WHEY PROTEIN HYDROLYSIS ABILITY OF PROTEASE EXTRACTED FROM KIWI (*Actinidia deliciosa*) FRUIT

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Approval Letter

This *dissertation* entitled *Whey Protein Hydrolysis Ability of Protease Extracted from Kiwi (Actinidia deliciosa) Fruit* presented by Roshan Bhattarai has been accepted as the partial fulfilment of the requirement for the B. Tech. degree in Food Technology.

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(Roshan Bhattarai)

Abstract

Plant protease are gaining interest for its application to food protein hydrolysate preparation to improve nutritional and functional properties. The main aim of this study is to exploit the partially purified protease from kiwi fruit to evaluate hydrolysis ability for whey protein and antioxidant activity of generated hydrolysate. For this work protease was extracted from kiwi (Actinidia deliciosa) fruit by using sodium phosphate buffer (pH 7, 50mM concentration) and subjected to partial purification applying ammonium sulphate precipitation method followed by dialysis. The partially purified protease was used to determine optimum temperature and pH for casein and whey protein and optimum substrate concentration (enzyme by substrate ratio) for whey protein hydrolysis. Further the changes in degree of hydrolysis and antioxidant activity at optimized condition for whey protein hydrolysis with respect of time of hydrolysis up to 6h was also evaluated.

The crude kiwi protease was extracted in 40-60% ammonium sulfate saturation level having maximum activity and after dialysis was found to be 1.154 fold improvement in purification having specific activity of 2.241. The optimum activity of the kiwi protease for casein hydrolysis was obtained at 50°C and pH 5 & 7 and for whey protein at 60°C and pH 6 respectively. The maximum degree of hydrolysis for whey protein for the enzyme was found to be at substrate concentration of 2.347mg/ml equivalent to enzyme by substrate ratio of 1:192. The increase in degree of hydrolysis and antioxidant activity with respect to time up to 6h was found for both the heated and non-heated whey protein hydrolysis by the enzyme. The degree of hydrolysis at 6h of hydrolysis was found to be 58.1815% in heated whey protein and was higher than that of non-heated i.e. 54.1846%. However, the antioxidant activity of heated whey protein was 9.30% which is less than that of the non-heated whey protein i.e. 15.80%.

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Lists of abbreviations

Abbreviations	Full form
ACE	Angiostension I- converting enzyme
BCCA	Branch chain amino acid
BOD	Biological oxygen demand
BSA	Bovine serum albumin
DF	Degree of filtration
DH	Degree of hydrolysis
DOE	Design of experiment
DPPH	2-2diphenyl1 picryl hydrazine
EAA	Essential amino acid
EDTA	Ethylene diamine tetra acetic acid
HCL	Hydrochloric acid
HIV	Human immune deficiency viruses
PER	Protein efficiency ratio
RSM	Research surface methodology
SEM	Standard error of mean
UF	Ultrafiltration

WPH	Whey protein hydrolysate
WPI	Whey protein isolate
WPC	Whey protein concentrate

Part I

Introduction

1.1 General introduction

Whey is the liquid that remains after casein and fat have been extracted from milk using an isoelectric process or rennet-induced coagulation. Global whey production is over 160 million tonnes per year, with an annual growth rate of 1-2%. Approximately 75% of whey is processed into various products in developed countries and less than 50% in other countries. Leftover whey is still used as animal feed, agricultural fertilizer, and is even dumped into water sources (Macwan *et al.*, 2016). Drainage of whey causes significant nutrient loss due to its high biological oxygen demand (BOD5) and chemical oxygen demand, creating a serious environmental problem (Sathish Kumar, 2004).

Whey is a nutrient-dense food and contain approximately 90% of the water, 98% of lactose, 25% of protein and 50% of inorganic salts respectively present in milk (Fox *et al.*, 2017). It can be utilized in new-born, geriatric and athletic meals because it has an excessive amount of nutrients (Macwan *et al.*, 2016).

Whey can be used in a variety of ways in the food industry, but is primarily dehydrated into whey powder or used to make whey protein concentrates, whey protein isolate and protein. Whey contains less than 1% protein consisting mainly of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulin and protease peptone, as well as several small proteins including including lactoferrin, lactollin glycoprotein, lactoperoxidase and transferrin (Kilara and Panyam, 2003). Whey protein as a part of whey- is still wasted even though it has high potential to transform in to various product such as whey protein hydrolysate. Whey protein can be hydrolysed by chemical and enzymatic processes. Enzymatic hydrolysis is preferred by food manufacturers because of the wide availability of protease that are considered safe and natural. Chemical modifications of proteins are possible, but because of the need to demonstrate the safety of these modifications in humans, they are generally not practiced as it cause- loses some essential amino acids, such as tryptophan, and can produce products containing large amounts of free amino acids, which can upset the body's osmotic balance (Jeewanthi *et al.*, 2015). Recently, whey protein and its components; α-lactalbumin and β-lactoglobulin have

also been shown to contain biologically active chains. Whey and whey protein after enzymatic hydrolysis not only improve its digestibility but also have been shown to contain several peptides which possess antioxidant activity , ACE inhibitory activity, anti-microbial activity and many other functional properties (Pihlanto, 2006).

Proteases from different sources like animals, microbial and plants can used for the hydrolysis of whey proteins. In recent years, interest in plant proteases have increased rapidly. The number of plant-based enzymes used industrially is still low but growing rapidly (Gonzalez-Rabade *et al.*, 2011). Proteases extracted from plant sources have been used in cheese making, meat tenderization, bioactive peptide production, and flour modification (Shah and Mir, 2019a). Papain and bromelain are formerly commercial enzymes from plant sources that have been used to produce dairy proteolytic products, including whey protein, and explored the potentiality of plant proteases as emerging field in the production of whey protein hydrolysates (Shah *et al.*, 2014). Plant proteases are superior to microbial-derived enzymes mainly due to safety issues such as pathogenicity or other adverse effects such as higher proteolysis and lower yield. Thus, compared with microbial and animal proteases, plant sources are an accessible alternative (Anwar and Saleemuddin, 1998).

Kiwi fruit is known to contain a highly active proteolytic enzyme, dominated by actinidin (Karki *et al.*, 2018). Actinidin is the major enzyme in kiwi fruit, similar to the proteolytic enzymes thiol papain (papaya), ficin (fig) and bromelain (pine cone) (Boland, 2013). Actinidin extracted and purified from kiwi has been exploited in cheese making (Sharma and Vaidya, 2018b), tenderization of different meat (Sharma and Vaidya, 2018c) and protein hydrolysate too (Kaur *et al.*, 2022). Based on these facts, the kiwi enzyme has been found to be a potential source of proteolytic enzymes and can be used as a source of whey protein hydrolysate to enhance nutritional and functional properties of hydrolysate such as antioxidant activity, ACE inhibition activity, antimicrobial activity and use of them for development of functional foods and pharmaceutical applications.

1.2 Statement of the problem

Increasing pollution and environmental problems have caused cheese and paneer manufacturers to stop dumping whey into waterways and sewers. A large amount of whey is wasted in the world, and direct whey disposal is always a burden to pollute the environment. Whey discharge is also a significant waste of potential nutrients and energy and has been taken seriously by ecologists and technologists due to its strong polluting effects (Macwan *et al.*, 2016). With global milk production increasing 2% annually and cheese production increasing 3% annually, evidence suggests that the volume of whey production will continue to increase in the coming years. The development of sustainable whey processing methods is urgently needed. In addition to the tremendous growth in the use of whey protein, much remains to be done to exploit the full potential of whey (Ryan and Walsh, 2016).

Whey proteins are magical, powerful, promising and active ingredients with a wide variety of nutritional and therapeutic benefits. Whey and its biological components have proven their effects in the treatment of chronic diseases of the cervix such as cancer, cardiovascular disease, HIV, etc. Since it is so rich in nutrients, it can also be used in infant drinks, aged food and sports. Environmental issues have forced governments to legislate regarding whey disposal, and as a result scientific challenges have led to the development of different technologies to take advantage of what was once considered a " waste" from cheese making into an important and economical raw material for the production of many ingredients/products for the food industry (Božanić *et al.*, 2014). However, the use of whey as a source of protein in many diets, including those of infants and the elderly, is not applicable due to poor digestion.

Proteolytic enzymes are usually derived from animals (e.g. pepsin, trypsin and chymotrypsin) and microorganisms (e.g. Alcalase® and Neutrase®) were being largely utilized for such a transformation. However, plant proteases (e.g. papain and bromelain) gaining an increased demand for proteolytic enzymes in several industrial applications as emerging sources of natural origin (Mazorra-Manzano *et al.*, 2020a). Recent studies focused that kiwi protease could also have wide application as milk coagulant, meat tenderization and for improving digestibility and functionality of various protein due to its broad proteolytic activity in various substrates and has shown the activity and functionality

is depend on substrate and reaction conditions too. So, the hydrolysis of whey protein by the use of kiwi protease can have tremendous opportunity to add value to the whey protein and their functionality. Thus, study regarding such an aspect is crucial for future use of kiwi protease for whey protein hydrolysis, development of whey protein hydrolysate having improved bio-functionality and add value on whey protein products.

1.3 Objectives

The objectives of the research was divided into two parts:

1.3.1 General objective

The general objective of this work is to evaluate whey protein hydrolysis ability of protease extracted from Kiwi (Actinidia deliciosa) fruit.

1.3.2 Specific objectives

- To extract the proteolytic enzyme from kiwi fruit and purify it.
- To optimize temperature and pH of kiwi protease in casein and whey protein as substrate.
- To evaluate the effect of substrate concentration on the degree of hydrolysis of whey protein.
- To determine degree of hydrolysis and antioxidant activity of heated and non-heated whey protein treated for 6h at optimized condition.

1.4 Significance of the study

Whey is a by-product of the cheese and casein industry and contains more than 50% of the original milk solids and more than 85% of the original milk volume (Guirguis *et al.*, 1993). On average, about 10 L of milk is used to make 1 kg of cheese, creating a by-product of 8-9 L of whey. This is equivalent to 5 million tons of whey produced annually. Disposing of large quantities of whey without proper pre-treatment causes not only environmental problems but also economic losses to this premium by-product to value added products. Currently, new applications of whey and its derivatives are attracting increasing interest in higher quality food products with health benefits (De Souza *et al.*, 2010). enzymatic

transformation of whey protein to whey protein hydrolysate for nutritional and functional importance is one of the seeking area for the value addition (Eberhardt *et al.*, 2021).

Enzymatic hydrolysis of whey proteins not only improves their functional properties but also provides a powerful technology in exploiting their biological properties for nutritional and functional food applications (Gauthier and Pouliot, 2003). Whey proteins are also considered "fast proteins". The result of this research will help to utilize selected plant as a source of protease for the whey protein hydrolysis and explore furthermore possibility of improvement in digestibility, bioavailability and functionality of hydrolysed whey protein.

1.5 Limitations of the study

Some of the hurdles that came during the thesis are listed as follows:

i) Method of enzyme purification like chromatography and electrophoresis was not carried out due to unavailability of required equipment.
ii) Molecular wt. of the enzyme was not carried out due to lack of equipment.

Part II

Literature review

2.1 Whey and whey protein

Whey is a liquid by-product from the precipitation of proteins in milk. Precipitation can be facilitated by microorganisms (e.g. cheese whey), acid addition (forming acid casein) or by adding enzymes (forming rennet casein) (Kilara and Vaghela, 2018). Total whey production in the world is currently estimated at 180-190 million tons/year (Khezri *et al.*, 2016). The type and composition of whey in dairy plants is largely dependent on the processing techniques used to remove casein from the whey (Macwan *et al.*, 2016). There are two types of whey; acid whey (pH 4.4 to 4.6) and sweet whey (pH 5.9 to 6.3). The acid whey is obtained on precipitating casein by acidification of milk to at pH 4.6, e.g. whey obtained during manufacture of paneer. The sweet whey is obtained on precipitating casein by enzymatic coagulation of milk, e.g. whey obtained by rennet coagulation of milk during manufacture of cheese (Ghanshyambhai *et al.*, 2015). It consists of 45-50% total milk solids, 70% lactose, 20% milk protein, 70%-90% milk minerals and almost all the water-soluble vitamins found in milk (Chatterjee *et al.*, 2015). Whey and whey preparations are known as "forgotten treasures", but they have been "rediscovered" due to their unique nutritional and functional properties and have been used successfully by scientists for various nutrients rich food production (Królczyk *et al.*, 2016).

Whey protein contains all 20 amino acids and all 9 essential amino acids, and it is a rich and balanced source of sulfur amino acids, which play an essential role as an antioxidant as a precursor to the antioxidant glutathione. Vigorous intracellular and in single carbon metabolism (Solak *et al.*, 2012). Whey protein is a high-quality protein with a rich amino acid profile. It contains many amino acids including essential amino acids (EAAs) and branched chain amino acids (BCAAs) that are important in tissue growth and repair. Leucine, isoleucine, and valine are amino acids that play a key role in BCAAs in protein synthesis and have recently been identified as having a role in muscle building and increased hormonal growth. It is easy to digest compared to other dairy ingredients like casein and has no fat which helps to increase lean muscle in the body (Kumari *et al.*, 2022).

Whey protein is one of the two main proteins in cow's milk, making up 20% of the milk's protein, while casein makes up the other 80%. The main whey proteins are β-LG, α-lactalbumin (α-LA), glycomacropeptide (GMP), immunoglobulin (Igs), bovine serum albumin (BSA), Lf, lactoperoxidase (LP) and proteose peptone (PP) (Mangano *et al.*, 2019). The four major whey protein are: β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulin (Ig) (Jovanović *et al.*, 2005).

2.1.1 β-lactoglobulin

β-lactoglubulin (β-Lg) is the most important protein in whey with ~ 18 kDa molecular weight. It represents 50% of whey protein and also 12% of total protein in milk. β-Lg is able to bind fatty acids and retinol (vitamin A) and because of this, it has great foaming and gelation properties (Heino, 2010).

2.1.2 α-lactalbumin

α-lactalbumin (α–La) is the second most important protein in whey and milk with a molecular weight of ~ 14 kDa. It comprises 20 % of total whey protein and also 3.5 % of total protein in milk. It has dependency on calcium (Ca^{2+}) ions and it is known as a metallo-protein. It has a high tryptophan (Trp) content that is very useful for human brain function. It also has high level of the amino acids useful in muscle protein synthesis (Thompson *et al.*, 1988).

2.1.3 Bovine serum albumin

Bovine serum albumin (BSA) with the molecular weight of 66 kDa is another cow milk proteins. BSA has significant biological effect on human health but its role in food and milk are not well known. BSA has only a slight effect on whey physiochemical properties due to its low concentration in milk (Heino, 2010).

