# EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF KINEMA PROTEASE PRODUCED FROM WHITE SOYABEAN

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# Extraction, Partial Purification and Characterization of Kinema Protease Produced from White Soyabean

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## Approval Letter

**This dissertation entitled *Extraction, Partial Purification and Characterization of Kinema Protease Produced from White Soyabean* presented by Tikaram Pahadi has been accepted as the partial fulfillment of the requirement for the B. Tech. degree in Food Technology.**

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# Abstract

Protease constitute a large group of highly demanded enzymes having huge application in various industries. This study was carried out with an aim to extract protease from kinema prepared from white soybean, partial purify the extracted protease by ammonium sulphate precipitation followed by dialysis and to determine general characteristics of the purified protease. The kinema, which has highest proteolysis activity after completion of three days fermentation was taken for the study. The data were analyzed by using one way ANOVA using Genstat at 5% level of significance level.

About 2.5 fold of purification with overall recovery of 71.7% was achieved after precipitation with ammonium sulphate at 30-70% saturation followed by dialysis of crude extracted protease. The specific activity was found to be 7.67 U/mg/min. The enzyme was found active over various natural substrates; showing highest activity on casein, followed by BSA, gelatin, hemoglobin and whey protein. Metal ions such as Iron, Mercury and aluminum shows the inhibition effect whereas presence of sodium, cobalt, zinc, copper, calcium, potassium shows the activation effect on protease activity. Furthermore, the enzyme activity was highly inhibited with EDTA and then PMSF at 5mM concentration, suggesting the protease from kinema was a metal dependent serine protease.

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## List of Abbreviations

| Abbreviations | Full form |
|---|---|
| % | Percentage |
| BSA | Bovine serum albumin |
| cfu | Colony forming unit |
| DB | Dry basis |
| DFP | Di-isopropyl flurophosphates |
| EAAs | Essential amino acids |
| EDTA | Ethylene diamine tetra-acetic acid |
| FAO | Food and Agricultura Organization |
| Fig | Figure |
| g | Gram |
| IDA | Ido acetic acid |
| LSD | Least significance difference |
| mg | Milligram |
| ml | Milli liter |
| MW | Molecular weight |
| nm | Nano meter |
| PA | Protease Activity |
| PGA | Poly gamma glutamic acid |
| pH | Potential of Hydrogen |
| PMSF | Phenylmethyl sulfonyl fluoride |

| Abbreviations | Full form |
|---|---|
| SD | Standard Deviation |
| SH | Sulphydryl |
| spp | Species |
| TCA | Trichloro acetic acid |
| TPP | Three phase partitioning |
| UNO | United Nation Organization |
| µg | Micro gram |
| Vol | Volume |
| WHO | World Health Organization |

# List of plates

# Part I

# Introduction

## 1.1 General introduction

A traditional product is a representation of a group, it belongs in a defined space, and it is part of a culture that implies the cooperation of the individuals operating in that territory. From this clearly sociological definition it can be derived that in order to be traditional, a product must be linked to a territory and it must also be part of a set of traditions, which will necessarily ensure its continuity over time (Jordana, 2000). People in Nepal are skilled at preparing and preserving locally available foods such as *Fulaura, Jilebi, Selroti, Kinema, Khalpi, Mesu, Dahi, Mahi, Ghiu,* and *Jand* for their food security for a very long time. The foods are prepared by natural or controlled mixed fermentation, principally due to lactic acid-producing bacteria, yeasts, and molds (Dahal *et al.*, 2005).

The soybean (*Glycine max (L.) Merrill*) is a yellow vegetable consisting of 37.69% protein, 28.2% crude fat/ oil, Ash 4.29%, Moisture 8.07%, Crude fiber 5.44% and Carbohydrate 16.31% (Etiosa *et al.*, 2017) that comes from China and constitutes the base of human and animal diets in many Oriental countries due to its high nutritional value and low cost. Moreover, soy products are increasingly being used in other countries because they are a good source of vegetable proteins, with a low-fat content. According to Nguyen and Nguyen (2020), the essential amino acids that the body cannot produce are found in soybeans. The nutritional value of the soybean is not the only factor enhancing its consumption, as it plays an important role in health. Many clinical studies have demonstrated the associated advantages of the use of soy products in preventing heart disease, obesity, blood cholesterol, cancer, diabetes, kidney disease, and osteoporosis (Garcia *et al.*, 1997).

Kinema is an indigenous non-salted fermented soybean food commonly consumed as a low-cost source of plant protein by the people of the Himalayan regions of the Darjeeling hills, Sikkim and north-eastern hills in India, Nepal and Bhutan (Tamang and Nikkuni, 1996). Traditionally kinema is prepared by cooking overnight-soaked whole soybeans, wrapped in leaves and fermented naturally for 1±3 days at ambient temperature (Tamang and Nikkuni, 1998). It has a pungent smell of ammonia, slimy texture, and short shelf-life.

The shelf life is often lengthened to one month by drying in the sun or by keeping on earthen ovens in kitchens.

The product is similar to Natto of Japan and Thau Nao of Thailand. Kinema is used to give a pleasant, nut-like flavor to curry (Tamang *et al.*, 1988). *Bacillus subtilis* is the most dominant fermenting organism in Kinema product (Nout *et al.*, 1998). Due to the production of specific hydrolytic enzymes such as proteases, α-amylase, cellulose, pectinase and β-glucosidase, *Bacillus* spp. have been used for solid state fermentation of plant matrix including agricultural by-products (Rai *et al.*, 2017). Several *Bacillus* spp. secrete two major types of protease, a subtilisin or alkaline protease and a metalloprotease or neutral protease, which are of industrial importance (Rao *et al.*, 1998).

During fermentation, the extracellular enzymes act on soybean to produce mucilage, which gives slimy and sticky nature to kinema. These gummy substances are exopolypeptides of D-isomeric glutamic acid. There is a significant decrease in protein nitrogen with a coincident increase in water-soluble and trichloroacetic acid-soluble nitrogen content (Weng and Chen, 2011). Protease produced by *B. subtilis* hydrolyze soy proteins into polypeptides. Kinema fermentation has shown an overall increase in free amino acids and ammonia by 60 and 40 fold, respectively (Sarkar *et al.*, 1997). Based on these facts kinema has shown to be a potential source of proteolytic enzymes and can be utilized as a source of hydrolyzing enzymes for various protein (Nout *et al.*, 1998).

Protease breaks the peptide bond to produce amino acids and other simpler peptides and thus facilitating their absorption by the cells and can be isolated from a variety of sources such as plants, animals and microbial (fungi and bacteria) (Rao *et al.*, 1998). The use of proteases in the food industry dates back to antiquity are being demanded for an increasing number of applications in food, life science, and chemical industries. Major sectors benefiting from protease utilization are the food, beverage, feed, detergent, pharmaceutical, chemical, leather, paper, pulp, and silk industries (Yegin and Dekker, 2013). They have been routinely used for various purposes such as cheesemaking, baking, preparation of soya hydrolysates, and meat tenderization (Rao *et al.*, 1998).

Protease represent one of three largest groups of industrial hydrolytic enzymes and their demand is increasing worldwide (Rao *et al.*, 1998). Furthermore, due to lack of sufficient animal production and other ethical consideration, forcing the researcher toward new sources

of proteases. This results a gaining interest toward microbial and plant sources of protease. Papain, bromelain, ficin and well-known protease from plants. Similarly alcalase, thermolysinand subtilisin from *Bacillus* species; corolase and flavorzyme from *Aspergillus* species are some of the example of commercial well characterized and exploited protease of the microbial origin (Ortiz *et al.*, 2016; Razzaq *et al.*, 2019). Various researcher todays has shown *Bacillus subtilis* fermented alkaline fermented food such as Japanese Natto, Thua nao from northern Thailand, Gembus from Indonesia could be the alternative source of protease and protease producing strains (Afifah *et al.,* 2014; Chantawannakul *et al.,* 2002; Fujita *et al.,* 1993). Protease form *Bacillus subtilis* strain 38 isolated from thua nao has been characterized and Nattokinase from Natto extract has been purified and characteristics has been well documented. Kinema being similar to Thua nao and Natto could also be potent source of protease and proteolytic strains which could have future potential to use in various pharmaceutical and food applications. However, extraction, purification and characterization of protease from kinema or protease producing proteolytic strain is very essential and will be first step toward exploitation of kinema protease.

## 1.2 Statement of the problems

Proteases covers up to 60% of total enzyme market and are valuable commercial enzyme that have biotechnological as well as industrial applications however the present known proteases are not sufficient to meet most of the industrial demands. So, it is desirable to have new proteases with novel properties from different sources (Gaur *et al.*, 2010). Therefore, search for new protease from different sources is crucial. The indigenously fermented soybean based fermented food such as Nepalese kinema, Japanese Natto and Thua nao, which consist the *Bacillus* species as a dominated microflora have been shown to possess proteolytic activity during fermentation. The protease enzyme have been already extracted purified and characterized from Japanese Natto and various proteolytic strain has been isolated and applied as source of protease enzyme from Thuanao and Natto. Kinema being similar food has not been studied so far as source of protease and proteolytic strain in spite of being such a potentiality. So, extraction and purification of kinema protease by using the suitable methods and their characterization not only provide a novel protease sources but also can contributes to fulfill the existing market demand to some extent and such a study could be a step forward for their usage in the specified field in future too. Alkaline proteases

produced are of special interest as they could be used in manufacture of detergents, food pharmaceuticals and leather.

## 1.3 Objectives of the study

### 1.3.1 General objectives of the study

The general objective of this study is extraction, partial purification and partial characterization of kinema protease prepared from white kinema.

### 1.3.2 Specific objectives

The specific objective of the study will be

i. To prepare kinema on laboratory by using traditional method.
ii. To study the proteolytic activity during kinema fermentation.
iii. To study the chemical composition of the prepared kinema.
iv. To extract and partially purify the protease from kinema.
v. To evaluate general characteristics (optimum pH, optimum temperature, substrate specificity, effect of metal ions & protease inhibitors, storage stability) of partially purified protease from kinema.

## 1.4 Significance of the study

The present known proteases are not sufficient to meet most of the industrial demands due to their widespread applications on food, feed and different pharmaceutical industries. This study is expected to provide novel and affordable sources of protease from alkaline, traditionally fermented soybean-based foods, called kinema, which possess the enormous commercial potential and have been used in several industrial processes, including food industry, leather industry processing, silk processing, detergent industry and therapeutic applications. Similarly, it can be use as protein hydrolyzing enzymes for the production of various protein hydrolysates with improved digestibility and bioavailability and also promote medicinally and functionally important bioactive peptides.

## 1.5 Limitation of the study

i. The molecular weight of the enzyme was not carried out.
ii. Further purification of the enzyme was not carried out.
iii. Extent of hydrolysis was not carried out.

# Part II

# Literature review

## 2.1 Kinema

Kinema is a popular fermented soy bean-based, gray tan coloured, slightly alkaline, sticky food having ammonical odour, which serves as cheap source of high protein food in a local diet. Kinema is similar to natto of Japan, chungkok-jang of Korea, thua-nao of Thailand, douche of China, pe-poke of Myanmar; hawaijar of Manipur, aakhoni of Nagaland, turangbai of Meghalaya and bekanihu of Mizoram in northeast India (Tamang, 2003). According to Nepali (2007), origin of Kinema was in Southern part of China. While it spread, this food settled into a niche as seasonings in East Nepal, North East India, Burma, Thailand and in Japan. Traditionally kinema is prepared by cooking overnight-soaked whole soybeans, wrapped in leaves and fermented naturally for 1±3 days at ambient temperature (Tamang and Nikkuni, 1998).

## 2.2 Raw materials of kinema

Soybean (*Glycine max*) is the main ingredient used in kinema production. According to Shrestha *et al.* (2010), Soybean, one of the nutritionally richest natural vegetable foods known to human kind, records of its food usage dates back to 2838 BC in China . It is locally known as *Bhatmas* in Nepali language, is traditionally used to prepare various fermented and non-fermented recipes. Soybean was probably introduced to India from China through the Himalayas several centuries ago and some believe that soybeans were also brought via Myanmar by traders from Indonesia.

According to Nguyen and Nguyen (2020), the essential amino acids that the body cannot produce are found in soybeans. The nutritional value of the soyabean is not the only factor enhancing its consumption, as it plays an important role in health. Many clinical studies have demonstrated the associated advantages of the use of soy products in preventing heart disease, obesity, blood cholesterol, cancer, diabetes, kidney disease, and osteoporosis (Garcia *et al.*, 1997). Additionally, the inhibitory activity of an angiotensin 1-converting enzyme (ACE) detected in soybean has been found to play an important role in regulating blood pressure and salt and water balance (Garcia *et al.*, 1997).

Mainly three varieties of soybean; yellow (white), dark brown and black are generally used in kinema preparations. Among three varieties, two indigenous varieties of soybeans 'yellow cultivar' and 'dark brown cultivar' are grown in between May and June and harvested in November. Locally grown soybeans are harvested and dry seeds of soybeans are naturally fermented into flavoursome and sticky product in eastern parts of Nepal, Darjeeling hills, Sikkim, North Eastern regions of India, and southern parts of Bhutan by the Mongolian races. Previously, in most of the studies about kinema, yellow seeded variety (white) soybean was used as raw material. But, Nepali (2007) made a study to compare the nutritional and sensory parameter of kinema made from different varieties of soybean including black variety and brown variety. The chemical composition of different varieties of soybean is given in the table 2.1.

**Table 2.1** Chemical composition of soybean

| Parameter | Soybean black | Soybean brown | Soybean white |
|---|---|---|---|
| Moisture(g) | 12.1 | 8.1 | 10.2 |
| Protein(g) | 33.3 | 43.2 | 33.3 |
| Fat(g) | 15 | 19.5 | 17.7 |
| Carbohydrate (g) | 31.3 | 20.9 | 29.6 |
| Minerals(g) | 4 | 4.6 | 5 |
| Fiber(g) | 4.3 | 3.7 | 4.2 |
| Energy(kcal) | 393 | 432 | 411 |
| Calcium(mg) | 213 | 240 | 226 |
| Phosphorous(mg) | 509 | 690 | 546 |
| Iron (mg) | 9.5 | 10.4 | 8.5 |
| Carotene (µg) | 10 | 426 | 10 |
| Vitamin (mg) | - | - | 0 |
| Thiamine (mg) | 6.65 | 0.73 | 0.66 |
| Riboflavin (mg) | 0.23 | 0.39 | 0.22 |
| Niacin (mg) | 2.8 | 3.2 | 2.2 |

Sources: DFTQC (2017)

## 2.3 Methods of prepararion of kinema

Kinema can be prepared by traditional method using banana leaves or sal leaves or powdered straw as the source of fermenting organism mainly *Bacillus subtlis*. Kinema can also be made from pure culture method making the isolation of *Bacillus subtilis* from old kinema samples. Traditionally, kinema is prepared by cooking overnight-soaked whole soybeans by boiling and cooled to room temperature. They are then crushed lightly with a wooden ladle

to split the kernels. A small amount of firewood ash is added and blended with the whole soybean grits which are traditionally wrapped with banana or Leucosceptrum canum Smith leaves; polythene bags are also sometimes used. The wrapped mass is covered with sackcloth and kept in a warm place, usually above an earthen oven in the kitchen, for 1-2 days during summer or 2-3 days in winter (Tamang and Nikkuni, 1998). *Bacillus subtilis* is the predominant microorganism in kinema and is solely responsible for kinema production (Sundus *et al.*, 2016).The desired state of fermentation is indicated by the formation of mucilage and an unpleasant ammoniacal aroma (Tamang *et al.*, 1988). The flow chart for traditional and pure culture method of kinema preparation is given in the figure 2.1 and 2.2 respectively.

