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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERISATION OF A PROTEASE EXTRACTED FROM KESINAI (StreblusasperLour.) LEAVES, AND ITS POTENTIAL APPLICATION IN THE PRODUCTION OF CHEDDAR CHEESE

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy.

January 2011

DEDICATION

This thesis is dedicated to

My father Kandia Mylvaganam

My mother Mylvaganam Paramesvari

My wife Pagthinathan Kumuthini

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

EXTRACTION, PARTIAL PURIFICATION AND CHARACTERISATION OF A PROTEASE EXTRACTED FROM KESINAI (*Streblus asper* Lour.) LEAVES, AND ITS POTENTIAL APPLICATION IN THE PRODUCTION OF CHEDDAR CHEESE

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Chair : Hasanah Mohd Ghazali, PhD

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Calf-rennet is a conventional milk-clotting enzyme, which is most widely used coagulant in cheese-making all over the world. The world wide reduced supply of calf rennet and increase of cheese production have led to a systematic investigation of calf-rennet substitutes. Extracts of 'kesinai' (*Streblus asper*) leaves have been investigated as a source of enzymes to be used in cheese-making as an alternative source of calf rennet. The overall aim of this study was to evaluate the properties and application of *Streblus asper* leaf protease as a rennet substitute in cheese-making.

A milk-clotting protease was extracted from kesinai leaves using 100 mM Tris-HCl buffer (pH 7.4) and several other buffers. Purification was carried out using acetone precipitation, and ion-exchange and size-exclusion chromatography. Results obtained showed that 100 mM Tris-HCl buffer (pH 7.4) was found to be the most effective

extraction buffer. A higher yield of the kesinai protease was obtained using acetone precipitation as compared to ammonium sulphate precipitation. The ratio of cold acetone to crude extract of 1.25:1 was found to be the most suitable ratio for the partial purification of the protease. After the final purification step, the enzyme was partially purified 3.3 fold with a 42.3% of recovery.

The partially purified protease was characterised and it showed an optimum activity at 60 ^{o}C and pH 7.4. The enzyme was stable up to 70 ^{o}C for one hour, and residual activities at pH 6.0 to 10.0 were 72.4% and 70.2% of the optimum activity, respectively. The enzyme was found to have a higher temperature stability at -10 ^{o}C and 4 ^{o}C. It retained more than 98% of its activity 7 days after storage at both -10 and 4^{0}C. The enzyme was inhibited by PMSF and trypsin inhibitor by 98% and 95.87% relative to the initial activity, respectively, suggesting the presence of serine residue at the active site. Ca^{2+} had a slight stimulating effect while, Hg^{2+}, Zn^{2+} and Pb^{2+} had strong inhibitory effects on the enzyme activity. The partially purified enzyme appeared as a single band on SDS PAGE with an apparent molecular weight of 75.8 kDa.

The maximum coagulation activity was observed at pH 6.0 and at temperature 70 ^{o}C. The presence of $CaCl_2$ up to 10 mM increased the milk-clotting activity while addition of NaCl decreased the milk-clotting activity. The milk-coagulation was also strengthened by increase enzyme concentration

Cheddar cheese was prepared using the acetone-precipitated enzyme and commercial enzyme (chymosin), and the physico-chemical, biochemical and sensory properties

were determined throughout two months of ripening. The yield of cheese produced using the commercial enzyme was 6% higher ($p<0.05$) than the yield obtained with kesinai enzymes. Cheeses made from kesinai enzyme and commercial enzyme were proximate compositionally alike except for moisture, protein contents and pH. The moisture content (42.67 ± 0.47%) of cheese made with commercial enzyme was lower than the moisture (43.88 ± 0.99%) of cheese made with kesinai enzyme, while the protein content (21.02 ± 0.41%) and pH (4.79 ± 0.03) were ($p<0.05$) higher in cheese made with commercial enzyme than the protein content (18.87 ± 0.32%) and pH (4.72 ± 0.01) obtained from cheese made from kesinai enzyme. In both cheeses, the moisture contents declined ($p<0.05$) with ripening, while the pH increased with ripening time. Non protein nitrogen (NPN) (0.04 ± 0.00%) and non-casein nitrogen (NCN) (0.09 ± 0.00%), were higher ($p<0.05$) in the whey obtained with kesinai enzyme compared to those (NPN and NCN were 0.03 ± 0.00% and 0.06 ± 0.00%, respectively), obtained with the commercial enzyme. Protein nitrogen (PN) (2.75 ± 0.06%) and casein nitrogen (CN) (2.56% ± 0.05) contents of cheese made with kesinai enzyme were ($p<0.05$) lower than those (CN and TN were 2.93 ± 0.11% and 3.29 ± 0.12%, respectively), made with the commercial enzyme. In general, all NPN and NCN values were found to increase ($p<0.05$) throughout the ripening period in both cheeses. Cheese made with the commercial enzyme exhibited a slightly higher level of free amino acids (27.06 ± 0.64 mg per g of cheese) compared to that of cheese made with kesinai enzyme (26.57 ± 2.05 mg per g of cheese). The principal component analysis (PCA) using a zNose® (Electronic Sensor Technology Co., Newbury Park, CA, USA) indicated the presence of seven volatile (aroma) compounds that are common in both cheeses. PCA analysis was performed to discriminate the cheeses in terms of aroma with their ripening time. Textural