2.1.4 Immunoglobulin

Immunoglobulin (Ig) is the immunological part of the milk. Immunoglobulins are antibodies that can protect people against a wide range of bacteria and viruses. Human milk has the highest amount of Ig but cow's milk has low level of Immunoglobulin. Immunoglobulin has a molecular weight of 150-1000 kDa. These proteins have immune-

active peptides and therefore the presence of this protein is beneficial for a whey product (Tovar Jiménez *et al.*, 2012).

2.1.5 Minor whey proteins

Lactoferin (LF) has a molecular weight of about 76.5 kDa and is a multi-functional protein from the transferrin family. It exists in different liquids like milk, nasal, saliva and others. LF has antibacterial activity in humans and interacts with nucleic acids (Yang *et al.*, 2013). Glycomacropeptide (GMP) is the C-terminal portion of kappa casein and is sometimes called casein macro peptide (CMP). It has a molecular weight of 6-10 kDa. The amino acid composition of this protein is very unique. GMP has various chemical attributes like extensive emulsifying properties and is stable in a wide range of pH (Sharma *et al.*, 2013).

2.2 Production and uses of whey protein

Whey proteins can be broadly defined as proteins that remain soluble after removal of casein from skim or whole milk at pH 4.6 and 20°C. Whey protein is now an important source of functional protein for the global food industry (Bottomley *et al.*, 1990). Whey protein is known for its high nutritional value and versatile functional properties in food. Estimates of global whey production put approximately 700,000 tons of true whey protein available as a valuable food ingredient. The nutritional and functional properties of whey proteins are related to the structure and biological functions of these proteins (De Wit, 1998). Whey protein is derived from cheese whey, an industrial by-product with a significant environmental impact. However, it can also be converted into value-added products. This is a successful processing strategy of its management (Abadía-García *et al.*, 2016).

Advances in processing technologies including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange, have resulted in development of several different finished whey products: whey protein concentrates (WPC) containing between 50 – 85% protein on a dry basis, whey protein isolate (WPI) containing between 90-98% protein and very low amounts of lactose and fat, reduced lactose whey, demineralized whey and hydrolysed whey. Whey ultrafiltration (UF) and diafiltration (DF) are standard processes in the dairy industry that allow protein recovery without significant loss of their functional properties and with a low salt content, making it suitable for human consumption (Mollea et al., 2013).Whey proteins have a high nutritional value, due to the high content of essential amino acids, especially

sulphur-containing ones. They are high quality proteins with a protein efficiency ratio (PER) of 3.4, higher than casein (2.8) and similar to egg albumin (Mollea et al., 2013). Whey protein concentrate (35-80% protein) and whey protein isolates (>90% protein) are major whey protein products (Jeewanthi *et al.*, 2015).

WPC and WPI have wide ranges of application as nutritional supplements mainly in sport nutrition. It is also widely used ingredients in various food and beverage preparation because of its good hydration (solubility, water binding, cohesion adhesion) properties, aggregation and gelation (gel formation, film formation, emulsification) properties, interfacial (fat binding, whipping, foaming) properties and a sensorial (colour, flavour and texture) properties (Jeewanthi *et al.*, 2015). Because of their nutritional and functional properties, researchers have intensified effort to expand utilization of whey protein as food and nutraceutical ingredients. Enzymatic modification of whey protein are now highly acceptable and applied in the industries not only to improve the nutritional quality but also to improve its functional improvement and enhancement of bioactive properties (Athira *et al.*, 2015).

The various forms of commercially available whey proteins are:

2.2.1 Whey protein concentrate (WPC)

Whey protein concentrate is one of the most cheaply available rich sources of quality proteins offering many health benefits, and it has the ability to improve the food products due to its various functional properties (Parate *et al.*, 2011a). Whey proteins are concentrated by removal of minerals and lactose from whey through UF, electrodialysis, ion exchange, or crystallization of lactose (Patel *et al.*, 2006). It is a processed form of whey protein which has the lowest level of fats and cholesterol as compared to other forms of commercially available whey but a high level of bioactive compounds. It also contains carbohydrates in the form of lactose and has protein content in the range of 65-70%. It has a mild to slightly milky taste (Ganju and Gogate, 2017).

Whey Protein Concentrate (WPC) is rich in essential amino acids such as lysine, tryptophan, cysteine and methionine (Pandiyan *et al.*, 2010). WPC generally recognized as a safe (GRAS) for food product application. The food manufacturing industry come to realize that whey proteins have potential to improve the quality of food products due to various

functional properties such as solubility, viscosity, water binding, whipping, emulsification and gelation. WPC has many health benefits and clinical importance also. WPC is useful in the treatment of various diseases and health problems such as cancer and high cholesterol. Whey proteins are easily and readily digested and therefore useful in postoperative care of patients. Whey protein also acts as a sport food as whey proteins helps in speedy repairing of injured and torn muscle during practice and performance. Whey proteins provide excellent nutritional values in nutrition foods formulated for infant, kids, adults and old aged people as growth tonic for body health maintenance (Parate *et al.*, 2011b).

2.2.2 Whey protein isolate (WPI)

WPI is whey protein which is further processed to remove fats and lactose. It has lower quantities of bioactive compounds but a higher protein content (>90%). Similar to WPCs, it has a mild to slightly milky taste (Ganju and Gogate, 2017).

2.2.3 Whey protein hydrolysate (WPH)

Protein hydrolysates are defined as "mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis". Hydrolysis is done by using food grade proteolytic enzymes, or suitable acids and alkalis. Enzyme hydrolysis is greatly preferred because acid and alkali hydrolysis are difficult to control (Abd El-Salam and El-Shibiny, 2017). WPH are pre-digested and partially hydrolysed whey proteins manufactured with an objective of easier metabolism. WPHs have anti-oxidative properties and a protein content of 70-80%.The added processing steps leads to WPHs being expensive than WPCs and WPIs (Ganju and Gogate, 2017). Enzyme derived protein hydrolysates is a mixture of peptides and has received wide attention in recent years due to their health-promoting properties. Plant proteases like papain and bromelain have been used to prepare protein hydrolysates from different food systems (Phadke *et al.*, 2016).

Whey protein modifications based on enzymatic hydrolysis or thermal polymerization have broad potential for functional designing for specific applications (Foegeding *et al.*, 2002). Acid and alkaline hydrolysis tends to be a difficult process to control and reduced nutritional quality. Chemical hydrolysis can form toxic substances such as lysine-alanin. Enzymatic hydrolysis developed under mild conditions of pH (6-8) and temperature (40-60°C) can also lead to the development of bioactive nutrient components to promote health

opportunities in the use of dairy ingredients (Sinha *et al.*, 2007). Enzymatic hydrolysis is an important bioprocess to improve physical, chemical, functional, and nutritional properties of natural proteins. It is an effective method for releasing active peptides which have many physiological properties. Partial hydrolysis of whey proteins leads to changes that may impart certain desired functional properties of the released peptides from this industrial by-product, thereby increasing their applications (Martínez-Araiza *et al.*, 2012). Enzymatic hydrolysis improves solubility and surface activity of protein ingredients. Several treatments have been suggested to modify whey proteins in order to improve their functional attributes. Enzymatic hydrolysis and heat treatment have effectively altered the structure and functions of whey proteins (Britten *et al.*, 1994).

2.3 Whey protein hydrolysate as a source of bioactive peptides

Hydrolysis of protein is termed as the process of breakdown of peptide bond to increase the free amino acids and carboxyl group with the objective to enhance the digestibility and solubility of resulting hydrolysate. Protein hydrolysis is a powerful tool in the modification of the functional properties of proteins in food systems, including solubility, gelation, emulsifying and foaming characteristics (Tavano, 2013). The degree of hydrolysis measures the percentage of peptide bonds hydrolysed during protein hydrolysis. The progress of protein hydrolysis is currently evaluated by measuring the degree of hydrolysis at intervals. Because a limited controlled hydrolysis of a food protein yields products of particularly interesting functional and organoleptic properties, the DH concept simplifies the evaluation of the progress of the hydrolysis (Navarrete del Toro and García Carreño, 2003a).

Proteins are hydrolysed in industry by chemical or enzymatic means, just as they are in the digestive system. Chemical techniques for protein hydrolysis are harsh and extensive, and are generally only useful for amino acid composition analysis (Navarrete del Toro and García Carreño, 2003a). Enzymatic hydrolysis of proteins is an important bioprocess to improve the physical, chemical, functional and nutritional properties of original proteins. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Korhonen and Pihlanto, 2006). Milk proteins are considered the most important source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products (Korhonen and Pihlanto, 2006). Enzymatic

hydrolysis is an effective method to prepare active peptides, which possess many physiological properties including mineral binding, opioid activity, growth enhancer for bifidobacteria, anticancer activity and regulation of the blood pressure or the immune system (Qi and He, 2006).

Enzymatic hydrolysis of whey can be used for improving the functional, nutritional, and immunological properties of proteins, reducing the allergenicity and antigenicity of the proteins. Bioactive components of the hydrolysed products of whey cover a wide spectrum of compounds with anti-carcinogenic, antifungal, antihypertensive, antimicrobial, antioxidative , anti-proliferative, anti-thrombotic, antiulcerogenic, antiviral, and immunomodulatory properties, while also having prebiotic activity (Popescu *et al.*, 2021). Milk proteins caseins are an important source of bioactive peptides which has been extensively studied. Whey hydrolysates obtained after proteolysis with different digestive enzymes has been shown to have ACE-inhibitory activity, and contain several active peptides (Pihlanto, 2006).

Recently whey proteins and its components; α-lactalbumin and β-lactoglobulin, were also shown to contain bioactive sequences. Peptides showing opioid and angiotensin I-converting enzyme (ACE) inhibitor activity were found in α-lactalbumin and β-lactoglobulin (Tomita, et al., 1991).

Derived lactorphines from α-lactalbumin and β-lactoglobulin as opoid antagonist (Chiba and Yoshikawa, 1986), lactoferricin from lactoferrin as antimicrobial (Tomita, et al., 1991) and lactokinins from α-lactalbumin and β-lactoglobulin as ACE inhibitory activity (Mullally, et al., 1996) were reported. Bioactive peptides can be incorporated in the form of ingredients in functional and novel foods, dietary supplements and even pharmaceuticals with the purpose of delivering specific health benefits (Korhonen and Pihlanto, 2006).

2.4 Protease enzyme

Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes. Proteases are also called as proteolytic enzyme or proteinases. Proteinases constitute a large family and divided as endopeptidases and exopeptidase on the basis of cleavage site at which they breakdown peptide chain (Barret, 1994). Proteolytic enzymes are

very important in digestion as they breakdown the peptide bonds in the protein foods to liberate the amino acids needed by the body. The optimum temperature and pH of protease ranged from 40°C to 50°C and pH 8, respectively (Naidu, 2011). Currently, most enzymes used in industrial processes are hydrolytic and are used for the degradation of various natural substances (Gat et al., 2019). Protease remains the dominant type of enzyme because of its extensive use in dairy and detergent industries. Proteases are very important enzymes, accounting for more than 60% of total global enzyme sales. Protease enzyme catalyses the hydrolytic reaction, which brings about the breakdown of protein molecules to amino acids and peptides (Ningthoujam et al., 2009).

2.5 Classification of protease enzyme

Protease refers to a group of enzymes whose catalytic function is to hydrolyse peptide bond of proteins i.e., proteases conduct proteolysis and they begin protein catabolism. Proteases are also called as proteolytic enzyme or proteinases. Proteinases constitute a large family and divided as endopeptidases and exopeptidase on the basis of cleavage site at which they breakdown peptide chain (Barrett, 1994). Proteases are generally categorized into two majors' groups based on their site of action, that is, exopeptidases and endopeptidases. Exopeptidases are those proteases that cleave the peptide bond proximal to the amino or carboxy termini of the substrate (cleave N- or C-terminal peptide bonds of a polypeptide chain), whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (cleave internal peptide bonds). Proteases are also classified into acid, alkaline, and neutral proteases based on the pH at which they are active (Mamo and Assefa, 2018). Proteases are divided into six different types based on the method of catalysis: aspartic, glutamic, metalloproteases, cysteine, serine, and threonine proteases. Among them cysteine, serine, aspartic and metalloprotease has been more studied but glutamic proteases have not yet been discovered in mammals (Lopez-Otin and Matrisian, 2007).

2.5.1 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 omegapeptidase (Barrett and Rawlings, 1995). Serine

proteases possess a serine residue in their 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). Microbial alkaline serine proteases are mainly involved in hydrolysis of proteins for preparation of hydrolysates having high nutritional value. The generated protein hydrolysates are widely used in fortification of infant food formulations, dietary products and processed foods; and are also being used as bioactive compounds in nutraceuticals (Matkawala *et al.*, 2021).

2.5.2 Cysteine Protease

Cysteine proteases also known as thiol proteases are found in both prokaryotes and eukaryotes (e.g., bacteria, parasites, plants, invertebrates and vertebrates). The catalytic mechanism of these enzymes involves a cysteine group in the active site. Cysteine proteases comprise a family of enzymes, consisting of papain and related plant proteases such as chymopapain, caricain, bromelain, actinidin, ficin, aleurain (Turk *et al.*, 1997a). Cysteine proteases have great potential in the food, biotechnology and pharmaceutical industries due to their property of being active over a wide range of temperatures and pH (Gonzalez-Rabade *et al.*, 2011).

2.5.3 Aspartic Protease

Aspartic acid proteases, also known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2) and the enzymes from pararetroviruses (A3). Most aspartic proteases show maximal activity at low pH (pH 3 to 4)) (Kaur, 2011). Most aspartic proteases have molecular weights in the range 30-45 kDa, and their isoelectric points are usually in the range pH 3.4-4.6. These enzymes are specific against aromatic or bulky amino acid residues on both sides of the cleavage point. Catalytic activities involve two aspartic acid residues. The catalytic mechanism of the aspartic proteases requires the initial binding of a water molecule at the active site before nucleophilic attack on the substrate peptide bond (Ellaiah et al., 2002). Aspartic protease from microbial source are found in the manufacturing of cheese where they are used as milk-clotting agents and they has also been a growing interest for their applications in several other industries (Theron and Divol, 2014).

2.5.4 Metalloprotease

All these enzymes have pH optima between pH 5-9 and are sensitive to metal-chelating reagents, such as EDTA, but are unaffected by serine protease inhibitors or sulphydryl agents. Many of the EDTA inhibited enzymes can be reactivated by ions, such as zinc, calcium and cobalt. These are widespread, but only few have been reported in fungi. Most of the bacterial and fungal metalloproteases are zinc-containing enzymes, with one atom of zinc per molecule of enzyme. The zinc atom is essential for enzyme activity. Calcium is required to stabilize the protein structure (Beoynom and Bond, 1994). Metalloproteases, in which zinc is an essential metal ion for the catalytic activity, are produced by various human pathogenic microorganisms (Miyoshi and Shinoda, 2000).