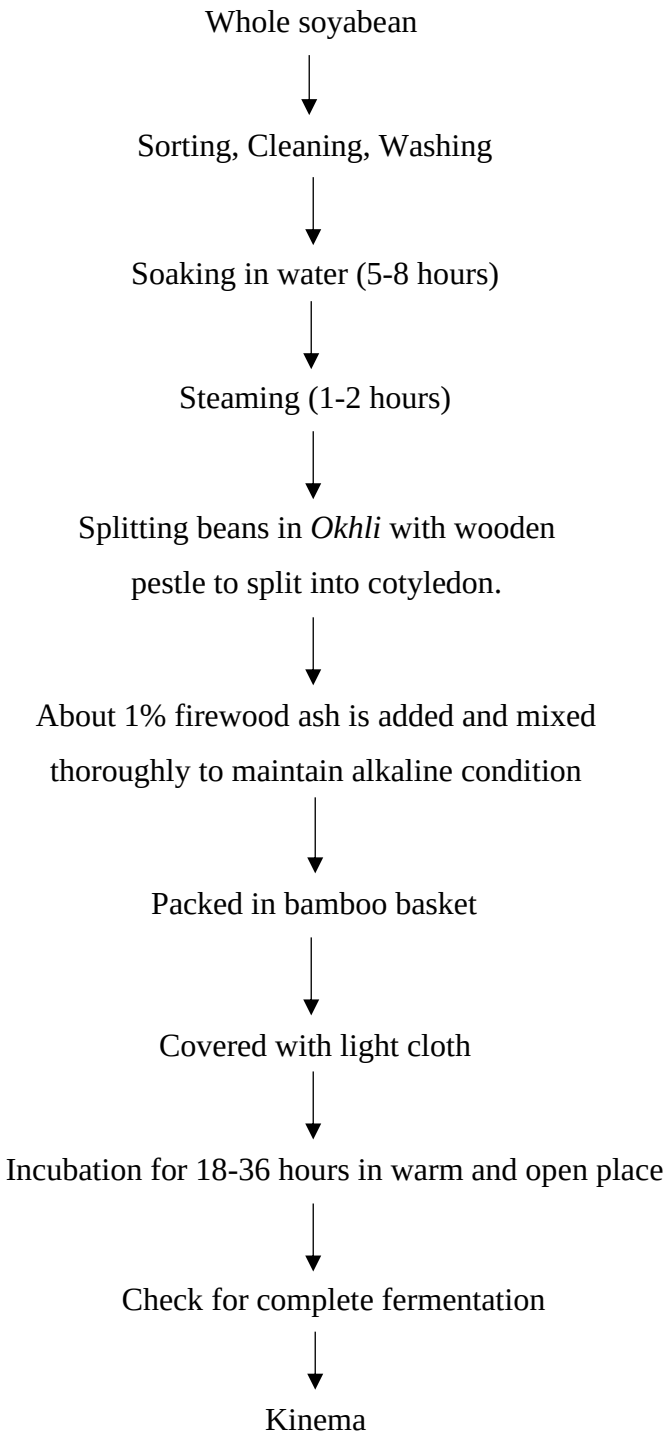


**Fig. 2.1** Flow sheet of kinema preparation by traditional method

Source: Shrestha (1989)

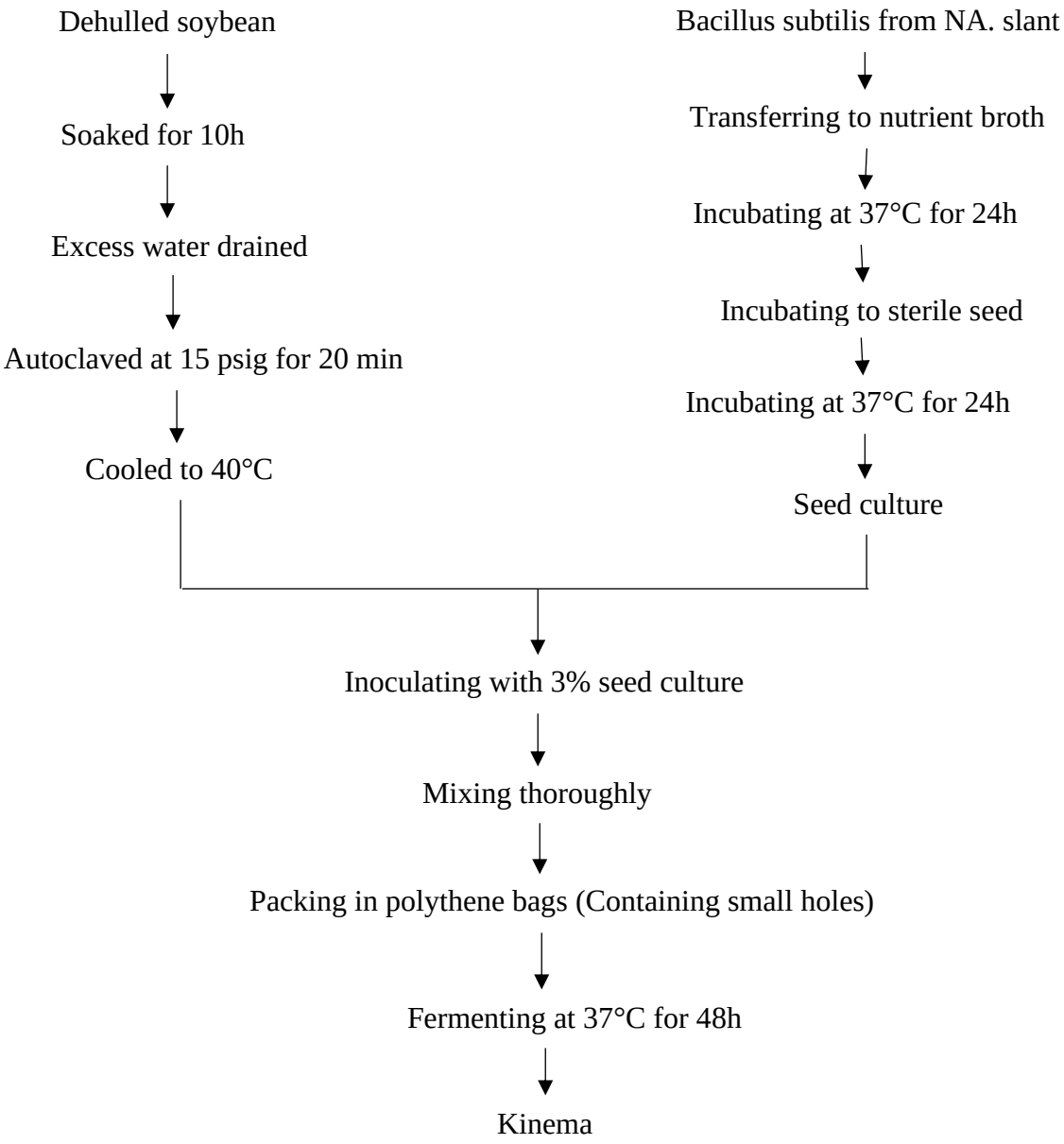


**Fig. 2.2** Flow sheet of kinema preparation by pure culture method.

Source: Shrestha (1989)

It is interesting to note the mountain women using their indigenous knowledge of food production prepare kinema. This unique knowledge of kinema-making has been protected as a hereditary right and passed from mother to daughter, mostly among the Limboo. Though there is good demand of kinema in the local markets, production of kinema is still confined to home production, there is no organized processing unit or factory of kinema production. Kinema making technology has not been recognized as small-scale industry till date

(Tamang, 2015). Production figure of kinema is not available. One obvious reason for limited kinema production is due to the virtual ignorance of this product. Another probable reason could be the typical ammonia odor which is not acceptable to other ethnic people living in other parts of the country.

## 2.4 Proximate composition of Kinema

The moisture content of kinema was 62%. Kinema contained about 48% protein, 17% fat, 28% carbohydrate and 7% ash; the energy value was 2.0 MJ/100g. The corresponding values in raw soyabean were 11%, 47%, 22%, 26%, 5% and 2.1 MJ/100g (Sarkar *et al.*, 1994).

**Table 2.2** Composition of kinema

| Properties | Kinema |
|---|---|
| Moisture% | 62 |
| Protein% | 48 |
| Fat% | 17 |
| Carbohydrate% | 28 |
| Ash% | 7 |

Sources: Sarkar *et al.* (1994)

One of the criteria for good quality of kinema is high stickiness of the product preferred by consumers (Tamang and Nikkuni, 1996). Relative viscosity and stickiness are probably due to production of PGA by *Bacillus* spp. (Chettri *et al.*, 2016). The production of a characteristic sticky material in kinema is called mucilage which is a mixture of poly gamma glutamic acid (PGA) and fructan produced by *Bacillus subtilis*. The mucilage produced from this type of fermentation has shown to have therapeutic potential against various chronic diseases. Sarkar *et al.* (1997) also compared the essential amino acid (EAA) profiles of Kinema with that of the reference patterns established by FAO/WHO/UNO which indicated the Kinema proteins as a good source of almost all EAAs (Essential Amino Acids) and that their score is as high as that of egg and milk proteins.

Hence, the fermented soybean can be utilized as a functional food material with potential applications in food, cosmetics, and medicines and also in the formation of characteristic aroma, flavor, and various compositional changes.

## 2.5 Kinema microflora and fermentation

Kinema is a non-salted, solid state fermented alkaline soyproduct. The major microflora reported to be associated in kinema fermentation are *Baciilus subtilis, Enterococus feecium* as bacteria and *Candida parapsilosis* and *Geotrichum candidum* as yeast. *Bacillus subtilis* has been reported to be most dominant in kinema fermentation. The duration of fermentation is reported to be 2-3 days depending on weather condition. The completion of fermentation is generally indicated by appearance of white viscous mass on the soybean seeds and typical kinema flavors with a slight odour of ammonia (Tamang, 2009).

## 2.6 Microbiological changes in kinema fermentation

*B. subtilis* was the most dominant organism during kinema fermentation. Its high initial count increased significantly at every 8 h interval during 48 h fermentation period. The load of *B. subtilis* DK-WI increased from 10 cfu $g^{-1}$ (wet weight) of soybeans at 0 h to 109 cfu $g^{-1}$ (wet weight) of kinema produced at 18 h. The increase in mean cfu of *E. faecium* was significant at every 8 h interval until the first 40 h of fermentation. Although the load of the only yeast, *C. parapsilosis* recovered from the laboratory made samples, was much less compared with the bacterial load, the mean cfu of the yeast cells increased throughout the period of fermentation; until 32 h, the increase was significant at every 8 h interval (Sarkar and Tamang, 1995).

The high initial load of *B. subtilis*, even at the onset of fermentation, was due to their presence on raw soybeans (Sarkar *et al.*, 1994) and their passage through soaking and cooking treatments. Although *E. faecium* and *C. parapsilosis* were not detected in raw beans, their recovery even at the start of fermentation indicates their entry through water. In many foods, *E. faecium* appears as a non-faecal contaminant. This species occurs predominantly in soak water of soybeans and is responsible for acidification of soak water (Sarkar and Tamang, 1995).

## 2.7 Physicochemical changes in Kinema Fermentation

Fermentation brings substantial chemical changes in kinema. During the first 16 h of traditional fermentation, as long as the organisms were growing exponentially, the pH of the fermenting beans decreased. Until the first 8 h, there was no significant rise in the contents of free fatty acids and non-protein nitrogen. Therefore it seems that sugars, not proteins or fats, were initially used as substrates for metabolism and growth of the organisms (Sarkar and Tamang, 1995).

Cooked soybeans contain sucrose, raffinose and stachyose, and *B. subtilis* was capable of producing acid from sucrose, raffinose and their hydrolytic products, glucose and fructose. *E. faecium* was capable of producing acid from galactose, another hydrolytic product of raffinose. *C. parapsilosis* was capable of utilizing sucrose and all the hydrolytic products of raffinose (Sarkar *et al.*, 1994). However, after this fall, the pH started rising significantly at every 8 h interval. This was due to strong proteolytic activities of microorganisms and consequent production of ammonia. While *B. subtilis* was capable of hydrolyzing proteins, *E. faecium* was unable to hydrolyze them (Sarkar and Tamang, 1995). Kinema fermentation reduces total sugar level of soybeans but increases the reducing sugar level. Crude fiber level of soybeans also decreased during fermentation due to hydrolysis by carbohydrate splitting enzymes from *B. subtilis* (Shrestha *et al.*, 2010). The organism was obligately aerobic and hydrolyzed gelatin and tributyrin (Owens *et al.*, 1997).

The protease produced by *B. subtilis* degraded soy proteins which resulted in the significant increase in non-protein and soluble nitrogen contents at every 8 h interval, starting from 8 h until the end of fermentation. Interestingly, total nitrogen increased significantly during fermentation (Sarkar and Tamang, 1995). Growth of *B. subtilis* on the cotyledons led to the formation of many additional volatile compounds and a greatly increased concentration of total volatile compounds. Some hydrolysis of *Bacillus* fermentation of soya beans fats and increase in free fatty acids also occurs (Owens *et al.*, 1997). Protease activity by the microorganism associated during fermentation can result in the release of bioactive peptides from the parent protein, which depending on the sequence of specific amino acids exhibits several functional properties (Rai *et al.*, 2017).

*B. subtilis* has high proteolytic activity that is evident by progressive increase in trichloroacetic acid-nitrogen (TCA-N) and ammonia nitrogen with Natto fermentation,

almost doubled after 48 h incubation. Visessanguan *et al.* (2005) also observed similar trend where *B. subtilis* during Thua Nao fermentation releases proteinases that play important role in proteolysis of soy proteins. These proteolysis reactions are reported to be responsible for the characteristic flavour of Thua Nao. It is reported three types of extra cellular enzymes namely: neutral proteases, alkaline protease and esterase play a major role during this activity and it was observed that most proteases are produced during exponential growth of microbes (Hu *et al.*, 2010).

