



UNIVERSITI PUTRA MALAYSIA

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

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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.

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

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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 purification 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 penggunaann 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.

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.

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 rennet 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 dituliskan menggunakan ultrafiltration (UF), Fast Protein Liquid Chromatography (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 telah dituliskan hanya memberikan satu jalur sahaja diatas SDS-PAGE dengan berat molekul 31.3 kDa. Pencirian keatas ekstrak kesinai yang telah dituliskan 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 aktiviti coagulasi adalah berkaitan.

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

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TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL SHEETS	x
DECLARATION FORM	xii
LIST OF TABLES	xvii
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xxi
 CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	4
2.1 Milk	4
2.1.1 Casein and Casein Micelles	4
2.1.2 Calcium Binding to Caseins	8
2.1.3 Milk Coagulation Mechanism	9
2.1.4 Syneresis	13
2.2 Rennets.	15
2.2.1 Calf Rennet.	15
2.2.2 Criteria for Calf Rennet Substitutes	18
2.2.3 Animal Rennets	19
2.2.4 Microbial Rennets.	20
2.2.5 Plant Rennets.	21
2.3 Proteases	23
2.4 Plant <i>Streblus asper</i> (kesinai)	28
2.4.1 Habitat.	28
2.4.2 Traditional Uses.	28
2.4.3 Potential Novel Uses	30
2.5 Extraction of Enzymes.	30
2.6 Enzymatic Browning.	32
2.6.1 Causes of Enzymatic Browning	33
2.6.2 Methods of Inhibition of Enzymatic Browning..	35
2.7 Purification of Proteases.	44
2.7.1 Purification Methods	45
2.7.2 Monitoring the Purification of enzymes	49
2.8 Gaps in <i>Streblus asper</i> Research	50
III GENERAL MATERIALS AND METHODS	
3.1 Materials	51
3.1.1 Plant Material	51

3.1.2 Milk	51
3.1.3 Chemicals	51
3.2 Methods	51
3.2.1 Preparation of <i>Streblus asper</i> Leaf Extract.	51
3.2.2 Ultrafiltration (UF)	51
3.2.3 Protease activity Assay	51
3.2.4 Milk Coagulation Activity Assay	53
3.2.5 Protein Determination	54
IV EXTRACTION AND INHIBITION OF THE ENZYMATIC BROWNING OF THE CRUDE LEAF EXTRACT	
4.1 Introduction	55
4.2 Materials and Methods	56
4.2.1 Plant Materials and Chemicals	56
4.2.2 Preparation of the Crude Leaf Extract.	57
4.2.3 Effect of Extraction Time on Properties of the Crude Extract	57
4.2.4 The Effect of Extraction Buffer pH on Properties of the Crude Extract	57
4.2.5 Ultrafiltration (UF)	58
4.2.6 Total Phenols	58
4.2.7 Measurement of Colour of the Crude Leaf Extract	58
4.2.8 Protease assay, Milk Coagulation Activity and Protein Content	59
4.2.9 Polyphenol Oxidase (PPO) Activity	59
4.2.10 The Effect of 10 mM Each of Citric Acid, Ascorbic Acid, L-cysteine and Metabisulphite on Browning of Crude Extract	59
4.2.11 Determination the Minimum Threshold of Inhibition of Enzymatic Browning by L-cysteine	60
4.2.12 Dialysis of the Decolourised Crude Extract.	60
4.2.13 Effect of Storage at Room Temp. and 4°C on Proteolytic Activity and Milk Coagulation Activity.	61
4.2.14 Effect of Added Calcium Chloride on Milk Coagulation Activity	61
4.2.15 The Effect of Temperature on Proteolytic Activity and Milk Coagulation Activity of the Crude Protease.	62
4.2.16 Temperature Stability of the Crude Extract	62
4.2.17 The Effect of pH on Proteolytic Activity and Milk Coagulation activity of the crude extract.	62
4.2.18 Effect of Crude extract Concentration on Milk Coagulation Time	63
4.2.19 Milk coagulation activity to proteolytic activity ratio.	63
4.3 Results	64
4.3.1 The Effect of Extraction Time on Properties of the Crude Leaf Extract	64
4.3.2 Effect of Extraction Buffer pH on Properties of the Crude Leaf Extract	66
4.3.3 Effect of 10mM Each of Citric Acid, Ascorbic Acid, L-cysein and metabisulphite on browning of the crude extract.	69
4.3.4 Minimum Threshold for Inhibition of Enzymatic Browning by L-cysteine and metabisulphite	71