2.6 Sources of protease

Proteases can be obtained from animals, plants and microorganisms (Singh *et al.*, 2016). Proteases fields of application are very diverse, including food science and technology, pharmaceutical industries and detergent manufacturing. The most well-studied plant proteases include papain from papaya latex, bromelain from pineapple stem, ficin from fig fruit, actinidin from kiwifruit, and zingibain from ginger rhizome and are currently the most well-studied plant proteases. Proteases are the object of renewed attention not only because of their proteolytic activity on a wide variety of proteins but also because often they are active over a range of temperatures and pH (Guevara *et al.*, 2018).

2.6.1 Protease from microorganisms

Microorganisms represent an excellent source of proteases owing to their broad biochemical diversity and their susceptibility to genetic manipulation (Sandhya *et al.*, 2005). As compared to plants and animals, microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods, and they produce an abundant, regular supply of the desired product (Sharma *et al.*, 2017). The optimum pH and temperature range of alkaline proteases is generally between pH 9-11 and 50-70C (Kumar and Takagi, 1999).

The use of some industrially important proteases producing microbes are *Streptomyces sp.*, *A. flavus*, *Bacillus amyloliquefaciens* and *Bacillus subtilis* (Banerjee and Ray, 2017).

Among different producers of proteases, *Bacillus sp.* Are mostly commercially exploited microbes for protease (Table 2.1). Protease produced by plants and animals are more labour intensive than microbiologically produced proteases. Microbial proteases are the most commercially exploited enzyme worldwide. A large number of intracellular proteases are produced by microbes playing a vital role in differentiation, protein turnover, hormone regulation, and cellular protein pool, whereas extracellular proteases are significant in protein hydrolysis (Razzaq *et al.*, 2019).

Table 2.1 Commercially available microbial protease

Microbial source of protease	Activity	Application
Bacillus licheniformis	Activated at high temperature from 45°C-65°C and moderate pH 7-8.5	Detergents, silk degumming
Aspergillus sp.	Activated between pH 4 and 7.5 and has optimum temp of 60°C	Not specified
Bacillus lentus	Activated at temperature up to 55°C and has high pH of 8-12.5	Detergents, food, silk degumming
Alkalophilic bacillus species	Activated at low temperature from 10°C to 65°C and high pH of 6-11	Detergents, textile

Source: Razzaq *et al.* (2019)

2.6.2 Protease from plant

Proteases are involved in most plant functions, such as protein processing, digestion, growth, reproduction, defence, apoptosis, senescence, etc. These are present in all kinds of plant tissues and, thus, can be extracted from their natural sources or can be prepared using in vitro technique. Production of plant proteases by in vitro techniques leads to higher enzyme yields and minimizes the extraction procedures used in extraction from natural sources (Shah and Mir, 2019a). The number of industrially used proteases of plant origin is small and some cysteine proteases (CPs) such as papain, bromelain, and ficin are still being

used in a variety processes (Feijoo-Siota *et al.*, 2011). 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 additive (Jinka *et al.*, 2009). Some of the well-known proteases such as papain, bromelain, and keratinases are produced from plant sources. Papain is produced from Papaya fruit (Carica papaya). It has the properties of milk clotting and protein-digesting with a wide pH range. Bromelain is a plant protease that is obtained from the leaf, juice, stem, and peel of pineapples (Naveed *et al.*, 2021).

Table 2.2 Plant protease in food industry

Plant protease	Sources
Actinidin	Actinidia spp.
Bromelain	Ananas comosus
Papain	Carica papaya
Zingibain	Zingiber officinale
Pomiferin	Maclura pomifera
Caricain	Carica papaya
Oryzasin	Oryza sativa

Source: Shah and Mir (2019b)

The most widely used plant proteases are papain, bromelain, ficin, actinidin, zingibain, and cardosins (Table 2.2). Plant proteases are being used in dairy processing, meat tenderization, bioactive peptide production, and baking industry (Shah and Mir, 2019a). The important sources of plant protease used in traditional medicine and industry are latex. Over 110 lattices of different plant families have been reported to contain at least one protease enzymes. Most of them are found to belong to the cysteine and serine endopeptidase (Domsalla and Melzig, 2008). Cysteine proteases, also known as thiol proteases, and the catalytic mechanism of 12 these enzymes involve a cysteine group in the active site (Gonzalez-Rabade *et al.*, 2011).

Cysteine proteases have great potential in the food, biotechnology, and pharmaceutical industries owing to their property of being active over a wide range of temperature and pH. Plants offer an attractive alternative for the production of cysteine protease as they occur naturally in different tissues, in some cases in excessive amount (Gonzalez-Rabade *et al.*, 2011). Serine proteases possess a serine residue in their active site and share a number of biochemical and physiological features (Mazorra-Manzano *et al.*, 2018). In spite of various proteases enzyme have been reported to be present in different plants, are characterized and use in various purposes in food preparation and processing, their exploitation in production milk protein and whey protein hydrolysate still limited (Mazorra-Manzano *et al.*, 2018).

2.6.3 Protease from animals

The most widely used animals derived proteases are pancreatic trypsin, chymotrypsin, pepsin and rennin. intestinal digestive enzymes chymotrypsin and rennin are extensively used in deallergenizing of milk protein hydrolysate and preparation of curd, respectively (Razzaq *et al.*, 2019). . In production of whey protein hydrolysate porcin trypsin (Pellegrini *et al.*, 1999) porcine pancreatic (Morais *et al.*, 2015)Bovine trypsisn and chymotrypsin (Pellegrini *et al.*, 2001) human gastric juice and duodenum (Almaas *et al.*, 2011) have been extensively studied.

2.7 Application of protease

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. The great majority of commercial enzymes have been obtained mainly from microbial sources but plant enzymes are becoming increasingly important, with applications in industrial processes, biotechnology and pharmacology. Proteases like papain, bromelain and ficin are employed in different industrial processes and medicine (Uhlig, 1998). Some of these papain-like proteases are currently used in the food industry for cheese, brewing and beverage industries for the preparation of highly soluble and flavoured protein hydrolysates (papain-like proteases), as a food complement to soften meats and dehydrated egg (Bailey & Light, 1989) and for the production of emulsifiers, among other uses (Pardo et al., 2000).

Uses in other industries include culture medium formulation (Headon & Walsh, 1994), isolation of genetic material (Genelhu et al., 1998) and the use of keratinases in the leather industry for dehairing and bating of hides to substitute toxic chemicals (Foroughi et al., 2006). Also, they are used in the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems (Rao et al., 1998). Proteases also have an important application in the pharmaceutical industry. Plant extracts with a high content of proteolytic enzymes have been used in traditional medicine for a long time. They have been used for the treatment of cancer (Batkin et al., 1988), as antitumorals (Otsuki et al., 2010), for digestion disorders (Kelly, 1975), and swelling and immune-modulation problems (Otsuki et al., 2010). A good example is bromelain, derived from pineapple, which has been shown to be capable of preventing edema, platelet aggregation and metastasis due to its capacity of modifying cell surface structures by peptide cleavage. Salas et al. (2008) reviewed the pharmacological activity of plant cysteine proteases, emphasizing their role in mammalian wound healing, immunomodulation, digestive conditions, and neoplastic alterations.

2.8 Plant proteases for whey protein hydrolysis

Proteases, proteinases or peptidases are a group of hydrolytic enzymes that carry out the hydrolysis of peptide bonds in proteins and peptides. They are widely distributed in nature from microorganisms to animals and plants. Enzymatic hydrolysis of whey protein still offers a promising and practical way to reduce its antigenicity and could provide a variety of new peptides that may offer many physiological benefits for humans (Shin *et al.*, 2007).

Enzymatic hydrolysis of milk proteins modifies the techno-functional and bio-functional properties of the hydrolysates depending on the enzyme(s) and hydrolysis conditions used (Abd El-Salam and El-Shibiny, 2017). Peptidases from latex of *Maclura pomifera* fruits hydrolyse bovine whey proteins (Bertucci *et al.*, 2015).

Enzymatic hydrolysis of whey proteins liberates fragments that can promote health benefits in the immune, cardiovascular, nervous and gastrointestinal systems (Dullius *et al.*, 2018). Protein hydrolysates are usually produced by limited enzymatic hydrolysis of protein molecules in foodstuff, yielding polypeptides that are smaller in molecular mass. Protein hydrolysis has several aims. The most common is to make the protein moiety of a

foodstuff soluble by reducing the size of the peptides (Navarrete del Toro and García Carreño, 2003b).

Whey hydrolysates from ultra-sonicated vegetable enzymes; papain and bromelain have shown increase antihypertensive activity but not the antioxidant, immunomodulatory, and antimicrobial activities as compared non treated enzymes (Abadía-García *et al.*, 2016).

Enzyme-catalyzed hydrolysis provides several advantages including fast reaction rate and high specificity. Also, enzyme hydrolysis is carried out under mild conditions resulting in minimum changes in proteins and generates food-grade protein hydrolysates (Abd El-Salam and El-Shibiny, 2017). Plant crude extracts from Citrus aurantium flowers, trompillo (*Solanum elaeagnif*olium) berries and melon (*Cucumis melo)* fruit are used for production of whey protein hydrolysate and has shown greater proteolytic action at pH 6.5 compared to pH 3.5 (Mazorra-Manzano *et al.*, 2020b). The author also revealed that the enzyme that hydrolyze β –lactoglbulin at higher degree compare to α-lactoalbumin had higher ACE inhibition activity.

Whey protein concentrate (WPC-80) and β-lactoglobulin were hydrolysed with a non-commercial serine protease isolated from Asian pumpkin (*Cucurbita ficifolia*) and has shown release of bioactive peptides having inhibitory effect against α-glucosidase, ACE and DPP-IV activity (Konrad *et al.*, 2014). The biological activity was reported to be higher in WPC hydrolysate then β-lactoglobulin hydrolysate and was more concentrated on peptide fractions having lower molecular weight (i.e., < 3KDa fractions).

2.9 Kiwi fruit

Kiwi fruit (*Actinidia deliciosa*) is native to Southern China (Ferguson, 1984) and was originally called “Yang Tao” in China and “Chinese Gooseberry” in rest of the world. Its cultivation was spread from China in the early 20th century, when Isabel Fraser introduced seeds to New Zealand (Dadlani *et al.*, 1971). Kiwi fruit is an oval berry about the size of a hen’s egg with a light brown hairy skin and emerald-green flesh with numerous tiny black seeds embedded in a juicy pericarp. Kiwi fruit have persistent long, hard, bristle like hairs, which are partially removed during grading and packaging. The green colour of the flesh of *A. deliciosa* is due to the presence of chlorophyll, which is retained during fruit

maturation and ripening. The flesh has a combination of tangy, sweet, and sour flavour (Padmanabhan and Paliyath, 2016).

Kiwi fruit is very popular in human diet due to its pleasant taste and high content of vitamin C, minerals (potassium, phosphorus, iron) and low calorific value. Kiwi fruit are good sources of folate, potassium, and contain high amounts of vitamin E in the seeds. Kiwi fruit also contain different pigments including chlorophylls, carotenoids, lutein and anthocyanins (Jung *et al.*, 2005). Kiwi fruit is known to contain a highly active proteolytic enzymes and dominated one is actinidin (Kaur *et al.*, 2010). Its inactive forms, also called thaumatin like protein and an unusual protein called kiwellin, literally has unknown function. Actinidin is the predominant enzyme in Kiwi fruit, similar to the thiol proteolytic enzymes papain (papaya), ficin (fig) and bromelain (pine apple) (Mike, 2013).

2.9.1 Sources of cysteine protease

Plant cysteine-proteases (CysProt) represent a well-characterized type of proteolytic enzymes that fulfil tightly regulated physiological functions (senescence and seed germination among others) and defence roles (Martínez *et al.*, 2012). Cysteine proteases are more abundant in plants even though the number of serine proteases (SPs) is higher in plant genome (Mazorra-Manzano *et al.*, 2018). Papain, bromelain and ficin from papaya, pineapple and fig respectively, are the most well-known plant proteases used in food processing, pharmaceutics and other industrial processes. These three enzymes represent 5% of the global sales of proteases. Other well-known cysteine protease includes zingipain from ginger rhizome and actinidin from Kiwi fruit.

The papain-like cysteine proteinases are the most abundant among the cysteine proteinases. The family consists of papain and related plant proteinases such as chymopapain, caricain, bromelain, actinidin, ficin, and aleurain (Boris Turk *et al.*, 1997b). Papain is an important plant peptidase due to its powerful proteolytic activity. It derived from the latex of unripe papaya fruit (Carica papaya, Caricaceae). It is characterized by its ability to hydrolyse large proteins into smaller peptides and amino acids. Bromelain is also a proteolytic enzyme which is a cysteine protease derived from pineapple fruit (*Ananas comosus*) which is a member of Bromeliaceae family (Eshamah, 2013). Proteases have generally been purified and characterized from various cereals: barley, wheat and maize (Ogbonna *et al.*, 2004). Cysteine Proteases from ginger rhizome have the potentials in

industrial processes, biotechnology and pharmacology (Nafi *et al.*, 2013) and (Hashim *et al.*, 2011). Cysteine protease from plant family Moraceae such as *Ficus carica sylvestris*, *Ficus religiosa* and *Ficus racemose* have been reported as sources of milk-clotting enzymes (Afsharnezhad *et al.*, 2019).

2.9.2 Protease from kiwi fruit

Actinidin (previously named actinidin, [EC 3.4.22.14]) is a cysteine peptidase purified from kiwifruit and a member of the papain superfamily, being similar to other plant cysteine peptidases such as papain, bromelain, and ficin in its structure and peptidase activity (Morimoto *et al.*, 2006). Kiwi fruit accumulates actinidin to very high concentrations, where it constitutes up to 60% of soluble protein in the fruit (Paul *et al.*, 1995).The amount of enzyme is greater in quantity with a higher level of activity in ripe kiwi, which represents a potential use for overripe and discarded fruit (Karki and Ojha, 2018).

Actinidin prefers β-casein, followed by κ-casein, which is hydrolysed into small number of larger peptides (Sharma and Vaidya, 2018b). Actinidin catalyses the hydrolysis of peptide bonds of proteins and also simple amides and esters. The pattern of hydrolysis was similar to that of papain: for 7 of the 10 bonds hydrolysed, the P2 residue (i.e., the residue second toward the N-terminal from the cleavage site) was hydrophobic (Leu, Val, or Phe, but not Tyr). For the other three bonds, it was suggested that the main specificity determinant was the P1 residue (Mike, 2013). Actinidin has no or limited proteolytic effect on globular proteins such as immunoglobulins including IgG, rabbit IgG, chicken IgG, bovine serum albumin (BSA), and whey proteins (α-lactalbumin and β-lactoglobulin). In contrast to globular proteins, actinidin can hydrolyse collagen and fibrinogen perfectly at neutral and mild basic pH. Moreover, this enzyme can digest pure α-casein and major subunits of micellar casein especially in acidic pH (Chalabi *et al.*, 2014a). The enzyme has been extensively characterized biochemically with the substrate specificity, kinetic parameters and catalytic site characteristics being well defined (Rawlings and Salvesen, 2013).