## 2.8 Protease Enzyme

Enzymes are biocatalysts essential for life that catalyze almost every biological process (Kirk *et al.*, 2002). Since ancient times, enzymes have been used in the manufacturing of different food products, including beer, wine, vinegar, cheese, and sourdough, and in the production of commodities like leather, linen and indigo. Bio-catalysis has evolved as a necessary tool in industrial production of active pharmaceuticals, agrochemical and pharmaceutical intermediates, bulk chemicals, and food ingredients. Currently, most enzymes used in industrial processes are hydrolytic and are used for the degradation of various natural substances (Sharma *et al.*, 2019).

Proteases are one of the most important classes of proteolytic enzymes widely distributed in the animal kingdom, plant and as well as microbes. These enzymes possess the enormous commercial potential and have been used in several industrial processes, including food industry, leather processing, silk processing, detergent industry and therapeutic applications (Srilakshmi *et al.*, 2015). Proteases are very important enzymes, accounting for more than 60% of total global enzyme sales. Proteases, particularly alkaline proteases, hold a great potential for application in the detergents, food, leather and various other industries due to the increasing trend to develop environmentally friendly technologies. With continuous increases in demand for alkaline proteases, there is a need to explore information on methods to increase commercial production from new sources like kinema (Sharma *et al.*, 2019). Alkaline protease can be produced by different fermentation techniques, including solid state fermentation and submerged state fermentation. To obtain commercially viable enzyme production, fermentation media has to be properly optimized. Different factors like pH, nitrogen source, carbon source, metal ions, temperature and inhibitors affect the production of enzymes (Sharma *et al.*, 2019).

## 2.9 Classification of protease enzyme

Proteases constitute a large group of enzymes that catalyze the cleavage of peptide bonds in other proteins as well as within proteins. The classification of proteases is based on either their origin, catalytic mechanism, specificity or the nature of reactive group in the catalytic site. Based on the site of action on polypeptide chains, proteases are divided into two groups, i.e. exopeptidases and endopeptidases (Razzaq *et al.*, 2019). Exopeptidases act at the amino or carboxyl ends and are further sub-classified as amino- and carboxypeptidases based on the site of action at the amino- or carboxyl terminus, respectively, while endopeptidases cleave peptide bonds in the inner regions of the polypeptide chains away from the both end terminus. Endopeptidases are further sub-classified into six groups i.e., serine, aspartic, cysteine, metallo, glutamic acid and threonine proteases based on the essential catalytic residue present in the active site. (Mazorra-Manzano *et al.*, 2018).

### 2.9.1 Serine Protease

Serine proteases are one of the largest groups of proteolytic enzymes, found in eukaryotes and prokaryotes. They exhibit different types of activities including exopeptidases, endopeptidase, oligopeptidase and omegapeptidas (Barrett, 1994). Serine proteases are characterized by the presence of a reactive serine residue in the active site and share a number of biochemical and physiological features. In humans, they are divided in major groups, including the chymotrypsin-like, the subtilisin like, the alpha/beta hydrolase, and signal peptidase groups (Borgoño *et al.*, 2007). In plants, they are widespread among taxonomic groups, from trees and crops to legumes and herbs and present in almost all plant parts, but most abundant in fruits. Serine proteases from cucurbits, cereals, and trees are usually classified together (Barrett, 1994).

These proteases are optimally active over a wide pH range 7-11 and have broad substrate specificities including amidase and esterolytic activity. Serine alkaline proteases have largest commercial application owing to their high activity and stability in extreme reaction conditions. This group of enzymes are generally inhibited by di-isopropyl fluorophosphates(DFP), phenylmethylsulfonyl fluoride (PMSF) (Singh *et al.*, 2016). The molecular masses of alkaline protease are in the range of 15 to 30 kDa (Rao *et al.*, 1998). Most of the neutral and alkaline protease that are commercial serine proteases are

produced from bacteria of the genus *Bacillus* (Mamo and Assefa, 2018). Some of the serine protease and their characteristics is shown in the table 2.3.

**Table 2.3** Characteristics of serine protease

| Protease | Alcalase (Subtilisn) | Serine type | Dubiumin serine | Nattokinase |
|---|---|---|---|---|
| Sources | *Bacillus licheniformis* | *Caesalpinia bonducell seed* | *Solanum dubium* Fresen seed | *Bacillus subtilis* |
| Molecular wt. (KD) | - | 63 | 66 | 23.5 |
| Optimum pH | 6.5-8.5 | 8 | 11 (3-12) | 9 (8-10) |
| Optimum Temp (°C) | 60 | 40 | 70 | 37 (35-39) |
| Effects of substrates | Goat whey | AzoCN>BSA> CN>Hb>gelatin | - | - |
| Effects of inhibitors | - | PMSF | PMSF and chymostatin | Drastically inhibited by PMSF and partially inhibited by EDTA |
| Effects of metal ions | $Fe^{+3}$, $Ca^{2+}$ have (+ve) effect | - | - | $Cu^{+2}$, $Zn^{2+}$ strongly (-ve) effect & Mg2+, Ca2+ and Mn2+ have negligible effect |
| References | (Shu *et al.*, 2015) | (Khan *et al.*, 2010) | (Ahmed *et al.*, 2009) | (Choi *et al.*, 2017; Thu *et al.*, 2020) |

### 2.9.2 Aspartic Protease

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. They are widely distributed in fungi, but are rarely found in bacteria or protozoa. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3), and have been placed in clan AA. The members of families A1 and A2 are known to be related to each other, while those of family A3 show some relatedness to A1 and A2. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa (Rao *et al.*, 1998). These proteases preferentially cleave peptide bonds between non-polar amino acids residues. These proteases are inhibited by pepstatin and, in the presence of copper ions by diazoketone compounds (Singh *et al.*, 2016).

### 2.9.3 Cysteine Protease

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. Though reducing agents such as HCN or cysteine, DTT, EDTA are required for stimulation of the catalytic activity of cysteine proteases but inhibited by sulfhydryl (SH) reagents such as 4-hydroxy mercuri benzoic acid (p-CMB), iodoacetic acid, iodoacetamide, etc. They have high potential in food and pharmaceutical applications due to their activity over a wide range of temperature and pH. Most of these enzymes are active at pH 5-8. They are involved in metabolic degradation of proteins and peptides such as scrapie protein degradation dendritic and neuronal cells. Papain is one the best known microbial cysteine protease, which is widely used in food industry (Rao *et al.*, 1998; Singh *et al.*, 2016).

### 2.9.4 Metallo Protease

Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion such as zinc, cobalt or manganese for their catalytic activity (Rao *et al.*, 1998). They contain enzymes from different origins, such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria. A total of about 30 families of metalloproteases have been

documented, out of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 contains both endo- and exopeptidases (Mamo and Assefa, 2018). These proteases are sensitive to chelating agents, such as EDTA, due to sequestering effect of chelating agents on the metal ions involved in the catalytic mechanism. They have a wide range of substrate specificity and find wide range of applications in different industries including drug development (Singh *et al.*, 2016).

## 2.10 Sources of protease enzyme

Proteases constitute a large group of enzymes that catalyze the cleavage of peptide bonds in other proteins as well as within proteins. Protease mainly involved two groups of hydrolytic enzymes known as exo and endo peptidase. Exopeptidase act at carboxyl and Aminoacid end respectively known as carboxy peptidase and aminopeptidase while endopeptidase act at interior sequence of the peptides and protein molecules (Mazarro-Manzano *et al.*, 2017). Proteases, one of the most valuable industrial enzymes, have potential applications in a wide number of industrial processes such as food, feed, leather, textile, pharmaceutical industries. Proteases can be obtained from animals, plants and microorganisms (Razzaq *et al.*, 2019; Singh *et al.*, 2016).

### 2.10.1 Protease from Microorganism

Microbes serve as a better source of proteases than plants and animals because they can be cultured in large amounts in a short duration, are relatively inexpensive, and can produce a continuous supply of the desired product (Boominadhan *et al.*, 2009). Proteases from microorganisms are the largest group of industrial enzymes. Microbial proteases account for greater than two- third (>60%) of total global sale of enzymes and are most widely studied protease (Razzaq *et al.*, 2019). Various microbial proteases have been commercialized, among them proteases from *Bacillus sp.* and *Aspergillus sp.* are extensively studied. Some fungal species like *Aspergillus sp*. have been also studied thoroughly for the production of alkaline protease (Singh *et al.*, 2016).

### 2.10.2 Protease from animals

The most widely used animals derived proteases are pancreatic trypsin, chymotrypsin, pepsin and rennin. Trypsin, an intestinal digestive enzyme, is utilized for biocontrol of insect

pests, microbial growth media and few medical applications. Chymotrypsin and rennin are extensively used in deallergenizing of milk protein hydrolysate and preparation of curd, respectively (Singh *et al.*, 2016).

### 2.10.3 Protease from plant

Protease obtained from plant sources are attractive because they can be used over wide range of temperature range and pH in presence of organic compounds as well as other additives (Jinka *et al.*, 2009). Plant proteases are widely used in food and pharmaceutical industry.
The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Most extensively explored plant proteases are bromelain, ficin and papin extracted from Ananas comosus, Ficus carica and Carica papaya, respectively. These proteases are utilized for different application such as brewing, tenderization of meat, milk coagulation, digestion, viral and cancer treatment. Keratinases, another important plant protease, hydrolyze hair and wool to produce essential amino acids and to prevent clogging of waste water system (Razzaq *et al.*, 2019). The incapability of the animal proteases to meet current world demands has led to an increased interest in plant proteases (Hamza, 2017). Although the use of plant proteases is still relatively limited to papain and bromelain from papaya and pineapple, respectively, the application of new plant proteases is increasing (Mazorra-Manzano *et al.*, 2018).

## 2.11 Kinema as a potential sources of protease enzyme

Soybean is rich in proteins and several other bioactive components including phenolic acids, isoflavones and flavanols, which can be converted to more bioavailable and bioactive form during fermentation (Cho *et al.*, 2011). Previous studies have shown that *B. subtilis* is the key organism responsible for fermentation of different varieties of alkaline traditionally fermented soybean products (Rai *et al.*, 2017). Due to the production of specific hydrolytic enzymes such as proteases, α-amylase, cellulose, pectinase and β-glucosidase, *Bacillus* spp. have been used for solid state fermentation of plant matrix including agricultural by-products (Rai *et al.*, 2017).

These hydrolytic bacteria are associated with utilization and reduction of indigestible oligosaccharides and polysaccharides. The organism has also shown to reduce the activity

of anti-nutrients that hinders availability of proteins and phytochemicals present in soybeans which makes the kinema a potential source of essential hydrolyzing enzymes (Sarkar *et al.*, 1997).

A 60 fold increase in free amino acid in kinema compared to raw soybean has also been reported. Kinema has been reported to possesses high antioxidant activity due to presence of bioactive peptides and phenolics compound release during fermentation. (Sanjukta and Rai, 2016) These findings well suggested the presence of high protease activity indicating kinema fermentation and associated organisms in kinema fermentation (mainly *Bacillus subtilis*) could be potential source of protease enzyme.

### 2.11.1 Protease from different alkaline fermented food

Various soyabean fermented alkaline food products, similar to kinema has been reported to be good source of protease. *Bacillus* species are predominant and expected to be responsible for the fermentation of soybean based fermented food, in which their proteases help to accelerate the hydrolysis of protein, thus releasing ammonia. Such a mechanism provides an alkaline condition. These fermented soybean products are therefore often termed “alkaline fermented soybeans”. Other related fermented soy products include Indian kinema, Chinese douchi, and Burmese chine pepoke. These products do have similar properties (i.e., stickiness and typical flavor). Interestingly, such products are located in a specific region referred to as a theoretical triangle, with an individual angle on Japan (natto) and Korea (chongkukjang), Sikkim and Nepal (kinema), and Thailand (thua nao) (Visessanguan *et al.*, 2005).

Thua nao is an indigenous alkaline-fermented soybean of Thailand. It is hypothesized that protein hydrolysis is possibly a major activity in Thua nao. Microbial proteases help to accelerate the protein degradation into various peptides and amino acids. In addition, there is a conversion of isoflavones, which are abundant in soybeans, by these microflora. The biochemical mechanisms of these fermented soybean products are similar to kinema. Other Bacillus species found are *B. licheniformis, B. megaterium,* and *B. pumilus*. These *Bacillus* species are very proteolytic. Enzymatic degradation of soy proteins is hence definitely a key process in thua nao fermentation. In general, the soy proteins are hydrolyzed into peptides, amino acids, and ammonia (Visessanguan *et al.*, 2005).

Natto is a traditional fermented soy food of Japan. The bacteria, identified as *Bacillus subtilis* is the organism responsible for this fermentation. Thus, natto possesses the characteristic odor and persistent musty flavor of this organism and is also covered with the viscous, sticky polymers that this organism produces. Hu *et al.* (2010) tested natto as a source of protein in the diets of infants and concluded that it could substitute, at least in part, for animal protein with no adverse effects on growth, digestibility, and nitrogen retention.

This proteolysis and other enzymatic activities are expected to contribute to a good source of protease in all soyabean based alkaline fermented food (Visessanguan *et al.*, 2005). So kinema being similar products can also have huge source of protease which could have different application but the extraction, purification and characterization of those protease is still lacking which should be carried out to enhance its application.

## 2.12 Application of Protease

The use of enzymes and microorganisms in processing raw materials from plant and animals has been practice for a long time. (Uhlig, 1998). Proteases being one of the most important group of enzymes that are efficiently involved in food industry for enhancing nutritional value, digestibility, palatability, flavour and reducing allergenic compounds as well as in management of domestic and industrial wastes. In addition to this, they occupy a pivotal position owing to their broad application in detergents, pharmaceuticals, photography, the leather industry, cosmetics and agriculture. Accordingly proteases account for about 60% of the total industrial enzyme sale in the world (Hamza, 2017). According to Contesini *et al.* (2018) some important applications of protease enzyme from *Bacillus* spp. are listed below:

### 2.12.1 Food industry

Protease use in food industries for protein modification and to improve palatability and storage stability of the available protein sources. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value with well-defined peptide profile. It also plays a great role in meat tenderization, especially of beef.

### 2.12.2 Pharmaceutical industry

Proteases of the subtilisin group are used for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses to speed up healing process by producing anti-

inflammatory response in patients have been reported. It is possible to use protease as a therapeutic agent for the treatment of pulmonary emboli and degradation of elastin, collagen. The purified protease from bacteria could be used for various purposes like antibacterial activity against clinical pathogens as well as it degrades slime and bio films to limit gram negative bacteria. In addition, it digests debris in blood like bacterial and viral proteins and as medicine in the field of oncology.

