UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

PUTRA SCIENCE PARK
PUTRA SCIENCE PARK

Ruj. Kami: UPM/100-45/2 (A) MY149878A

Tarikh: 17 Mac 2014

Prof. Dato' Dr. Mohd Yazid Abd. Manap,
Dekan,
Fakulti Sains Dan Teknologi Makanan, UPM

Y. Bhg. Dato',

Salinan Sijil Paten bagi "Extract having Protease Activity" MY149878A

Dengan hormatnya saya merujuk perkara di atas.

2. Sukacita dimaklumkan bahawa hasil penyelidikan di atas telah berjaya mendapat sijil paten di **Malaysia** pada **31 Oktober 2013**. Bersama ini disertakan salinan sijil untuk rujukan. Sila lawati laman web harta intelek untuk rujukan http://tncpi.upm.edu.my/upmip/index.php

3. Pihak kami mengucapkan tahniah di atas kejayaan ini dan semoga ia akan meningkatkan lagi kualiti penyelidikan dan perlindungan harta intelek UPM.

Sekian, terima kasih.

"BERILMU BERBAKTI"

Yang menjalankan tugas,

DR. ZAHIRA MOHD ISHAN
Timbalan Pengarah
Bahagian Perlindungan Harta Intelek
Putra Science Park

TERIMA
26 MAR 2014

s.k. Timbalan Dekan
Siswazah, Penyelidikan & Inovasi,
Fakulti Sains Dan Teknologi Makanan, UPM

Pejabat Timbalan Naib Canselor (Penyelidikan & Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
603-8947 1291/ 1850/ 1286 603-8946 1421 http://tncpi.upm.edu.my

MALAYSIA

CERTIFICATE OF GRANT OF A PATENT

In accordance with Section 31 (2) of the Patents Act 1983 a patent for an invention having grant number MY - 149878 - A has been granted to UNIVERSITI PUTRA MALAYSIA, in respect of an invention having the following particulars :

TITLE	:	EXTRACT HAVING PROTEASE ACTIVITY
FILING DATE	:	14 NOVEMBER 2008
PRIORITY DATE	:	NONE
NAME OF INVENTOR	:	MOHD YAZID ABD MANAP ABDULLAH BIN SIPAT YOUSIF MOHAMED AHMED IDRIS NASSIM NADERI
PATENT OWNER	:	UNIVERSITI PUTRA MALAYSIA 43400 UPM SERDANG SELANGOR DARUL EHSAN MALAYSIA
DATE OF GRANT	:	31 OCTOBER 2013
DURATION OF PATENT	:	14 NOVEMBER 2008 UNTIL 14 NOVEMBER 2028
END OF PROTECTION	:	30 OCTOBER 2014 (SUBSEQUENT ANNUAL FEE SHALL FOLLOW AS STATED IN THE SCHEDULE OF FEES AT THE BACK OF THIS PAGE)

Dated this 31 day of OCTOBER 2013

(SHAMSIAH BINTI KAMARUDDIN)
for Registrar of Patents
MALAYSIA

PROIP
managing intellectual assets

Our Ref : 080714 MBA/JS
Your Ref : UPM/TNCPI/ICC/3.5.83
Date : 5 March 2014

Bahagian Perlindungan Harta Intelek
Putra Science Park
Pejabat Timbalan Naib Canselor (Penyelidikan & Inovasi)
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan

By Hand and E-mail

Attn: Dr. Zahira Mohd Ishan

Dear Madam,

MALAYSIAN PATENT NO. MY-149878-A
"EXTRACT HAVING PROTEASE ACTIVITY"
IN THE NAME OF UNIVERSITI PUTRA MALAYSIA

We refer to the above matter.

We are pleased to inform you that the above-mentioned patent application has been granted on **31 October 2013** and assigned with the patent number **MY-149878-A.**

Important particulars of the patent are as follows:

- Patentee : Universiti Putra Malaysia
- Grant No. : MY - 149878 - A
- Date of Grant and Publication: 31 October 2013
- Priority Data : None
- Filing Date : 14 November 2008
- Maximum Term : 14 November 2028 (20 years from filing date)
- Renewal Fees : Due annually, payable before the anniversary of the date of grant. Next renewal fee for 2nd year is due on **30 October 2014.**

Enclosed for your reference and safekeeping is a notice of grant, a certificate of grant with a copy of the patent and the examiner's final report.

Copies of the patent were made available to the public on the date of grant. A reference to the grant will be published in the Gazette as soon as possible.