2.10 Characterization of kiwi protease (Actinidin)

Proteases are the large group of enzymes that catalyses the hydrolysis of peptide bond in protein and poly-peptide. They differ in properties such as substrate specificity, active site, and catalytic mechanism, pH and temperature optima and stability profile.

2.10.1 Molecular weight

Proteases with various molecular mass varies from 21 kDa to 29 kDa were reported to be exist in kiwifruit (Sun *et al.*, 2016). The protein, with a molecular mass of 21− 24 kDa, was identified as an allergen in *Actinidia chinensis* (Maddumage *et al.*, 2013). The actinidin is a common name given to kiwi protease. Dhiman *et al.* (2021) and Kazem and Habeeb (2020) reported that the molecular weight of the actinidin extracted from *Actinidia delicosa* and *Actinidia Chinesis* is 27 kDa and 24.5 kDa respectively.

2.10.2 Optimum temperature and pH

Enzymes deactivate under a range of conditions such as extremes of temperature or pH value, physical forces and aqueous–organic or gas–liquid interfaces (Homaei and Etemadipour, 2015). Kiwifruit showed the highest milk clotting activity in the temperature range of 40-60˚C (Mazorra-Manzano *et al.*, 2013). Ketnawa *et al.* (2011) And Vallés *et al.* (2007) explained that actinidin shows strong caseinolytic activity in a broad pH range of 5-9 but Grozdanovic *et al.* (2013) explained that cysteine protease from kiwifruit has wide pH activity range (4-10) and wide substrate specificity. Sharma *et al.* (2018a) reported the maximum activity of actinidin extracted from immature kiwi as compared to ripen one and highest activity was achieved at 8 pH and 45 ºC. Furthermore, actinidin has shown to have a broad pH optimum from 5 to 7 for benzoyl-L-arginine ethyl ester and benzoyl-L-arginine ethyl ester as substrate (Lewis and Luh, 1988b) and (Mike, 2013). These facts suggest that the optimum pH and temperature depending on substrate and type of sources (i.e. maturation stage of fruit) can vary to some extend in case of Kiwi protease.

2.10.3 Effect of substrate on actinidin activity

Actinidin is a cysteine protease from kiwifruit (Actinidia deliciosa) with a wide pH activity range (4-10) and wide substrate specificity (Grozdanovic *et al.*, 2013). Proteolytic effect of actinidin on casein was pH-dependent and the enzyme is more effective at lower pH

values. Actinidin has narrow substrate specificity with the highest enzymatic activity for the collagen and fibrinogen substrates whereas papain showed broad specificity towards the fibrous and many globular proteins (Chalabi *et al.*, 2014a). However recent studies have also shown actinidin could be important in hydrolysis of milk proteins; casein and whey protein (Kaur *et al.*, 2022). Actinidin has optimum activity at 5-7 pH and 4- 4.3 pH respectively for the substrate; benzoyl-L-arginine ethyl ester (BAEE) and 0.4 % gelatin (Lewis and Luh, 1988b).

2.10.4 Influence of metal ions

The actinidin activity was enhanced with Ca^{2+} while it was inhibited by Cd^{2+} and Hg^{2+} ions. The minimum concentrations of Hg^{2+} and Cd^{2+} induced substantial inhibition of actinidin, whereas Na^{2+}, K^{+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, and Ba^{2+} caused moderate inhibition of enzyme activity (Dhiman *et al.*, 2021). Inhibitors of cysteine proteases include natural inhibitors such as cystatins and exogenous inhibitors such as Cu^{2+}, KCl, NaCl and potato proteins (Bekhit *et al.*, 2014). The metallic ions have shown substantial inhibitory effects on cysteine oxidase activity. The order of potency of inhibition by metallic ions was found to be $Zn^{2+} > Cu^{2+} > Li^{+} > Ca^{2+} > C0^{2+} > K^{+} > Mn^{2+}$ (Misra, 1983).

2.10.5 Stability

The stability of proteins and enzymes is usually the factor that limits their usefulness. Proteases undergo various changes both in function and structure under varying conditions of pH, temperature and denaturants as well as in presence of organic solvents and the extent of changes may vary from protein to protein (Dubey and Jagannadham, 2003). The activity of cysteine protease remained almost constant for 150 days of storage at −20°C (Fahmy *et al.*, 2004). Actinidin retained 85% residual activity after being frozen at −20°C for 3 days (Nam *et al.*, 2006). Proteolytic activities of fresh pineapple and kiwi juices were maintained efficiently with less than 10% loss during storage for 30 days at −20°C and protease stability of pineapple juice was reported better than that of kiwi juice during storage at 4°C (Park *et al.*, 2016).

2.11 Exploitation of kiwi protease

The cysteine proteases of plants and animal are of considerable commercial importance due to their strong proteolytic activity against a broad range of protein substrates. Some major industrial applications are presented below (Grzonka *et al.*, 2007). Actinidin can have applications in the food industry replacing other plant cysteine proteases such as papain and ficin that are used as meat tenderizers, aiding removal of chill haze in beer and plant milk clotting enzymes for novel dietary products as well as to improve the processing quality of cereals (Yuk *et al.*, 2017).

2.11.1 Application as milk coagulating agent

The cysteine protease actinidin purified from kiwifruit was characterized in view of its potential use as coagulant enzyme of bovine milk. Actinidin exhibits the ability to form milk clots in which the casein coagulum is separated away from the whey proteins. The milk-clotting activity of the kiwi aqueous solution seems exclusively due to its component actinidin and is significantly higher than that reported for pure actinidin (Puglisi *et al.*, 2014). Actinidin totally retains the ability to degrade the casein fractions also in the presence of cream fat up to 5% thus suggesting a potential application in the manufacture of cheese with optimized fat content (Puglisi *et al.*, 2012). The partially purified kiwi protease at 0.5% has been used for the production of cottage cheese considerable quality equivalent to rennet cheese (Sharma and Vaidya, 2018b).

2.11.2 Application as meat tenderizer

The use of cysteine plant/fruit-derived proteases in meat tenderization enhances the overall texture and palatability of meat, especially beef. Tenderization with natural plant proteases is relevant for consumers with regard to nutritional, healthy and sensory properties and for meat industry stakeholders as it affects their profitability and allows diversifying the range of their products by adding value to low-value meat cuts (Gagaoua *et al.*, 2021). Kiwi protease (Actinidin) can digest muscle protein when they are mixed with meat. They also can hydrolyse the proteins of collagen and elastin, which lessens the toughness of meat (Sharma and Vaidya, 2018c). Lewis and Luh (1988b) reported that the actinidin hydrolysed myofibrillar protein of beef streaks less than papain and does not produce over tenderization at the meat surface. Actinidin tenderizes pork M. biceps femoris by affecting

both the myofibrils and connective tissue and can be applied as a meat tenderizer without affecting other sensory attributes (Christensen *et al.*, 2009).

2.11.3 Application in milk protein hydrolysis

The kiwi protease (Actinidin) has been exploited to hydrolyse the commercial whey and pure whey proteins (β-lactoglobulin and α-lactoalbumin) as a function of pH (2-5) and temperature (41-58) and incubation time of 120 min. It has been reported that pH and temperature has simultaneous effect of whey protein degradation showing maximum degradation of whey at pH 4 and 41.6ºC and actinidin degraded α-lactoalbumin more efficiently then β-lactoglobulin (Vázquez-Lara *et al.*, 2003).

The kiwi protease (actinidinh) has shown to hydrolyse whey protein isolate, whey protein concentrate and milk protein concentrate with high affinity to α-lactoalbumin and αs-casein. The author reported higher degree of hydrolysis with low enzyme by substrate ratio and the degree of hydrolysis has shown to increase with increasing temperature (from 15ºC to 60ºC) and incubation time (from 0 to 5h) (Kaur *et al.*, 2021). In the second study, the author has shown an progressive increase in degree of hydrolysis of milk proteins and whey protein and significant reduction of the antigenicity of β-lactoglobulin (46%) on 5 h of hydrolysis (Kaur *et al.*, 2022).

2.12 Extraction method of protease

Determination of suitable protease extraction protocol is considered to be a preliminary requirement to study its properties. Nevertheless, there are many types of extraction protocols used to extract from the sample ranging from simple solvent-based extraction method to a complex method associated with purification of the protease. For instance, simple extraction methods using distilled water and buffer solution are applied to extract protease from the plant leaves of *Canavalia ensiformis* where the leaves are first treated with the liquid nitrogen to form powder (Yahya et al., 2019).

2.12.1 Sodium phosphate buffer solvent-based extraction

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.

2.12.2 QB solvent-based extraction

QB method was carried according to the procedures described by Abdullah and Chua (2017) .100-150mg of plant leaves were ground in a pre-cooled mortar. Approximately, 100-150 mg of ground tissue powder was incubated with freshly prepared 1 mL cooled extraction buffer (2M KPO_4, 0.5MEDTA, 1% Triton X 100, 80% glycerol, 1M DTT and distilled water) and vortexed vigorously for 30 seconds. Lysate was centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was collected and were transferred to new tube. If there is still tissue seen, lysate was spin for another 10 min at same speed and tem. Supernatant were kept at -80°C for long term storage.

2.12.3 HBA solvent-based extraction

The HBA method described by Abdullah and Chua (2017) uses 100 mM Tris (pH 7.5) with 10% sucrose for extraction. The solvent was then spiked with 0.28 % β-mercaptoethanol. In a 15 mL centrifuge tube, 150 mg leaves were incubated with 1 mL extraction buffer. The mixture was vortexed for 30 seconds before being placed in an ice bath for 15 min using a refrigerated centrifuge, the mixture was centrifuged for 10 min at 4°C and 12000 rpm after incubation. The supernatant was collected after centrifugation and stored at -20°C for further investigation.

2.13 Precipitation method of protease

2.13.1 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 h. A refrigerated centrifuge

was used to centrifuge the mixture for 15 min 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 colourless. Finally, the pellet was dried and re-suspended in a pH 7.0 0.05 M sodium phosphate buffer.

2.13.2 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 Further purification method of protease

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).

2.14.1 Ammonium sulphate precipitation

Salt precipitation is a very useful and convenient tool for protein purification. Ammonium sulfate is the most commonly used salt used for this process, as it is cheap, highly soluble in water, and interacts readily with water. Ammonium sulfate is either added directly as a solid, or added as a (usually) saturated solution to precipitate desired proteins (Mukherjee, 2019). Ammonium sulphate precipitation protocol has been described in (Sharma *et al.*, 2018b) where the crude extract of kiwifruit was precipitated by ammonium sulphate using different concentrations (0-90%). Precipitation was carried out at 0-5°C and the precipitate was recovered by centrifugation. The supernatant was discarded and the sediment from

each concentration was dissolved in phosphate buffer solution (pH 8.0) and dialyzed over night against the same buffer. The dialyzed enzyme was used for further studies.

2.14.2 Ion-exchange chromatography

Ion-exchange chromatography protocol has been described in (Kumar *et al.*, 2005) the precipitated enzyme obtained from the ammonium sulphate precipitation was further purified by passing through a column (25 cm × 2.6 cm) of activated DEAE-cellulose previously equilibrated with 50 mM phosphate buffer, pH 6.0. The fractions of 3 ml each were eluted at the flow rate of 35 ml/h with linear gradient of 0–0.4 M KCl and analyzed for enzyme activity and protein content.

2.14.3 Gel permeation chromatography

Gel permeation chromatography using Sephadex G75 was carried according to the procedures described by (Kazem and Habeeb, 2020) . The enzymatic solution resulting from ion exchange on the gel surface has been passed quietly and gradually on the sides of the column in order to make sure that the enzymatic solution has been distributed homogeneously on the surface of the gel. The gel is prepared according to the instructions of the Swedish Pharmacia company. Then, elution was done by sodium acetate buffer and separated parts from the column were then collected in tubes. The absorption was estimated for that separated parts at 280 nm wavelength. Also, the enzyme activity was measured to the separated peaks after plotting the relationship between the numbers of discrete parts with respect to absorption at 280 nm. Finally, the active parts were collected, measured in size and estimated their effectiveness and protein concentration.

2.15 Factor affecting activity of enzyme

The factors affecting the enzyme activity can be classified as physical-chemical (pH, temperature, ionic strength, water activity etc.), chemical (activators, inhibitors, stabilizers etc.), and physical (pressure, shear forces, attrition etc.) (Vitolo, 2020). When temperature is high, the rate of the enzyme activity is high because substrates collide with active sites on the enzyme more frequently as the molecules move rapidly. Similarly in the case of pH, it affects ionic and hydrogen bonds which are important to enzyme shape and finally effect on the enzyme activity (Eed, 2012). Other factors – such as ionic strength and water activity (Aw) – can more or less affect enzyme activity depending on the particular type of

enzyme, and the intensity and duration of their action along the reaction. The activator is a compound that increases enzyme activity. It can be bound to the enzyme molecule (prosthetic group) or dissolved in the reaction medium, which, in turn, bounds to the enzyme at the moment of catalysis. Inhibitor is a substance that decreases the enzyme reaction rate through binding specifically to some domain of the enzyme molecule. Unspecific factors can act negatively on enzyme catalysis through purely mechanical effects, such as the shear forces originated by the agitation of the reaction medium (Vitolo, 2020).

2.16 Antioxidant activity of hydrolysate

Chicken breast protein was hydrolysed by papain and it was observed that hydrolysate showed strong reducing power, as well as an ability to scavenge DPPH radicals (Sun *et al.*, 2012). Hydrolysis of heat-treated WPI with Flavourzyme for 0.5 or 1 h produced the most active antioxidant hydrolysates (Peña Ramos *et al.*, 2004). Protein hydrolysates obtained from defatted peanut kernels with esperase treatment for 2h exhibited higher antioxidative activity toward linolenic acid peroxidation than other proteases (including neutrase, pepsin, protease A and protease N) (Hwang *et al.*, 2010). Whey protein hydrolysates which were produced by Flavorzyme had higher DPPH radical scavenging activity than the native whey protein (Yoo and Chang, 2016). Mann *et al.* (2015) reported that the hydrolysis of whey protein by microbial proteases (alcalase, flavourzyme, protamex and neutrase) increased antioxidant activity of whey protein from 7–19.8 to 40–54.2%. Bioactive peptides with increased antioxidant activity than proteins can be obtained after hydrolysis of egg proteins with proteolytic enzymes from animal, plant or bacterial origin as well as by chemical hydrolysis or gastrointestinal digestion (Benedé and Molina, 2020).