### 2.12.3 Textile and leather industry

Proteases are useful in dehairing for the purpose of leather manufacture. Since, the beginning of human civilization the conventional method of dehairing involves the use of lime and sodium sulphide as the lack of technology. But currently it is possible to replace chemical dehairing with enzyme based dehairing processes using proteases. This avoiding the use of lime and sulphide are being developed because of their environmental benefits. Thus, enzyme based dehairing processes using proteases help to reduce or even avoid those chemical and offer enormous environmental benefits. In addition, to improve the quality of leather produced.

### 2.12.4 Detergent industry

The idea of using detergent enzymes dates back to 1914 when two German scientists, Rohm and Haas, used pancreatic proteases and sodium carbonate in washing detergents. However, it was only in 1963 alkaline protease was effectively incorporated in detergent powder. Due to this its economic importance became well known. However, currently proteases constitute the largest product segment in the global industrial enzymes market. The detergent enzyme market has grown nearly 10-fold during the past 20 years. Removal of proteinaceous stains such as blood, milk, egg, grass and chocolate are very difficult using conventional detergent method. However, removal of such stain is achieved by using bacterial proteases. In addition, the use of protease supplementation to detergent formulation significantly improves the cleansing of proteinaceous stain and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies.

### 2.12.5 Application of protease in waste management

The scarcity of natural resources and the accumulation of pollution caused by human activity have required the development of production technology that is less harmful to the environment. One well-established application of modern biotechnology is the use of bacterial protease for treatment of waste or the bioremediation of hydrocarbons. In addition to that these enzymes also offer advantages over the use of conventional chemical catalysts for numerous reasons. Bacterial proteases have interesting potential applications in the management of wastes from households and processing.

## 2.13 Extraction and purification of protease enzymes

Biomolecules from plant and microorganisms have been used for centuries, and their demands have increased in food, medicinal, and chemical industries due to their unique biological activities. Bioactive compounds are constituents other than nutrients that generally occur in small quantities of foods, and whose intake has been associated with protective effects against adverse health or physiological disorders, for instance, cardiovascular, diabetes, or cancer. One of the main challenges is to extract these biomolecules from their respective natural sources. Different techniques have been reported on these aspects in the literature, each having their own pros and cons. The choice of technique mainly depends on the type of raw material, environmental concerns, process conditions, and future applications of the bioactives.

Many studies have been published in literature about purification of protease with different method. The recovery and purification of enzyme typically involves numerous process steps including; precipitation with ammonium sulphate and/or organic solvents, filtration or centrifugation, dialysis, often followed by several chromatographic separation techniques such as ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography or affinity chromatography. At the end of the purification process steps, the enzymes must be concentrated by lyophilization or ultra-filtration (Nadar *et al.*, 2017). The retention of catalytic activity for enzymes is important for the success of the extraction processes. In this context, significant efforts have been focused on the development of new or adapted technologies for the purification of enzymes, with lower costs, as well as they should be sustainable, efficient and biocompatible with the enzyme conformational structure. So, there is a need to develop easy, fast, scalable and

economically feasible methods to extract enzymes either from fermentation or natural sources.

## 2.14 Extraction and purification Techniques

Over the years, different extraction techniques have been reported and used for the extraction and purification of the biomolecules from the natural resources. The low content of active molecules in the source material and the complexity of raw material makes it necessary to find alternative methods of effective extraction. It is worthwhile to understand the conventional methods of extraction before discussing enzyme-aided extractions. Traditional methods to isolate and purify proteins involve several steps such as ammonium sulfate precipitation, ionic and affinity chromatography, dialysis and final concentration of the product, which are time and cost consuming, induce loss of their biological activity and reduce the yield of the whole process. The purification process is a difficult and important matter, owing to the complexity of the protein mixtures and the necessity to retain their biological activity after purification. It has been shown that the 50–90% of the total production cost of a biological product is determined by its purification grade (Porto *et al.*, 2008). Some of the extraction and purification methods are listed below:

### 2.14.1 Solvent Extraction Methods

Solvent extraction is the oldest and traditional method of extraction which mainly depends on factors such as nature of the solvent, energy input, and agitation to improve the chemical solubility and efficiency of mass transfer. The selection of solvent for the extraction depends on the raw material to be used and the product of interest. Lipophilic compounds can be well extracted using nonpolar organic solvents such as hexane or dichloromethane, whereas hydrophilic compounds can be extracted using polar solvents such as acetone, methanol, or ethanol. Based on these study made by Marathe *et al.* (2017) some methods of extractions methods are:

#### 2.14.1.1 Buffer extraction

The suitable buffer can be used for the extraction of protease depending on its nature. The protease gets dissolved in buffer and gets extracted on it. Balqis & Rosma (2011) demonstrated an extraction method where leaf samples were extracted using 1 mL of 0.05

M sodium phosphate buffer, pH 7.0. 100 mM sodium chloride (NaCl), 10 mM ascorbic acid, and 0.75 g polyvinylpolypyrrolidone were used to make the sodium phosphate buffer (PVPP). The homogenates were filtered with Whatman No 1 filter paper before centrifugation in a refrigerated centrifuge for 20 minutes at 4 °C and 10000 rpm. The supernatant (crude enzyme) was transferred from the 15 mL centrifuge tube and stored at 4 °C for further protease activity assay and total protein determination.

### 2.14.2 Precipitation method of protease

#### 2.14.2.1 TCA-acetone precipitation method

TCA-acetone precipitation protocol has been described in Abdullah et al., (2017) where the extraction solution was freshly prepared in cold acetone with 10% TCA and 0.07% β-mercaptoethanol. In a 15 mL centrifuge tube, 150 mg of leaves were extracted using 2 mL of extraction solvent. At 4°C, the mixture was incubated for 24 hours. A refrigerated centrifuge was used to centrifuge the mixture for 15 minutes at 10000 rpm and 4°C. The supernatant was removed from the tube and transferred to other tubes, leaving only the pellet inside. Using ice-cold acetone with 0.07 percent β-mercaptoethanol, the pellet was rinsed twice until it became colorless. Finally, the pellet was dried and re-suspended in a pH 7.0 0.05 M sodium phosphate buffer.

#### 2.14.2.2 Ethanol/ Acetone precipitation

Methanol, ethanol, isopropanol and acetone are important precipitants. Ethanol precipitation is an attractive technique because it has good physicochemical properties, complete miscibility with water, high volatility, low cost and low toxicity. Precipitation by adding organic solvents is caused by a decrease in the dielectric constant of the aqueous solution. This reduction triggers an increase in the electrostatic interaction of oppositely charged regions of proteins, which enables aggregation and, subsequently, precipitation. Moreover, hydrophobic regions of the protein are exposed by the addition of these organic solvents due to the displacement of water surrounding the biomolecule, which favours aggregation (Gimenes *et al.*, 2021).

### 2.14.3 Methods of partial purification

The purification of enzymes usually involves several methods depending on their size, charge, hydrophobicity or capability to bind to particular compounds. Protease purification has been commonly performed by precipitation with organic solvents or salts and sequential chromatography (Gimenes *et al.*, 2021).

#### 2.14.3.1 Ammonium sulphate precipitation

All of the purification procedures were operated at 4°C and conducted according to the report of (Wang *et al.*, 2009). Ammonium sulfate was added to the supernatant of crude enzyme inorder to obtain the solution of different saturations ranging from 30 to 80% (in increments of 10%). The precipitate was collected by centrifugation (5000 rmp, 15 min). The pellet was dissolved in small volume of sodium phosphate buffer (pH 7, 50 mM) and the solution was dialyzed against the same buffer overnight (Ueda *et al.*, 2007).

#### 2.14.3.2 Dialysis

In separation by means of diffusion (dialysis) using a dialyzer, the clearance rate of low molecular weight solutes is higher. When the objective is separation, diffusion presents a convenient means of separating low and high molecular weight solutes, but the low membrane permeability rate is a major reason that this phenomenon is not widely used in industry. Membranes may be distinguished on the basis of pore size as reverse-osmosis (0.2-0.5 nm), dialysis (2-5 nm), ultrafiltration (5-50 nm) and microfiltration ( l00-5,000 nm) membranes (Sakai, 1994).

#### 2.14.3.3 Further purification method

Kumar *et al.* (2005) and Kazem and Habeeb (2020) reported chromatographic method of separation (anion exchange chromatography and affinity chromatography), Electrophoresis, Three phase portioning (TPP) in their various studies.

## 2.15 Characterization of protease

Proteases are a complex group of enzymes which differ in their properties such as substrate specificity, active site, and catalytic mechanism. Their exquisite specificities provide a basis for their numerous physiological and commercial applications. Despite the extensive research on several aspects of proteases from ancient times, there are several gaps in our knowledge of these enzymes and there is tremendous scope for improving their properties to suit projected applications (Rao *et al.*, 1998).

Homogeneity and characterization of purified proteases are prerequisites for studying their mechanism of action and exploitation in production value added products. Characteristic properties of the purified enzyme, such as optimum pH and temperature, organic solvents, surfactants and oxidizing agents, substrate specificity, stability, effects of metal ions and effects of inhibitors on purified enzyme, needs to be optimized in order to find its commercial potential, which prove beneficial for use of human kind and can be useful addition to the existing enzyme world. In general, industrial use of proteases highly depends on their stability during isolation, purification and storage in addition to their robustness against solvents, surfactants and oxidants (Sari *et al.*, 2015).

# Part III

# Materials and methods

## 3.1 Materials

### 3.1.1 Collection of samples

The local variety of white soyabean was collected from the local market of Dharan. The collected grains were cleaned and sorted to remove dust, foreign matters and damaged ones.

### 3.1.2 Chemicals

Whey protein concentrate, sodium phosphate dibasic heptahydrate, bovine serum albumin, sodium phosphate basic monohydrate, ammonium sulphate, Bradford reagent, coomassie brilliant blue G-250, HCl acid, ethanol, tris buffer, phosphate buffer, tyrosine.

### 3.1.3 Glassware

Petri dish, watchglass, burette, pipette, test tubes, volumetric flask, funnel, conical flask, measuring cylinder and micropipette.

### 3.1.4 Equipments

Hot air oven, electric balance, thermometer, heating mantle (burner), Buchner funnel, uv-visible spectrophotometer, hot water bath, incubator, filter paper, micropipette, centrifugation machine, petri-plates, Bunsen burner, weighing machine, mortar and pestle, magnetic stirrer, pressure cooker.

### 3.1.5 Other equipments

Banana leaves, fern leaves, muslin cloth, bamboo basket, wood ash, tray etc.

## 3.2 Methods

### 3.2.1 Research frame work

Preliminary study was made by preparing kinema and monitoring its daily proteolysis activity during fermentation to confirm optimal proteolysis activity. Then the study deals with partial characterization of protease extracted from kinema. When protease activity was at maximum during the fermentation period, protease from kinema, was extracted in sodium phosphate buffer pH-7 and the crude extracted protease was confirmed by performing caseinolytic assay using casein as substrate. Once the conformation was done, the extracted protease was further purified by ammonium sulphate precipitation method followed by dialysis against the same buffer. Partial characterization of the extracted proteases was done to identify optimum temperature and optimum pH of the protease activity, substrate specificity, effect of protease inhibitors (PMSF, IDA and EDTA) to determine probable type of protease, effect of monovalent, divalent and trivalent ions like $K^{+}$, $Na^{+}$, $Hg^{+}$, $Ca^{++}$, $Co^{++}$, $Zn^{++}$, $Mg^{++}$, $Fe^{+++}$, $Al^{+++}$ of the enzyme, and storage stability of protease as according to method applied by Sari *et al.* (2015) with some modifications.

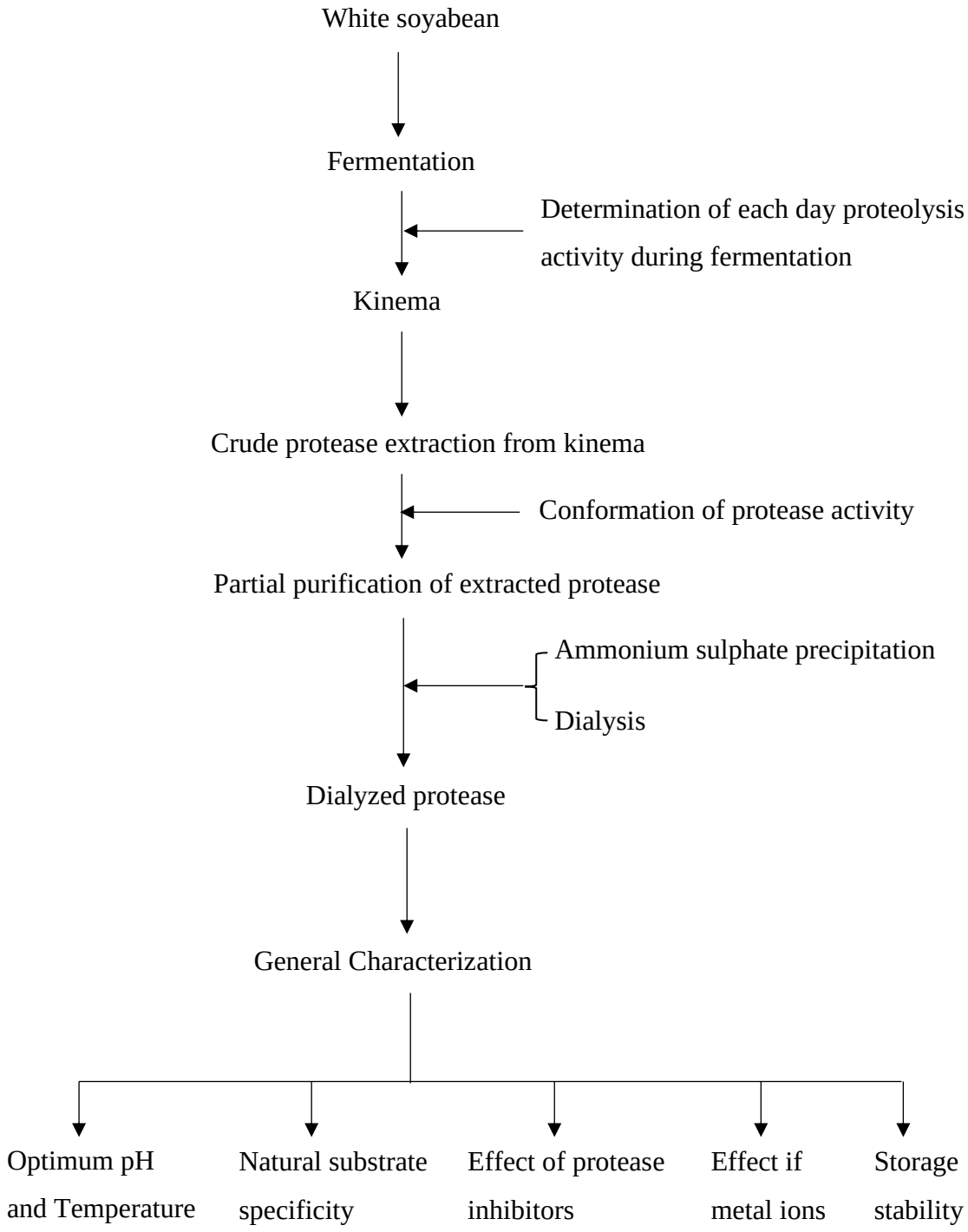


**Fig 3.1** General Frame work diagram after preliminary study

### 3.2.2 Preparation of kinema

White variety of soybean (*Glycine max (L.) Merrill)* was collected from the local market of Dharan. The soybean was then sorted manually so as to remove any straw, large soil and stones, wires, metals, and other visible foreign matters. Then cleaning with water was done so as to remove any adhered dirt, dust and mud. Then soybean was soaked overnight in water (8- 25 addition of clean water; addition 1% firewood ash, 10h). Excess water was drained after the completion of soaking. Soybeans were then steamed for 1 to 2 hours in a well-covered vessel till the beans become soft enough to be splitted by the use of two fingers. The seeds were then cooled to temperature around 40°C and macerated with hands so as to expose the cotyledons from seed coat. 1% firewood ash was also incorporated at this stage. Cracked beans was then kept in a bamboo basket lined with banana leaves or ferns fords and wrapped with same leaves. It was further covered by muslin cloth. The cracked seeds were then left for fermentation at room temperature (30±3°C) for 48-72h.