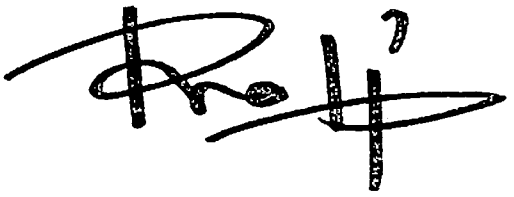
While we do make a practice of sending reminders in regard to the date on which renewal fees become due for payment, we cannot accept any responsibility for failure to do so or for the fact that a reminder may not be received by the due date.

Ahide, Notification & Records File 14/3

We would like to take this opportunity to thank you for entrusting our firm with the prosecution of the captioned patent. We look forward to providing a continued service to you.

Kindly acknowledge safe receipt of this letter and the enclosure.

Yours faithfully
PRO IP SDN BHD



encl.

PERBADANAN HARTA INTELEK MALAYSIA
INTELLECTUAL PROPERTY CORPORATION OF MALAYSIA
(Diperbadankan)
Unit 1-7 & Mezzanine, Aras 12–19
Tower B, Menara UOA Bangsar
No. 5, Jalan Bangsar Utama 1
59000 KUALA LUMPUR
MALAYSIA

Tel : +603 - 2299 8400
Faks (Fax) : +603 - 2299 8989
Laman Web (Web) : www.myipo.gov.my

APPLICATION NO.	:	PI 20084588
GRANT NO.	:	MY - 149878 - A
OWNER	:	UNIVERSITI PUTRA MALAYSIA
DATE OF GRANT AND PUBLICATION	:	31 OCTOBER 2013
APPLICANT'S/AGENT'S REF.	:	080714 MBA

NOTICE OF GRANT

The purpose of this notice is to advise you that a patent/utility innovation has been granted on the above application.

Please find enclosed a certificate of grant with a copy of the patent/utility innovation together with a copy of the Examiner's final report (if not previously provided) in accordance with Section 31 (2)(a) of the Patents Act.

Copies of the Patent/utility innovation were made available to the public on the date of grant. A reference to the grant will be published in the Gazette as soon as possible.

Your attention is drawn to the need to pay annual renewal fees in order to keep the patent/utility innovation in force (see Section 35(2) and (3) of the Patents Act and Schedule 1 of the Regulations).

Date : 12 FEBRUARY 2014

(SHAMSIAH KAMARUDDIN)
for Registrar of Patents
shamsiah@myipo.gov.my
Tel: 03-22998403

To : MOHD BUSTAMAN HJ ABDULLAH
C/O MESSRS BUSTAMAN
LOT C9-3, JALAN SELAMAN 1
DATARAN PALMA, AMPANG
68000 SELANGOR DARUL EHSAN
MALAYSIA

PERBADANAN HARTA INTELEK MALAYSIA
INTELLECTUAL PROPERTY CORPORATION OF MALAYSIA
(Diperbadankan)
Unit 1-7 & Mezzanine, Aras 12-19
Tower B, Menara UOA Bangsar
No. 5, Jalan Bangsar Utama 1
59000 KUALA LUMPUR
MALAYSIA

Tel : +603 - 2299 8400
Faks (Fax) : +603 - 2299 8899
Laman Web (Web) : www.myipo.gov.my

APPLICATION NO.	:	PI 20084588
APPLICANT	:	UNIVERSITI PUTRA MALAYSIA
FILING DATE	:	14 NOVEMBER 2008
AGENT'S OR APPLICANT'S FILE REF.	:	080714 MBA
DATE OF MAILING	:	30 SEPTEMBER 2013

SUBSTANTIVE / ~~MODIFIED SUBSTANTIVE~~ EXAMINATION
CLEAR REPORT - Section 30(1)/30(2)

The Examiner has ~~reported~~ / further reported* that the above application complies with the requirements of the Patents Act 1983 and Patents Regulations 1986. The examination was carried out on the following application documents:

Description:	Pages	1 - 11	filed on	14/11/2008
Claims:	Pages	12	filed on	7/11/2012
Drawings:	Figure	1A - 4	filed on	14/11/2008
Abstract:	Pages	13	filed on	14/11/2008
	Figure	1	filed on	14/11/2008

Attached the bibliographic data of the application. Kindly check and update, if any and revert to us preferably via e-mail to nurazliana@myipo.gov.my or nazira@myipo.gov.my within 7 days from the date of mailing of this report. If no response is received within the time given, the application will now proceed to grant. Notice of Grant will be issued once the application is granted.

A search report is attached *~~Yes~~ / No
* Delete if not applicable.