Part III

Materials and methods

3.1 Materials

3.1.1 Collection of samples

The kiwi fruit (*Actinidia deliciosa*) was purchased from the local market of Dharan. The collected fruits was then cleaned to remove dust, foreign matters and damaged parts.

3.1.2 Glass Wares

Burette, pipette, test tubes, volumetric flask, funnel, conical flask, measuring cylinder, petridish and micropipette were obtained from the laboratory of central campus of technology.

3.1.3 Chemicals

Whey protein concentrate, sodium phosphate dibasic heptahydrate, bovine serum albumin, sodium phosphate basic monohydrate, ammonium sulphate, Bradford reagent, coomassie brilliant blue G-250, coomassie brilliant blue R-250, HCl acid, ethanol, tris buffer, phosphate buffer and tyrosine were obtained from the laboratory of central campus of technology.

3.1.4 Equipments

Hot air oven, electronic balance, thermometer, heating mantle (burner), Buchner funnel, uv-visible spectrophotometer, hot water bath, shaking incubator, filter paper, micropipette, centrifugation machine, petri-plates, Bunsen burner, weighing machine, mortar and pestle, and magnetic stirrer were obtained from the laboratory of central campus of technology.

3.2 Methods

3.2.1 Extraction of crude kiwi protease

For the preparation of crude enzyme extract, at first collected kiwi fruit having 10.1 $\pm 1^{\circ}$BX were washed properly with sterile water and chopped into small pieces and then grinded in

mortal pestle. Then sodium phosphate buffer (50mM) having pH 7 was added in the ratio of 1:2 and the solution is stirred in magnetic stirrer at refrigeration temperature (15°C) for 20 min It was then filtered using muslin cloth. The filtrate was centrifuged at 7000 rpm for 15 min and the obtained supernatant was collected as crude enzyme extract after filtration through whatmann 41 filter paper. Thus, obtained crude protease was kept at refrigeration temperature until further analysis and purification.

3.2.2 Partial purification by Ammonium Sulphate

The crude extract of the protease was first precipitated by mixing with increase gradient ammonium sulphate saturation concentration ranges from 20-80% at refrigerated condition followed by centrifugation at 7000 rpm for 15 min to precipitate. The precipitate was collected and dissolved on sodium phosphate (buffer pH 7.0, 50mM) assayed for protease activity and protein content. The appreciable activity was found in each fraction from 40% to 60%. Hence, precipitate obtained from 40-60% fraction was collected and subjected to dialysis and further study.

3.2.3 Dialysis

At first, preparation the dialysis bag (LA395) was carried out by following the method, 8-10 cm of dialysis membrane was taken and it was treated with 0.3% (W\V) sodium sulphate solution of 80°C for 1min with the objective to remove sulphur compound. The membrane was decanted from sodium sulphate solution and washed with hot water (60°C) for 2min followed by acidification with a 0.2% (V/V) solution of sulphuric acid, then rinse with hot water to remove the acid and allowed to cool.

The membrane was taken and tied with nylon thread tightly on one side of the membrane. The ammonium sulphate precipitated protease was placed in a dialysis bag and checked for the leakage of the sample in it. The dialysis bag was then dipped in a beaker containing 50mM of sodium phosphate buffer solution. This setup was kept in refrigerator/cool conditions overnight with three times exchange of buffer.

3.2.4 Determination of protein content

The protein content of enzyme was determined by Bradford method (Bradford, 1976). Bradford assay was performed by adding 3ml of Bradford reagent to 100µl of each standard solutions or protease extract, and homogenization by using vortex mixer and incubated at 37°C for 30min and the absorbance was read at 595 nm. The protein content of the extract was estimated by comparing with BSA standard solution where the final concentration of the solution gradually increases from 10µg/ml to 100µg/ml. The BSA standard curve is shown in appendix C.

3.2.5 Determination of protease activity

The protease assay (PA) using casein as substrate was done following procedure given by Cupp-Enyard and Aldrich (2008) to confirm the presence of protease enzyme and to determine the protease activity. 0.1ml of crude/ partially purified protease 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 40°C for 30 min and stopped by addition of 3.6 ml of 5% trichloroacetic acid (TCA), cooled at refrigeration condition followed by centrifuge at around 7000 rpm for 15 min., The centrifuge content was filter though the Whatmann no.41 filter paper. The blank was prepared in similar manner except enzyme was added after addition of TCA. The obtained 1ml filtrate was mixed with 5ml of 2% Sodium carbonate reagent and allowed to stand for 10min then the addition of 0.5ml Folin and ciocalteus phenol (FC) reagent in the reaction mixture was done. The reaction mixture was incubated at 37°C for 45min and absorbance was measured spectrophotometer at 700nm. The activity of protease samples was calculated in terms of Units, which is the quantity of tyrosine equivalents released from casein per min in micromoles.

$$\text{Protease activity (Units/ml enzyme)} = \frac{(\mu\text{mol tyrosine equivalent release}) \times V_T}{V_E \times t \times V_C \times 181}$$

Where, V_T is the total assay volume in ml, V_E is the volume of enzyme used in ml, t is time of assay in minute, and V_C is the volume used in colorimetric determination in ml.

3.3 Determination of optimum temperature and pH on casein protein as substrate

Optimum temperature and pH of the partially purified kiwi protease was carried out with some modifications (Ketnawa *et al.*, 2012).

3.3.1 PH optimization on casein protein as substrate

Caseinolytic assay was done at different pH ranging from 4 to 10 used to determine the optimum pH of partially purified protease. Buffers (50mM) used were citrate buffer having pH 4.0, acetate buffer having pH 5.0, sodium phosphate having pH 6.0, sodium phosphate buffer having pH 7, tris– HCl buffer having pH 8.0 and 9 and carbonic acid buffer having pH 10. Optimum pH of the partially purified protease was confirmed by performing protease assay using casein protein as substrate following procedure.

0.1ml of partially purified protease extract was added to 2.2 ml of (5mg/ml) 0.5% casein solution prepared in above mention different buffer. The reaction mixture was incubated at temperature 45°C for 15min and stop by addition of 3.6 ml of 5% tri-chloroacetic acid (TCA), cooled at refrigeration condition followed by centrifuge at around 5000 rpm for 15 min, The centrifuge content was filter though the Whatmann no.41 filter paper. After filtration, both each 1ml filtrate 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 1h and absorbance was measured spectrophotometer at 700nm. Finally, the protease activity versus pH graph was plotted to determine the optimum pH on casein protein as substrate.

3.3.2 Temperature optimization on casein protein as substrate

Casein solution (5mg/ml) was prepared at optimum pH and the activity of enzyme solution was assayed at various temperatures ranging from 0°C to 90°C. Optimum temperature of the partially purified protease was determined by performing protease assay by applying procedure as mention in 3.3.1. Finally, the protease activity versus temperature graph was plotted to determine the optimum temperature on casein protein as substrate.

3.4 Determination of optimum temperature and pH on whey protein as substrate

3.4.1 pH optimization on whey protein as substrate

Different pH values ranging from 4 to 10 were used to determine the optimum pH of protease enzymes. Buffers used were 50mM citrate buffer having pH 3.0, acetate buffer having pH 5.0, sodium phosphate having pH 6.0 and tris-buffer pH 8.0 and 9, and carbonic acid buffer pH 10. Optimum PH of the partially purified protease was confirmed by performing protease assay using whey protein as substrate following procedure.

0.1ml of crude/ partially purified protease extract was add to 2.2 ml of (5mg/ml) 0.5% whey protein solution prepared with sodium phosphate buffer (50mM, pH 7). The reaction was incubated at temperature 40°C for 1 h and stopped by heating 95°C for 15min followed by cooling to room temperature and centrifuge at around 5000 rpm for 15 min. The centrifuge content was filter though the whatmann no.41 filter paper. For the blank preparation, enzyme was first treated to 95°C for 15 min to inactivate and used to allow whey protein hydrolysis in similar manner. The obtained filtrate as blank and test sample were diluted 3 times with 1ml of each respective filtrate and absorbance was measured spectrophotometrically at 280nm. The absorbance vs pH graph was plotted to determine the optimum pH for whey protein hydrolysis.

3.4.2 Temperature Optimization on whey protein as substrate

Whey protein solution (5mg/ml) was prepared at optimum pH and the activity of enzyme solution was assayed at various temperatures ranging from 0°C to 90°C. Optimum temperature of the partially purified protease was confirmed by applying procedure as mentioned in 3.4.1. Finally, the protease activity versus temperature graph was plotted to determine the optimum temperature on whey protein as substrate.

3.5. Optimization of Substrate whey protein concentrate

Whey protein hydrolysis for 60°C for 1h in shaker incubator has been carried out varying substrate (0.78mg/ml, 1.17mg/ml, 1.56mg/ml, 1.95mg/ml, 2.37mg/ml, 3.13mg/ml, 4.70mg/ml and 7.82mg/ml) concentration keeping enzyme (protein content= 0.28mg/ml, enzyme activity= 0.630u/ml and keeping enzyme concentration (0.0122 mg/ml) same to determine optimum substrate concentration. The reaction was stopped by heating 95°C for

15min followed by cooling to room temperature and centrifuge at around 5000rpm for 15 minute. The centrifuge content was filtered through the whatmann 41 filter paper and kept on refrigeration for the analysis of degree of hydrolysis. Degree of hydrolysis was measured by O-pthaldehyde (OPA) method with some modification as mentioned in 3.6.1. Finally, the degree of hydrolysis versus substrate concentration graph was plotted to determine the optimum substrate concentration and enzyme: substrate concentration ratio for highest catalytic efficiency.

3.6 Extent of hydrolysis and antioxidant activity of whey protein

Hydrolysis of heated (95°C for 15min) and non-heated whey protein was carried out at optimized enzyme to substrate ratio (1:192) for 6h and sample taken at different time were taken to evaluate changes in degree of hydrolysis and antioxidant activity of the hydrolysate. The reaction was performed at 60°C in a shaking incubator set at 200 rpm. About 3ml of sample at selected time interval (0, 15, 30, 1h, 2h, 3, 4, 5 and 6h) was withdrawn from the reaction mixture and immediately heated to 95°C for 15min to stop the reaction. After heating the samples were centrifuged at 5000 rpm for 15min filtered through whatmann 41 filter paper and kept at refrigeration. The sample collected at each time interval were subjected to analyse degree of hydrolysis and antioxidant activity.

3.6.1 Determination of degree of hydrolysis

O-pthaldehyde (OPA) method with some modification was carried out to determine the degree of hydrolysis (Nielsen *et al.*, 2001). This method is based on the development colour due to reaction between primary amino groups with OPA reagents and measurement colour intensity spectrophotometric ally at 340nm. OPA reagent was prepared just before the analysis by adding 160 mg o-phthaldialdehyde (OPA, 97%) in 4 ml ethanol, was transferred quantitatively to 150 ml di-Na tetraborate decahydrate buffer (consist of 7.620 g di-Na tetraborate decahydrate and 200 mg Na-dodecyl-sulfate) followed by adding 176 mg dithiothreitol (DTT, 99%) and final volume was made up to 200 ml.

The 40 µL of filtrate obtained from whey protein hydrolysis reaction mixture was added to 3ml o-pthaldehyde (OPA) reagent and absorption was read by using spectrometer at 340 nm after 2 min of incubation at room temperature. Blank was performed with the same

amount of distilled water instead of sample. L-serine 0.1mg/ml (0.9516 meqv/L) was used as standard for the quantification. Degree of hydrolysis was calculated as:

$\%DH = h/h_{tot}$

Where $h= (Serine\text{-}NH_2\text{-}\beta)/\alpha$ [meqv/g protein] and

$serineNH2= [(A_{sample}\text{-}A_{blank}) /(A_{standard}\text{-} A_{blank})] \times (0.9516 \text{ meqv/L}) \times 0.1 \times (100/X)$

Where $Serine\text{-}NH_2$= meqv $serineNH_2$/g protein; X= g sample; P= % protein in sample and 0.1 = sample volume in liter (L)
The value of α, β and h_{tot} were obtained from Nielsen *et al.* (2001).

3.6.2 Determination of antioxidant activity

Antioxidant activity by DPPH radical scavenging method according to the method described by Brand-William et al. (1995) with some suitable modification was used to evaluate antioxidant activity of the whey protein hydrolysate.

200ml of 0.1mM stock solution of DPPH was prepared by using 0.00985gm DPPH and 80% ethanol. 0.3ml of filtrate collected from whey protein hydrolysis reaction was mixed with 3ml of DPPH solution and were kept at dark condition for 30min at room temperature. After the incubation, the absorbance at 517nm was measured by using the spectrophotometer. Blank was performed by taking all the reagents except the filtrate. The antioxidant activity was expressed as percentage radical scavenging activity using the following equation.

% Radical scavenging activity = $[1 - \frac{A}{A0}] \times 100$, where A and A_0 are absorbance of sample and blank respectively.

3.7 Data analysis

All the test was conducted in triplicate. The results were expressed in average value ± SEM (Standard Error of Mean). The raw data were first processed by Microsoft Excel 2013 and was subjected to analysis of variance at 5% confidence level was conducted by using IBM SPSS version 20.

Part IV

Result and discussion

In this research work, protease was extracted, partially purified and dialyzed from kiwi fruit. The temperature and pH of the extracted protease was optimized on casein and whey protein as substrate and effect of substrate concentration on the degree of hydrolysis of whey protein was evaluated. Finally, bulk hydrolysis of extracted protease was carried out at optimized condition to determine degree of hydrolysis and antioxidant activity of whey protein with respect to time of hydrolysis.

4.1 Purification fold of kiwi enzyme after partial purification

The crude enzyme from kiwi fruit was subjected to ammonium sulphate precipitation, at different concentrations ranging from 20-80% saturation level (Sharma and Vaidya, 2018a). Protease precipitation at 30-40%, 40-50% and 50-60% saturation level, give maximum protease activity as shown in fig 4.1.

Hence 40-60% ammonium Sulphate saturation fraction was taken for further purification by dialysis and study. It is reported that, highest enzyme activity, yield and purification fold was found in 40-60 percent precipitation of ammonium sulphate (Santos *et al.*, 2018; Sharma and Vaidya, 2018a), while 0-60% ammonium sulphate saturation for lower and higher cut off has also reported (Dhiman *et al.*, 2021), which is almost near to support of my finding.

