EVALUATION OF DIPEPTIDYL PEPTIDASE-IV INHIBITORY POTENTIAL OF HYDROLYSATES OF β-LACTOGLOBULIN AND α-LACTALBUMIN ISOLATED FROM *GIR* COW MILK

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF TECHNOLOGY
IN
DAIRY TECHNOLOGY

By

Ashok Kumar

B. Tech. (Dairy Technology)

DAIRY TECHNOLOGY SECTION

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This is to certify that the thesis entitled, **"EVALUATION OF DIPEPTIDYL PEPTIDASE-IV INHIBITORY POTENTIAL OF HYDROLYSATES OF β-LACTOGLOBULIN AND α-LACTALBUMIN ISOLATED FROM *GIR* COW MILK"** submitted by **Mr. Ashok Kumar** towards the partial fulfillment for the award of the degree of **M. Tech.** in **DAIRY TECHNOLOGY** of the **ICAR-NATIONAL DAIRY RESEARCH INSTITUTE**, Karnal (Haryana), India, is a bonafide research work carried out by his under my guidance and no part of the thesis has been submitted for any other degree or diploma.

(Dr. Sathish Kumar M.H.)
MAJOR ADVISOR

DEDICATED TO

MY

BELOVED FAMILY

AND

RESPECTED GUIDE

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Date **Ashok Kumar**

Abstract

Food derived bioactive peptides are known to have peptide sequence corresponding to DPP-IV enzyme inhibitors. The present study was conducted to evaluate DPP-IV inhibitory potential of whey proteins viz., beta-lactoglobulin and alpha-lactalbumin. Whey was separated from Gir cow milk by precipitation of casein, it was then subjected to ultrfiltration (UF) to remove lactose and salts as permeate. Around 27.62% of UF retentate and 69.35% UF permeate were observed from whey. The retentate of UF, largely consisted of whey proteins, was then separated to beta-lactoglobulin (β-lg) and alpha-lactalbumin (α-La) by salting out technique. The resultant protein fractions were further purified by dialysis against water. The total solids (%) of whey, UF retentate, UF permeate were found to be 6.45±0.17, 6.07±0.12 and 4.66±0.07, respectively. The protein content of purified β-lg and α-La were found to be 76.17±2.06% and 72.91±1.91%, respectively. The SDS-PAGE analysis confirmed β-lg and α-La in isolated fractions. These fractions were hydrolysed with pepsin and trypsin at their optimal temperature and pH for 12 h at enzyme to substrate (E:S) ratios of 1:25, 1:50 and 1:100. The degree of hydrolysis (%DH) was measured using OPA method. The degree of hydrolysis was found to increase with duration. Pepsin treated β-lg showed maximum hydrolysis (28.48±1.29%) after 12h at 1:25 E:S ratio. Trypsin treated β-lg showed maximum 22.07±0.95 % DH at 12h and only marginal increase was observed after 6h. Rapid rise in % DH up to 6h was observed in α-LA with both trypsin and pepsin, and afterwards only marginal increase was observed in % DH. The α-La hydrolysis with pepsin and trysin showed maximum 23.07±1.61%, and 22.17±1.31% DH, respectively. Maximum DPP-IV inhibition (87.81±0.84) was observed in α-La hydrolysates treated with pepsin (at 12h and 1:50 E:S ratio) and no significant difference ($p>0.05$) was observed between 1:25, 1:50 and 1:100 E:S ratio at this duration. The IC_{50} value of this hydrolysate was found to be 0.78mg/ml. In β-lg hydrolysates maximum inhibition (77.62±0.98) was observed with pepsin (at 12h and 1:50 E:S ratio) and no significant difference ($p>0.05$) was observed between 1:25 and 1:50 E:S ratio. The IC_{50} value of this hydrolysate was found to be 3.78mg/ml. Significant ($p<0.05$) difference was observed between DPP-IV inhibitory activity of α-La hydrolysates obtained with pepsin and trypsin enzyme. Hence, this study revealed that potential DPP-IV inhibitors can be prepared from α-La hydrolysates treated with pepsin at 12h and 1:50 E:S ratio.

सारांश

खाद्य व्युत्पन्न बायोएक्टिव पेप्टाइड्स को डीपीपी-चतुर्थ एंजाइम अवरोधकों के समान पेप्टाइड अनुक्रम के लिए जाना जाता है। मट्ठा प्रोटीन बीटा-लैक्टोग्लोबुलिन और अल्फा-लैक्टाल्बुमिन का डीपीपी-चतुर्थ निरोधात्मक क्षमता का मूल्यांकन करने के लिए वर्तमान अध्ययन किया गया था। कैसिइन को तलछट करके मट्ठा को गिर गाय के दूध से अलग किया गया था, फिर इसे लैक्टोज और लवण को परमिट के रूप में हटाने के लिए अल्ट्राफिल्ट्रेशन (यूएफ) के अधीन किया गया था। यूएफ के लगभग 27.62% रेटिनेट और 69.35% यूएफ परमिट मट्ठे से देखे गए। यूएफ का रेटिनेट, जिसमें मोटे तौर पर मट्ठा प्रोटीन शामिल होता है, में से तब नमक तकनीक से बीटा-लैक्टोग्लोब्युलिन और अल्फा-लैक्टलबुमिन को अलग किया गया। परिणामी प्रोटीन अंशों को पानी के खिलाफ डायलिसिस द्वारा आगे शुद्ध किया गया था। मट्ठा, यूएफ रेटिनेट, यूएफ परमिट में कुल ठोस (%) क्रमशः 6.45±0.17, 6.07±0.12 और 4.66±0.074 पाया गया। शुद्ध बीटा-लैक्टोग्लोब्युलिन और अल्फा-लैक्टाल्बुमिन में प्रोटीन % क्रमशः 76.17±2.06% और 72.91±1.91%, पाया गया । एसडीएस-पेज विश्लेषण ने अलग-अलग अंशों में बीटा-लैक्टोग्लोब्युलिन और अल्फा-लैक्टाल्बुमिन की पुष्टि की। इन अंशों को उनके इष्टतम तापमान और पीएच पर पेप्सिन और ट्रिप्सिन के साथ विभिन्न (ई: एस) अनुपात 1:25, 1:50 और 1:100 पर अधिकतम 12 घंटे के लिए हाइड्रोलाइज किया गया था। हाइड्रोलिसिस (% डीएच) की डिग्री को ओपीए विधि का उपयोग करके मापा गया था। अवधि के साथ हाइड्रोलिसिस की डिग्री में वृद्धि पाई गई। पेप्सिन ने बीटा-लैक्टोग्लोब्युलिन के साथ 12h के बाद 1:25 ई:एस अनुपात में अधिकतम हाइड्रोलिसिस (% डीएच 28.48±1.29%) दिखाया। ट्रिप्सिन में बीटा-लैक्टोग्लोब्युलिन के साथ 12h के बाद अधिकतम 22.07±0.95 % डीएच 12h बाद दिखाया गया और केवल 6h के बाद सीमांत वृद्धि देखी गई। % डीएच में 6h तक तेजी से वृद्धि α-LA में ट्रिप्सिन और पेप्सिन दोनों के साथ देखी गई थी, और बाद में केवल% डीएच में मामूली वृद्धि देखी गई थी। पेप्सिन और ट्राइसीन के साथ α-La हाइड्रोलिसिस क्रमशः 23.07±1.61%, और 22.17±1.31% डीएच दिखाया गया है। अधिकतम डीपीपी-चतुर्थ अवरोध (87.81±0.84) अल्फा-लैक्टाल्बुमिन हाइड्रोलिसिट्स में पेप्सीन (12h और 1:50 ई:एस अनुपात पर)के साथ दिखाया गया था और इस अवधि में 1:25, 1:50 और 1:100 में कोई महत्वपूर्ण अंतर ($p>0.05$) नहीं दिखाया गया। इस हाइड्रोलाइजेट का IC50 मान 0.78mg/ml पाया गया। बीटा-लैक्टोग्लोब्युलिन हाइड्रोलिसेट्स में अधिकतम अवरोध (77.62±0.98) पेप्सीन (12h और 1:50 E: S अनुपात पर) के साथ देखा गया और कोई महत्वपूर्ण अंतर ($p>0.05$) 1:25 और 1:50 के बीच नहीं देखा गया। इस हाइड्रोलाइजेट का IC_{50} मान 3.78mg / ml पाया गया। α-La हाइड्रोलिसिस की डीपीपी-निरोधात्मक गतिविधि में महत्वपूर्ण ($p <0.05$) अंतर देखा गया था जो पेप्सिन और ट्रिप्सिन एंजाइम के साथ प्राप्त थी। इसलिए, इस अध्ययन से पता चला है कि संभावित डीपीपी-चतुर्थ अवरोधकों को अल्फा-लैक्टाल्बुमिन हाइड्रोलिसिस से 12h और 1:50 E: S अनुपात में पेप्सिन के साथ तैयार किया जा सकता है।

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ABBREVIATIONS

%	:	Percentage
%DH	:	Percent Degree of hydrolysis
<	:	Less Than
>	:	Greater Than
±	:	Plus minus
µM	:	Micro mole
ACE	:	Angiotensin-converting Enzyme
ACOG	:	American College of Obstetricians and Gynecologists
ADA	:	American Diabetes Association
AOAC	:	International or Association of Analytical Communities
APS	:	Ammonium Per Sulphate
BCAAs	:	Branched Chain Amino Acids
BMI	:	Body mass Index
BSA	:	Bovine Serum Albumin
CoPP	:	Corolase PP
Da	:	Dalton
DH	:	Degree of Hydrolysis
DM	:	Diabetes mellitus
DPP-IV	:	Dipeptidyl peptidase IV
E:S	:	Enzyme To Substrate
fig	:	Figure
g/L	:	Gram per Liter
GDM	:	Gestational Diabetes Mellitus
GI	:	Gastrointestinal
GIP	:	Glucose-dependent insulinotropic peptide
GLP-1	:	Glucagon-like peptide 1
HCL	:	Hydrochloric Acid
HPLC	:	High Performance Liquid Chromatography
IC_{50}	:	Half Inhibitory Concentration
IDDM	:	Insulin-Dependent Diabetes Mellitus