Date : 30 SEPTEMBER 2013

(Khairul Syafiq Bin Mohd Shukri)
For Registrar of Patents
✉khairulsyafiq@myipo.gov.my
☎03-2299 8849

To : MOHD BUSTAMAN HJ ABDULLAH
C/O BUSTAMAN
LOT C9-3, JALAN SELAMAN 1
DATARAN PALMA, AMPANG
68000 SELANGOR DARUL EHSAN
MALAYSIA

(12) **MALAYSIAN PATENT**

(11) **MY - 149878 - A**

(21) **Application No. :** PI 20084588

(22) **Filing Date :** 14 November 2008

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31 October 2013

(30) **Priority Data :**
None

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C07K 14/415(2006.01)
A23J 3/14(2006.01)
C12N 9/50(2006.01)
A01H 1/00(2006.01)

(56) **Prior Art :**
As per WO 2010/056108 A3

(72) **Inventors :**
Mohd Yazid Abd Manap
Abdullah Bin Sipat
Yousif Mohamed Ahmed Idris
Nassim Naderi

(73) **Patent Owner :**
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

(74) **Agent :**
Mohd Bustaman Hj Abdullah
C/O Messrs Bustaman

(54) **Title :** Extract Having Protease Activity

(57) **Abstract :**

The present invention discloses a composition comprising a proteinaceous extract of *Streblus asper* having substantially protease activity that degrades proteins by hydrolysis of peptide bonds. The proteinaceous extract of *Streblus asper* according to the present invention is suitable for us a as a meat quality-improving agent and a milk coagulant in food processing industries, as well as an additive in the manufacture of detergents.

Figure 1A

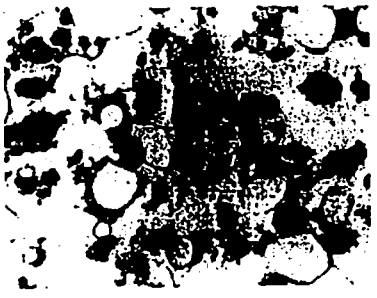


Figure 1B

EXTRACT HAVING PROTEASE ACTIVITY

The present invention relates generally to proteinaceous compositions. More particularly, the present invention relates to a composition comprising proteinaceous extracts of *Streblus asper* that is substantially having protease activity.

BACKGROUND TO THE INVENTION

Proteases are enzymes that degrade proteins by hydrolysis of peptide bonds. Practical uses of proteolytic enzymes are in medicine, softening of leather, laundry detergents and food processing. In food industry protease are being used in baked goods, beer and wine, cereals, milk, meat tenderization, fish products, legumes and for production of protein hydrolysates and flavour extracts.

Among the proteases used in food processing are the milk-clotting enzymes for cheese production. In order for milk to coagulate and eventually form cheese, a milk coagulating enzyme must be added to breakdown the proteins that keep milk a liquid. More particularly, when proteins are denatured or otherwise modified, milk loses its liquid structure and begins to coagulate.

Rennets, milk coagulating enzymes traditionally obtained from the abomasums (the fourth stomach of the calf) have long been used in the production of cheese. The main enzyme extracted from the calf rennet is chymosin. Calf-rennet, however, is expensive and is difficult to obtain due to a chronic shortage of calves to provide chymosin raw materials.

Various milk-coagulating enzymes of animal, plant and microbial origin have been identified as substitutes for chymosin and tested in cheese production. Still, the only-milk-clotting enzymes to be utilized in practice as alternatives for chymosin are pepsin (animal origin) and microbial rennet derived from various types of filamentous fungi, for example Endothia parasitica, Mucor pusillus and Mucor miehei.

U.S. Patent No. 4,526,792 discloses the use of *R. miehei* as microbial rennet in the production of cheese. *R. miehei* does not contain chymosin, but instead acid proteases, which are similar in function to chymosin.

A number of methods to extract and purify milk-coagulating enzymes are known to those skilled in the art. The methods include affinity gel chromatography and subsequent elution of the adsorbed enzymes. For example, Kobayashi, *et al.*., "Rapid isolation of microbial milk-clotting enzymes by N-acetyl-(or N-isobutyryl)-pepstatin-aminohexylagarose" Anal, Biochem., 122: 308-312 (1982) teaches purification of microbial rennet from *R. miehei* by use of affinity gel column using N-acetylpepstatin as affinity ligand. Enzymes can also be separated on affinity gel columns using Cibacron Blue F3GA ("CB") as disclosed by Dead, *et al.*, "Protein purification using immobilized triazine dyes," J. Chromatogr., 165: 301-319 (1979) and Burgett, *et al.*, "Cibacron Blue F3GA affinity chromatography", Am. Lab., 9(5): 74, 78-83 (1977). Both describe separation of enzymes on CB columns, including for example, kinases and nucleases. U.S. Patent No. 4,743,551 describes the use of a blue dye affinity ligand and elution of the adsorbed rennet to produce purified *R. miehei* rennet.

Recent research has been focused on the discovery of a new milk-coagulating enzyme that is a plant derivative and environmental friendly. It has been shown that the leaf extract of *Streblus asper* (plant Kesinai) contains protease, i.e. a milk coagulating factor, which can be a potential rennet substitute.