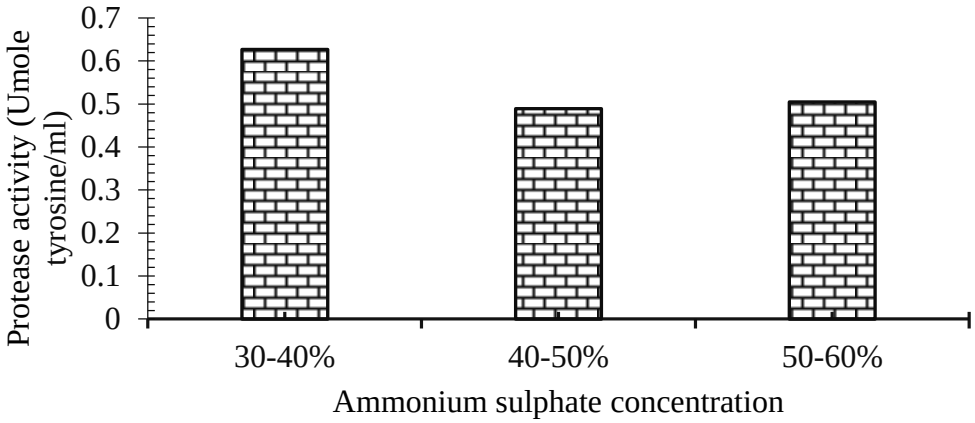

Fig.4.1 Purification fold of kiwi enzyme after partial purification

The purification fold, total yield and specific activity of extracted kiwi protease after dialysis is presented in the table 4.1. Almost 1.5 times of purification fold, and 54.98 % recovery of total activity as compared to crude extract was achieved after ammonium sulphate precipitation followed by dialysis. The specific activity of the dialysed kiwi protease was found as 2.241U/mg protein.

Table 4.1 Protein concentration, protease activity, specific activity, Total activity, purification fold and percentage yield of kiwi protease

Purification step	Protein content (mg/ml)	Protease activity (U/ml)	Specific activity (U/mg protein)	Total activity (U)	Purification fold	Yield (%)
Crude enzyme	0.151	0.219	1.451	26.34	1	100
40-60% Ammonium sulphate precipitation	0.382	0.6274	1.642	12.40	1.1312	47.07
Dialyzed	0.281	0.630	2.241	14.483	1.1544	54.98

4.2 Temperature and pH optimization on casein protein as substrate

4.2.1 Temperature optimization of kiwi protease on casein protein

The kiwi protease was found to be active at temperature range of 8°C to 70°C and the activity on casein was increased from 8°C to 50°C of incubation temperature and then started to decrease; showing maximum activity at 50°C (Fig. 4.2). The value for protease activity obtained at 50°C was not significantly different from the value obtained for 40°C but was found to be significantly different with value obtained for other incubated temperature ($P < 0.05$). The finding indicate that optimum activity of kiwi protease lies in between 40°C to 50°C. The decrease in activity at higher temperature might be due to enzyme being protein can denature and loss their activity at higher temperature. When the temperature is elevated over the ideal range, the kinetic energy between the water molecules and the enzyme is so high that the structure of the enzyme molecule begins to break down (Ketnawa *et al.*, 2011). As a result, when the temperature rise, less activity was seen (Lewis and Luh, 1988a).

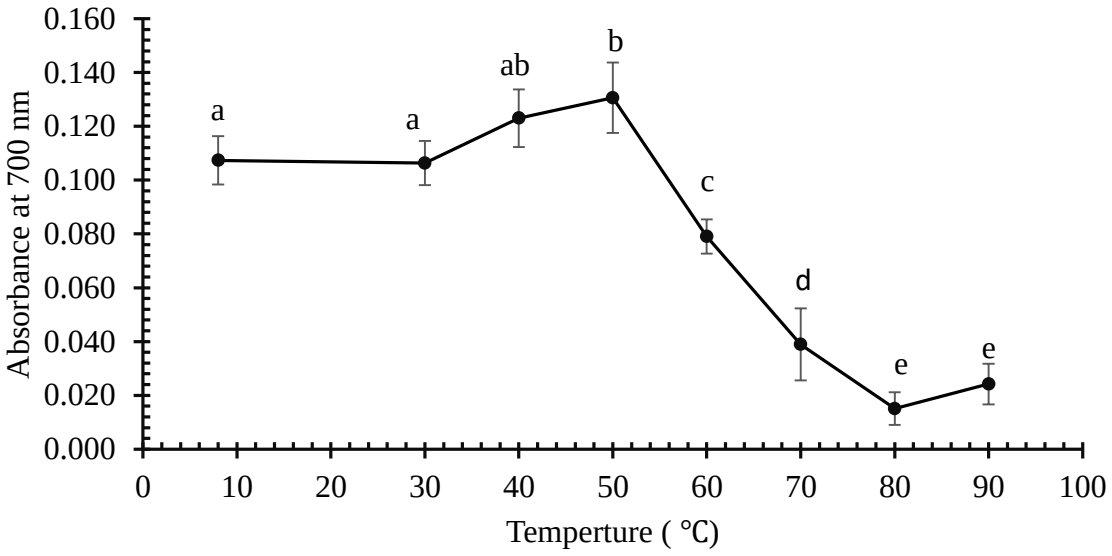

Fig.4.2 Temperature optimization of kiwi protease on casein protein

It is reported that, protease extracted from matured fruits like Kiwi, Apple and Grape showed the highest protease activity in the temperature range of 50-60°C (Koak *et al.*,

2011). Koak et al. (2011) and Lewis and Luh (1998a) also reported similar result of optimum tem for activity of kiwi protease at 58-62°C. While Sharma *et al.* (2018a) has shown the maximum activity at 45°C and Dhiman *et al.* (2021) has reported the maximum activity at 35°C to 40°C. The temperature at which maximum activity found in this study is in between as reported by these researcher (Dhiman *et al.*, 2021; S. Sharma *et al.*, 2018a).

4.2.2 pH optimization of kiwi protease on casein protein

The effect of pH on caseinolytic activity of kiwi protease at different pH ranging from 4-10 is shown in figure 4.3 The protease activity of kiwi has increased at pH 5&7 and then gradually decrease; showing maximum activity at pH 7 (fig 4.3). The protease activity observed at pH 7 was not significant different with pH 5 but significantly different with other pH ($p<0.05$). This shows that the optimum pH of kiwi protease lies at pH 5&7 but start to decline from pH 7. The decline in protease activity in between pH 5 and 7 might be due to the impurities present in enzyme. The enzymes activity abruptly decreased in acidic pH (by around 70%), probably as a result of denaturation and Charge repulsion which weakens electrostatic connections. proteolytic effect of actinidin on casein was pH-dependent and the enzyme is more effective at lower pH values (Maryam Chalabi *et al.*, 2014b).

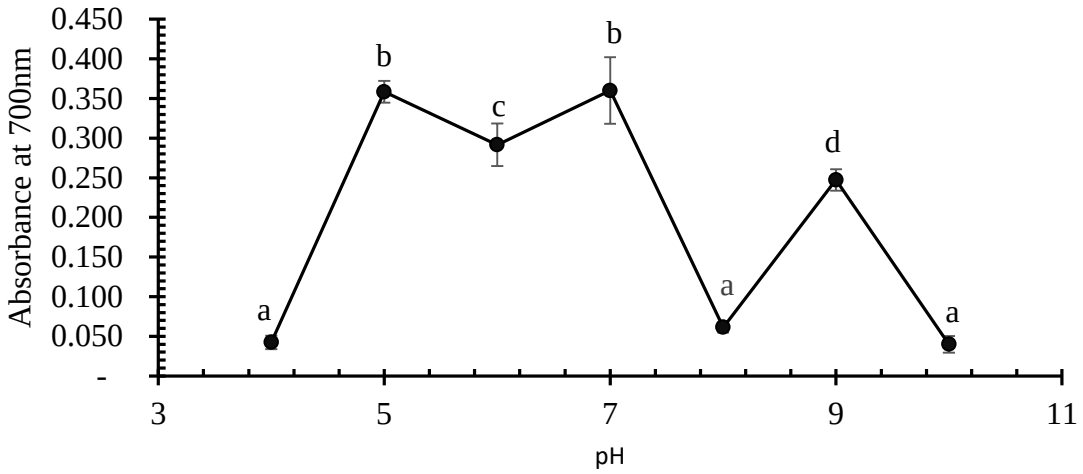

Fig. 4.3 pH optimization of kiwi protease on casein protein

It is noteworthy that proteolytic effect of actinidin on casein was pH-dependent and the enzyme is more effective at lower pH (Chalabi *et al.*, 2014a). Actinidin has a broad pH optimum from 5 to 7 for benzoyl-L-arginine ethyl ester and benzoyl-L-arginine ethyl ester as substrate (Lewis and Luh, 1988b) and (Mike, 2013). According to Ketnawa *et al.* (2011) cysteine protease like bromellin shows strong caseinolytic activity in a broad pH range of 3-9 while Grozdanovic *et al.* (2013) mentioned that proteolytic enzyme from ripe fruit of bromelia antiacantha bertol. shows maximum activity at pH 5-9. Thus, it can be concluded that protease from kiwifruit being cysteine in nature, its maximum protease activity can be found at pH 5&7.

4.3 Temperature and pH optimization on whey protein as substrate

4.3.1 Temperature optimization of kiwi protease on whey protein

The absorbance of the hydrolysate prepared by hydrolysing whey protein by partially purified kiwi protease at different temperature ranging from 20°C-90°C is shown in figure 4.4. The kiwi protease was found to be active at temperature 30°C-70°C. The activity on whey protein was increased from 30°C-60°C of incubation temperature and then gradually started to decrease; showing maximum at 60°C (fig4.4). This might be due to inactivation of the enzyme at high temperature. The value obtained at temperature 60°C was found to be significantly different with the value obtained for other incubated temperature ($p<0.05$).

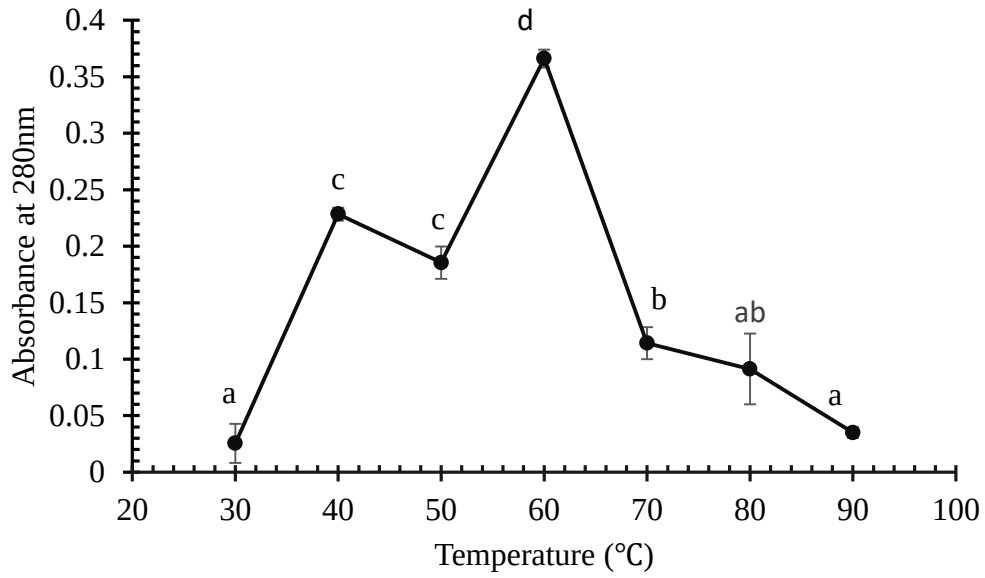

Fig.4.4 Temperature optimization of kiwi protease on whey protein

According to different researcher Yamaguchi *et al.* (1982), Demir *et al.* (2008) and Chalabi *et al.* (2014a) the optimum temperature of kiwi protease for maximum hydrolysis was varied on the basis of substrate used. Kaur *et al.* (2021) mentioned that actinidin was temperature dependent and maximum hydrolysis was achieved at 60°C while Vázquez-Lara *et al.* (2003) reported the best condition for the proteolysis of whey protein by actinidin was 41.6°C. So, on the basis of these information, it can be concluded that 60°C is optimum temperature of kiwi protease for whey protein.

4.3.2 pH optimization of kiwi protease on whey protein

The absorbance of the hydrolysate prepared by hydrolysing whey protein by partially purified kiwi proteases at different pH ranging from 4-10 is shown in figure 4.5. The activity on whey protein hydrolysate using kiwi protease has increased from pH 4-6 and the gradually decreases; showing maximum activity at pH 6 (fig 4.5) and these might be due to charge repulsion at highly acidic and alkaline pH.

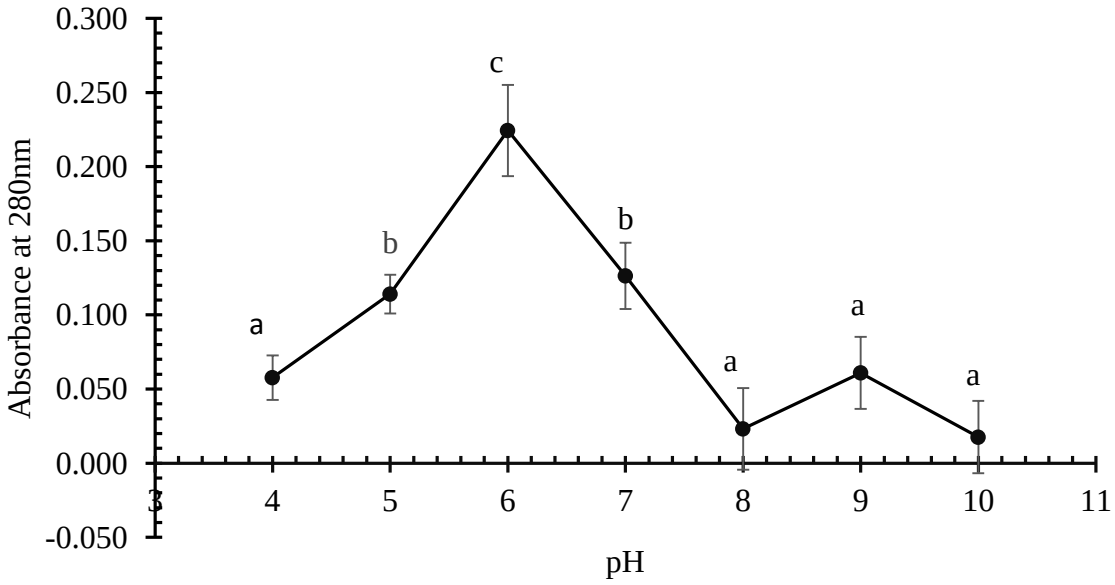

Fig.4.5 pH optimization of kiwi proteases on whey protein

The protease activity at pH 6 was significantly different with the values obtained for other pH. It suggests that kiwi protease have optimum pH 6 for whey protein as substrate.