IDF	:	International Diabetes Federation
IPI	:	Isoleucine Proline Isoleucine
KDa	:	Kilo Dalton
LC-MS	:	Liquid chromatography–mass spectrometry
MALDI-TOF	:	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
mg/dL	:	Milligram per Deciliter
mg/ml	:	Milligram per Milliliter
mmol/ml	:	Milimole per Milliliter
MS	:	Mass spectrometry
NaCl	:	Sodium Chloride
OPA	:	O-phthaldialdehyde
ORAC	:	Oxygen Radical Absorbance Capacity
PPG	:	Postprandial Glycaemia
RP-HPLC	:	Reverse phase high Performance Liquid Chromatography
RSM	:	Response Surface methodology
SD	:	Standard Deviation
SDS	:	Sodium Dodecyl Sulphate
SDS-PAGE	:	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
SPI	:	Soy Protein Isolate
T1D	:	Type 1 diabetes
T2D	:	Type 2 diabetes
T2DM	:	Type 2 diabetes mellitus
TS	:	Total Solid
UF	:	Ultrafiltration
WHO	:	World Health Organization
WPC	:	Whey Protein Concentrate
WPH	:	Whey Protein Hydrolysate
WPI	:	Whey Protein Isolate
WSE	:	Water Soluble Extracts
WSE	:	Water Soluble Extract

α-La	:	Alpha-Lactalbumin
α_{S1}- CN	:	Alpha s1 Casein
β-CN	:	Beta- Casein
β-Lg	:	Beta-Lactoglobulin
K-CN	:	Kappa Casein

Chapter-1

INTRODUCTION

1. INTRODUCTION

Food protein derived bioactive peptides are gaining attention in recent time owing to their nutraceutical and therapeutic properties. Milk derived Bioactive peptides have shown various health benefits viz., antioxidant, antimicrobial, mineral binding, immune-modulatory, antihypertensive (ACE inhibitory), cardio-protective, opioid and others. In recent time, attempt has been made to identify bioactive peptides which improves insulin secretion or decreases insulin resistance. Since, diabetes mellitus is a metabolic disorder and considered as one of the major global health problems because of its rising incidence and prevalence rates (WHO, 2016). It is mainly specified by a condition of hyperglycemia in which free glucose is available within blood stream. Hyperglycemia mainly results from absolute reduction of insulin secretion, resistance to action of insulin, or both. According to the *International Diabetes Federation* (IDF) it is assessed that globally about 425 million people were living with diabetes in 2017 and also predicted that if the demographic growth continues, almost 642 million peoples will be affected with this metabolic illness by 2040 (IDF, 2018). In type 2 diabetic patient, glucose level after meal could be better indicator of glycemic control then blood glucose level during fasting (Abrahamson, 2004; Avignon *et al*., 1997). It is important to have both fasting and postprandial glucose at normal level, but in case of type 2 diabetes postprandial glucose level control is very essential (Ben-Avraham *et al*., 2009). Various strategies are being used to manage type 2 diabetes such as change in diet, lifestyle, physical exercise, therapy using antidiabetic medication etc. (Bantle *et al*., 2008; Hawley and Gibala, 2012). Among the various stratergies, inhibition of DPP-IV and carbohydrate digestive enzymes such as alpha-amylases and alpha- glucosidases is also a promising treatment for type 2 diabetes (Holman *et al*., 1999; Bennett *et al*., 2001; Tahrani *et al*., 2011). DPP-IV enzyme is a serine protease mainly cleaves dipeptide, Xaa-proline or Xaa-alanine from the N-terminus side of a long polypeptide chain (Xaa denotes amino acid). This enzyme inactivates gut hormones, such as GLP-1 and GIP. These hormones are released in body in response to the food consumption and helps in secretion of insulin to maintain normal blood glucose level in body. However, after 5-7 minutes, naturally these hormones get inactivated due to inactivation by DPP-IV enzyme in our body. In case of people suffering from diabetes, if any delay in the inactivation of GLP-1 and GIP would help to release more insulin and hence assist to reduce the glucose level. Based on this mechanism, several synthetic drugs

such as sitagliptin, saxagliptin, and linagliptin are being prescribed by medical practitioner to inhibit DPP-IV enzyme. However, these synthetic drugs cause some proven side effects such as nausea, heart attack, stomach pain, diarrhea, etc. In order to reduce the side effect incurred by synthetic drugs as well as to treat type 2 diabetes using natural sources, many researcher came up with certain speculations that food derived dietary proteins, and bioactive peptides obtained from food derived proteins are able to reduce to glucose level specially in case of postprandial hyperglycemia (Oseguera, 2015). It has been reported that milk bioactive peptides, positively regulates postprandial glycaemia as well as promotes insulin secretion in both normal and type 2 diabetic patients (Nongonierma and FitzGerald, 2013). It is evidenced in some recent studies that milk derived peptides have both DPP-IV inhibitory and alpha-glucosidase inhibitory properties (Lacroix and Li-Chan, 2013; Konrad *et al*., 2014). In many recent studies, it has been reviewed that several food proteins contains identical peptides sequence as DPP-IV inhibitory peptides. Whey proteins and their hydrolysates also showed their ability to inhibit DPP-IV enzyme. Jakubowicz and Froy (2013) have stated that orally admitted whey protein and its hydrolysates in humans are positively affect blood glucose level and insulinotropic responses. The DPP-IV inhibitory activity of milk proteins from exotic cattle belonging to *Bos taurus* has been proven in recent research work.

In the current study, an attempt will be made to evaluate DPP-IV inhibitory potential of whey proteins hydrolysates prepared from indigenous cattle, *Gir* (belonging to *Bos indicus*). *Gir* cow is selected as it is widely prevailed across India and also it is one of the higher milk yielding animal amongst Indigenous cattle. Hence, this study is planned with the following objectives.

1) Preparation of β-lactoglobulin and α-lactalbumin hydrolysates from *Gir* cow milk using select enzymes

2) Evaluation of β-lactoglobulin and α-lactalbumin hydrolysates for dipeptidyl peptidase-IV inhibition (antidiabetic potential)

Chapter-2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Overview

Recent advances in food and nutrition sciences have been exploring foods for nutraceutical and therapeutic benefits. Food derived bioactive peptides is widely proven area which confer health benefits to consumer. In near future our food will be the best medicine to keep our body healthy. So mining of bioactive peptides from foods especially from milk is attracting researchers in recent time.

2.2 Diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder specified by a condition of hyperglycemia, in which free blood sugar level high in blood stream over prolonged period. Diabetes mellitus mostly instigated due to absolute of both conditions i.e. absence of insulin secretion and action of insulin resistance. According to the International Diabetes Federation (IDF), it is estimated that about 425 million people were living with diabetes globally in 2017 and that the number will be 642 million by 2040 (IDF, 2018). The World Health Organization (WHO) estimates that 90 percent of people living with diabetes have type 2 disease (WHO, 2018).

2.2.1 Types of diabetes

The three main types of Diabetes

- Type-1 Diabetes (T1D),
- Type-2 Diabetes (T2D) and
- Gestational Diabetes Mellitus

a) Type-1 diabetes (T1D)

T1D is also known as Insulin-Dependent Diabetes Mellitus (IDDM) or “juvenile diabetes”. It results from the autoimmune destruction of the β-cells presents in the body by T-cells which then further leads to the permanent termination of insulin production (Elham *et al*., 2009). In the condition of type-1 diabetes pancreas produce very minute or no insulin due to loss of β-cells (Chou, 2004). T1D represents approximately 10-15% of all types of diabetes (Smith *et al*., 2008).

b) Type-2 diabetes (T2D)

Type 2 Diabetes is a chronic illness mainly characterized by an insufficiency in either secretion of insulin or its action, both of these two symptoms were seen in the diabetic individual. In the early stage of this disease, normal or even high level of insulin produces, but the body becomes insensitive to this essential hormone, and this condition is referred as insulin resistance. In the advanced stages, the predominant abnormality is the reduced secretion of insulin by the pancreatic β-cells (DeFronzo, 1997; Reaven, 1988; Virally *et al.,* 2007).

This is the most prevalent form of diabetes and approximately 90% of all diabetes cases can be attributed to T2D worldwide. Resistance of insulin is correlated with obesity and can be recognized to release of free fatty acid and inflammatory cytokines from the expanded adipose tissue mass are the casualties of T2D. Cabot and Jasinska (2007) reported that 80 to 90 % of T2D affected patients are obese or overweight. T2D is largely seen in adults but there is significant increase of its prevalence in children also registered (Rosenbloom *et al*., 2009). T2DM can be a leading cause of many disorders such as kidney failure, cardiovascular disease, blindness, and can lead to lower limb amputation, if left untreated.

c) Gestational diabetes

It is defined as glucose intolerance diagnosed during the pregnancy period and is usually cured after delivery within a few months, but mothers with gestational diabetes mellitus (GDM) during pregnancies remains at high risk to be affected by T2D (ADA, 2004). GDM occurs in about 3-10% of pregnancies and account for 90% of cases of diabetes mellitus in pregnancy (Berkowitz *et al*., 1992). Kuhl (1998) has reported that to maintain blood glucose level within normal limits, a healthy pregnant woman's insulin secretion should elevate by approximately by 2–4 times. The ability to increase the insulin secretion is highly impaired in GDM affected pregnant women and due to that their increased insulin resistance cannot be compensated. Exercise for 30 minutes in a day has been suggested by (ACOG Committee, 2002) and (ADA, 2003) as an ancillary therapy for GDM. Chu *et al*. (2007) based on their meta-analysis study conducted, it is observed that overweight, obese, and severely obese pregnant women have around 2.14, 3.56 and 8.56-fold higher chances of affecting with GDM. The diagnosis of GDM is directly correlated with increase in body fatness. In case of pregnant women the increase in body

fatness is indexed as pre-pregnancy Body Mass Index (BMI). It has been found that each unit increase in BMI, raises the occurrence of GDM by 0.92% (Torloni, *et al*., 2009).

2.2.2 Postprandial hyperglycemia

Postprandial hyperglycemia which is early governing stage of T2D specified by increase in glucose level just after consumption of food (Postprandial Glucose level i.e. PPG). The absorption of carbohydrates in the upper gastrointestinal (GI) tract results an increase in plasma glucose concentrations, which usually starts about 10–20 min after having meal, and variety of factors are responsible for it such as, rate of absorption of carbohydrate, secretion of insulin, glucagon pathway, metabolism of glucose and the schedule, quantity, composition of food, and also alterations in microbiota of the upper GI tract (Tibaldi, 2009). Commonly, postprandial rise in glucose level is closely regulated by rapid augment in insulin secretion so that it rarely exceeds 140 mg/dL (7.8mmol/ml). Glucose excursion is therefore maintained within normal range through an action of insulin on its target organs.

2.2.3 Glucose-dependent insulinotropic peptide (GIP)

GIP is produced by K cells present in the mucosa of proximal small intestine (duodenum and jejunum) after food intake (Costanzo, 2014). It is also called as gastric inhibitory peptide and induces insulin secretion, primarily by hyper osmolarity of glucose in the duodenum (Thorens, 1995). The half-life of GIP is less than 7 min as it acted upon by DPP-IV which cleaves the active form of GIP-(1-42) subsequently leading to inactive form of GIP-(3-42) by cleaving the dipeptide (Tyr-Ala) from 1^{st} and 2^{nd} positions (Vilsboll *et al*., 2006). So, active form of GIP should be preserved in order to enhance insulin secretion. Another possibility is that bioactive peptides released from whey protein can cause higher GIP half-life.