characteristics such as hardness (811.8 ±36.6 g for cheese made from kesinai enzyme and 984.5 ± 24.2 g for cheese made from commercial enzyme), gumminess (622.8 ± 39.2 g for cheese made from kesinai enzyme and 820.9 ± 20.9 g for cheese made from commercial enzyme), and chewiness (534.7 ± 17.1 g mm for cheese made from kesinai enzyme and 735.3 ± 14.6 g mm for cheese made from commercial enzyme), increased in both types of cheese during the early stage of ripening and decrease gradually at end of ripening whereas springiness and cohesiveness showed similar changes in both cheeses during the ripened period.

It is concluded that kesinai protease could be used for the production of Cheddar cheese or it can be used together with another commercial enzyme in cheese production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGEKSTRAKAN, PENULENAN SEPARA DAN PENCIRIAN PROTEASE DARIPADA DAUN KESINAI (*Streblus asper* Lour.), DAN POTENSI PENGGUNAANNYA DALAM PENGELUARAN KEJU CEDAR

Oleh

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‘Calf-rennet’ ialah enzim pengkoagulasi susu konvensional, yang digunakan secara meluas sebagai koagulan dalam pembuatan keju di seluruh dunia. Kekurangan bekalan ‘calf-rennet’ di seluruh dunia dan peningkatan penghasilan keju telah membawa kepada kajian sistematik pengganti ‘calf-rennet’. Ekstrak daun ‘kesinai' (*Streblus asper*) telah dikenalpasti sebagai sumber enzim yang digunakan dalam penghasilan keju sebagai sumber alternatif ‘calf-rennet’. Objektif keseluruhan kajian ini ialah untuk menilai kesesuaian protease *Streblus asper* sebagai pengganti rennet dalam pembuatan keju.

Protease pengkoagulasi-susu telah diekstrak dari daun kesinai menggunakan 100 mM penimbal Tris-HCl (pH 7.4) dan beberapa larutan penimbal lain. Penulenan telah dijalankan menggunakan pemendakan aseton, kromatografi penukaran ion dan kromatografi ‘size-exclusion’. Keputusan menunjukkan bahawa 100 mM penimbal Tris-HCl (pH 7.4) telah didapati menjadi penimbal pengekstrakan paling berkesan.

Hasil protease kesinai yang lebih tinggi telah diperolehi melalui pemendakan aseton berbanding pemendakan ammonium sulfat. Nisbah aseton sejuk dan ekstrak mentah iaitu 1.25:1 telah didapati menjadi nisbah yang paling sesuai untuk penulenan separa protease. Selepas langkah terakhir penulenan, enzim ditulenkan dengan 3.3 lipatan penulenan dan menghasilkan 42.3 % perolehan.

Enzim separa tulen telah dicirikan dan ia mempamerkan suhu optimum 60°C dan pH 7.4. Enzim stabil sehingga 70°C selama satu jam, dan aktiviti residual pada pH 6.0 dan 10.0 dengan aktiviti optimum 72.4% dan 70.2%, masing-masing. Enzim telah didapati mempunyai kestabilan suhu yang tinggi pada -10°C dan 4°C. Ia mengekalkan lebih dari 98% aktivitinya selepas 7 hari penyimpanan pada -10°C dan 4^{0}C. Enzim telah direncat oleh PMSF dan perencat tripsin dengan 98% dan 95.87% relatif terhadap aktiviti permulaan, masing-masing, mencadangkan kehadiran residu serin pada tapak aktif. Ca^{2+} mempunyai kesan rangsangan lemah manakala, Hg^{2+}, Zn^{2+} dan Pb^{2+} mempunyai kesan rencatan kuat pada aktiviti enzim. Enzim separa tulen muncul sebagai satu jalur tunggal pada SDS PAGE dengan berat molekul ketara 75.8 kDa.