4.3.5	The Effect of Dialysis on Colour, Proteolytic Activity and Milk coagulation activity of the crude extract.	77
4.3.6	The effect of Storage at Room Temp. and 4°C on Proteolytic Activity and Milk Coagulation Activity of the Crude Extract	78
4.3.7	Effect of Added Calcium Chloride on Milk Coagulation Activity.	82
4.3.8	Optimum Temperature for Proteolytic Activity and Milk Coagulation Activity of the Crude Extract.	84
4.3.9	Temperature Stability of the Crude Leaf Extract.	84
4.3.10	The Effect of Buffer and Milk pH on Proteolytic Activity and Milk Coagulation Activity.	87
4.3.11	Effect of Crude Extract Concentration on Milk Coagulation Time.	90
4.3.12	The Milk Coagulation Activity to Proteolytic Activity Ratio of the Crude Leaf Extract.	90
4.4	Discussions	93
4.5	Summary	103
V	PHYSIOCHEMICAL PROPERTIES OF THE MILK COAGULUM OF THE CRUDE LEAF EXTRACT	
5.1	Introduction	105
5.2	Materials and Methods	106
5.2.1	Preparation of Enzyme Samples.	106
5.2.2	Preparation of the Milk Coagulum.	107
5.2.3	Preparation of Samples for SEM and TEM Examination.	107
5.2.4	Quantification of Coagulum Porosity.	109
5.2.5	Texture Measurements.	110
5.2.6	Measurement of Syneresis.	112
5.2.7	Electrophoretic Profile of Milk Coagulum and Whey Proteins.	112
5.2.8	Statistical Analysis	113
5.3	Results.	114
5.3.1	Microstructure.	114
5.3.2	Quantification of Coagulum Porosity.	114
5.3.3	Testure and Syneresis of the Milk Coagulum.	117
5.3.4	SDS-PAGE Protein Profile of Milk Coagulum and Whey.	121
5.4	Discussion	126
5.5	Summary	130
VI	PURIFICATION AND CHARACTERISATION OF STREBLUS ASPER PROTEASE	
6.1	Introduction	132
6.2	Materials and Methods	133
6.2.1	Materials	133
6.2.2	Preparation of Crude Leaf Extract	134
6.2.3	Ultrafiltration	134

6.2.4. Purification Procedure.	134
6.2.5 Polyacrylamide Gel Electrophoresis	136
6.2.6 Zymogram.	137
6.2.7.Characterisation of the Purified Protease.	137
6.3 Results.	141
6.3.1 Purification results	141
6.3.2 Characterisation Results	148
6.4 Discussion	158
6.5 Summary	161
VII SUMMARY AND CONCLUSIONS	
7.1 Summary	163
7.2 Conclusions	166
7.3 Recommendation	169
REFERENCES	170
APPENDICES	186
BIO DATA	188

List of Tables

Table	Page
Table 2.1: World whole cow's milk and cheese production	15
Table 2.2: Some general properties of the four classes of proteases.	24
Table 2.3: Representative inhibitors of enzymatic browning.	36
Table 2.4: Protein purification methods	46
Table 4.1: The effect of blending time on colour, total phenols, protease activity and milk coagulation activity of crude leaf extract	65
Table 4.2: The effect of extraction buffer pH on colour, protease activity and milk coagulation activity of crude leaf extract.	67
Table 4.3: The effect of 10mM each of ascorbic acid, citric acid, L-cysteine and sodium metabisulphite on browning of crude leaf extract	70
Table 4.4: The effect of L-cysteine at various concentrations on browning of crude leaf extract.	73
Table 4.5: The effect of sodium metabisulphite at various concentrations on browning of crude leaf extract.	74
Table 4.6: The effect of dialysis on stability of colour of the crude leaf extract	77
Table 4.7: The ratio of milk coagulation activity to proteolytic activity of crude leaf extract at pH 6.7 assayed at 37°C and 70°C	92
Table 5.1: Assessment of porosity of the milk coagulum of kesinai, calf rennet and Fromase using SEM micrographs.	118
Table 5.2: Assessment of porosity of the milk coagulum of kesinai, calf rennet and Fromase using TEM micrographs.	119
Table 5.3: Assessment of texture of kesinai, calf rennet and Fromase coagulum	120
Table 5.4: Whey volume of kesinai, calf rennet and Fromase milk coagulum	120
Table 6.1: Summary of purification of protease from crude leaf extract	145
Table 6.2: The effect of metal ions on proteolytic activity of the purified protease	156
Table 6.3: The effect of protease inhibitors on proteolytic activity of the protease.	157