2.2.4 Glucagon-like peptide 1 (GLP1)

GLP-1 is a potent anti-hyperglycemic incretin hormone released from intestinal L cell as a gut hormone (Skibicka, 2013). It induces the β-cells of the pancreas to release the hormone insulin in response to rising glucose in blood while suppressing glucagon secretion but, the half-life of GLP-1 is less than 2 minutes because of rapid degradation of this hormone by the DPP-IV enzyme (Shubrook, 2014). The biological active forms of GLP-1 are: GLP-1-(7-37) and GLP-1-(9-36) NH_2 but when DPP-IV acts on it, it yields the inactive form i.e. GLP-1-(9-36) by selectively cleaving dipeptide (His-Arg) from 7^{th}

position (Patil *et al*., 2015). The complete mechanism is depicted in Fig. 2.1. The synthesis of pro-insulin and insulin stores in β-cells are improved by enhanced GLP-1 levels (Nauck *et al*., 2009; Portha *et al*., 2011).

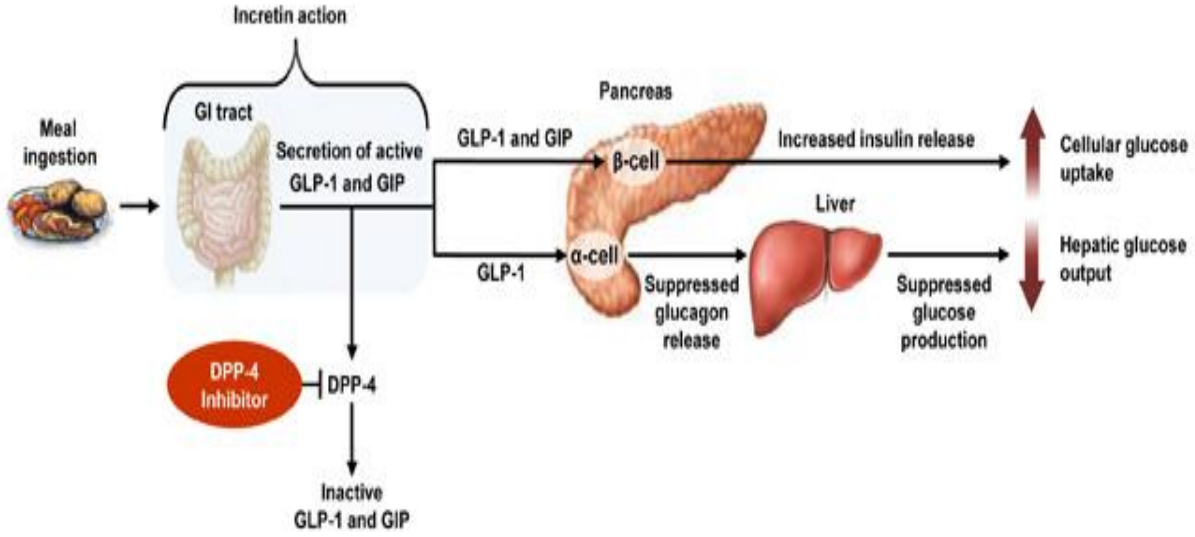

Fig.2.1. Functions of the incretin hormones and mechanism of action of Dipeptidyl peptidase-IV inhibitors (*adopted from Diabetes in Control, 2011*)

2.2.5 Dipeptidyl peptidase IV

DPP-IV is an enzyme, consists of 766 amino acids, has molecular weight of 110kDa and it is a serine protease. It has dipeptidases activity at the N-terminal end. The dipeptides which contain proline or alanine residues at penultimate position are selectively cleaved by DPP-IV (Fan *et al*., 2003). It is a multifunctional transmembrane glycoprotein and also known as adenosine deaminase complexing protein 2 (ADCP2) or cluster of differentiation 26 (CD26). DPP-IV resides in endothelial plasma membrane of all human organs and tissues in the body (Shubrook *et al*., 2011). It plays diverse role in physiological processes such as enzymatic incretin degradation, cell adhesion, endocrine and immune activity and resists cancer growth (Krushner and Gorrell, 2010; Wang *et al*., 2005). DPP-IV is renowned for its inhibiting the activity of two major gastrointestinal hormones, including GIP and GLP-1, which induces glycemic homeostasis after glucose uptake (Baggio and Drucker, 2007).

2.2.6 Diabetes management

The vital action is required to control blood sugar level, as it emerged as one of the essential strategy, to control the complications of T2D with the importance of postprandial glucose level. Influx of glucose in main blood stream from gastrointestinal track can be reduced by inhibiting the two key enzymes involved in carbohydrate digestion and absorption i.e. alpha-amylase and alpha-glucosidase. Higher glucose level in blood stream can also be reduced by increasing the half-life of incretin hormones which are released following ingestion of meal and stimulates the glucose-dependent insulin secretion in pancreatic beta-cells by inhibiting DPP-IV enzyme (Deacon *et al*., 2004; Mentlein, 2005). The inhibition of DPP-IV is one of the proven strategies for reducing postprandial hyperglycemia and thereby managing complications arising due to T2D. In recent years many novel strategies has been developed for diabetes treatment, which are mainly focus on therapies using incretin such as GLP-1 analogues and DPP-IV inhibitors.

2.2.7 Present medications approach and its consequence

The present medications available in market include insulin injections and inhalers, sensitizers, secretagogues, injectable incretin mimetics, and synthetic DPP-IV inhibitors (such as sitagliptin, saxagliptin, vildagliptin, aloliptin). Among all the medications DPP-IV inhibitors act effectively on reducing the postprandial hyperglycemia. But synthetic DPP-IV inhibitors available in the market also impart certain side effects such as sore throat, headache, pancreatic and upper respiratory tract inflammation.

2.3 Milk proteins and T2D

Milk in terms of nutrition is recognized as almost complete food having all the major macro and micro nutrients and it is also devoid of toxins and anti- nutritional factors (McSweeney and Fox, 2003). The 80% of milk protein is casein and rest 20% is whey. The major constituents of casein are α-s_1, α-s_2, β, κ and γ casein, while β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and glycomacropeptide are the major constituents of whey proteins. Bioactive peptides derived from milk have been proven to have numerous functionalities or health benefits. Milk derived proteins are one of the richest source of bioactive peptides (Korhonen and Pihlanto, 2007). Dairy protein as a dietary component has been received recent interest that may aid in prevention of type 2 diabetes (Jensen and Newburg, 1995). The study was conducted on obese, pre-

diabetic, diabetic, and healthy individuals and it has been observed that consumption of casein and whey proteins has increased the insulin secretion. These proteins also help in controlling the blood glucose level in T2D patients (Frid *et al*., 2005; Manders *et al*., 2005). The release of milk protein-derived peptides can be occurred by following four ways (i) Gastrointestinal enzymatic hydrolysis (ii) Milk fermentation using proteolytic starter cultures (iii) Microorganisms or plant derived enzymatic hydrolysis and (iv) Combining both hydrolysis and fermentation (Phelan *et al*., 2009). These peptides derived from milk are highly sought out components for the manufacturing of novel and functional foods, dietary supplements and pharmaceuticals. The most regularly used enzymes are pepsin, trypsin, and chymotrypsin, of both animal and microbial origin. Bioactive peptides are also generated in cheese and fermented milk due to the synthesis of metabolites with proteolytic capability (Fitzgerald and Murray, 2006).

2.3.1 Casein

Casein derived bioactive peptides are reported to have many physiological functions (Meisel and FitzGerald, 2003). Koopman *et al*. (2009) reported that on the basis of study conducted on ten healthy males, hydrolysate obtained from casein significantly accelerated the digestion of protein, gut absorption and also, increased secretion of postprandial insulin.

2.3.2 Whey proteins

The whey proteins mainly consists of globular proteins such as β-Lg and α-La followed by minor components such as BSA, lactoferrin, Immunoglobulins and proteosepeptone. The whey protein has the ability to reduce postprandial glycaemia through different complementary processes which includes; an increase in secretion of insulin and incretin hormone, by inducing gastroparesis and thereby reducing energy consumption and appetite (Fig. 2.2). Jakubowicz and Froy (2013) have concluded that orally admitted whey protein and its hydrolysates in humans are positively affect blood glucose level and insulinotropic responses. Gunnerud *et al*. (2013) demonstrated that whey protein has positive effect on insulinaemia, glycaemia and plasma amino acids in twelve health individuals in a dose-dependent manner. Whey proteins have been identified as a potential source of DPP-IV inhibitors.

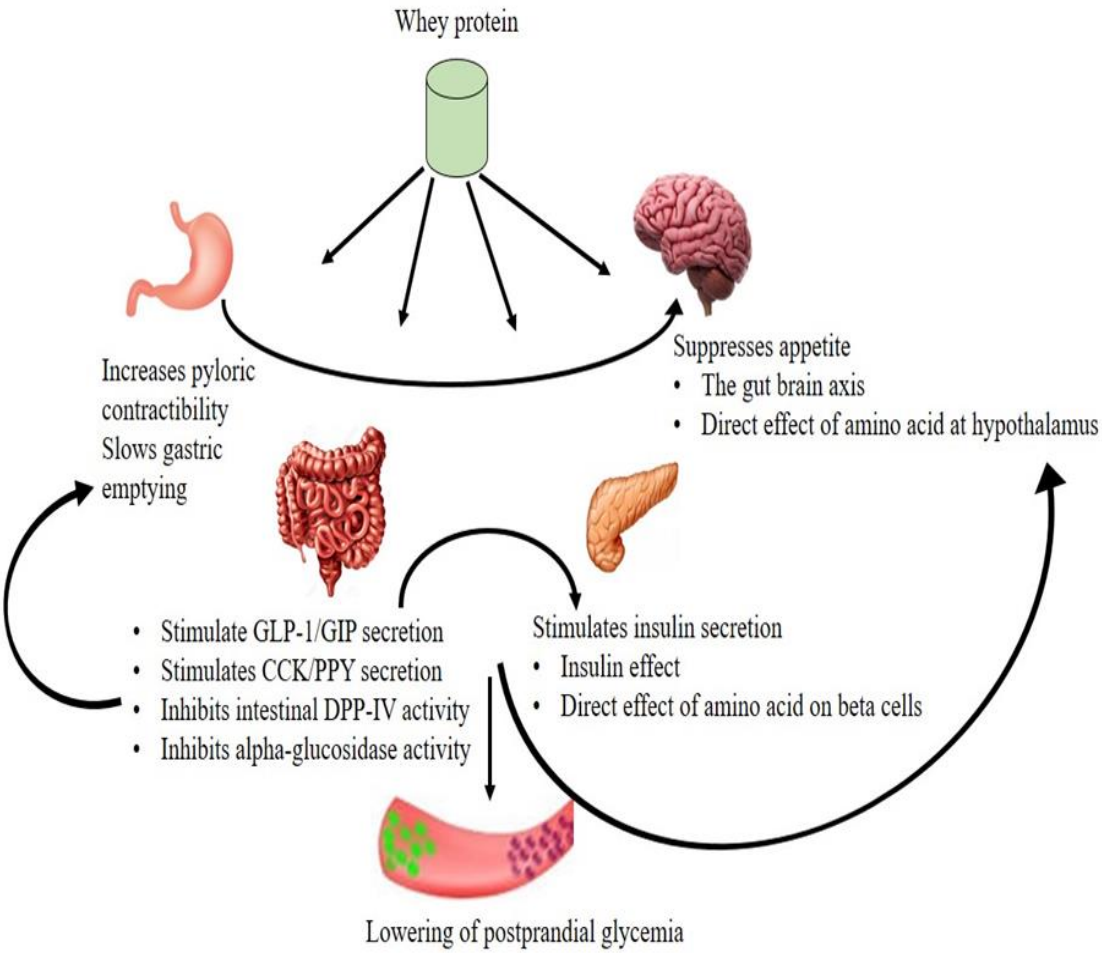

Fig.2.2. Mechanism by which whey protein can reduce postprandial glycaemia and increase insulin, GLP-1 and GIP secretion