Therefore, is advantageous to provide a composition comprising extracts of *Streblus asper* that is substantially having protease activity.

SUMMARY OF THE INVENTION

The present invention is directed to a composition comprising a proteinaceous extract of *Streblus asper* having substantially protease activity that degrades proteins by hydrolysis of peptide bonds.

It is an advantage of the present invention to provide a proteinaceous extract of *Streblus asper* that is suitable for us a as a meat quality–improving agent and a milk coagulant in food processing industries, as well as an additive in manufacturing of detergents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a scanning electron micrograph (SEM) of a proteinaceous extract of *Streblus asper* (Kesinai) at x 7000;

Figure 1B is a transmission electron microscopy (TEM) of a proteinaceous extract of *Streblus asper* (Kesinai) x 30,000;

Figure 2 is a sodium dodecyl sulphate polyacrylamide gel electrophoretic profile (SDS-PAGE) of purified protease;

Figure 3A is a graph that shows optimum temperature for proteolytic activity of the purified proteas;

Figure 3B is a graph that shows temperature stability of the purified protease; and

Figure 4 is a graph that shows the effect of added calcium chloride concentration on milk coagulation time of proteinaceous extract of *Streblus asper* (Kesinai).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a proteinaceous leaf extract of plant kesinai, i.e. *Streblus asper,* which is substantially having protease activity.

Preparation of the crude leaf extract results in an undesirable, very dark brown color and inhibition of this browning may enhance the use of the leaf extract. Browning inhibitors such as citric acid, L-cystein and sodium metabisulphite are used for prevention of browning and to obtain a crude extract with an acceptable color. This solved the main problem of Kesinai leaf extract and enhanced its potential use as a milk coagulant, meat tenderizer and as additive for the detergent industry.

Metabisulphite was found to be an effective inhibitor of the enzymatic browning of the leaf extract. At 2 mM concentration, it inhibited browning and the extract obtained resulted in a white milk coagulum compared to the brown colored 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. 6 mM added calcium chloride was optimum for its milk coagulation activity.

The successful inhibition of the enzymatic browning and characterization of the crude extract makes the basis for examining the physiochemical characteristics of its milk coagulum, purification and characterization of the milk coagulating protease. The use of milk coagulating protease is an essential step in cheese making. Strength, syneresis and yield of the milk coagulum are largely affected by the type of the rennet used. Texture is one of the most important characteristics of cheese, which can be influenced by the type of the coagulant. Textural differences are related to the structural network of the milk coagulum. To study the textural properties, microstructure and syneresis of crude extract is useful in evaluation of the potential suitability of a milk coagulating protease as a rennet substitute. To this extent, milk coagulum was prepared from fresh cow's milk by *Streblus asper* (kesinai) for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations. These examinations were done to quantify the coagulum porosity, texture and syneresis.

Finally, the crude enzyme extracts from plant kesinai were 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.

Referring to Figure 2, the purified protease appeared as a single band on SDS-PAGE with a molecular weight of 31.3 kDa. Characterization 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 isoelectric point (pI) is 5.2. It is thermostable up to 85°C, with an optimum temperature of 70°C. Zymogram analysis showed that protease activity is associated with milk coagulation activity. Kesinai protease could be used in the production of short ripened cheese varieties.

The present invention will now be described in greater detail by way of examples, which are not intended limit the scope of the invention.

EXAMPLE 1

Preparation of Leaf Extracts

Fresh *Streblus asper* leaves were washed and homogenized in 200 ml 100 mM Tris-HCL buffer with pH 6-9 including 0.5-10 mM sodium metabisulphite at room temperature. The homogenate was filtered and centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was collected as crude enzyme extract. Crude enzyme extract was ultrafiltrated and concentrated at room temperature with 43 mm disc membranes using stirred cell Amicon 8050. Retentates and filtrates were collected separately. Then, protease activity was determined using azo-casein in 100 mM Tris-HCL; pH 7.2 as the substrate (0.05%, weight/volume). 100 µl of enzyme was incubated with one ml substrate for one hour at room temperature. The reaction was terminated by the addition of 300 µl trichloroacetic acid. Then, the mixture was centrifuged and the supernatant was collected and its absorbance was measured against a mixture of substrate and buffer as the blank. The change in the absorbance was measured at 410 nano meter and the enzyme activity expressed as 1.0 unit = change of 0.01 absorbance unit.