LIST OF FIGURES

Figure	Page
Figure 2.1: Coat-core model of casein micelles (Pyenes, 1966)	6
Figure 2.2: Internal structure model of casein micelles (Garnier and Ribadeau-Dumas, 1970)	6
Figure 2.3: Casein sub-micelle model proposed by Schmidt (1982)	7
Figure 2.4: Casein sub-micelle model proposed by Walstra (1984)	7
Figure 2.5: Schematic diagram of the attack by chymosin on casein micelles	12
Figure 2.6: <i>Streblus asper</i> (Kesinai) Tree	29
Figure 2.7: <i>Streblus asper</i> (Kesinai) Leaves	29
Figure 2.8: Scheme of initiation of enzymatic browning by polyphenol oxidase (McEvily et al., 1992)	34
Figure 2.9: Scheme for proposed mechanism of the inhibition of polyphenol oxidase by metabisulphite (Valero et al., 1991)	38
Figure 2.10: The mode of action of sulfhydryl compounds in the inhibition of enzymatic browning (McEvily et al., 1992)	40
Figure 4.1: Typical <i>Streblus asper</i> brown extract.	68
Figure 4.2: Decolourised (A) and brown (B) <i>Streblus asper</i> crude leaf extract	75
Figure 4.3: Milk coagulum produced by brown (A) and decolourised (B) <i>Streblus asper</i> crude extract	76
Figure 4.4: The effect of storage at room temperature on proteolytic activity of decolourised and brown leaf extract.	80
Figure 4.5: The effect of storage at 4°C on proteolytic activity of decolourised and brown leaf extract.	81
Figure 4.6: The effect of added calcium chloride concentration on milk coagulation time of crude extract.	83
Figure 4.7: Optimum temperature for proteolytic activity and milk coagulation activity of crude leaf extract	85

Figure4.8: Temperature stability of crude leaf extract.	86
Figure4.9: Optimum pH for proteolytic activity of crude leaf extract	88
Figure4.10: The effect of milk pH on milk coagulation activity of crude leaf extract.	89
Figure4.11: The effect of crude leaf extract concentration on milk coagulation time	91
Figure 5.1: Test Grid superimposed on a TEM micrograph	111
Figure 5.2: SEM micrograph of Kesinai milk coagulum (x7, 000).	115
Figure 5.3: SEM micrograph of Calf rennet milk coagulum (x7, 000)	115
Figure 5.4: SEM micrograph of Fromase milk coagulum (x7, 000)	115
Figure 5.5: TEM micrograph of Kesinai coagulum (x30, 000).	116
Figure 5.6: TEM micrograph of calf rennet milk coagulum (x30, 000).	116
Figure 5.7: TEM micrograph of Fromase milk coagulum (x30, 000).	116
Figure 5.8: SDS-PAGE of the milk coagulum of kesinai extract and calf rennet	122
Figure 5.9: SDS-PAGE of the milk coagulum of kesinai extract and Fromase	123
Figure 5.10: SDS-PAGE of the whey of kesinai extract and calf rennet	124
Figure 5.11: SDS-PAGE of the whey of kesinai extract and Fromase	125
Figure 6.1: Elution profile of the protease on Superose-6	142
Figure 6.2: Protein elution profile of the protease on Mono Q HR 5/5	143
Figure 6.3: IEF profile of the protease.	144
Figure 6.4: SDS-PAGE of the purified protease.	146
Figure 6.5: Zymogram of the purified protease.	147
Figure 6.6: Estimation of the molecular weight of the protease by SD-PAGE.	149
Figure 6.7: Optimum pH of the purified protease.	150
Figure 6.8: pH stability of the purified protease.	152

Figure6.9: Optimum temperature for proteolytic activity of the purified protease. 153

Figure6.10: Temperature stability of the purified protease. 154

LISAT OF ABBREVIATIONS**Abbreviation**

AU	Absorbance unit.
β-CD	β-Cyclodextrin.
BDMA	n-Benzyl dimethylamine
BSA	Bovine serum albumin.
CCP	Colloidal calcium phosphate.
DDSA	Dodecyl 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.
<i>p</i> -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 (Stefansson, 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 $1,4 \times 10^7$ 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).



proteinases from *Endothia parasitica*, *Mucor pusillus* and *Mucor miehei*, in the United States in the 60s (Nelson, 1975).

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