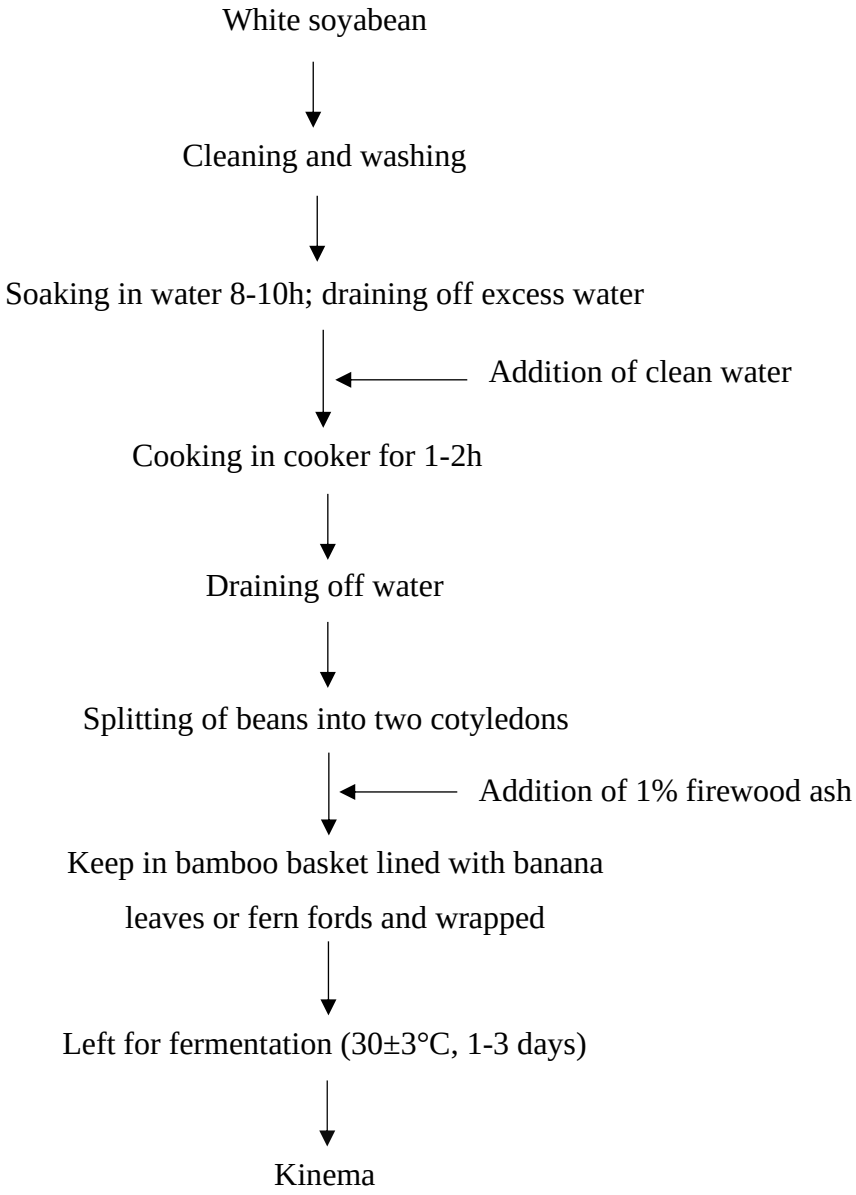


**Fig 3.2** Indigenous method of kinema production in Nepal

Source: (Tamang, 2015)

### 3.2.3 Determination of proteolysis during kinema fermentation

In order to determine maximum proteolysis during kinema fermentation, sample was taken at every interval of 24h during the fermentation period 0 to 4 days. Samples (3g) was homogenized in 4ml of deionized water on magnetic stirrer for 25min at refrigerated condition. The homogenate was filter through the clean muslin cloth and volume made up to 4ml followed by centrifugation at about 7000rpm for 15 min. After centrifugation,

supernatant was filtered through whatmann 41 filter paper, the obtained filtrate was collected in clean test tube and volume was adjusted to 4 ml by same deionized water. At each step, the protease activity was measured in an ultraviolet spectrophotometer as described in section 3.4.5.

## 3.3 Analysis of chemical component of soybean and kinema

### 3.3.1 Moisture content

Moisture content of the sample was determined by heating in an oven at 130 ± 2 °C to get constant weight (Ranganna, 1986). Weighing of the sample was done on tared Petri plates using electronic balance.

### 3.3.2 Crude protein content

Crude protein content of the samples was determined indirectly by measuring total nitrogen content by micro Kjeldahl method. Factor 6.25 was used to convert the nitrogen content to crude protein (Ranganna, 1986).

% protein = % nitrogen × 6.25

### 3.3.3 Crude fat content

Crude fat content of the samples was determined by solvent extraction method using Soxhlet apparatus and solvent petroleum ether (Ranganna, 1986).

### 3.3.4 Total ash content

Total ash content of the samples was determined by following the method given by (Ranganna, 1986) using Muffle furnace.

### 3.3.5 Crude fiber content

Crude fiber content of the samples was determined by the method given by (Ranganna, 1986).

### 3.3.6 Carbohydrate

The carbohydrate content of the sample was determined by difference method (Shrestha, 1989) as follows:

%Carbohydrate (dry basis) = 100-% protein - % fat- % ash- %crude fiber

## 3.4 Extraction and partial purification of kinema protease

### 3.4.1 Extraction of crude protease

For the preparation of crude enzyme extract, enzyme was extracted from three days fermented kinema with the solvent Sodium phosphate buffer (pH-7, 50mM) keeping kinema: solvent ratio of 1: 1.5 (w/v). For this, 80g of kinema was mixed with 120 ml of sodium phosphate buffer (pH 7.0, 50mM) then, the mixture was stirred by magnetic stirrer for 25 minutes. After stirring, the whole stirred content was filter through the clean muslin cloth and volume made up to 120 ml followed by centrifugation for 15 minutes at around 7000rpm on centrifuge tube. And the centrifuged sample content was filter through the muslin cloth and filtrate made up to 120ml by same buffer (Nafi *et al.*, 2013).

### 3.4.2 Partial purification by Ammonium Sulphate

The crude extract (120ml) of the protease was precipitated by mixing with gradual increasing concentration of 30%, 40%, 50%, and 60%, 70%, 80% saturated ammonium sulphate at refrigerated condition followed by centrifugation at about 7000rpm for 15 minutes to obtain partially purified protease enzyme. After centrifugation precipitated was collected and dissolved with sodium phosphate buffer (pH 7, 50mM) to make total volume of 12ml, according to the method given by Ueda *et al.* (2007) with suitable modification. At each step, the protease activity and total protein content were measured in an ultraviolet spectrophotometer.

### 3.4.3 Partial purification by Dialysis

The ammonium sulphate purified protease was further purified by a Dialysis Memberane-110 (LA395), of 8-10 cm, permeable, regenerated seamless cellulose tube having molecular weight cut off between 12,000 to 14,000. In order to stop enzyme leakage, the determined volume of partially purified enzyme was stored in a dialysis bag that was knotted at both

ends with thread. The enzyme-containing bag was then immersed for a day in a solution of sodium phosphate buffer (pH 7, 50mM), with three times buffer exchanges. Following this procedure, the enzyme is dialyzed, and the final volume was measured. This source of dialyzed enzyme was used for further enzyme characterization. At each step, the protease activity and total protein content were measured in an ultraviolet spectrophotometer.

### 3.4.4 Determination of protein content

The protein content of crude extract and partially purified fraction of enzyme was determine by Bradford method (Bradford, 1976). Bradford assay was performed by adding 3 ml of Bradford reagent to 100µl of each protease extract, and homogenization by using vortex mixer and incubate at 37°C for 30 minutes and the absorbance was read at 595 nm (Kruger, 2009). Bovine serum albumin (BSA) standard (0.1 to 1.2 mg/ml) solutions were used to prepare the standard curve. The protein content was determined by comparing the absorbance of sample with the BSA standard curve and expressed as mg per ml.

### 3.4.5 Protease activity of the extracted enzyme

The presence of protease in the extract was conformed by performing protease assay using casein as substrate according to the procedure given by (Cupp-Enyard, 2008) . 0.1ml of crude/partially purified protease extract was added to 2.2 ml of (5mg/ml) casein solution prepared with sodium phosphate buffer (50mM, pH 7). The reaction was incubated at temperature 35°C for 10 min and stop by addition of 3.6 ml of 5% Trichloroacetic acid (TCA), cooling at refrigeration condition followed by centrifuge at about 7000 rpm for 15 min. The supernatant was filter though the Whatman no.41 filter paper and collected in a clean test tube. The blank was prepared in similar manner by addition of same of same volume of enzyme extract after addition of 5% TCA followed by incubation, store at refrigeration condition, centrifugation and filtration. When casein is digested by protease, tyrosine gets liberated along with other amino acids and peptide fragments. The tyrosine thus liberated was determined as protease activity after allowing to react with Folin and ciocalteus phenol reagent. For this both each 1ml filtrate of blank and test sample were mixed with 5ml of 2% Sodium carbonate reagent and 0.5ml FC-reagent in separate test tube. The mixed reagents and samples were incubated at 37°C for 45min and absorbance was measured by spectrophotometer at 700nm.

The absorbance values produced by the protease's activity was compared to a standard curve created by reacting known amounts of tyrosine with FC reagent. This helps to correlate variations in absorbance with the amount of tyrosine in micromoles. The activity of protease samples was be calculated in terms of Units, which is the quantity of tyrosine equivalents released from casein per minute in micromoles. One protease unit is defined as the amount of casein hydrolyzed to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per minute at 37°C and 7.0 pH. The protease activity (unit/ml enzyme extract) was calculated by using the formula:

$$\text{Protease activity}\left(\frac{\text{Units}}{\text{ml enzyme}}\right)=\frac{(\mu\text{mol tyrosine equivalent released}) \times V_t}{(V_e \times V_c \times t \times 181)}$$

Where, $V_t$ is the total assay volume in ml, $V_e$ is the volume of enzyme used in ml, t is the time of assay in minute, and $V_c$ is the volume used in colorimetric determination in ml.

### 3.4.6 Stability of the enzyme

Crude enzyme was extracted and partially purified by ammonium sulphate precipitation method. The partially purified enzyme solution was stored at freezing (at -4±2°C) condition. Protease activity was determined from 0-7 days to find out the ability of enzyme to hydrolyze the same casein from 0 days to 7 days.

## 3.5 General characterization of protease

General characterization of the extracted and partially purified protease was work to identify optimum temperature and pH of the enzyme, types of enzyme, storage stability and effect of metal ions ($K^+$, $Na^+$, $Hg^+$, $Ca^{++}$, $Co^{++}$, $Zn^{++}$, $Mg^{++}$, $Fe^{++}$, $Al^{+++}$) in the enzyme according to method applied by Adinarayana *et al.* (2003) with some modifications.

### 3.5.1 Determination of optimum pH

Different pH values ranging from 4 to 10 were used to determine the optimum pH of partially purified enzymes. Buffers used were 50mM citrate buffer for pH 4.0 and 5.0, 50mM, Sodium phosphate buffer for pH 6.0 and 7.0, 50mM tris– HCl buffer for pH 8.0 and 9.0, and Carbonic acid buffer for pH 10. Casein was dissolved in each buffer to obtain 5mg concentration and

then subjected to assay the protease activity of partially purified enzymes as described earlier on section 3.4.5.

### 3.5.2 Determination of optimum temperature

To determine the optimum temperature, casein solution (5mg/ml) was prepared on that optimum pH (50mM), the activity of enzyme solution was assayed at various incubation temperatures ranging from 30°C to 100°C maintaining in water bath. Before making the reaction mixture, the enzyme and substrate solution were kept on respective temperature for 5 min. The protease activity at each of the incubation temperature was determined in the similar manner as described on section 3.4.5.

### 3.5.3 Analysis of substrate specificity

Substrate specificity of partially purified enzyme was analyzed using natural substrates; casein, Bovine Serum albumin (BSA), whey protein, hemoglobin and gelatin by preparing 0.5mg/ml solution of each substrate with sodium phosphate buffer (pH 7, 50 mM). And then the protease assay was performed as like in section 3.4.5 at optimum temperature and pH conditions.

### 3.5.4 Inhibition or Activation assay by Metal ions

Caseinolytic assay for the partially purified protease was conducted after treating with 5mM concentration of various monovalent, divalent and trivalent metal ions. The metal ions used in the experiment were $Na^{+}$, $Cu^{++}$, $Fe^{++}$, $Hg^{+}$, $Co^{++}$, $Al^{+++}$, $Ca^{++}$, $K^{+}$ and $Zn^{++}$. Firstly, each of the metal ions solution of concentration 10mM were prepared, then 100µl metal ions solution was mixed with 100µl dialyzed enzyme, to maintain final concentration of 5mM. The mixture was incubated for 1 hour at 40°C. Then residual activity of the treated enzyme was determined using casein (5mg/ml) as substrate at optimum temperature and pH as described earlier on section 3.4.5 to find out the activation or inhibition effect of metal ions.

### 3.5.5 Inhibition by protease inhibitors

Residual protease activity of the partially purified enzyme in presence of 5mM concentrations protease inhibitors like PMSF (for serine inhibitor), IDA (for cysteine inhibitor), EDTA (for metalloenzyme inhibitors) was conducted. The 100µl enzyme was

first treated with 10mM concentration of each of the inhibitors and incubated at 37°C for 1 h. After then, protease activity of treated enzyme by using casein as substrate (5mg/ml) was monitored to find out the activation or inhibition effect of protease inhibitors at optimum temperature and pH described in similar way as described on section 3.4.5.