According to Vázquez-Lara *et al.* (2003) the best condition for the proteolysis of whey protein by actinidin was pH 4 but the researcher only perform from pH 2-4 while the other researcher like Boland (2013) and Ha *et al.* (2012) reported that the optimum pH for the optimum activity of kiwi protease varied on the basis of substrate used. Additionally Scott and Reed (1975) reported that papain (a thiol protease, very closely related to actinidin), has a broad pH activity profile (from 5 to 9) depending on the substrate. The optimum pH at which maximum activity found in this study is in between by these researcher (Scott and Reed, 1975; Vázquez-Lara *et al.*, 2003)

4.4 Effect of substrate concentration on whey protein hydrolysis

The result of degree of hydrolysis reaction achieved with different substrate concentration keeping the enzyme (protein content= 0.281mg/ml and protease activity=0.630U/ml/min) concentration constant (0.0122 mg/ml) is shown in the fig 4.6 The degree of hydrolysis at 2.347mg/ml substrate concentration was significantly different with 0.782, 1.173, 1.565, 1.956, 3.13, 4.695 and 7.826 mg/ml substrate concentration ($p<0.05$). The degree of hydrolysis was gradually increased at the low substrate concentration and became maximum at 2.347 mg/ml and the gradually starts to decrease as shown fig 4.6. This might be due to the decrease in the concentration of peptide bond susceptible to be hydrolysis. This result suggest that the optimum substrate for maximum degree of hydrolysis is 2.347mg/ml and the optimum activity can be achieved by keeping E:S = 1:192. S. Kaur *et al.* (2021) mentioned that the lower E:S i.e. 1:100 represented greater DH but the researcher only apply 1:100 and 1:500 E:S ratio. The obtained E:S of my work lies in between of the value mentioned by the researcher. Thus, it can be concluded that by maintaining E:S i.e 1:192 maximum degree of hydrolysis can be achieved.

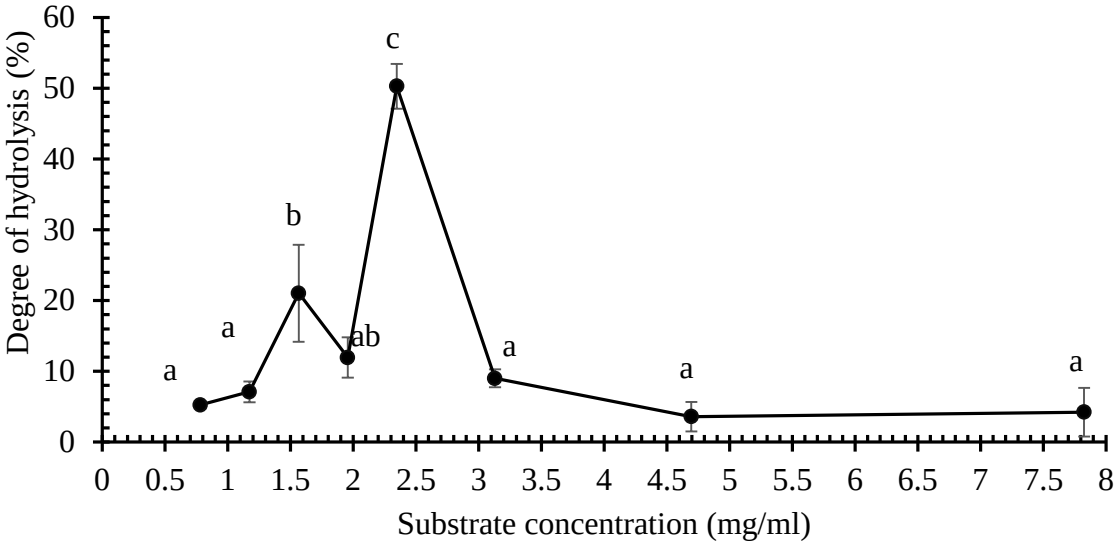

Fig.4.6 Effect of substrate concentration on whey protein hydrolysis

The decrease in the rate of hydrolysis at higher substrate concentrations has been shown in several cases: for the hydrolysis of casein, for rapeseed protein isolate, for pure bovine haemoglobin by Alcalase, and for hydrolysis of whey protein concentrate (WPC) for three enzyme preparations (MKC Protease, Alcalase, and PEM). In all cases, an increase of the substrate concentration at constant enzyme concentration resulted in a decrease in the rate of hydrolysis (Butré *et al.*, 2012). On the other hand, similar effect was found for the FLA WPHs where the highest DH values were reported at intermediated protein concentration and decreased when substrate concentration was out of this corresponding values (Eberhardt *et al.*, 2021).

4.5 Degree of hydrolysis on heated and non-heated whey protein

The percentage degree of hydrolysis between heated and non-heated whey protein at different time interval ranging from 0-6 hour is shown in figure 4.7.

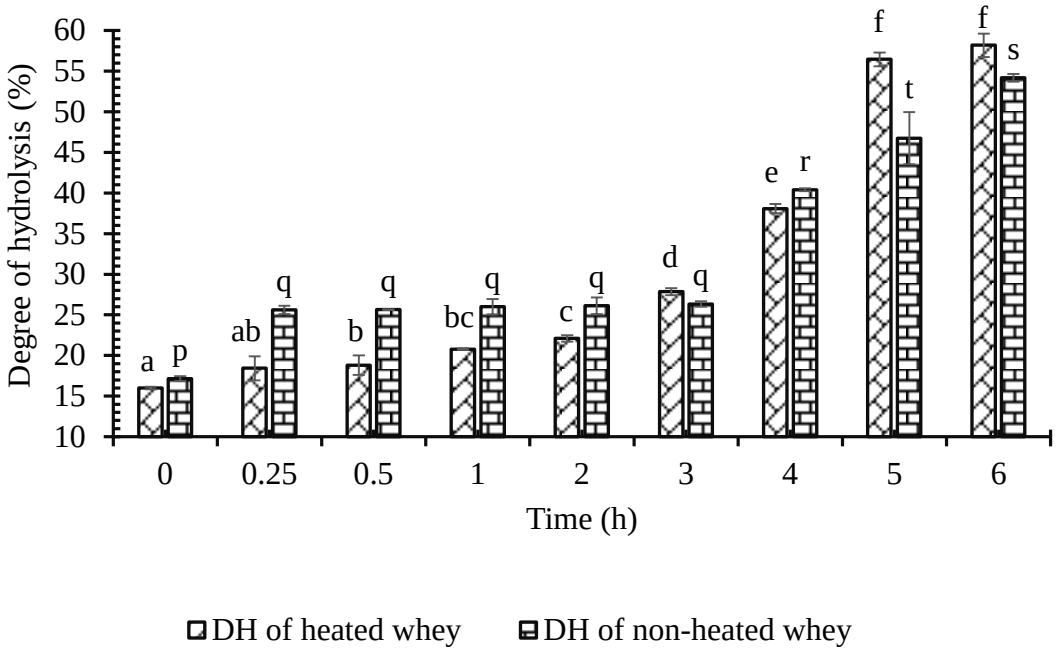

Fig.4.7 Degree of hydrolysis on heated and non-heated whey protein

Initially, low DH values in whey protein was found and gradually increases with the time, it might be due to the fact that cysteine rich whey protein react weakly and unstable with OPA at the beginning but more number of small peptides and free amino acid appeared with the increase in hydrolysis time. Statistical analysis at 5% level of significance shows that the degree of hydrolysis at 5 hrs and 6 hrs was significantly different with 0, 0.25, 0.5, 1, 2, 3 and 4h. This shows that the more DH can be obtained with the increase in reaction time.

Proteolysis patterns of heat denatured whey protein by different protease may differ from those of native protein (Mutilangi *et al.*, 1995). Loughlin *et al.* (2013) reported that the susceptibility of whey protein to heat induced structural change has the potential to alter hydrolysis by exposing previously inaccessible amino acid to cleavage, which support the zigzag pattern of DH on heated and non-heated whey protein at different time. Progressive increase in % DH with elevation of time indicates the availability of cleavage sites, leading to rise in number of free amino groups and smaller peptide chains after hydrolysis (Kaur *et al.*, 2021). Silva and Silveira (2013) reported that there was marked increase in degree of hydrolysis when the reaction time increase from 1-2h but maximum value was obtained at 5 hour of hydrolysis and similarly Kaur *et al.* (2021) mentioned that higher degree of hydrolysis was increased with increasing temperature (from 15ºC to 60ºC)

and incubation time (from 0 to 5h). In the second study, the author explained that the degree of hydrolysis of milk proteins and whey protein significantly reduced the antigenicity of β-lactoglobulin (46%) on 5h of hydrolysis (Kaur *et al.*, 2022). Thus, the obtained result lies almost near to the finding of above mention researcher and it is also confirmed that heat treatment of the protein solutions increased DH and (Pena-Ramos and Xiong, 2001).

4.6 Antioxidant activity of whey protein hydrolysate

Effect of time on the radical scavenging activity of heated and non-heated whey protein at different time interval ranging from 0-6 hrs is shown in fig.4.8

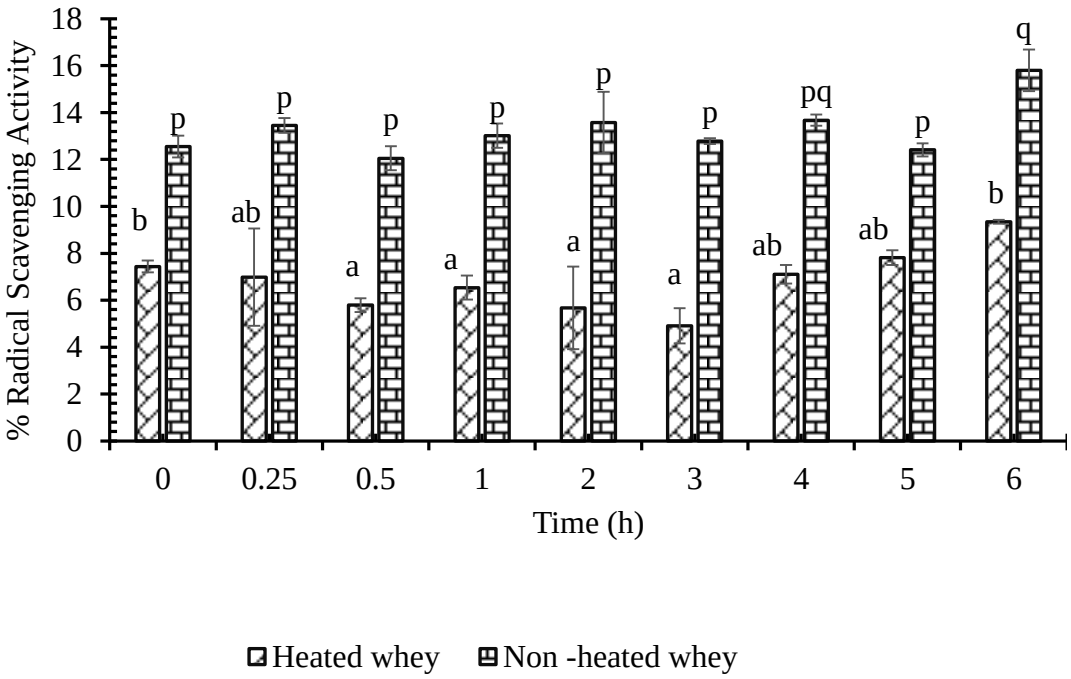

Fig. 4.8 Antioxidant activity of whey protein hydrolysate

The radical scavenging activity of whey protein increases to a great extent after hydrolysis for 3h (4.90% and 12.78%) and steadily increased until 6h (9.30% and 15.80%) of hydrolysis and it might be due to the fact that the structural change in the protein, leading to a great radical quenching ability.

Statistical analysis at 5% level of significant shows that maximum radical scavenging activity was observed at 6h of hydrolysis which was significantly different with other.

Hydrolysates prepared with protease exhibited an antioxidant activity, which increased with the hydrolysis time up to 6h. (Pena-Ramos and Xiong, 2001).

The radical-scavenging activity of WPI hydrolysates increased to a great extent after hydrolysis for 1h. and then steadily increased until 5h of hydrolysis (Salami *et al.*, 2010). Peptides with low molecular weight exhibited higher antioxidant activities compared to high molecular weight peptides Liu *et al.* (2015) thus, maximum radical scavenging activity can be observed with the increase in time of hydrolysis. The peptides fraction of different size obtained after partial hydrolysis with trypsin and chymotrypsin of both bovine and camel whey protein display the lowest and highest antioxidant activity (Salami *et al.*, 2010) which supports the results of my finding. Athira *et al.* (2015) and del Mar Contreras *et al.* (2011) reported that the WPH and WPC using alcalase and thermolysin showed the maximum antioxidant activity at 8 h. Thus from these researcher Athira *et al.* (2015), del Mar Contreras *et al.* (2011) and Salami *et al.* (2010) it can be assumed that the maximum antioxidant activity of whey protein using actinidin can be achieved at 6h of hydrolysis.

Part V

Conclusions and recommendations

5.1 Conclusions

Determination of whey protein hydrolysis ability of protease extracted from kiwi fruit was done as research work and based on the research work following conclusion can be drawn.

1. A purification fold of 1.15 times can be achieved on kiwi protease extracted from 40-60% saturated ammonium sulphate solution followed by dialysis with specific activity of 4.2418 U/mg protein and yield of 54.98%
2. The optimum temperature and pH was found to be 50°C and 5-7 respectively on casein protein while optimum temperature of 60°C and pH 6 was found on whey protein.
3. Optimum substrate concentration was found to be 2.347mg/ml with 50.30% degree of hydrolysis.
4. Degree of hydrolysis was found to be increased with the increase in hydrolysis time from 0-6h showing maximum of 58.18% and 54.184% respectively on heated and non-heated whey protein.
5. Antioxidant activity was found to be raised with the increment on hydrolysis time ranging from 0-6h showing maximum of 9.340% and 15.805% respectively on heated and non-heated whey protein.

5.2 Recommendations

1. Various purification method of kiwi protease like chromatography and electrophoresis can be applied.
2. Degree of hydrolysis can be prolonged more than 6h.

Part VI

Summary

The main objective of this research work is to extract, partially purified and dialyzed proteolytic enzyme from kiwi fruit to evaluate hydrolysis ability for whey protein and antioxidant activity of generated hydrolysate. The protein content, protease activity, yield and purification fold along with optimum temperature and pH of the dialyzed enzyme using casein and whey protein was determined. Moreover, the effect of substrate concentration on the degree of hydrolysis was evaluated. Finally, the change in degree of hydrolysis and antioxidant activity at optimized condition for whey protein hydrolysis with respect to time of hydrolysis up to 6h was evaluated.