Ciri-ciri pengkoagulasi susu bagi kesinai protease mentah, protease separa tulen dan rennet komersial telah dikaji. Aktiviti koagulasi sangat bergantung kepada pH dan suhu susu, dan aktiviti koagulasi maksimum telah didapati pada pH 6 dan 70°C. Kehadiran $CaCl_2$ sehingga 10 mM meningkatkan aktiviti pengkoagulasi-susu manakala penambahan NaCl menurunkan aktiviti pengkoagulasi-susu. Pengkoagulasian susu juga dipertingkatkan dengan penambahan kepekatan enzim.

Keju cedar telah disediakan dengan menggunakan enzim hasil pemendakan aseton dan enzim komersial (chymosin), seterusnya ciri-ciri fiziko-kimia, biokimia dan sensori telah ditentukan sepanjang dua bulan tempoh pematangan keju cedar. Hasil perolehan keju yang disediakan dengan menggunakan enzim komersial adalah 6% lebih tinggi ($p<0.05$) berbanding keju yang dihasil dengan menggunakan enzim kesinai. Komposisi bagi kedua-dua keju ini adalah sama kecuali kelembapan, kandungan protein dan pH. Kandungan kelembapan (42.67 ± 0.47%) keju yang dibuat menggunakan enzim komersial adalah lebih rendah daripada kandungan kelembapan (43.88 ± 0.99%) keju dibuat menggunakan enzim kesinai, manakala kandungan protein (21.02 ± 0.41%) dan pH (4.79 ± 0.03) adalah lebih tinggi dalam keju yang dibuat menggunakan enzim komersial berbanding kandungan protein (18.87 ± 0.32%) dan pH (4.72 ± 0.01) diperolehi dalam keju yang dibuat daripada enzim kesinai. Pada kedua-dua keju, kandungan kelembapan adalah berkurangan ($p<0.05$) dengan masa pematangan, manakala nilai pH pula bertambah dengan masa pematangan. Kandungan nitrogen bukan-protein (NPN) (0.04 ± 0.001%) dan nitrogen bukan-kasein (NCN) (0.09 ± 0.001%), adalah lebih tinggi ($p<0.05$) dalam 'whey' yang diperolehi dengan enzim kesinai berbanding dengan enzim komersial (NPN dan NCN ialah 0.03 ± 0.002% dan 0.06 ± 0.002%, masing-masing). Kandungan nitrogen protein (PN) (2.75 ± 0.06%) dan nitrogen kasein (CN) (2.56% ± 0.05) dalam keju yang dibuat menggunakan enzim kesinai adalah ($p<0.05$) lebih rendah daripada enzim komersial (CN dan TN ialah 2.93 ± 0.11% dan 3.29 ± 0.12%, masing-masing). Secara keseluruhannya, nilai NPN dan NCN meningkat ($p<0.05$) sepanjang tempoh pematangan dalam kedua-dua keju. Keju yang dibuat menggunakan enzim komersial menunjukkan tahap asid amino bebas (27.06 ± 0.64 mg per g keju) yang tinggi sedikit berbanding dengan keju dibuat menggunakan

enzim kesinai (26.57 ± 2.05 mg per g keju). Perubahan pada kandungan asid lemak bebas adalah sama bagi kedua-dua keju tersebut semasa tempoh pematangan. Analisis komponen utama (PCA) menggunakan zNose® (Electronic Sensor Technology Co, Newbury Park, CA, USA) menunjukkan kehadiran tujuh sebatian meruap (aroma) yang biasa dijumpai dalam kedua-dua keju tersebut. Analisis PCA dijalankan bertujuan untuk membezakan aroma bagi kedua-dua keju semasa pematangan berlaku. Ciri-ciri tekstur seperti kekerasan (811.8 ± 36.6 g untuk keju dibuat daripada enzim kesinai dan 984.5 ± 24.2 g untuk keju dibuat daripada enzim komersial), kebergetahan (622.8 ± 39.2 g untuk keju dibuat daripada enzim kesinai dan 820.9 ± 20.9 g untuk keju dibuat daripada enzim komersial), dan kekenyalan (534.7 ± 17.1 g mm untuk keju dibuat daripada enzim kesinai dan 735.3 ± 14.6 g mm untuk keju dibuat daripada enzim komersial) bertambah dalam kedua-dua jenis keju semasa peringkat awal pematangan dan berkurang secara beransur-ansur pada peringkat akhir pematangan, manakala keanjalan dan kekohesifan menunjukkan perubahan yang sama dalam kedua-dua keju semasa tempoh matang.

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I certify that a Thesis Examination committee has met on 13th of January, 2011 to conduct the final examination of Mylvaganam Pagthinathan on his thesis entitled “Extraction, partial purification and characterisation of a protease extracted from kesinai (*streblus asper*) leaves, and its potential application in the development of Cheddar cheese ” in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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MYLVAGANAM PAGTHINATHAN

Date: 13th January 2011

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