The effect of calcium chloride on milk coagulation time was studied by dissolving calcium chloride in fresh milk to obtain a calcium chloride concentration of 1 to 10 mM. Fresh milk without added calcium chloride was used as control. The milk (2 ml) was tempered for 5 minutes in a water bath at 65°C, then 200 µl crude leaf extract was added. The milk and enzyme mixture was incubated at the set temperature without shaking the water bath. Referring to Figure 4, the addition of calcium chloride in 1, 2, 4, 6, 8 and 10 mM concentration to fresh milk has increased milk coagulation activity. Milk coagulation activity increased with an increase in added calcium chloride up to a concentration of 6 mM, above which an increase in milk coagulation activity was small.

In this example the effect of sodium metabisulphite for inhibition of enzymatic browning of crude leaf extract was studied. For this reason, the crude extract, prepared by this method, was assayed for color (by measuring the absorbance units with spectrophotometer) and milk coagulation activity.

EXAMPLE 2

Determination of Milk Coagulation Activity

Milk coagulating activity was determined by measuring the time taken by the leaf extract to coagulate 12.5% reconstituted milk. Sample pre-incubated at 65°C for 5 minutes after which 200 µl leaf extract was added and the mixture was incubated at 65°C. The tube was tilted approximately 45° every 15 seconds. The time taken to form the first visible sign of milk coagulation was recorded as milk coagulation time. One unit milk coagulation activity is that which coagulates 1 ml milk in 1 min under the assay conditions and specific milk coagulation activity is activity unit/mg protein. Boiled enzyme was used as the control.

The result, as shown in Table 1, concludes that a crude extract of an acceptable color was obtained using 10 mM sodium metabisulphite in the extraction buffer. Extract prepared using metabisulphite showed high milk coagulation activity in maintaining protease activity. Sodium metabisulphite is widely used in the food industry as a multifunctional additives and recognized as safe (GRAS) for use as chemical preservation. The level of sulphite used in this study for inhibition of the browning of the leaf extract is low and will not be organoleptically detectable in milk and leaf extract mixture as the level would be ~ 38 ppm (part per million) and the minimum threshold for organoleptic detection of sulphite is about 50 ppm (part per million).

The crude leaf extract obtained has an optimum pH of 7.2 and stable in a wide pH range. It is thermostable and has an optimum temperature of 70° C.

Table 1 Effect of sodium metabisulphite at various concentrations on browning of crude leaf extract

Concentration (milli Mole)	Color (absorbance at 420 nano meter)	Protease specific activity	Specific milk coagulation activity
0.5	1.08	13.62	0.567
1.00	0.667	17.04	0.739
2.00	0.519	17.22	0.754
3.00	0.126	17.46	0.786

4.00	0.118	17.64	0.821
5.00	0.114	17.70	0.836
10.0	0.103	17.82	0.854

EXAMPLE 3

Preparation of milk coagulum

For preparing milk coagulum, 0.2 mM calcium chloride and 2 mg of decolorized *Streblus asper* (kesinai) extract were added to 100 ml fresh cow milk. Then, the mixture was incubated till a coagulum is formed. The prepared coagulum was cut into small pieces and was subjected to scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations. Porosity of the milk coagulum was determined by quantification of pores fractional area of SEM and TEM micrographs. For texture, coagulum strength was determined by using a texture analyser.

The extent of syneresis was determined by measuring sample volume and then measuring the volume of whey that could be separated from the coagulum by filtration.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run for the milk coagulum. The results were observed as follow:

The microstructure of the milk coagulum of the leaf extract appeared as a sponge-like when examined under scanning electron microscopy (SEM). The formation of a sponge like structural network by leaf extract was attributed to the nature and proteolytic specificity of the leaf extract in addition to new cross-linkages between casein micelles caused by the phenolic compounds in the leaf extract.

The leaf extract was found to produce a milk coagulum with a lower porosity and a denser casein network. Referring to Figures 1a and 1b, both the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) porosity quantification results showed low porosity of kesinai milk coagulum. This is a desirable property in cheese production as casein contribution to cheese yield

includes its own weight plus associated moisture and minerals. Also it has expelled less whey, which is the serum phase of milk.

Sodium dodecyl sulphate polyacrylamide gel electrophoretic profile of coagulum and whey (SDS-PAGE) showed that leaf extract has high proteolytic activity.

EXAMPLE 4

Purification and characterization of kesinai milk coagulating protease

(i) Fast protein liquid chromatography (FPLC) Gel Filtration Chromatography

The prepared crude enzyme extracts from the Kesinai leaves with sodium metabisulphite was loaded on a superpose-6 fast protein liquid chromatography (FPLC) column with a bed volume of 25 ml which was equilibrated with 100 mM Tris-HCL; pH 7.2 prior to filtration.