The bioactive peptide *Ile-Pro-Ala* (IPA) obtained as a product of β-Lg hydrolysis acts as a mild DPP-IV inhibitor and this also has a positive influence in glycemic control (Tulipano *et al*., 2011). It has been demonstrated in many studies that whey proteins are more easily digested than casein. Morifuji *et al*. (2009) concluded that insulin plasma concentration was increased after digestion of dipeptides of branched chain amino acids (BCAAs) *viz., Val-Leu, Ile-Leu,* and *Leu-Leu* obtained from whey proteins of β-Lg and α-La. Morifuji *et al*. (2010) reported that whey proteins are highly efficient in T2D management than caseins because of its properties such as quick digestion, more solubility in the intestinal acidic condition and higher BCAAs content compared to casein. (Lacroix and Li-Chan, 2014) demonstrated that whey proteins contains promising fragments in their primary structure similar to synthetic DPP-IV inhibitory peptides.

2.4 Bioactive peptides

Bioactive peptides are specific protein fragments which ultimately influence health by creating a positive impact on body functions or conditions (Kitts and Weiler, 2003). The bioactive peptides usually contain 3-20 amino acids per molecule and are generally inactive in the parent or native protein. They get activated after enzymatic hydrolysis taking place during processing of food and/or during gastrointestinal digestion. Anti-obesity and satiety bioactive peptides have positive impact on the gastrointestinal system while antihypertensive, antithrombotic, antioxidant and hypo-cholesterolemic bioactive peptides exert positive effect on the cardiovascular system. Whereas, antimicrobial, cyto-modulatory and immune-modulatory bioactive peptides have positive influence on the immune system and opioid peptides exerts positive impact on nervous system (Fig. 2.3).

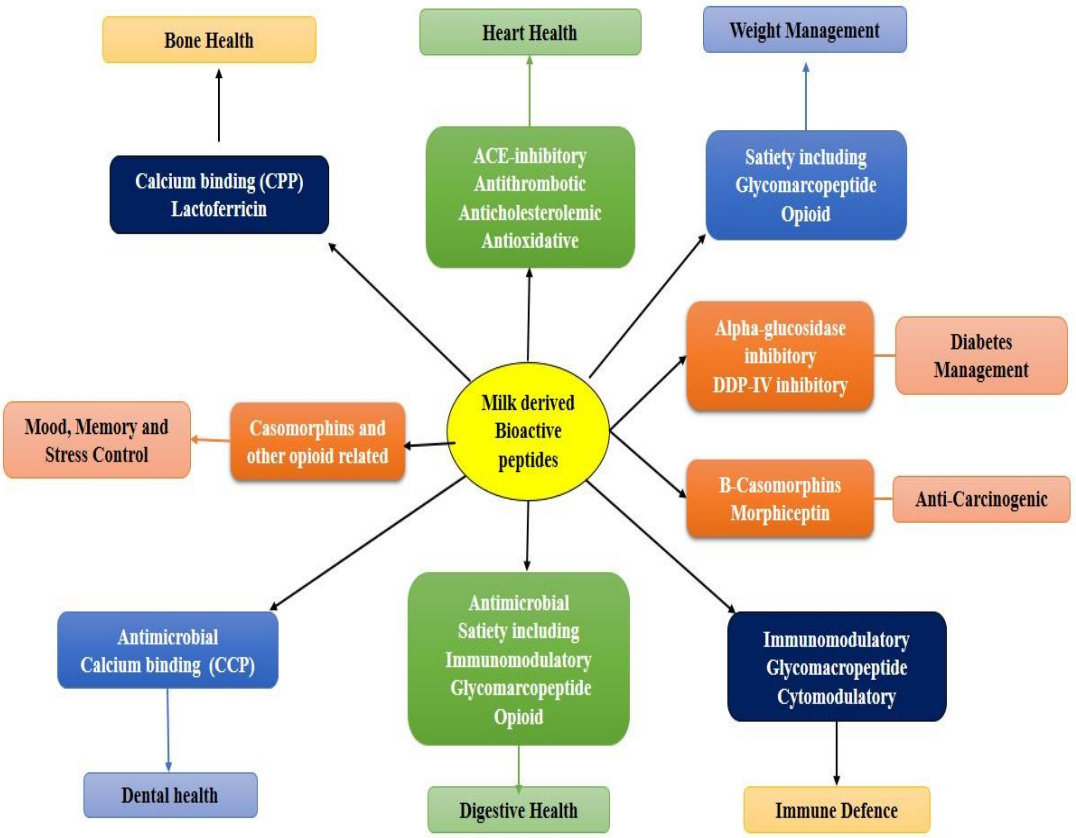

Fig.2.3. Functions of the bioactive peptides

2.5 Food derived bioactive peptides as a source of DPP-IV inhibitors

Inhibition of DPP-IV enzyme preserve the action of incretin hormones, increasing their half-life and circulating concentration so, ultimately increases the production of insulin and its action. In the past few years various studies have been reported several food derived bioactive peptides which showed DPP-IV inhibition to great extent.

Many studies concluded that peptides with proline or alanine at their penultimate position (i.e *Xaa-Pro* or *Xaa-Ala,* where *Xaa* is an amino acid) from their N terminus are most potent DPP-IV inhibitors irrespective of their mode of action as a substrate or prodrug type of inhibitory action (Rahfeld *et al*., 1991). According to Umezawa *et al*. (1984), *Ile-Pro-Ile* (Diprotin A) is the most potent DPP-IV inhibitory peptide that is originally identified from filtrates of *Bacillus cereus* (BMF673-RF) culture, followed by *Val-Pro-Ala* (Diprotin B).

2.5.1 DPP-IV inhibitory peptides from sea proteins

Neves *et al*. (2017) studied the DPP-IV inhibitory, ACE inhibitory, and ORAC activities of peptides released from Atlantic salmon proteins (extracted by using pH shift method). Hydrolysates obtained from Atlantic salmon proteins with corolase PP had maximum DPP-IV inhibitory, ACE, and ORAC activities. They found that peptide *Tyr-Pro* had maximum ACE inhibitory activity (IC_{50} value 5.21 ± 0.94 µM), Tyr had maximum DPP-IV inhibitory potential (IC_{50} value 75.15 ± 0.84 µM) and *Val-Pro* had highest ORAC activity (19.45 ± 2.15 µmol of TE/g). Li-Chan *et al*. (2012) studied the DPP-IV inhibitory potential of Atlantic salmon skin gelatin. Three different enzymes namely, alcalase, bromelain and flavourzyme were used to hydrolysed the Atlantic salmon skin gelatin. Hydrolysate obtained with flavourzyme at 6% enzyme to substrate ratio has been revealed maximum DPP-IV inhibitory activity. Upon fractionation of hydrolysates by using UF, they found that less than 1kDa fraction had showed maximum DPP-IV inhibition with IC_{50} value of 1.35 mg/ml. Further, fraction F-1 was isolated by using HPLC and it displayed higher DPP-IV inhibition with IC_{50} value 57.3 µg/ml. Two DPP-IV inhibitory peptides were identified using mass spectrometry analysis, namely *Gly-Pro-Gly-Ala* (300.4 Da) and *Gly-Pro-Ala-Glu* (372.4 Da) with IC_{50} values of 41.9 and 49.6 µM respectively.

Huang *et al*. (2012) studied the DPP-IV inhibitory activity of Tuna cooking juice proteins. Juice samples were hydrolysed by protease XXIII (PR) and Orientase (OR).

Highest DPP-IV inhibition was displayed by the peptides with molecular weight more than 1422 Da. Three novel DPP-IV inhibitory peptides were identified through MALDI-TOF MS/MS viz., *Pro-Gly-Val-Gly-Gly-Pro-Leu-Gly-Pro-Ile-Gly-Pro-Cys-Tyr-Glu* (1412.7 Da), *Cys-Ala-Tyr-Gln-Trp-Gln-Arg-Pro-Val-Asp-Arg-Ile-Arg* (1690.8 Da) and *Pro-Ala-Cys-Gly-Gly-Phe-Try-Ile-Ser-Gly-Arg-Pro-Gly* (1304.6 Da) with IC_{50} values of 116.1, 78.0, 96.4 µM respectively. This study concluded that Tuna cooking juice can be used as an efficient source of DPP-IV inhibitory peptides. Cheung and Li-Chan (2017) assessed DPP-IV inhibitory and ACE inhibitory activity of hydrolysates obtained from steelhead skin gelatin with enzymes pepsin, CorolaseN and papain. Highest DPP-IV and ACE inhibitory activities had been shown by a less than 3kDa fraction, obtained through ultrafiltration of hydrolysates. Harnedy *et al*. (2015) isolated DPP-IV inhibitory peptides from purified macroalga palmaria palmata. In this study, firstly, the palmaria palmata proteins were extracted and then hydrolyzed by using corolase PP enzyme. The hydrolysates were fractionated using semi-preparative RP-HPLC. Fraction SP RP-HPLC 25_F28 had been emerged as most efficient DPP-IV inhibitory fraction. Thirteen peptides were recognized from this fraction using mass spectrometry. Three peptides namely *Ile-Ala-Pro, Leu-Leu-Ala-Pro* and *Met-Gly-Val-Asp-His-Ile* (IC_{50} values ranging from of 43-159 µM) were emerged as novel DPP-IV inhibitory peptides among all thirteen peptides.