### 3.6 Data Analysis

The analyses were carried out in triplicate. The results are expressed in average value ± SEM (Standard Error of Mean). Obtained experimental values was processed by Microsoft Excel 2013. Analysis of variance (ANOVA) was carried out for data obtained during characterization. The significant differences between them were studied by Bonferroni method using L.S.D. at 5% level of significance using Genstat release 12.1 software program developed by VSN International Ltd.

# Part IV

# Result and discussion

This research involved the traditional method of preparing kinema in the lab, analyzing the proteolytic activity during kinema fermentation to determine time of fermentation when highest protease activity reach during fermentation. The kinema when reach highest protease activity was taken as a source for extraction and partially purification by ammonium sulphate precipitation followed by dialysis to obtain partially purified kinema protease and used for determination of optimum pH and temperature, substrate specificity, effect of metal ions, effect of inhibitors and storage stability for the purpose of general characterization. The results obtained from the study are presented and discussed on the following headings.

## 4.1 Proximate composition of kinema after three days

Kinema was prepared from white soyabean according to the traditional method given by Tamang (2015). Then the prepared kinema and white soybean used was subjected to proximate analysis in the laboratory. The analytical result of the proximate composition of the kinema after three days of fermentation and white soybean is given in table 4.1.

**Table 4.1** Proximate composition of kinema and white soybean

| Parameters (%) | White soybean | White kinema (after 3 days fermentation) |
|---|---|---|
| Moisture | 8.89±0.215 | 7.4±0.115 |
| Crude protein | 40.2±0.167 | 45±0.121 |
| Crude fat | 20.42±0.241 | 23.5±0.431 |
| Total ash | 4.43±0.132 | 6.1±0.155 |
| Crude fiber | 4.55±0.305 | 3.2±0.062 |
| Carbohydrate | 30.4±0.398 | 22.20±0.192 |

Data are expressed in mean of triplicate analysis ± S.D. on dry matter basis.

## 4.2 Proteolysis during kinema fermentation

Based on preliminary study, different days of kinema fermentation from 0 days to 6 days, protease activity and protein content was determined and specific activity was calculated which can be observed in fig 4.1.

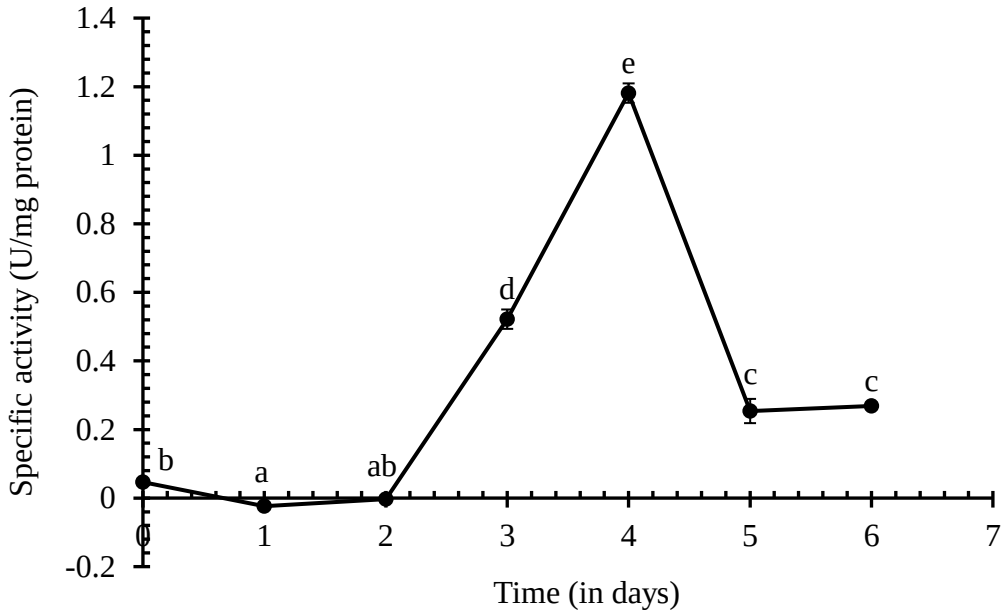


**Fig 4.1** Specific activity of crude enzyme extracted from kinema of white Soybean

Higher Specific activity 1.18 U/mg protein (Protease activity 0.2746 U/ml and protein content 0.2325 mg/ml) was observed was observed on 4$^{th}$ days of fermentation. In many fermented product *B. subtilis* has shown to increase with fermentation. So, it can be expected that *B. subtilis* plays the important role in secretion of protease enzyme on 4$^{th}$ day of fermentation. The result indicated that kinema isolated organism (*Bacillus subtilis*) probably played an essential role in protein degradation. Proteases secreted by *B. subtilis* growing on soybean surface might hydrolyze soy proteins to oligopeptides and amino acids. Hence the proteolytic activity increase and the protein concentration gets decreased during the kinema fermentation (Visessanguan *et al.*, 2005).

## 4.3 Partial purification of crude enzyme

Extracted crude sample was purified with different concentration of ammonium sulphate solution followed by determination of protease activity, protein content, specific activity,

total activity, purification fold and yield percentage. of those partially purified enzyme samples.

**Table 4.2** Different parameters of partially purified enzymes

| Steps of purification | Protease activity (U/ml) | Protein content (mg/ml) | Specific activity (U/mg protein) | Total activity (U) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|
| Crude | 0.484 | 0.158 | 3.0676 | 16.0931 | 1 | 100 |
| (0-30) % Ammonium sulphate | 0.252 | 0.125 | 2.0158 | 1.2584 | 0.6571 | 7.4326 |
| (30-70) % Ammonium sulphate | 1.891 | 0.377 | 5.0188 | 9.4546 | 1.6361 | 55.84 |
| Dialyzed 70% Ammonium sulphate | 1.685 | 0.220 | 7.6763 | 12.1350 | 2.5024 | 71.6724 |

For the selection of partial purification, the precipitated crude enzyme fraction obtained at (30-70) % ammonium sulphate precipitated fraction was selected because this partially purified protease has highest specific activity (5.0188 U/mg protein), and highest purification fold (1.6361) as compare to 0-30% ammonium sulphate fraction.

Upon dialysis, ammonium sulphate pellet (30-70) % has found increase in specific activity to (7.6763 U/mg protein) with increase in purification fold to 2.5402. The dialyzed enzyme was used for the further characterization of enzyme whose protease activity, specific activity, total activity, purification fold and yield percentage were found to be 1.685, 7.6763, 12.1350, 2.5024 and 71.6724 respectively.

### 4.4 Partial characterization of extracted enzyemes

The different properties of extracted enzymes including pH, temperature, susbstrates specificity, effect of metal ions, effect of inhibitors and storage stability was observed from the partially purified enzymes of white kinema.

#### 4.4.1 Optimization of pH of kinema protease

The absorbance of the hydrolysates prepared by hydrolyzing casein by partially purified kinema protease at different pH is shown in figure 4.2.

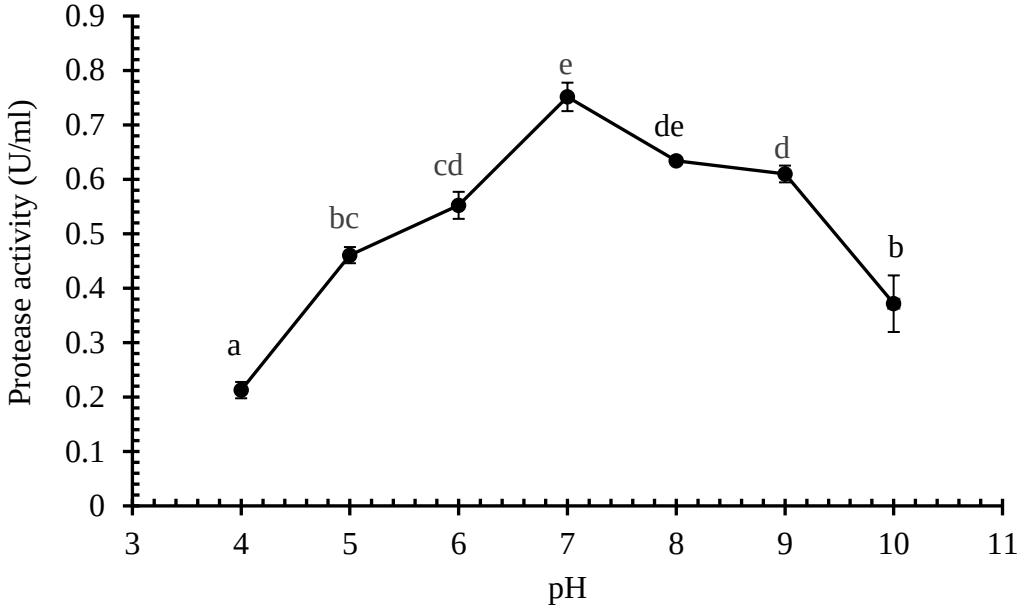


**Fig. 4.2** Protease activity of partially purified kinema protease at different pH

Statistical analysis at 5% level of significance shows that the protease activity observed at pH 7 was significantly different with pH 4, 5 ,6 ,9 and 10 but not significantly different with pH 8. This shows that optimum pH range of kinema protease was 7-8 however it remains active in broad pH range (pH 6 to pH 9).

*Bacillus subtilis* is the predominant microorganism in kinema (Sundus *et al.*, 2016) and protease from *Bacillus subtilis* have been categorized into neutral and alkaline protease where the former exhibit optimal pH at 7.0, whereas the latter have pH optima between 9-11 Chantawannakul *et al.* (2002). The protease activity of partially purified enzyme

gradually increased up to pH 7.0 and gradually decreased after 7. Previous study made by Wang *et al.* (2009) has shown that the amino acid composition of protease plays an important role in its optimal reaction pH value. He also reported that that an acid condition made the enzyme activity decrease more rapidly than an alkaline condition did as the acidic condition leads to enzyme denaturation. Furthermore, it can be expected that protease from kinema in this finding may contain more basic amino acid than acidic amino acid. Similarly study made by (Boominadhan *et al.*, 2009) shows that various *Bacillus* spp. was able to grow in a pH range of 7-12 with better protease production which is similar to our finding.

### 4.4.2 Optimization of temperature of kinema protease

Protease activity of hydrolysates prepared by hydrolyzing the casein with extracted enzyme at different temperature is shown in figure 4.3.

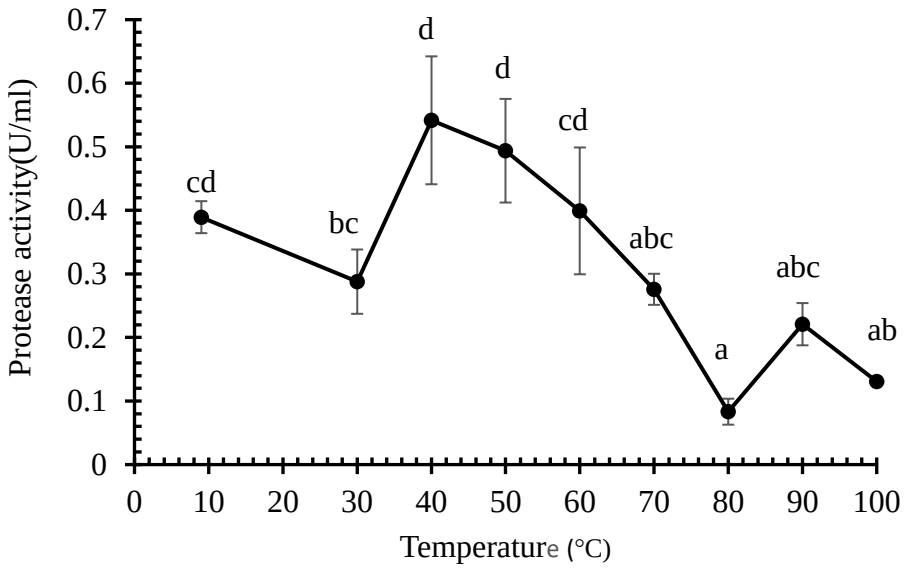


**Fig. 4.3** Protease activity of hydrolysis of casein by partially purified kinema protease at different temperature.

The protease activity of kinema has increased from 30°C to 40°C and then gradually decrease. Statistical analysis at 5% level of significance shows that the protease activity observed at temperature 40°C was significantly different with temperature 9, 30, 70, 80 90 and 100°C but not significantly different with temperature 50 and 60°C. This shows that optimum temperature range of kinema protease might be (40-60) °C but starts to decline from 60°C.

Gençkal and Tari (2006) reported that the decrease in proteolytic activity above 60°C is due to the denaturation of protein at higher temperature. The optimum temperature of the *Bacillus subtilis* isolated from natto, a product similar to kinema was also reported to be 40°C (Wang *et al.*, 2009). As the temperature was raised above 60°C, the activity of protease showed a rapid decline although slight activity was observed even after incubation at 80°C for 10 min. These results suggest that protease obtained from *B. subtilis* can be considered as a thermostable enzyme according to (Pant *et al.*, 2015). The protease activity at 90°C was again increased which may be due to the presence of other types of protease i.e., the protease was only partially purified. Several other authors has also reported the highest activity at 45°C and 50°C in case of several other strain of Bacillus such as B. licheniformis and Bacillus subtilis respectively (Boominadhan *et al.*, 2009).

### 4.4.3 Substrate specificity of kinema protease

Substrate specificity of partially purified enzyme at optimum temperature and pH was conducted with different five substrate including casein which is shown in figure 4.4.

