thank members of Lab 202 (Department of Biochemistry and Microbiology, UPM), Mr. Murthi, Geok Yong, Ainu, Filza, Ong, Yadi and Aneela and members of Probiotics lab (Department of Food Technology, UPM), Suhaimi, Mariam, and Lim, for their cooperation. Thanks are also due to members of Enzymology lab (Department of Biotechnology, UPM), to all the staff of the Department of Biochemistry and Microbiology and the Department of Food Technology (UPM).

I am grateful to Sudan University of Science and Technology for the scholarship, which enabled me to study for this degree and to the Ministry of Science Technology and Environment, Government of Malaysia, For IRPA grant that financed this study.

To my wife Badria and my children Nazim, Hala, Mawadda and Mohamed, I am indebted for their love, forbearance and support throughout the period of studies.

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LISAT OF ABBREVIATIONS

Abbreviation	
AU	Absorbance unit.
β-CD	β-Cyclodextrin.
BDMA	n-Benzyldimethylamine
BSA	Bovine serum albumin.
CCP	Colloidal calcium phosphate.
DDSA	Dodoenyl Succinic Anhydride
DHAA	Dehydroascorbic acid.
DIECA	Diethyldithiocarbamate.
DMSO	Dimethylsulphoxide.
DOPA	3,4-dihydroxyphenylalanine.
EDTA	Ethylene diamine tetra acetic acid.
FAO	Food and Agriculture Organization.
FASEB	The Federation of American Societies for Experimental Biology.
FDA	Food and Drug Administration.
FPLC	Fast Protein Liquid Chromatography.
GRAS	Generally Regarded As Safe.
IDF	International Dairy Federation.
IEF	Isoelectric Focusing.
kda	Kilo Dalton.
MCA	Milk coagulation activity.
MNA	Methyl nadic anhydride

MWCO	Molecular weight Cut-off.
O.D	Optical Density.
p-CMBA	para-Chloromercuriobenzoic acid.
pI	Isoelectric point.
PMSF	Phenylmethyl sulphonyl fluoride.
PPO	Poplyphenol oxidase.
PVP	Polyvinylpyrrolidone.
PVPP	Polyvinylpolypyrrolidone.
R_f	Relative mobility.
SAPP	Sodium acid pyrophosphate
SAS	Statistical Analysis System.
SDS	Sodium dodecyl sulphate.
SDS-PAGE	Sodium dodecyl sulphate polyacryl amide gel electrophoresis.
SEM	Scanning Electron Microscopy.
SHMP	Sodiumhexametaphosphate.
TCA	Trichloroacetic acid.
TEM	Transmission Electron Microscopy.
TEMED	N, N', N'-Tetramethyl-ethylene diamide.
Tris	Tris (hydroxymethyl) aminomethane.
UF	Ultrafiltration.

CHAPTER I

INTRODUCTION

Proteases are enzymes that degrade proteins by hydrolysis of peptide bonds. They play an important role in the life cycle of proteins in the cell. They are investigated in fields such as protein chemistry and engineering as well as for applied purposes. Practical uses of proteolytic enzymes are in medicine, softening of leather, laundry detergents and food processing. Food industry uses proteases as processing aids for many products including baked goods, beer and wine, cereals, milk, meat tenderisation, fish products, legumes and for production of protein hydrolysates and flavour extracts (Stefensson, 1988; Haard, 1990; Haard and Simpson, 1994). Among the proteases used in food processing are the milk-clotting enzymes for cheese production. This thesis describes a protease from kesinai (*Streblus asper*) plant, with the potential of being a rennet substitute.

World cheese production amounts to approximately $1{,}4x10^7$ tonnes per annum and is growing at a rate of 2.5% annually (Guinee and Wilkinson, 1992). The milk coagulant traditionally used for cheese making in most parts of the world is the rennet extracted from the abomasa of 10 to 30-day-old milk-fed calves. Rennet is also required for the manufacture of rennet casein. The declining supply of calves for slaughter and the resulting chronic shortages and price increases fuelled the search for alternative rennet sources. This also led to the introduction of gastric proteinases and microbial-derived proteinases from *Endothia parasitica*, *Mucor pusillus* and *Mucor miehei*, in the United States in the 60s (Nelson, 1975).

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There are many organisms from which milk-coagulating enzymes can be extracted, including plants. Some of the plants, which are investigated as potential sources of rennet substitutes, include Cardo flowers (De Sa and Barbosa, 1972), Sodom apple leaves (Aworh and Muller, 1987) and Jubbain berries (Mohamed and Habbani, 1996).

In Malaysia the leaf extract of plant *Streblus asper* (Kesiani) is reported to contain a milk coagulating factor, which could be a potential rennet substitute (Manap et al., 1992). However, its potential as rennet substitute has not been investigated, and this study aims to achieve this end.

Literature review for this study will cover, chemistry of milk, milk coagulation mechanism, and the milk coagulating enzymes, rennet and rennet substitute, proteases and kesinai (*Streblus asper)* plant. The topics of enzymatic browning of plant extract , and methods of enzyme purification will also be covered.

Research Objectives

The overall aim of this study is to evaluate the suitability of *Streblus asper* protease as a rennet substitute. The specific objectives of the study are:

1- To find a means to inhibit the enzymatic browning of the leaf extract of kesinai