2.5.2 DPP-IV inhibitory peptides from meat proteins

Blue mussel meat protein extract and its hydrolysates were evaluated for its DPP-IV inhibition, ACE inhibition and antioxidant activity by Neves *et al*. (2016). In this study they used four different enzymes, alcalase, corolase PP, flavourzyme and promod 144MG (from carica papaya) for hydrolysis. Hydrolysates obtained with a combination of alcalase + flavourzyme and with corolase PP alone have been showed maximum DPP-IV inhibitory, ACE inhibitory, and antioxident activity. IC_{50} values for DPP-IV and ACE were distributed in range of 0.33-2.43 and 1.03-3.34 mg/ml respectively, and ORAC values ranged from 66.26-121.56 µmol trolox equivalents per gram. Jin *et al*. (2015) used an unified approach of LC-MS and *in silico* analysis to characterize DDP-IV inhibitory peptides derived from deer skin hydrolysates. Deer skin protein was hydrolyzed by using pepsin, pepsin + trypsin and pepsin + alcalase to obtain a total number of 203, 244 and 60 peptides respectively, which were identified by using LC-MS. Among all 5.9, 20.9 and 20.0% of peptides respectively possessed proline residue at penultimate position. *Gly-Pro-Gly-Ser-ProGly-Gly-Pro-Leu*, *Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys*, *Gly-*

Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys, *Gly-Pro-Val-GlyPro-Ser-Gly-Pro-Hyp-Gly-Lys* and *Gly-Pro-Ala-Gly-Pro-Hyp-GlyVal-Hyp-Gly-Leu*, these five peptides were selected to evaluate the DPP-IV inhibitory activity with a proline residue at the penultimate position and found IC_{50} values of 1638.3 ± 233, 83.3 ± 3.2, 226.9 ± 8.9, 93.7 ± 0.9 and 318.1 ± 2.5 µM respectively.

2.5.3 DPP-IV inhibitory peptides from vegetable proteins

Wang *et al*. (2015) studied the peptides released from oat, highland barley and buckwheat protein and evaluated there *in vitro* inhibitory effect on DPP-IV enzyme. Maximum DPP-IV inhibitory activity has been shown by oat gelatin (hydrolysed with alcalase) with IC_{50} value of 0.13 mg/ml, whereas minimum activity showed by highland barley albumin (hydrolysed with trypsin) with IC_{50} value of 8.15 mg/ml. The tryptic hydrolysates derived from oat globulin were evaluated using LC-MS. Two peptides namely *LQAFEPLR* and *EFLLAGNNK* were synthesized and checked for their DPP-IV inhibitory potential, *LQAFEPLR* has been displayed maximum DPP-IV inhibition with IC_{50} value of 103.5µM. Potential of quinoa protein isolate was studied for their DPP-IV inhibitory and antioxidant potential by Nongonierma *et al*. (2015). Quinoa protein isolate was digested by papain and papain like microbial enzyme. Hydrolysates of quinoa protein isolate produced using papain and papain like microbial enzyme have been showed almost similar DPP-IV inhibitory and ORAC activities. Hydrolysis was also observed in control sample of quinoa protein isolate due to endogenous proteinases, this hydrolysate also displayed higher DPP-IV inhibitory activity but lower ORAC activity than hydrolysates produced from papain and papain like microbial enzyme. Hydrolysate obtained using papain had a DPP-IV IC_{50} value of 0.88 ± 0.05 mg/ml and ORAC activity of 501.60 ± 77.34 mmol Trolox equivalent (T.E.)/g. DPP-IV IC_{50} value and ORAC activity value for hydrolysate obtained using papain like microbial enzyme were similar as hydrolysate obtained using papain.

Hatanaka *et al*. (2012) used defatted rice bran for the production of DPP-IV inhibitory peptides. In this study defatted rice bran protein was hydrolysed separately with Umamizyme G and Bioprase SP. The results were concluded that hydrolysates produced with Umamizyme G displayed 10 times more DPP-IV inhibitory potential than hydrolysates produced with Bioprase SP. Two peptides such as *Leu-Pro* and *Ile-Pro* obtained with Umamizyme G were found as most potent DPP-IV inhibitors. Among all 15 dipeptides tested, Ile-Pro was found as strongest inhibitor of DPP-IV. Kinetic study

revealed that *Ile-Pro* also competitively inhibit DPP-IV enzyme. The half-maximum inhibitory concentration (IC_{50} value) found for rice bran hydrolysates was 2.3 ± 0.1 mg/ml. Based on study conducted by Velarde-Salcedo *et al*. (2013) on the DPP-IV inhibitory activity of peptides released from amaranth seed protein after enzymatic digestion it was reported that highest and dose dependent DPP-IV inhibitory activity was observed in amaranth peptides after *in vitro* gastrointestinal digestion with an IC_{50} of 1.1 mg/ml.

2.5.4 DPP-IV inhibitory peptides from milk proteins

In recent past, several researchers identified milk protein derived DPP-IV inhibitory peptides. The source of these peptides, sequences and IC_{50} values are detailed in table 2.1. Uchida *et al*. (2011) Isolated novel DPP-IV peptides obtained from trypsin treated β-lactoglobulin hydrolysate. These peptides showed a concentration dependent DPP-IV inhibition with an IC_{50} value of 210 µM. An active hexapeptide *Val-Ala-Gly-Thr-Trp-Tyr* (β-La, f15-20) emerged as potent DPP-IV inhibitor (IC_{50} value 174 µM), which also exhibit concentration dependent DPP-IV inhibitory effect. Tulipano *et al*. (2012) studied the difference between DPP-IV inhibitory potential of β-lactoglobulin hydrolysates obtained from goat, sheep and bovine whey by using *in silico* and *in vitro* approaches. Combined results indicated that hydrolysates obtained from sheep and goat whey had more short chain amino acid sequence than bovine whey, and both sheep and goat whey had weak DPP-IV inhibitors than bovine whey. Uenishi *et al*. (2012) extracted and identified DPP-IV inhibitory peptides from Gouda type cheese and observed the effect of these peptides on plasma glucose level in rats. Water soluble extracts (WSE) of Gouda type cheese were prepared and studied for their DPP-IV inhibitory potential. Twelve months ripened WSE had maximum DPP-IV inhibitory activity as compared to six months ripened and un-ripened WSE, but the peptides concentration was found more in six months ripened WSE than twelve months. Further twelve months ripened WSE fractioned through RP-HPLC and obtained four fractions were analysed for their constituent peptides using mass spectrometry. Among all peptides obtained, four peptides were identified with X-Pro structure has been displayed IC_{50} value below 200µM. Highest DPP-IV inhibitory activity was showed by peptide residues obtained from β-Casein (f(70-77); LPQNIPPL). Further, synthesised peptide LPQNIPPL was orally administered to rats at the rate of 30mg per 100gm weight of rat and tested for their glucose tolerance by

using cross-over experimental design. It showed significant reduction in postprandial area blood glucose curve as compared to placebo-treated group.

Table 2.1. Milk derived peptides known for DPP-IV inhibition

Source	Peptide Sequence	IC_{50} value (μM)	References
β-Lg	LKPTPEGDL	45	Lacroix and Li-Chan, 2014
	LKPTPEGDLE	42	
	LKPTPEGDLEIL	57	
	TPEVDDEALEK	320	Silveira *et al.*, 2013
	VLVLDTDYK	424	
α-La	*WLAHKALCSEKLDQ*	141	Lacroix and Li-Chan, 2013
	LAHKALCSEKL	165	Lacroix and Li-Chan, 2014
Goat κ-CN	INNQFLPYPY	40	Zhang *et al.*, 2015
β-CN	YPVEPF	125	Nongonierma and FitzGerald, 2016
β-CN	VLGP	580	Nongonierma and FitzGerald, 2013c
Lactoferrin	WK	41	Nongonierma and FitzGerald, 2013c
	WQ	120	
Milk protein	*Val-Pro*	380.3	Nongonierma and FitzGerald, 2016
	Arg-Pro	657.2	
	Phe-Pro	682.5	
	His-Pro	902.8	
β- CN	*Leu-Pro-Val-Pro-Gln*	43.8	
	Val-Pro-Gly-Glu-Ile-Val-Glu	224.5	
	Val-Pro-Gly-Glu-Ile-Val-Glu	224.5	
	Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr	205.2	
	Ile-Pro-Pro-Leu-Thr-Gln-Thr	465.1	
	Thr-Pro-Val-Val-Val-Pro-Pro	1408.9	
	Tyr-Pro-Val-Glu-Pro-Phe	124.7	
	Leu-Pro-Leu-Pro-Leu-Leu	371.5	
	Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr	1754.8	
	Gln-Pro-Leu-Pro-Pro-Thr	1013.8	
	Ile-Pro-Pro-Leu	428.9	
	Leu-Pro-Pro	563.3	
β-Lactoglobulin	*Asn-Leu-Gly-Ile-Ile-Leu-Arg*	86.3	Song *et al.*, 2017
	Thr-Gln-Met-Val-Asp-Glu-Glu-Ile-Met-Glu-Lys-Phe-Arg	68.8	

Nongonierma and FitzGerald (2013c) investigated the DPP-IV inhibitory, DPPH radical scavenging and superoxide (SO) activity of hydrolysates and dipeptides obtained from

milk protein. Five, four and three hydrolysates were obtained from whey proteins, caseins and lactoferrin were obtained using gastrointestinal digestion. Five out the twelve hydrolysate and eight out of the twelve dipeptide studied were showed DPP-IV inhibition. The most efficient DPP-IV inhibitory peptide reported was *Trp-Val* (IC_{50} value 65.69 ± 2.95 µM). But the reverse peptide *Val-Trp* did not inhibit DPP-IV. The IC_{50} value of the most potent dipeptide rported (*Trp-Val*) was just about 15 times lesser than that for Diprotin A (8.49±0.15 µM). Nongonierma and FitzGerald (2013b) studied the effect of simulated gastrointestinal digestion, fractionation and food-drug interaction on DPP-IV inhibitory activity of whey protein hydrolysates (WPH). The IC_{50} value of WPH was 1.33 ± 0.17 mg/ml. After fractionation with 5kDa and 2kDa (permeate) the IC_{50} value was decreased to 0.95 ± 0.16 and 0.48 ± 0.05 respectively mg/ml that indicates increase in DPP- IV inhibitory potential, also when WPH subjected to *in vitro* gastrointestinal digestion activity, IC_{50} value was found to be 1.02 ± 0.05 mg/ml. It shows that DPP-IV inhibitory property increases with gastrointestinal digestion. Peptides obtained from WPH and its fractions inhibited enzyme through direct interaction with its active site. When sitagliptin was used with bioactive peptides an additive effect was observed.