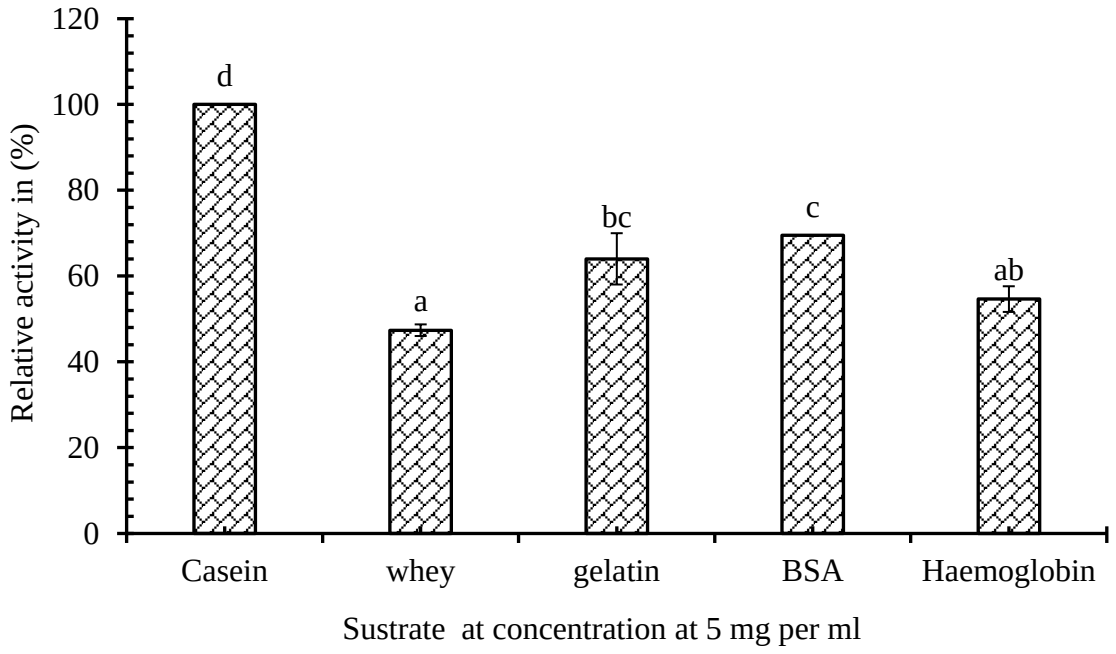


**Fig 4.4** Substrate specificity of partially purified enzyme

Among the five substrates (casein, whey, bovine serum albumin, hemoglobin and gelatin), highest relative activity was found on Casein (100%) followed by Bovine serum

albumin (69.47%), gelatin protein (64.0177%), Hemoglobin (54.637%) and whey protein (47.3828%) respectively. Statistical analysis at 5% level of significance shows that the relative protease activity on casein is significantly different with other substrates but no significant different was found in case of BSA and gelatin and also in case of gelatin and hemoglobin. The highest activity was found with casein while lowest activity observed in whey protein among the tested substrates. These results suggest that partially purified protease from kinema has broad substrate specificity but has different catalyzing activity depending on the substrates.

According to Gupta *et al.* (2002) alkaline proteases have broad substrate specificity and are active against a number of synthetic substrates but in many cases their activity was reported to be highest in casein than against azocasein, hemoglobin or BSA. In case of *B. circulans* M34 protease, casein, ovalbumin and BSA has shown to be specific substrates for serine protease although activity was also found for gelatin (Sari *et al.*, 2015). So based on these facts we can conclude that the kinema protease might be alkaline, serine protease. So far, several researchers in various literature have reported Casein, ovalbumin and BSA were specific substrates for serine proteases (Ghafoor and Hasnain, 2010).

### 4.4.4 Effect of Meatal ions on kinema protease

Effect of metal ions on protease activity of partially purified enzyme at optimum temperature and pH was conducted with nine different metal ions $Na^{+}$, $Cu^{++}$, $Fe^{+++}$, $Hg^{+}$, $Co^{++}$, $Al^{+++}$, $Ca^{++}$, $K^{+}$ and $Zn^{++}$. The activities with these metal ions is shown in fig. 4.5.

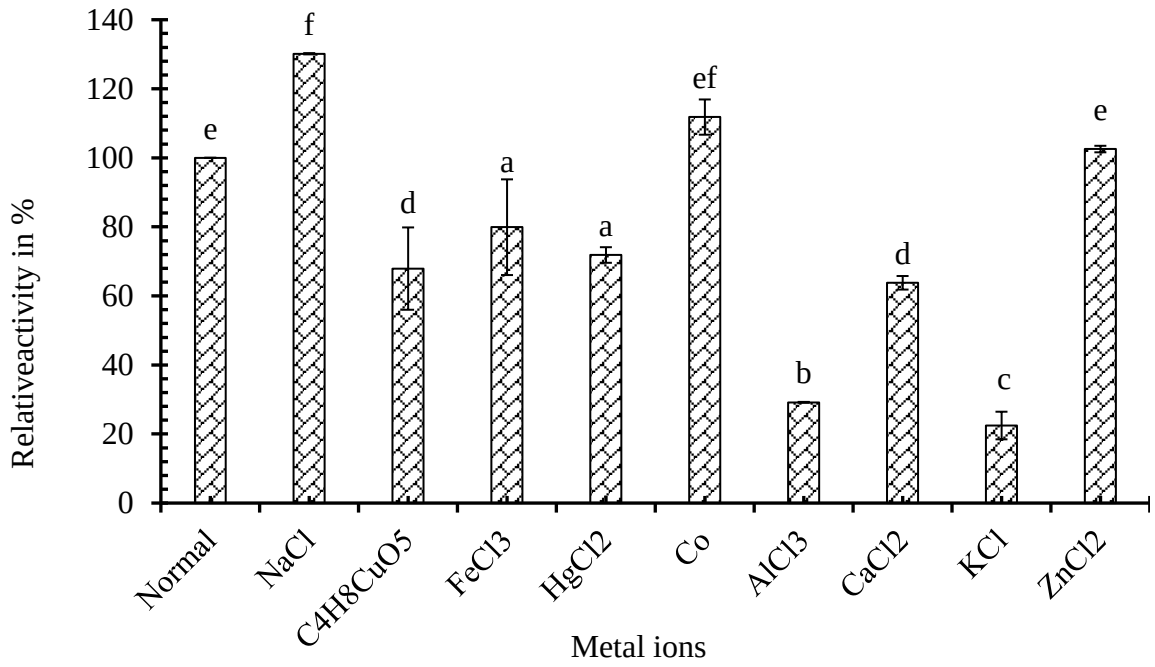


**Fig. 4.5** Effect of Metal ions on protease activity

Statistical analysis at 5% level of significance shows that the residual protease activity by NaCl was significantly different with metal ions but not significantly different with cobalt. sodium, cobalt, zinc, copper, calcium and potassium show the positive effect whereas iron, mercury and aluminium show the negative effect on proteolysis at normal optimum conditions. Sodium enhanced the protease activity by 30% while cobalt and zinc slightly raise the activity by 11% and 2% only.

Sinha and Khare (2013) reported that monovalent $Na^+$ ion participate in regulating the activity and assist in refolding of the enzyme activity on serine protease isolated from a moderately halophilic protease producer of *Bacillus* sp. strain. Furthermore, it was also observed that enzymatic activity was completely inhibited by $Hg^{2+}$, enhanced in the presence of $Na^+$ and almost 100% activity retained in the presence of $Zn^{2+}$. Wang *et al.* (2009) also reported that enzyme was activated by $Zn^{2+}$ but inhibited by $Fe^{3+}$, and $Al^{3+}$ in purified nattokinase isolated from natto, a product similar to kinema.

The study made by Qureshi *et al.* (2011) on biosynthesis of protease by *B. subtilis* when grown on sodium chloride, potassium chloride and zinc chloride shows that protease production was enhanced with sodium chloride and protease production was very low with

potassium chloride. Divalent metal ions such as cobalt, zinc reported to have slightly higher activity according to (Singh *et al.*, 2016).

### 4.4. 5 Effect of inhibitors on protease from kinema

Effect of protease inhibitors such as EDTA, IDA and PMSF on protease activity of partially purified enzyme at optimized temperature and pH is presented in figure 4.6.

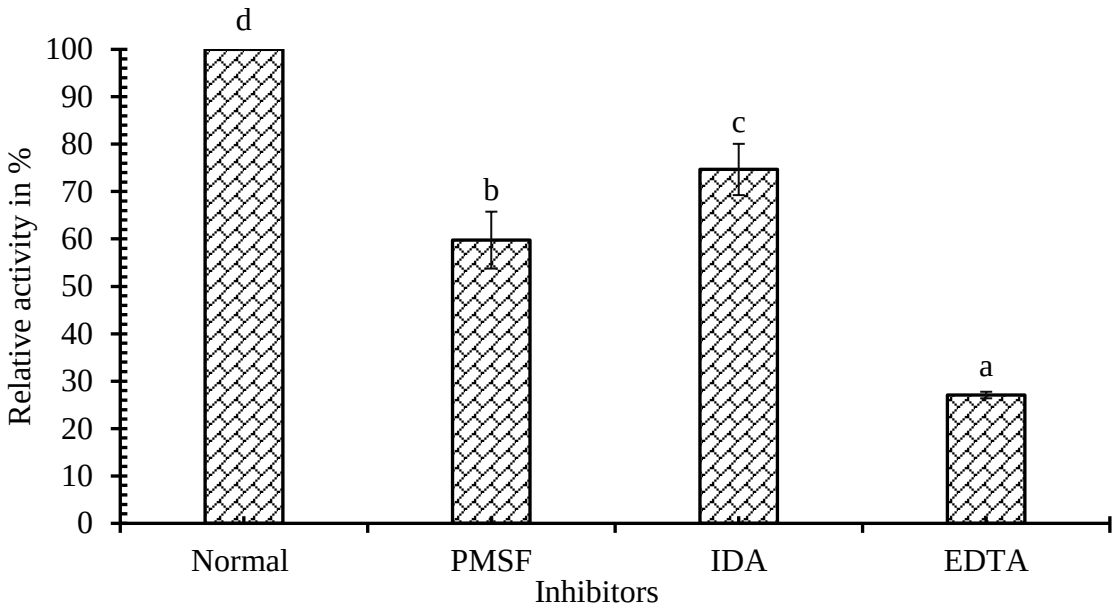


**Fig. 4.6** Effect of inhibitors

Statistical analysis at 5% level of significance shows that the protease activity observed among different inhibitors (PMSF, IDA and EDTA) was significantly different with each other. The protease activity with EDTA was found to be less which suggest that the highest inhibition effect has obtained by EDTA (73%) followed by PMSF (40%). IDA only shows upto 25% inhibition.

These result shows that inhibitors of metallo protease (EDTA) had significant effect on the activity of the purified kinema protease indicating that the kinema protease was metallo protease. This finding is in accordance with the study made by (Sellami-Kamoun *et al.*, 2008). Similarly, the study made by (Yang *et al.*, 2000) on "Production and purification of protease from a Bacillus subtilis that can deproteinize crustacean wastes" also found that the EDTA was the most effective inhibitor that caused nearly complete inhibition of protease. And he concluded that enzyme was a metal-chelator-sensitive neutral protease. Mamo and

Assefa (2018) also reported that most of the neutral and alkaline protease that are commercial serine proteases are produced from bacteria of the genus *Bacillus*. So, on the basis of different studies in previous literature we can conclude that the kinema protease was metal depending serine protease.

### 4.4.6 Storage stability of partially purified enzyme

The protease activity of kinema protease stored in refrigerated condition of (-4±2) °C and analyzed at normal room temperature (23-28) °C from prepared (0) day to seventh day is presented in fig. 4.7.

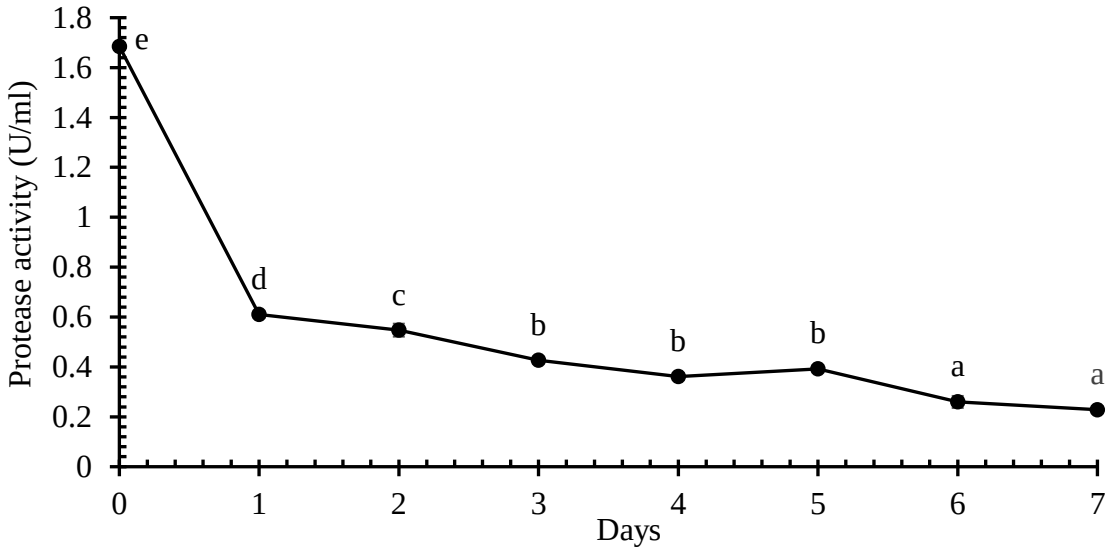


**Fig.4.7** Stability of partially purified enzyme by dialysis

Statistical analysis at 5% level of significance shows that the protease activity observed at prepared (0) day was significantly different from other days. The optimum activity was observed at prepared day while minimum activity was at seventh day.

Protease activity had been decreased from (1.685 U/ml) to (0.2285 U/ml) from prepared (0) to 7$^{th}$ day. There is rapid decline in protease acitivity from first to second day. 86% of the activity had been decreased within seven days even at refrigeration condition. The research on partially neutral protease from a newly isolated strain of *Bacillus subtilis* KIBGE-HAS by Anwar *et al.* (2009) also found that the enzyme lost its activity within ten days at refrigerated conditions. Our results are also consistent with those observed for other industrial enzymes by Misset (1993).

# Part V

# Conclusions and recommendations

## 5.1 Conclusions

Based on the overall results from this study the following conclusions are drawn:

i. The protease activity of kinema is highest after completion of three days fermentation.
ii. An increase of 2.5 purification fold and 77% of recovery of total activity with specific activity of 7.67 U/mg/min can be achieved when crude extract of kinema protease is subjected to purification by ammonium sulphate precipitation followed by dialysis.
iii. The partially purified kinema protease was found to be active in broad temperature and pH range showing optimum activity at 40°C and 7.0 pH.
iv. The partially purified kinema protease have broad substrate activity but activity is highly depended on tested natural substrate; indicating highest activity on casein followed by BSA, gelatin, hemoglobin and then whey protein.
v. Iron, Mercury and aluminium along with EDTA and PMSF shows the inhibition effect whereas sodium, cobalt, zinc, cupper, calcium, potassium shows the activation effect on protease activity indicating kinema protease is metal dependent serine protease.
vi. The storage stability of enzyme gradually decreased upto three days and rapidly decreased after fourth days during storage at refrigerated condition.

## 5.2 Recommendations

Present work is carried out to study extraction of crude plant protease and hydrolysis on milk protein. Based on this research following recommendation can be made.

i. General and complete characterization of kinema protease after purification by TPP and chromatography method can be done.
ii. Kinema protease and its hydrolysis capacity on various other substrate can be performed.

iii. As this study shows that kinema could be important source of protease, the specific strain having highest protease activity can be screened and further study for the characterization and utilization can be done.