Proteins were eluted with the equilibrating buffer at a flow rate of 0.3 ml per min. Filtration resulted in 4.26 fold purification with a 69.84% yield. The protease containing fractions were pooled and further purified by fast protein liquid chromatography (FPLC) ion exchange chromatography on Mono Q HR 5/5 column. The enzyme was eluted from the column with a salt concentration of 0.35 -0.40 M. Ion exchange purification step resulted in 23.76 fold purification with 24.34 per cent yield. Protease active fractions eluted from the ion exchange chromatography step were pooled, dialyzed against distilled water and purified by isoelectric focusing using the Rotofor apparatus. The 25.10 fold purification was achieved with a final yield of 18 per cent.

On the basis of protein, a protein recovery of 140 fold was achieved.

(ii) Characterization of the Kesinai protease

Molecular mass determination was estimated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A standard curve of log molecular mass versus relative mobility of the standard proteins was plotted and the molecular

mass of the purified protease was then estimated from the standard curve using its relative mobility.

The results suggest that the protease is probably a monomer consisting of a single subunit. Referring to Figure 2, electrophoresis experiments to determine molecular mass of the purified protease (lanes 6 and 7) showed a single band with a molecular weight corresponding to about 31.3 kDA. The low molecular weight of the protease is similar to that of serine proteases, which are generally of low molecular weight, usually between 15,000 and 30,000.

Isoelectric point of the purified protease was determined from isoelectric focusing elution profile of the protease, where the protease was eluted as a single peak at pH 5.2, and based on this; its isoelectric point (pI) was estimated to be pH 5.2. Results also showed that the purified protease from Kesinai can coagulate milk even after electrophoresis at pH 8.3 at room temperature.

Optimum activity pH for proteolytic activity of the purified protease over a range of pH values of 5 to 9 for an incubation time of one hour at 37° C showed a pH optimum for azocasein hydrolysis of 7.2. Under these conditions, the enzyme had 60% of its maximum activity at pH 6.2, which is considered as the pH at which cheese milk is acidified by starter culture.

In order to determine pH stability of the purified protease from kesinai, the enzyme from kesinai was incubated in various pHs from 4.5 to 9.5 for one hour at room temperature. After that, the residual protease activity was determined. Results showed that protease was stable at a pH range of 5 to 8.5 when incubated for one hour at room temperature. In this condition, protease maintained 10% of its activity at pH 5.

In order to determine the effect of temperature on protease activity, the purified protease was equilibrated for 5 minutes at a temperature ranging from 5 to 95°C. Then, a substrate (azocasein 0.05% w/v in Tris-HCL buffer, pH=7.2) was added and the mixture was incubated at the test temperature for one hour and assayed for proteolytic activity according to standard assay method. Results as shown in Figure 3A revealed that the optimum temperature of the purified protease was around 70°C.

In order to determine temperature stability of the purified protease from kesinai, the enzyme was incubated at various temperatures in the range of 5 to 95°C for one hour and then immediately cooled in ice. Residual proteolytic activity was assayed at 37°C using azocasein (0.05% w/v) as the substrate. The temperature stability of the protease is shown in Figure 3B. Referring to Figure 3B, the enzyme was stable up to 75°C when incubated for one hour. The enzyme activity was 44%, 26% and 14% of its full activity after one hour of incubation at 80°C, 85°C and 90°C, respectively.

Thermostability of the purified protease indicates that it would be capable of surviving conventional milk and whey pasteurization conditions, which is an undesirable property in rennet substitutes.

INDUSTRIAL APPLICATION

The crude extract from kesinai according to the present invention can be suitably used as a meat quality–improving agent capable of modifying a meat at an appropriate softness and imparts no undesirable after taste to the meat treated. Moreover, a meat quality-improving agent, the enzyme inactivation temperature of which is relatively low and therefore, which is highly usable for domestic and industrial purposes while easily controlling temperature or inactivation.

Also thermostability and the high proteolytic activity of the crude extract are desirable properties in detergent industry and the purified enzyme could be useful in these processes. Use of enzyme in detergent products can save energy by enabling a lower wash temperature and they are biodegradable, leaving no harmful residues. It will not possess negative environmental impact on sewage treatment processes and also does not present a risk to aquatic life.

The browning of *Streblus asper* leaf extract indicates that it is rich in phenolic compounds and polyphenoloxidase (PPO), both having potential industrial uses. Polyphenoloxidase is potentially useful in many future industrial applications, including production of flavonoids-derived colorants as antioxidants and the removal of oestrogenic substances from aquatic environments. Being a rich source of phenolic compounds, *Streblus asper* leaf extract could be useful in improving the thermal and colloidal stability of concentrated milk, as new evident suggests that

plant extracts rich in phenolic compounds markedly increase the heat and colloidal stability of milk.