Nongonierma and FitzGerald (2014) investigated using *in silico* approach for the DPP-IV inhibitory potential of milk proteins (casein, whey protein and lactoferrin) derived hydrolysates and synthetic peptides obtained from casein. Firstly *in silico* digestion of milk proteins (casein, whey protein and lactoferrin) was carried out by using pepsin, trypsin and chymotrypsin. Further these hydrolysates were incubated with DPP-IV enzyme at 37°C for 24h with two different E:S ratio (0.2 and 2%). Peptide suspended in Tris-HCL buffer at 10mg/ml concentration without DPP-IV used as a control. Five synthetic peptides *(Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu, Tyr-Pro-Tyr-Tyr, Leu-Pro-Tyr-Pro-Tyr* and Ile-Pro-Ile) obtained from casein were evaluated for DPP-IV inhibition. Peptide suspended in Tris-HCL buffer (10mg/ml) and DPP-IV at pH 8 and incubated at 37°C for 18h at two different E:S ratios (1 and 10%). In this study, it was concluded that, *Ile-Pro-Ile-Gln-Tyr, Tyr-Pro-Tyr-Tyr* and *Leu-Pro-Tyr-Pro-Tyr* were used as substrate by DPP-IV enzyme. Nongonierma and FitzGerald (2013a) used an *in silico* approach to figure out proline containing peptides derived from casein as DPP-IV inhibiting peptides. In this study theoretical digestion of casein was carried out by using prolyl oligopeptidase activity. Out of the total peptides generated, 15 peptides were selected for *in vitro* analysis of DPP-IV inhibitory activity. Further *in silico* approach was

used to check stability of these peptides under gastrointestinal conditions by using peptide cutter programme and resulting peptides were checked for DPPH scavenging and DPP-IV inhibition properties. Among all the peptides maximum DPP-IV inhibition was showed by *Leu-Gln-Pro* with IC_{50} value of 65.3 ± 3.5 µM.

Whey protein rich in β-Lg was hydrolyzed using trypsin to obtain hydrolysate with DPP-IV inhibitory activity, on purification of hydrolysate using HPLC, six fractions emerged out of which two fractions F_2 (IC_{50} value = 367.3 µg/ml) and F_3 (IC_{50} value = 86.0 µg/ml) showed better DPP-IV inhibitory activity. Upon HPLC coupled mass spectrometry analysis of these fractions, fragment IPAVFO corresponding to β-Lg f (78-82) has been emerged as the most potent DPP-IV inhibitor with IC_{50} value of 44.7 µM (Silveira *et al*., 2013). Power *et al*. (2014) demonstrated the effect of fractionation of tryptic hydrolysates of β-Lg using ultrafiltration on antioxidant, DPP-IV and ACE inhibitory activity. Ultrafiltration increased the bio-functional properties of β-Lg hydrolysates. Permeate obtained from 1 kDa polyethersulfone membrane had 1.7 fold more antioxidant property and ACE IC_{50} value was reduced by 2 fold. A threefold reduction was observed in DPP-IV IC_{50} of 1 kDa permeate i.e. decreased from 1.6 ± 0.31 to 0.53 ± 0.05 mg/ml. Tulipano *et al*. (2015) studied DPP-IV inhibitory potential of β-Lg and α-La using *in silico* and *in vitro* studies. In this study seventy-one DDP-IV inhibitory peptides considered for the *in silico* analysis that were reported in the literature. *In vitro* simulated gastrointestinal digestion of β-Lg and α-La was carried out by using pepsin and trypsin at their respective optimum pH and temperature. Both *in silico* and *in vitro* studies suggested that hydrolysate of β-Lg have more DPP-IV inhibitory potential than that of α-La.

Lacroix and Li-Chan (2012) studied DPP-IV inhibitory properties of milk protein hydrolysates. A 3% solution (on protein basis) of sodium caseinate, milk protein concentrates, WPI and skim milk powder were subjected to *in vitro* gastrointestinal digestion. Among all four hydrolysates obtained during *in vitro* gastrointestinal digestion, hydrolysate obtained from WPI with pepsin after 60 min hydrolysis showed maximum DPP-IV inhibitory activity with 0.075mg/ml IC_{50} value. Hydrolysates obtained from hydrolysis of sodium caseinate using 11 proteases, exhibited higher DPP-IV inhibitory activity than most WPI hydrolysates. However among all the hydrolysates, peptic digested WPI hydrolysate showed maximum DPP-IV inhibitory activity. Lacroix and Li-Chan. (2014) studied DPP-IV inhibitory potential of peptides obtained from bovine whey proteins after pepsin treatment. WPI and α-La were hydrolysed by using pepsin and

purified through chromatographic steps, and then analysed by using mass spectrometry. Among total recognized peptides, sequences 24 from α-La and 11 from β-Lg were formed and then studied for DPP-IV inhibitory potential. Two fragments, *LKPTPEGDL* and *LKPTPEGDLEIL* (IC_{50} value 45 and 57 µM respectively) showed maximum inhibition. Lacroix and Li-Chan. (2013) hydrolysates of WPI and whey proteins namely β-Lg, α-La, serum albumin, and lactoferrin were obtained by peptic digestion and then for their potential as a natural sources of DPP-IV and α-glucosidase inhibitors. Although all the hydrolysates showed DPP-IV inhibitory activity, the α-La hydrolysate came up as the most potent DPP-IV inhibitor (IC_{50} value = 0.036 mg/ml). α-glucosidase inhibition displayed only by hydrolysates of β-Lg and WPI isolates (IC_{50} value = 3.5 ± 0.4 mg/ml and 4.5 ± 0.6 mg/ml respectively). WPI isolates showed both DPP-IV and α-glucosidase inhibition.

Zhang *et al.* (2015) compared the DPP-IV inhibitory potential of caprine and bovine milk casein hydrolysates by using *in silico* and *in vitro* analysis. The *in silico* study predicted that caprine casein has lesser DPP-IV inhibitory potential than bovine casein. Higher DPP-IV inhibition has been shown by trypsin treated casein hydrolysates. Fractionation of trypsin treated caprine casein hydrolysates displayed significantly higher activity for less than 5kDa and notably higher activity for more than 5kDa peptide fractions as compare to bovine counterpart. Four novel DPP-IV inhibitory peptides were identified from *in silico* hydrolysis of caprine casein with trypsin, including GPFPILV and HPINHR (IC_{50} = 163.7 ± 1.33 and 452.2 ± 7.15 µM respectively).

Nongonierma *et al*. (2016) used *in silico* approach to identify DPP-IV inhibitory peptides and identified 5 peptides (*GY, GL, GI, NY*, and WL) from bovine α-La. An experimental design used with 3 factors (temperature, pH and E:S ratio) at 2 levels to hydrolyse α-La using elastase enzyme. The highest DPP-IV inhibition (75.8 ±3.7%) was observed by peptides at 3.1 mg/ml concentration of hydrolysate released at pH 8.5, 50°C and E:S 2.0% (w/w) (H_9). Nongonierma *et al*. (2017a) used a multifactorial experimental design to study the release of DPP-IV inhibitory peptides from bovine milk protein isolate. In this study bovine MPI was hydrolysed using trypsin enzyme at different E:S ratio viz: 0.50, 1.25 and 2.00%, hydrolysis time (60, 150 and 240 min) and temperature (40, 50 and 60°C) to produce 15 hydrolysates (H_1 to H_{15}). It was concluded that temperature had no significant effect on DPP-IV IC_{50} value. However, an increase in time or E:S ratio levels significantly decreased the DPP-IV IC_{50} value. The predicted DPP-IV IC_{50} value (0.69

mg/ml) by RSM (to be obtained with hydrolysate H_{16} at 2% E:S, 5.5°C and 231 min) was achieved by actual experiment (0.66 ± 0.10 mg/ml) as well.

Nongonierma *et al*. (2018) investigated the DPP-IV inhibitory potential of camel milk protein hydrolysates obtained from trypsin digestion of camel milk. Nine different DPP-IV inhibitory peptide (*FLQY, FQLGASPY, ILDKEGIDY*, *ILELA*, *LLQLEAIR*, *LPVP*, *LQALHQGQIV, MPVQA* and *SPVVPF*) obtained from tryptic digestion were identified through LC-MS/MS and sequential approach, among them two peptides (LPVP and MPVQA) identified as most potent DPP-IV inhibitors with IC_{50} value of 87.0 ± 3.2 and 93.3 ± 8.0 µM, respectively.

Chapter-3

MATERIALS AND METHODS

MATERIALS AND METHODS

This chapter deals with various materials and methodologies used during present study relating to evaluation of dipeptidyl peptidase inhibitory potential of β-Lg and α-La hydrolysates isolated from *Gir* cow milk.

3.1 Materials

In the present study, the following materials were used in various methods for isolating β-Lg and α-La from *Gir* cow milk and evaluating their DPP-IV inhibitory properties.

3.1.1 Raw Materials

3.1.1.1 *Gir* cow milk

Gir cow milk was procured from M/S Madhuban farm located near Kanakapura, Karnataka.

3.1.1.2 Enzymes

These following enzymes were used for hydrolysis of β-Lg and α-La.

a) Pepsin

Pepsin (P7012, EC 3.4.23.1), ≥ 2500 units/mg protein was procured from M/s. Sumana Enterprises (Brand: Sigma-Aldrich, St. Louis MO, USA), Bengaluru.

b) Trypsin

Trypsin (P4799, EC 3.4.21.4), 1000-2000 BAEE unit/mg solid was procured from M/s. Sumana Enterprises (Brand: Sigma-Aldrich, St. Louis MO, USA), Bengaluru.

3.1.1.3 DPP-IV

Dipetidyl Peptidase IV (D4943), ≥ 10U/mg protein was procured from M/s. Sumana Enterprises (Brand: Sigma-Aldrich, St. Louis MO, USA), Bengaluru.

3.1.1.4 Diprotin A (Ile-Pro-Ile)

Diprotin A (I9759), was used in DPP-IV inhibition assay as a positive control, was procured from M/s. Sumana Enterprises (Brand: Sigma-Aldrich, St. Louis MO, USA), Bengaluru.

3.1.1.5 Substrate (Gly-pro-p-nitroanilide)

Gly-pro-p-nitroanilide was used as a substrate in DPP-IV inhibition assay, was procured from M/s. Sumana Enterprises (Brand: Sigma-Aldrich, St. Louis MO, USA), Bengaluru.

3.1.1.6 Dialysis Membrane (Cellulose)

Dialysis membrane used for purification of β-Lg and α-La samples was procured from M/s. Sumana Enterprises (Brand: Sigma-Aldrich, St. Louis MO, USA), Bengaluru.

3.1.2 Equipment

3.1.2.1 Analytical weighing balance

A weighing balance (Make: Mattler Toledo, ME – 204) was used for weighing samples and chemicals used in various analysis.

3.1.2.2 pH meter

The pH meter (Make: INFRA DIGI) was used for determination of pH. The electrode was calibrated using standard buffers of pH 4.0 and 7.0.