# Part VI

## Summary

The study was carried out to evaluate general characteristic of protease enzyme extracted from indigenously fermented alkaline food; kinema prepared in laboratory from white variety of soybean. The kinema having highest proteolytic activity after completion of 3 days fermentation was selected for the protease extraction. Crude protease was extracted with sodium phosphate buffer (pH 7, 50Mm) maintaining kinema to solvent ration of 1:1.5 and stirring time of 25 min by using magnetic stirrer at speed of 300±50 rpm. The crude extracted was subjected to further purification by ammonium sulphate precipitation method followed by dialysis. Highest amount of crude protease was precipitated by 30-70% saturated ammonium sulphate fractions, was collected and subjected to dialysis with same buffer (pH-7) for one day with 3 times exchange of buffer.

The purification of crude enzyme with ammonium sulphate precipitation followed by dialysis was found to increase purification fold by 2.5 time with retention of 77% of total activity. The specific activity of the partially purified enzyme was achieved to be 7.67 U/mg/min. The kinema protease thus obtained was active to different pH range (4 to 9) and temperature range 30 to 80°C but the optimum activity was found at temperature and pH 40°C and at 7.0 respectively. This showed the enzyme is either neutral or alkaline in nature. The protease was found active against different tested natural substrate however activity was found to largely influence by the individual substrate. Substrate specificity was highest on Casein followed by Bovine serum albumin, gelatin, hemoglobin and whey protein. The enzyme treated with 5mM of metal ions such sodium, cobalt, zinc, copper, calcium and potassium were not inhibited the enzyme activity but was found to be inhibited mercury and aluminum, whereas magnesium, calcium were found to have activation effect. The effect of protease inhibitors on activity of the kinema protease showed that protease activity is highly inhibited by EDTA (72% inhibition) followed by PMSF (40% inhibition) showing the probability of being metal dependent serine protease.

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# Appendices

## Appendix A

**Table A.1 Equipment and utensils**

| | |
|---|---|
| Incubator | Centrifuge machine (220V, 10% Hz) |
| Micropipette | Conical flask |
| Petri-plates | Measuring cylinder |
| Bunsen burner | Test tubes |
| Pipettes | Magnetic stirrer (SH-4C) |
| Mortar and pestle | Spectrophotometer (60UV-Vis) |
| Weighing balance | Water bath |
| Thermometer | Filter paper |
| Glass rod | Glass vials |
| Beaker | |

# Appendix B

**Table B.1 Chemicals used during research work**

| | |
|---|---|
| Ethylene diamine tetra acidic acid (M.W.= 372.24) | Comassie brilliant blue G-250 (M.W = 854.04) |
| Indole-3-Acetic acid (M.W= 175.15) | Sodium Acetate glacial (M.W= 136.08) |
| Phenyl-methane sulphonyl fluoride (M.W= 174.20) | Acetic Acid (M.W= 60.05 g/mol) |
| Tris-buffer (M.W= 121.14) | Ortho-pthalaldehyde AR (M.W= 134.14) |
| Di-thiothretol (M.W.= 154.28) | Tri-chloroacidic acid (M.W= 163.39 |
| Ammonium sulphate (M.W= 132.14) | Sodium dihydrogen phosphate (M.W=156.01) |
| Sodium phosphate dibasic (M.W= 177.99) | 2-2 diphenyl-1-picryhydrazyl (M.W= 394.32) |

# Appendix C

## C.1 Clibration curve for protease activity

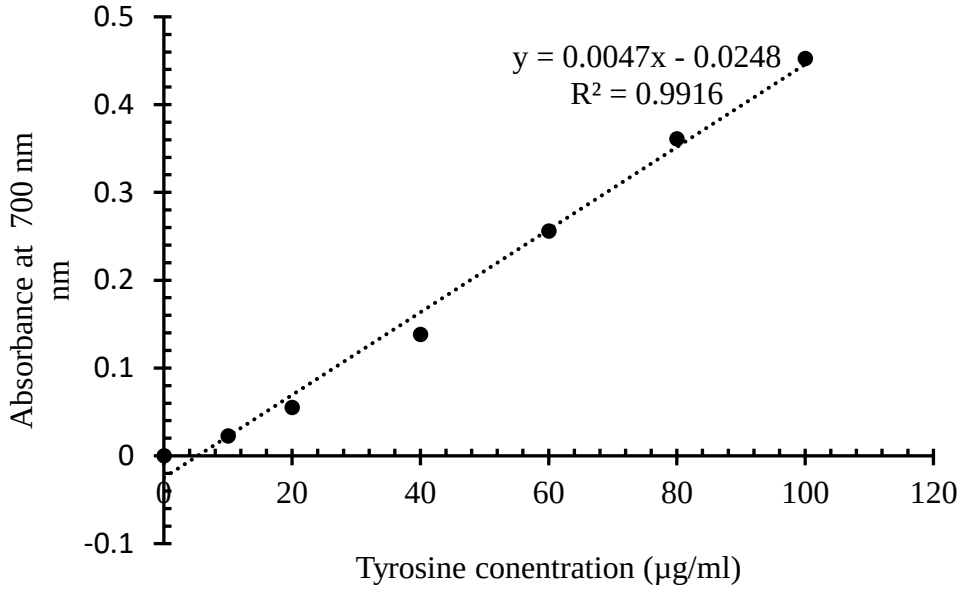



**Fig.C.1** Standard curve of tyrosine for protease activity

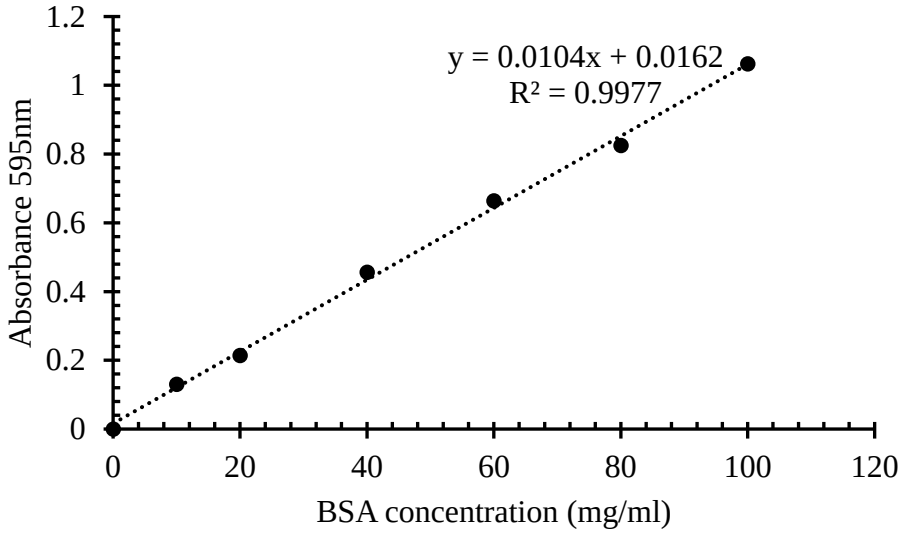



**Fig.C.2** Standard curve of BSA for protein determination

# Appendix D

## D.1 Preparation of reagents

1. Preparation of Phosphate buffer

- For pH 6: Mixing 87.7ml 0.2M $NaH_2PO_4.2H_2O$ and 12.3ml 0.2M $Na_2HPO_4.\ 2H_2O$
- For pH7: Mixing 39.0ml 0.2M $NaH_2PO4.2H_2O$ and 61.0ml 0.2M $Na_2HPO_4.\ 2H2O$

2. Preparation of Acetate buffer

- For pH4.0: Mixing 8.2ml 0.2M $CH_3COOH$ and 1.8ml 0.2M $CH_3COONa$
- For pH5.0: Mixing 3.0ml 0.2M $CH_3COOH$ and 7.0ml 0.2M $CH_3COONa$

3. Preparation of Tris-hydrochloride buffer

- For pH 8: Mixing 26.8ml 0.2M HCl and 50ml 0.2M Tris(hydroxymethyl)-aminomethane
- For pH 9: Mixing 5.00ml 0.2M HCl and 50ml 0.2M Tris(hydroxymethyl)-aminomethane

4. Preparation of Bradford reagent

- 0.01 g Coomassie Brilliant Blue G-250 is added to 5 ml ethanol (95%) and 10 ml phosphoric acid (85%).
- Volume make up is done with distilled water to 100 ml.

5. Preparation of Bovine serum albumin (BSA)

- 0.2 g BSA is added to 100 ml distilled water.

6. Preparation of casein solution (0.5 % w/v)

- 0.5 g casein is dissolved in 100 ml 50 mM potassium phosphate buffer. The solution is heated with gentle stirring to 60-65$^{o}$C for 10 minutes.

# Appendix E

**Table E.1** One way ANOVA at 5% level of significance for proteolysis during fermentation

| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Day | 6 | 2.2391274 | 0.3731879 | 2708.00 | <.001 |
| Residual | 7 | 0.0009647 | 0.0001378 | | |
| Total | 13 | 2.2400920 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| 0 | 0.0467 | 0.00063 | b |
| 1 | -0.0236 | 0.00070 | a |
| 2 | 0.000 | 0.00070 | ab |
| 3 | 0.5168 | 0.02828 | d |
| 4 | 1.2013 | 0.02828 | e |
| 5 | 0.2489 | 0.03536 | c |
| 6 | 0.2743 | 0.00707 | c |

**Table E.2** One way ANOVA at 5% level of significance for ph optimization

| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Ph | 6 | 0.3906119 | 0.0651020 | 97.15 | <.001 |
| Residual | 7 | 0.0046910 | 0.0006701 | | |
| Total | 13 | 0.3953028 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| 4 | 0.2127 | 0.01499 | a |
| 5 | 0.4607 | 0.14849 | bc |
| 6 | 0.5521 | 0.02481 | cd |
| 7 | 0.7514 | 0.02616 | e |
| 8 | 0.6339 | 0.00254 | de |
| 9 | 0.6098 | 0.01541 | d |
| 10 | 0.3715 | 0.05197 | b |

**Table E.3** One way ANOVA AT 5% level of significance for temperature optimization

| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Temperature | 8 | 0.588712 | 0.073589 | 18.58 | <.001 |
| Residual | 18 | 0.071277 | 0.003960 | | |
| Total | 26 | 0.659989 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| 9 | 0.3893 | 0.02517 | cd |
| 30 | 0.2878 | 0.05060 | bc |
| 40 | 0.541 7 | 0.10062 | d |
| 50 | 0.4938 | 0.08153 | d |
| 60 | 0.3993 | 0.09976 | cd |
| 70 | 0.2758 | 0.02444 | abc |
| 80 | 0.0832 | 0.02043 | a |
| 90 | 0.2113 | 0.03324 | abc |
| 100 | 0.1318 | 0.00360 | ab |

**Table E.4** One way ANOVA at 5% level of significance for substrate specificity

| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Substrats | 4 | 3283.293 | 820.823 | 88.14 | <.001 |
| Residual | 5 | 46.565 | 9.313 | | |
| Total | 9 | 3329.857 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| Whey | 47.38 | 1.35542 | a |
| Haemoglobin | 54.64 | 2.99321 | ab |
| Gelatine | 64.02 | 5.98033 | ac |
| BSA | 69.48 | 0.06227 | c |
| Casein | 100 | 0 | d |

**Table E.5** One way ANOVA at 5% level of significance for metal ions

| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Metal ions | 9 | 160908.68 | 17878.74 | 438.84 | <.001 |
| Residual | 20 | 814.82 | 40.74 | | |
| Total | 29 | 161723.49 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| $FeCl_3$ | -79.90 | 13.85830 | a |
| $HgCl_2$ | -71.85 | 2.25898 | a |
| $AlCl_3$ | -26.51 | 0.12302 | b |
| KCl | 22.46 | 3.99995 | c |
| $CaCl_2$ | 63.80 | 1.96206 | d |
| $C_4H_8CuO_5$ | 67.91 | 11.9235 | d |
| Normal | 100 | 0 | e |
| $ZnCl_2$ | 101.88 | 0.94152 | e |
| Co | 111.80 | 5.10534 | ef |
| NaCl | 130.15 | 0.18422 | f |

**Table E.6** One way ANOVA at 5% level of significance for inhibitors

| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Inhibitors | 3 | 8351.32 | 2783.77 | 169.27 | <.001 |
| Residual | 8 | 131.56 | 16.45 | | |
| Total | 11 | 8482.89 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| EDTA | 27.07 | 0.69324 | a |
| PMSF | 59.75 | 6.00328 | b |
| IDA | 74.66 | 5.40909 | c |
| Normal | 100 | 0 | d |

**Table E.7** One way ANOVA at 5% level of significance for enzyme storage stability

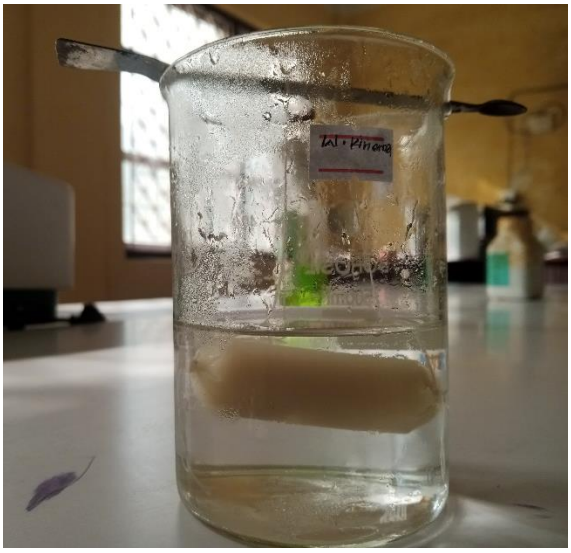
| Source of variation | d.f. | s.s. | m.s. | v.r. | F.pr. |
|---|---|---|---|---|---|
| Days | 7 | 3.1147956 | 0.4449708 | 2107.78 | <.001 |
| Residual | 8 | 0.0016889 | 0.0002111 | | |
| Total | 15 | 3.1164844 | | | |

| Treatment | Mean | Standard Deviation | Significance |
|---|---|---|---|
| 0 | 1.6860 | 0.002828 | e |
| 1 | 0.6107 | 0.01422 | d |
| 2 | 0.5274 | 0.02599 | c |
| 3 | 0.4241 | 0.00049 | b |
| 4 | 0.4117 | 0.00343 | b |
| 5 | 0.3925 | 0.01373 | b |
| 6 | 0.2597 | 0.02403 | a |
| 7 | 0.2285 | 0.00539 | a |

## Color plates

**P.1 Separation of enzyme purified by ammonium sulphate precipitation**

**P.2 Partially purified enzyme by Ammonium sulphate precipitaion**



**P.3 Dialysis of partially purified enzyme**

**P.4 Temperature optimization**

**P.5 Analysis of Protease Activity & Protein content**

**P.6 Weighing in weighing balance**