While the illustrative embodiments of the invention have been described with particularly, it will be understood that various other modifications will be apparent to and can be readily made by those skilled in the art without departing from the scope of the invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the examples and descriptions set forth hereinabove but rather that the claims be construed as encompassing all the features of patentable novelty which reside in the present invention, including all features which would be treated as equivalents thereof by those skilled in the art to which the invention pertains.

CLAIMS

1. Use, as a meat quality improving agent, of a composition comprising a proteinaceous extract of *Streblus asper* substantially having protease activity.

2. Use according to claim 1, wherein the proteinaceous extract of *Streblus asper* has a molecular weight of 31.3 kDA.

3. Use according to claim 1, wherein the proteinaceous extract of *Streblus asper* has an isoelectric point of pH 5.2.

4. Use according to claim 1, wherein the proteinaceous extract of *Streblus asper* has a protease activity within a pH range of 5 to 9.

5. Use according to claim 1, wherein the proteinaceous extract of *Streblus asper* has a protease stability within a pH range of 5 to 8.5.

6. Use according to claim 1, wherein the proteinaceous extract of *Streblus asper* has an optimum temperature of activity of 70°C.

ABSTRACT

EXTRACT HAVING PROTEASE ACTIVITY

The present invention discloses a composition comprising a proteinaceous extract of *Streblus asper* having substantially protease activity that degrades proteins by hydrolysis of peptide bonds. The proteinaceous extract of *Streblus asper* according to the present invention is suitable for us a as a meat quality–improving agent and a milk coagulant in food processing industries, as well as an additive in the manufacture of detergents.

Figure 1

Figure 1A

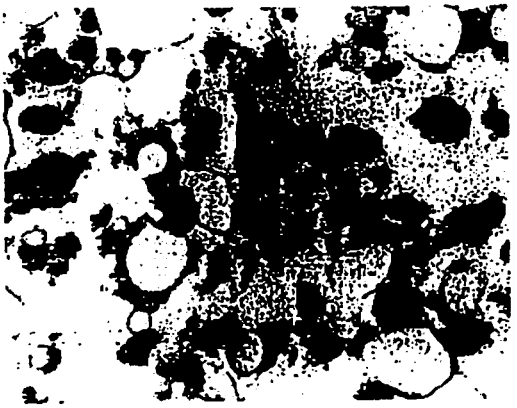
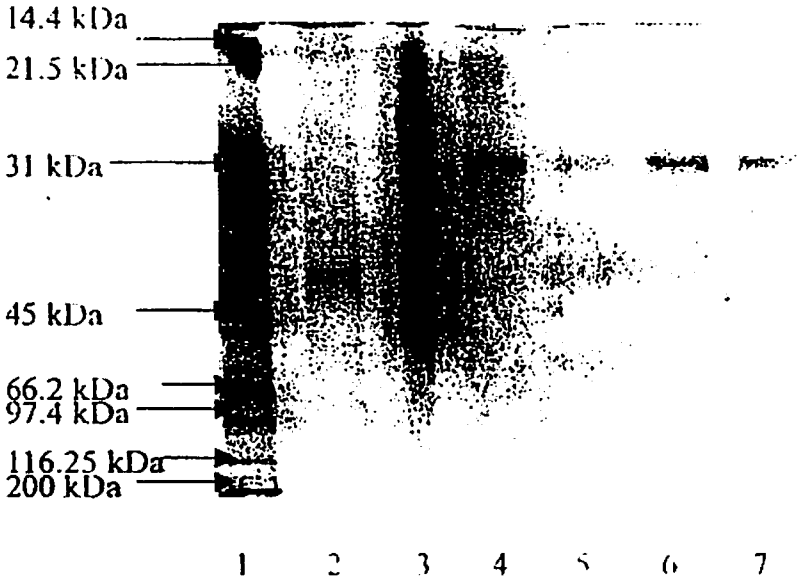


Figure 1B



Lanes 1: marker proteins, Lane 2: Crude extract, Lanes 4 and 5: partially purified protease, Lanes 6 and 7: purified protease