3.1.2.3 Laboratory centrifuge

Laboratory centrifuge (Make: REMI INSTRUMENTS LTD.) was used to centrifuge whey samples during separation of β-Lg and α-La from whey.

3.1.2.4 Hot air oven

Hot air oven (Make: Apollo Scientific Co.) was used for determination of moisture and total solid content of various samples.

3.1.2.5 Kjeldahl analyser

Protein digestion and distillation of samples were done using Kjeldahl analyser unit (Make: Gerhardt, Germany, Tubotherm Model: TT 125 for digestion and VAPODEST

for distillation). Titration of distillate was done using digital bottle top burette (Make: Brand Tech Scientific Inc., USA)

3.1.2.6 Micro Plate Reader

Micro Plate Reader (Make; Bio Tek Instruments, Inc., USA) was used to check DPP-IV Inhibition Activity of β-Lg and α-La using 96-well plate.

3.1.2.7 Mini – PROTEAN® Tetra Cell

The Mini – PROTEAN tetra cell Electrophoresis Unit (M/s Bio – Rad Laboratories, Inc., USA) was used for SDS-PAGE Electrophoresis of β-Lg and α-La. The specifications of the unit are given below in table .1

Table 3.1 Specification for Mini – PROTEAN® Tetra Cell

Glass plate size (W x L)	Short plate: 10.1 x 7.3 cm
	Spacer plate : 10.1 x 8.2 cm
Approximate Gel size (W x L)	8.3 x 6.4 cm
Total volume of buffer for 2 gels	800 ml
Recommended Power supply	PowerPac^{TM}universal
Maximum power	75W
Maximum voltage	300 V
Maximum current	400

3.1.2.8 UF Membrane

UF membrane plant at experimental dairy plant of SRS of ICAR-NDRI was used for fractionation of β-Lg and ɑ-la.

3.1.2.9 Freeze dryer/ Lyophilizer

Lyophilizer (Make: Lark Innovative Fine Teknowledge, Channai) was used for drying of purified samples of β-lactoglobulin and α-lactalbumin.

3.1.2.10 Laboratory Fermenter

Fermenter (Make: Lark Innovative Fine Teknowledge, Channai) with data logger and provision of maintaining constant temperature and pH was used for hydrolysis of β-lactoglobulin and α-lactalbumin samples.

3.1.3 Chemicals

All chemicals used during this study were of analytical grade and procured from M/s. Vasa Scientific (Brand: SD Fine), and M/s. Sumana Enterprises (Brand: Sigma Aldrich) Bengaluru.

3.1.4 Glassware

Glassware of BOROSIL and SCHOTT DURAN were used for processing and chemical analysis of whey samples

3.1.5 Plasticware

Plasticware (Make: TARSON) used during processing and chemical analysis of whey samples, were procured from M/S. VASA Scientific Bangalore.

3.2 METHODS

3.2.1 Preparation of whey

Whey was prepared from skim milk by acid coagulation method. Detailed procedure for preparation of whey is given in following paragraph.

Fresh *Gir* cow milk was collected from M/S Madhubhan Farm and immediately subjected to pasteurization at 72°C for 15 sec and then cooled to 45°C for cream separation. The cream was separated and skim milk was added with 1M Hcl at room temperature till the pH of 4.6 (isoelectric point of casein) was reached. The precipitate was allowed for 30 min, and the contents were filtered through four fold muslin cloth.

3.2.2 Fractionation of whey using ultrafiltration

Ultrafiltration technique was used to fractionate whey proteins. It removes lactose, minerals and water from whey by employing molecular weight cut off membrane. Whey samples were heated to 45°C and passed through cream separator for removing fat, casein

and other suspended particles. Ultrafiltration was done by using UF membrane unit. The inlet pipe and outlet pipe for retentate were kept in sample reservoir and permeate was collected separately. The inlet pressure of the ultrafiltration unit was maintained around 15 psi throughout the process. The filtration process was continued until the volume of whey sample reduced to 1/4-1/5 time of its original volume, further the concentrated sample was diafiltrated by adding water (quantity equal to permeate removed during process) to the retentate and again concentrated 5 times. After filtration, the membrane unit was cleaned as per manufacturer's instructions. The retentate samples were analysed for total solids and protein as per AOAC (2005) methods. Finally the concentrated samples were stored at refrigerated temperature until (≤ 7°C) further use.

3.2.3 Determination of total solids

Total solids (TS) content of whey protein samples was determined as per (BIS, 1981) with a slight modification. Sample of 2.0-2.5 g was taken in a dried and pre-weighed aluminum dish. It was transferred to a drying oven at 100 ± 2 ^{0}C for 3 hours. Further the dishes were cooled in a desiccator and weighed. The same procedure of heating, cooling and weighing was repeated till the successive weight difference did not exceed 0.5 mg. TS content was calculated with the following formula:

$$TS\% = \frac{(\mathrm{W2} - \mathrm{W})}{\mathrm{W1} - \mathrm{W}} * 100$$

Where,

W = weight in g of empty dish

W1 = weight in g of the dish with the sample before drying

W2= weight in g of the dish with the sample after drying

3.2.4 Total protein by Kjeldahl method

Protein content in whey sample was determined by standard Micro Kjeldahl method described in AOAC (2005) with slight modification.

3.2.4.1 Digestion

Digestion was carried out in 300 ml Kjeldahl digestion tubes, samples were weighed accurately according to their protein content and transferred carefully to the digestion tube. Then 5g digestion mixture (K_2SO_4:$CuSO_4$=100:2), was added to the each tube followed by 12.5 ml concentrated sulphuric acid. The contents were digested in Kjeldahl digestion unit for 3 hours until the clear and colorless solution was obtained.

3.2.4.2 Distillation

Distillation was carried out in Kjeldahl distillation unit. Digestion tubes were attached to the distillation unit after cooling. Sodium hydroxide solution (40%) was added in order make solution alkaline. The contents of tubes were steam distilled and liberated ammonia from each tube was collected into 25ml boric acid solution (2%) containing 3-4 drops of mix indicator (mixture of methylene blue and methylene red). The distillation process was continued until about 100-120 ml of distillate was collected.

3.2.4.3 Titration

The distillate was titrated against 0.1N H_2SO_4. Titration was continued until the light pink color was obtained. Simultaneously a blank test carried out using all the reagents without the test material and the percent protein was calculated using following.

$$\%Protein = \frac{6.38 \times 1.4007 \times (\text{Sample Reading} - \text{Blank Reading}) \times \text{Normality of H2SO4}}{Weight\ of\ sampleing(g)}$$

3.2.5 Separation of β-lactoglobulin and α-lactalbumin from concentrated whey

Whey proteins can be separated based on their solubility, by using salting out method with some modifications (Alomirah and Alli, 2004). β-lactoglobulin was separated with NaCl at 30% final concentration and at pH 2.0, while α-lactalbumin was separated with tri-chloro acetic acid at 0.5% final concentration.

3.2.5.1 Clarification of whey

Fractionated whey was clarified by adjusting the pH 7.0 using 1M NaOH solution, and then centrifuged at 5000g for 15 min to precipitate calcium and phosphate complexes, and fine particles of casein. Clarified whey was used for further separation of β-lactoglobulin and α-lactalbumin

3.2.5.2 Separation of β-lactoglobulin

Sodium chloride was added at the rate of 7 g per 100ml to the clarified whey, and allowed to dissolve completely. Then the pH of the whey was adjusted to 2.0 by using 3.5 M HCl and allowed to stand for 20 min at room temperature. The content was centrifuged at 10,000g for 15 min to separate the β-lactoglobulin as soluble supernatant from the precipitated whey proteins. Sodium chloride was added at 23g per 100ml of decanted supernatant to give a final concentration of 30% (w/v). Then pH of the supernatant was readjusted to 2.0 by using 3.5 M HCl and allowed to stand for 20 min at room temperature afterwards, samples were centrifuged at 10,000g for 15 min and precipitated β-lactoglobulin was collected. Precipitate was resuspended in water and dialyzed against distilled water for two times at room temperature each four hours. Purified β-lactoglobulin samples were dried using freeze dryer. The process flow chart for β-lactoglobulin preparation is shown as Fig 3.1.

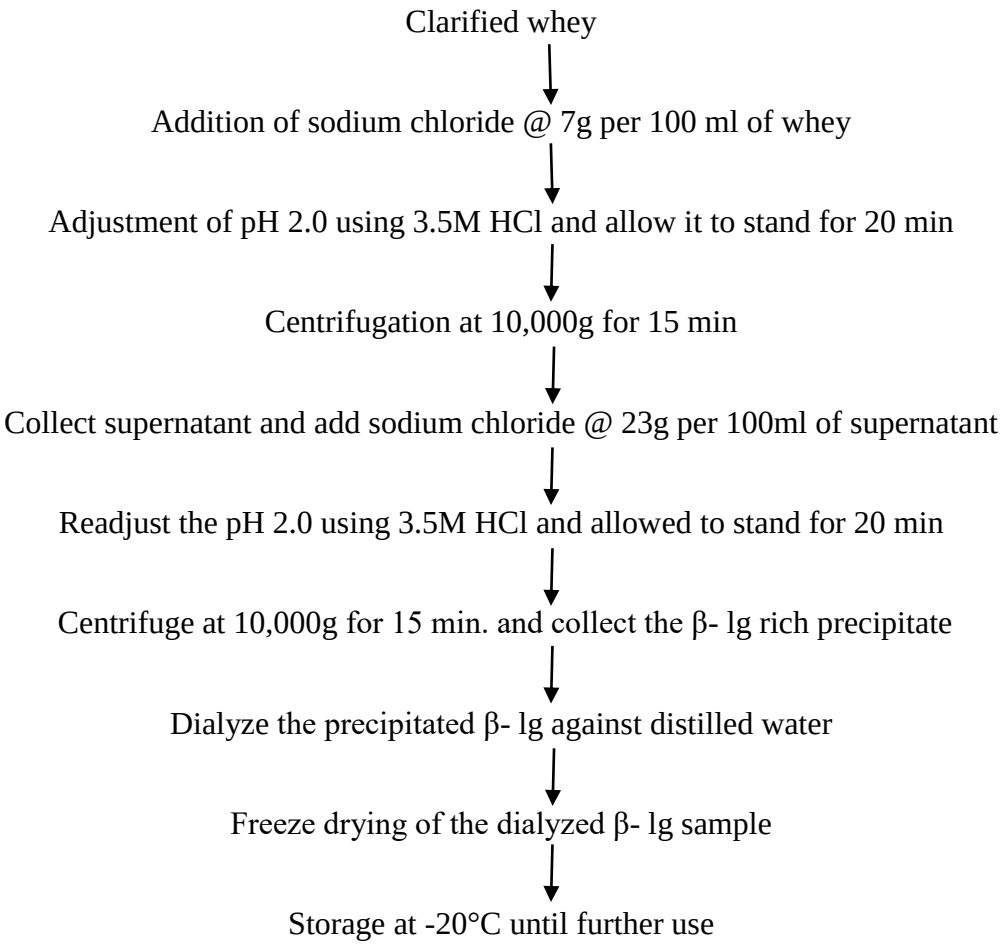

Fig 3.1 Separation of β- lactoglobulin from whey

3.2.5.3 Separation of α-lactalbumin

For the separation of α-lactalbumin, 20% trichloroacetic acid was added at the rate of 2.5 ml per 100 ml to the clarified whey, and allowed to stand at room temperature for 20 minutes. Afterwards content were centrifuged at 10,000g for 15 min and precipitated α-lactalbumin was collected. Precipitate was resuspended in water and dialyzed against distilled water for two times at room temperature each four hours. Purified α-lactalbumin samples were dried using freeze dryer. The process flow chart for α-lactalbumin preparation is shown as Fig 3.1.