The present work showed that the crude kiwi protease in 40-60% saturated ammonium sulphate solution gives maximum specific activity of 2.2418 U/mg protein along with 1.154 fold improvement in purification. The kiwi protease showed optimum activity at temperature 50°C and pH 5&7 for casein protein and similarly temperature 60°C and pH 6 for whey protein. The maximum degree of hydrolysis for whey protein was found at substrate concentration of 2.347 mg/ml (equivalent to 1:192 enzyme by substrate ratio). Degree of hydrolysis and antioxidant activity was found to be increased with time up to 6h of hydrolysis. Degree of hydrolysis was found to be higher in heated whey protein i.e. 58.181% as compared to non-heated whey protein i.e. 54.184%. Similarly maximum antioxidant activity of heated and non-heated whey protein was found at 6h of hydrolysis i.e. 9.34 % for heated and 15.80% for non-heated whey protein.

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Appendices

Appendix A

Table A.1 Equipment and utensils

Pipettes	Filter paper
Beaker	Water bath
Motar and pestle	Spectrophotometer (60UV-Vis)
Weighing balance	Test tubes
Portable digital thermometer	Weighing machine
Micro pipette	Measuring cylinder
Shaking Incubator (CLE-126)	Conical flasks
Refractometer (RBX0032)	Centrifugation machine (220v, 10%Hz)
Magnetic stirrer (SH-4C)	Glass rod

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-sodium tetraborate Decahydrate (M.W.= 381.37)	Sodium lauryl sulphate needles (M.W. = 288.38)
Di-thiothreitol (M.W.= 154.28)	Tri-chloroacetic 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-picrylhydrazyl (M.W.= 394.32)

Appendix C

C.1 Calibration curve for proteases activity

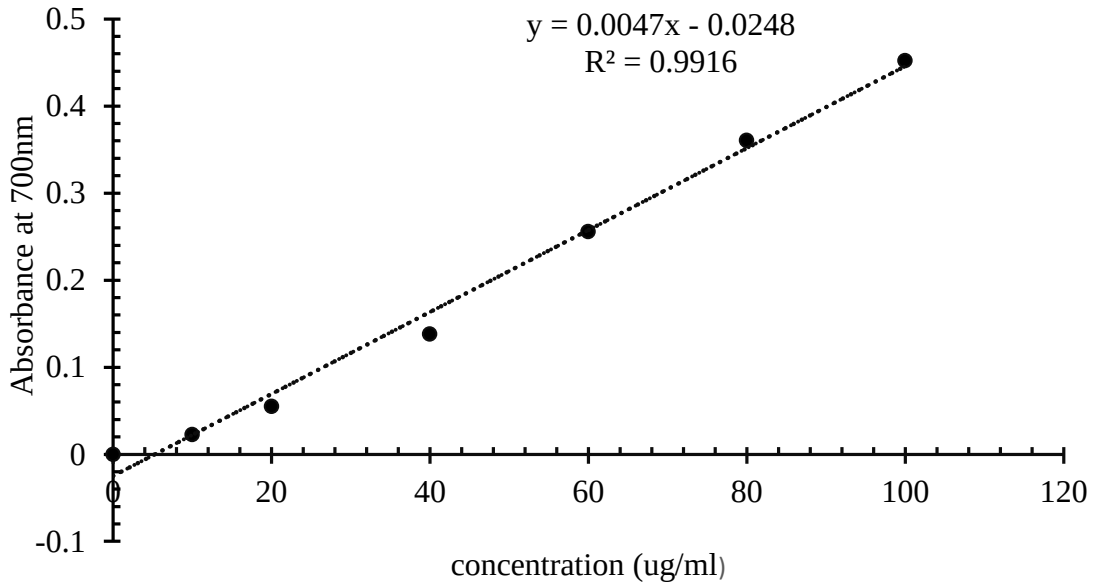

Fig.C.1 Standard curves of tyrosine for protease activity.

C.2 Standard curve of BSA for protein determination

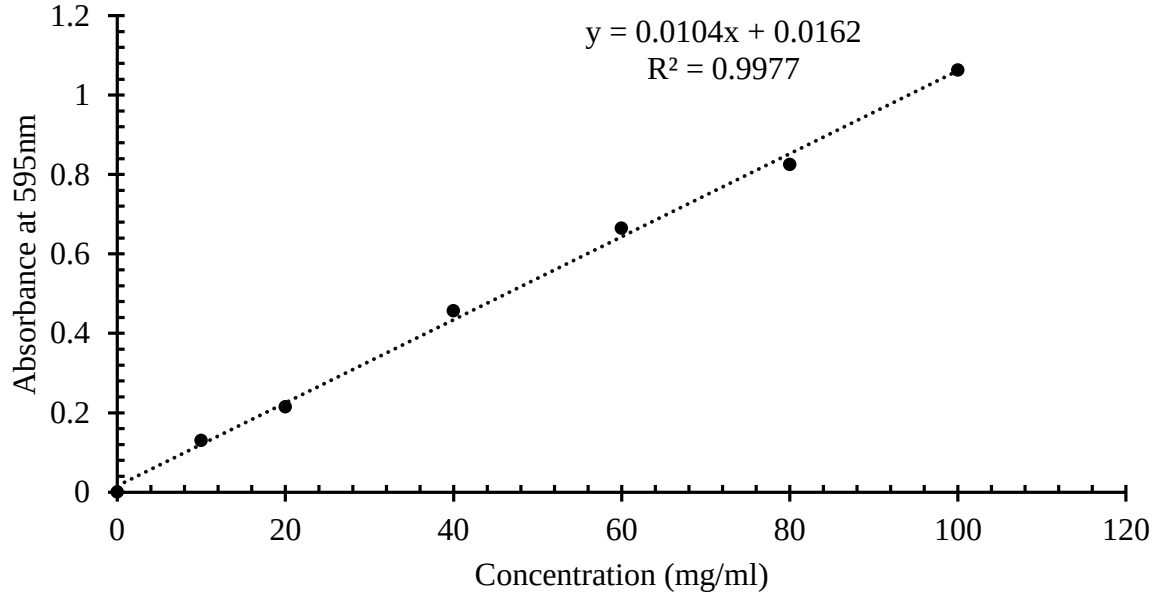

Fig.C.2 Standard curve of BSA for protein determination.

Appendix D

Table D.1 One way ANOVA at 5% level of significance for temperature on casein protein.

Source of variation	D.F	Some of square	Mean square	F	Sig.
Between group	7	0.045	0.006	68.341	0.000
Within group	16	0.001	0.000		
Total	23	0.046			

Treatment	Mean value	Significance	Standard deviation
8	0.10700	a	0.00900
30	0.10666	a	0.00838
40	0.12333	ab	0.01050
50	0.13066	b	0.01270
60	0.07900	c	0.00655
70	0.03900	a	0.01374
80	0.01533	e	0.00577
90	0.78125	e	0.00754

Table D.2 One way ANOVA at 5% level of significance for pH on casein protein.

Source of variation	D.F	Some of square	Mean square	F	Sig.
Between group	6	0.393	0.065	148.649	0.000
Within group	14	0.006	0.000		
Total	20	0.399			

Treatment	Mean value	Significance	Standard deviation
4	0.04240	a	0.00837
5	0.35830	b	0.01369
6	0.29163	c	0.02689
7	0.36007	b	0.04201
8	0.06143	a	0.00658
9	0.24730	d	0.01360
10	0.03993	a	0.01045

Table D.3 One way ANOVA at 5% level of significance for temperature on whey protein.

Source of variation	D.F	Some of square	Mean square	F	Sign.
Between group	6	0.263	0.044	15.086	0.000
Within group	14	0.041	0.003		
Total	20	0.303			

Treatment	Mean value	Significance
30	0.02550	a
40	0.22840	c
50	0.18550	c
60	0.36620	d
70	0.11420	b
80	0.09130	ab
90	0.03500	a

Table D.4 One way ANOVA at 5% level of significance for pH on whey protein.

Source of variation	D.F	Some of square	Mean square	F	Sig.
Between group	6	0.090	0.015	11.359	0.000
Within group	14	0.018	0.001		
Total	20	0.108			

Temperature	Mean value	Significance	Standard deviation
4	0.05772	a	0.01506
5	0.11410	b	0.01309
6	0.22440	c	0.03079
7	0.08520	b	0.07705
8	0.02320	a	0.02756
9	0.06090	a	0.02430
10	0.01760	a	0.02433

Table D.5 One way ANOVA at 5% level of significance for effect of substrate concentration on the degree of hydrolysis of whey protein

Source of variation	D.F	Some of square	Mean square	V.R	F.PR
Substrate conc.(mg/Ml)	7	5176.27	738.75	69.43	<.001
Residual	16	170.23	10.64		
Total	23	5341.51			

Treatment	Mean value	Significance
0.782	5.24	a
1.173	7.10	a
1.565	21.02	b
1.956	11.94	ab
2.347	50.29	c
3.130	9.02	a
4.695	3.58	a
7.826	4.22	a

Table D.6 Two way ANOVA at 5% level of significance for degree of hydrolysis of whey protein

Treatment		Sum of square	D.F	Mean square	F	Sig.
H	Contrast	6483.783	8	810.473	1023.979	.000
	Error	14.247	18	0.719		
N	Contrast	4660.805	8	582.601	1548.549	.000
	Error	6.772	18	0.376		

Homogeneous substrate

Treatment = H

Time	N	Subset					
		1	2	3	4	5	6
0h	3	15.99933					
0.25h	3	18.42000	18.42000				
0.5h	3		18.80067				
1h	3		20.77500	20.77500			
2h	3			22.10333			
3h	3				27.84367		
4h	3					38.06967	
5h	3						56.44200
6h	3						58.18167
Sign.		0.070	0.083	0.665	1.00	1.00	0.343

Treatment = N

Time	N	Subset 1	2	3	4	5
0h	3	17.15633				
0.25h	3		25.61533			
0.5h	3		26.54333			
1h	3		25.99533			
2h	3		26.11200			
3h	3		26.33767			
4h	3			40.40367		
5h	3				54.18433	
6h	3					56.44200
Sign.		0.070	0.083	0.665	1.00	1.00

Table D.7 Two way ANOVA at 5% level of significance for antioxidant activity of whey protein

	Sum of square	D.F	Mean square	F	Sig.
Contrast	598.601	1	598.601	829.823	0.000
Error	25.969	36	0.721		

Homogeneous substrate

Treatment = H

Time	N	Subset	
		1	2
3h	3	4.66533	
2h	3	5.33233	
0.5h	3	5.82800	
1h	3	6.35933	
4h	3	6.90733	6.90733
0.25h	3	6.93767	6.93767
0h	3	7.40933	7.40933
5h	3	7.55133	7.55133
6h	3		9.34533
Sig.		0.58	0.155

Treatment = N

Time	N	Subset 1	Subset 2
0.5h	3	12.024467	
5h	3	12.47400	
0h	3	12.65133	
3h	3	12.77333	
1h	3	12.84233	
0.25h	3	13.30100	
2h	3	13.39200	
4h	3	13.62633	
6h	3		17.18100
Sig.		0.091	1.000

Appendix E

Preparation of reagent

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 $NaH_2PO_4.2H_2O$ and 61.0ml 0.2M $Na_2HPO_4.2H_2O$

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

   - 0.01gm 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 (1% w/v)

   - 1 g casein is dissolved in 100 ml 50 mM potassium phosphate buffer. The solution is heated with gentle stirring to 80-85oC for 10 minutes

7. Preparation of Trichloroacetic acid
   - 5 ml stock TCA was diluted with 10 ml distilled water in the ratio of 1:2

8. Preparation of sodium carbonate reagent

   - Addition of 2 ml sodium potassium tartrate and 2 ml copper sulphate solution in 97 ml sodium carbonate reagent

9. Preparation of DPPH solution (0.125mM)

   - 0.00985 gm DPPH in 80 part methanol and 20 part D/W.
   - 0.429 gm of whey protein in 200 ml pH=6 phosphate buffer.

Appendix F

Colour plate

Plate 1: Ammonium sulphate purification of protease

Plate 2: Extraction of crude protease

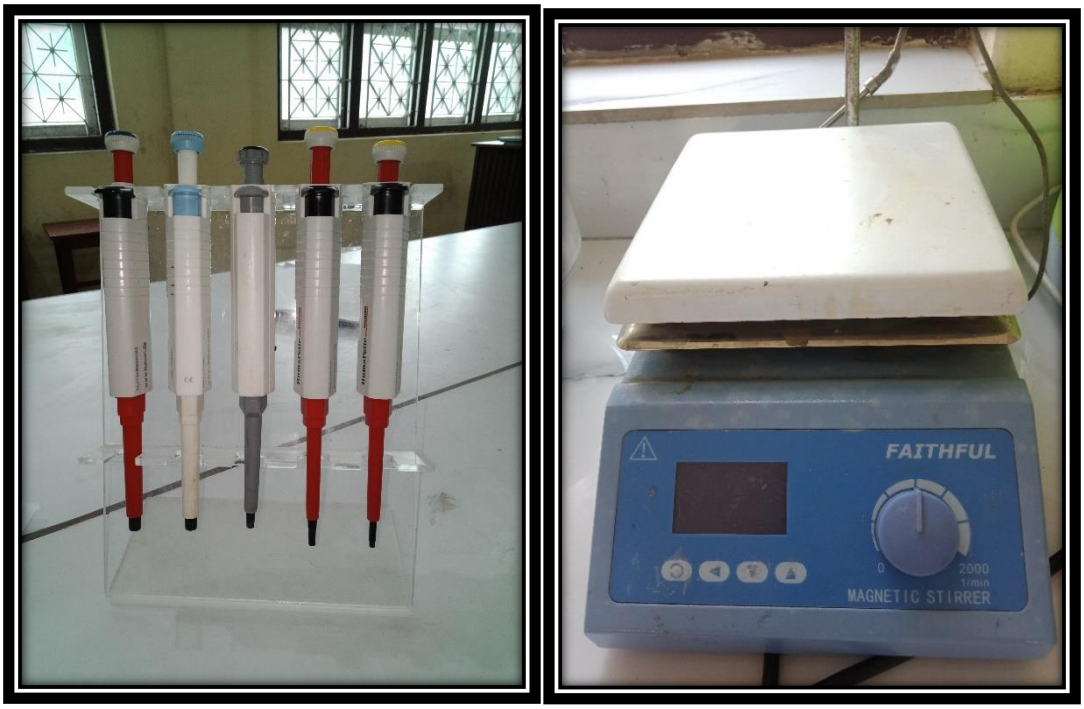

Plate 3: Micropipette and magnetic stirrer used during lab work

Plate 4: Crushing of kiwi fruit for crude enzyme extraction