Figure 2

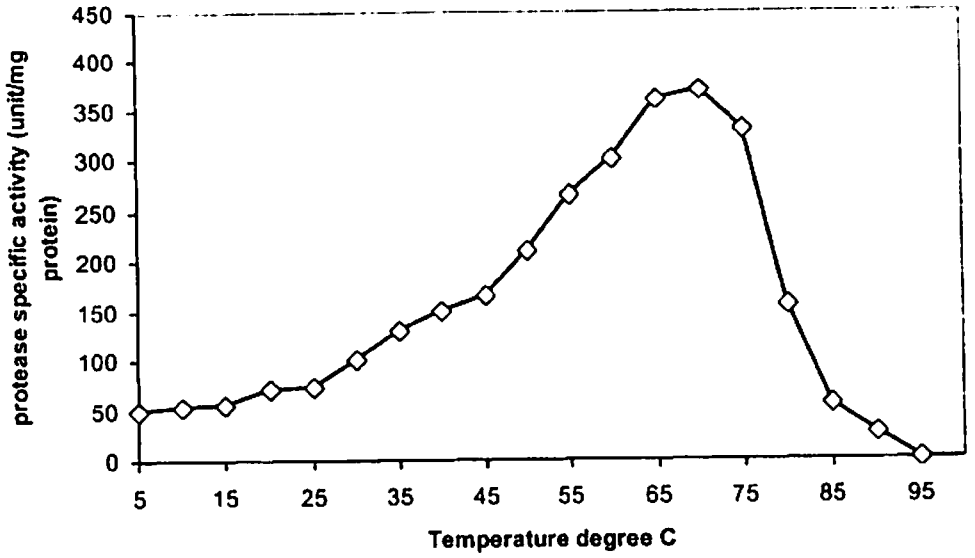


Figure 3A

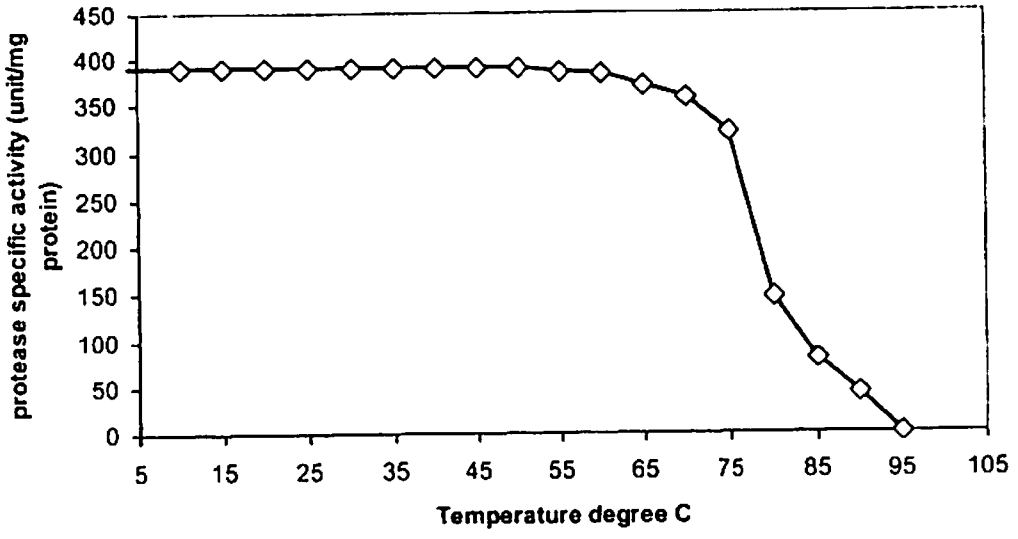


Figure 3B

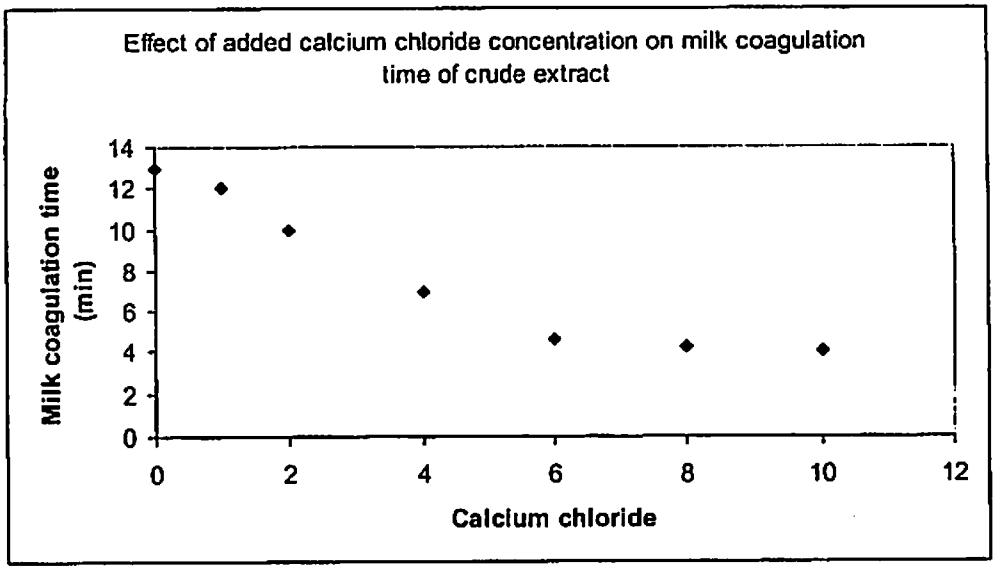


Figure 4