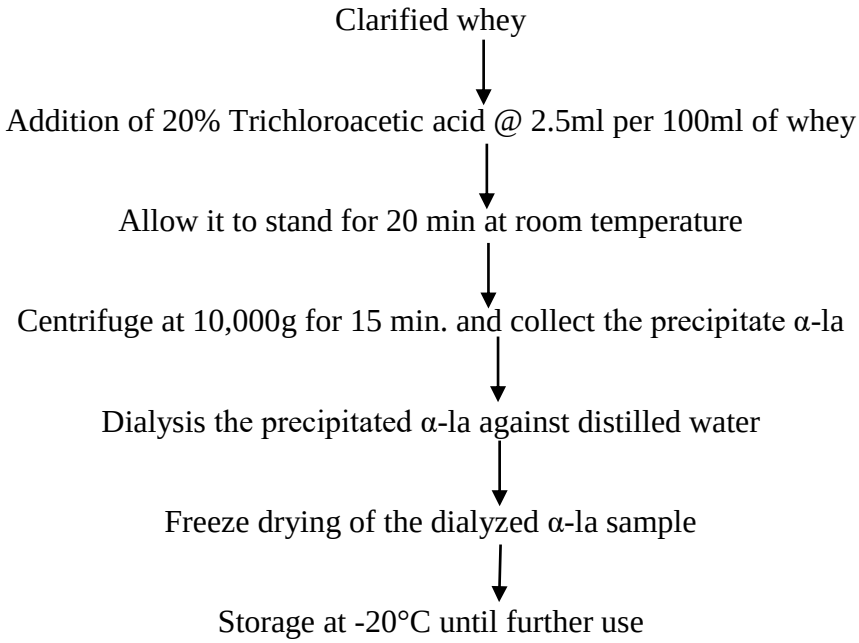

Fig. 3.2 Separation of α-lactalbumin from whey

3.2.6 Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS PAGE)

SDS-PAGE in vertical slab gel electrophoresis system was carried out as per the method of Laemmli (1970)

3.2.6.1 Reagents for SDS-PAGE

a) Acrylamide / bisacrylamide (30%)

29.2g of acrylamide and 0.8g of bis acrylamide were dissolved in distilled water and total volume was made upto 100ml. The solution was filtered and stored at 4^0C.

b) 1.5M Tris-HCl (pH 8.8)

18.15g of Tris base was dissolved in 60ml of distilled water. The pH was adjusted to 8.8 with 1N HCl and total volume was made upto 100ml with distilled water. The solution was stored at 4^0C.

c) 1.0 M Tri-HCl (pH 6.8)

12.1g of Tris base was dissolved in 60ml of distilled water and pH was adjusted to 6.8 using 1N HCl. Finally the total volume was made upto 100ml with distilled water. The buffer solution was stored at 4^0C.

d) 10% SDS

10g of SDS (Sodium dodecyl sulphate) was dissolved in distilled water and total volume was made upto 100ml. This solution was stored at room temperature.

e) Electrode buffer (5X, pH 8.3)

Tris	: 15 g
Glycine	: 72 g
SDS	: 5 g

Total volume was made upto 1litre with distilled water. The pH of buffer was 8.3. As recommended in method, pH was not adjusted with acid or alkali. The buffer was diluted five times just before use.

f) Sample buffer

Sample buffer was prepared by mixing following solutions:

Glycerol	:	2.4ml
20% SDS	:	4ml
2-Mercaptoethanol	:	0.80ml
1M Tris-HCl pH 6.8)	:	1.0ml

4 mg coomassive BBG (Tracking dye) was added to give blue colour to the sample.

g) 10% APS

Ammonium persulphate solution was prepared by dissolving 100mg APS in 1.0ml distilled water. This solution was always prepared fresh.

h) Gradient gel solutions:

Ingredients for preparation of stacking and separating gel are given below in table 3.2

Table 3.2 Ingredients for preparation of stacking and separating gel

Sr.No	Reagents	Stacking gel 4%	Separating gel 15%
1	Distilled water	3.4ml	2.3ml
2	Acrylamide/bisacrylamide	850µl	5ml
3	1M Tris-HCL (pH 6.8)	625µl	nil
4	1.5M Tris-HCL(pH 8.8)	nil	2.5ml
5	10%SDS	50µl	0.1ml
6	10% APS	50µl	0.1ml
7	TEMED	5µl	4µl

3.2.6.2 Gradient gel preparation

Fully cleaned gel plates of the size 8x7cm were assembled on casting stand of vertical slab gel unit using one mm spacers. The separating gel mix was poured down to the level of 2-3 cm from top into the gel plates. Distilled water was layered over the separating gel gently with the help of a syringe and the gel is allowed to stand until polymerization is complete. After the water is removed, stacking gel mix was layered over the previous layered polymerized separating gel and a comb of 10 wells was placed in position and the gel was allowed to polymerize. After the stacking gel polymerized, the comb was removed and the gel plates were transferred to the gel unit and electrode buffer was poured to both upper and lower chamber of the assembly.

3.2.6.3 Sample preparation

Protein samples free from insoluble material were mixed with equal volume of 2x sample buffer. These samples were heated in a dry bath at 90^{o} C for 5 min to denature the protein. Then samples were cooled and spinned for 1 min before loading into wells.

3.2.6.4 Electrophoresis

The samples (~10 µl, 50 µg protein) were loaded per well. After application of the samples, the electrophoresis was started at a constant voltage of 80V until samples pass through the stacking gel buffer and at 100V through the separating gel buffer until the tracking dye front is close to the bottom of the gel slab.

3.2.6.5 Gel-staining and de-staining

The gels were removed from the electrophoresis unit at the end of the electrophoresis, and kept for staining in the staining solution (CBB-R250- 0.25%, methanol- 25% and glacial acetic acid – 10%). After staining for 1 hour, the gels were tra nsferred into the destaining solution (5% methanol and 7.5% glacial acetic acid) at room temperature. Destaining was done overnight, till the bands appeared and background becomes clear. After destaining the gels were transferred into Milli-Q water for further destaining of bands. Staining and destaining were performed on orbital shaker.

3.2.7 Enzymatic hydrolysis of β-lactoglobulin and α-lactalbumin

Fresh solutions of β-lactoglobulin and α-lactalbumin were prepared @0.5% (w/v) on protein basis. Solutions were kept for 30 minutes for hydration before addition of enzyme. Two different enzymes were added at three different Enzyme:Substrate ratio. Further details are in table 3.1. Optimum pH and temperature for different enzymes were maintained throughout the experiment.

Table 3.3 Optimal pH and Temperature of enzymes used.

Enzymes	Optimum pH	Optimum temperature
Pepsin	2	37°C
Trypsin	8.0	37°C

3.2.8 Dipeptidyl peptidase-IV (antidiabetic) inhibition assay

DPP-IV inhibition assay of samples was checked by using method given by Lacroix and Li-Chan (2013).

3.2.8.1 Reagents

i) 100mM Tris-HCl buffer, (pH 8.0)

0.0546M HCl was added to 0.1M Tris, and then adjusted the pH to 8.0

ii) Gly-pro-p-nitroanilide (1.59mM)

52.27mg of Gly-pro-p-nitroanilide (substrate) was dissolved in 10 ml Tris-HCl buffer and final volume made up to 100ml using Tris-HCl buffer.

iii) DPP-IV (0.01U/ml)

Stock solution of DPP-IV (enzyme) was made by diluting the vial of 10U in 1ml of Tris-HCl buffer. A working solution of 0.01U/ml was made by diluting stock solution with Tris-HCl buffer.

iv) 1M Sodium acetate buffer, pH 4.0

0.852M acetic acid was added to 0.148M sodium acetate, and adjust the pH to 4.0

v) Diprotin A (500µM)

170.72µg of Diprotin A was dissolved in 1ml Tris-HCl buffer to make a stock solution of 500µM. Different concentration of Diprotin A were prepared using stock solution.

3.2.8.2 Procedure

DPP-IV inhibitory activity was determined for hydrolysates of β-Lg and α-La by using method described by Lacroix and Li-Chan (2013). Samples were reconstituted using 100 mM Tris-HCL buffer (pH 8.0) to the different concentration, then 25µL of diluted sample was preincubated with 25µL of substrate (Gly-Pro-p-nitroanilide, 1.59mM) at 37°C for 10 min in 96-well microplate reader. Immediately after incubation 50 µL of DPP-IV (0.01 U/mL) was added and the mixture was incubated at 37°C for 60 min. The enzymatic reaction was terminated by the addition of 100 µL of 1M sodium acetate buffer pH 4.0 and the absorbance of the released p-nitroanilide was measured at 405nm using microplate plate reader. The positive control (DPP-IV activity with no inhibitor) and negative control (no DPP-IV activity) were prepared by using Tris-HCl buffer (100 mM, pH 8.0) in place of the sample and in the place of the sample and DPP-IV solution,

respectively. For sample blanks in which DPP-IV was replaced with Tris-HCl buffer (100 mM, pH 8.0) the percent DPP-IV inhibition was calculated using formula.

$$\text{DPP} - \text{IV inhibition } \% = (1 - \frac{(absorbance\ of\ sample - absorbance\ of sample\ blank)}{(absorbance\ of\ positive\ control - absorbance\ of\ negative\ control)})100$$

Where,

Absorbance of sample = Diluted sample + Substrate + Enzyme + Sodium acetate buffer

Absorbance of sample blank = Diluted sample + Substrate + Tris-HCL buffer + Sodium acetate buffer

Absorbance of positive control = Tris-HCL buffer + Substrate + Enzyme + Sodium acetate buffer

Absorbance of negative control = Tris-HCL buffer + Substrate + Tris-HCL buffer + Sodium acetate buffer

The IC_{50} values were determined from linear regression equations generated by fitting the data from the plot of percent DPP-IV inhibition against hydrolysate concentrations (hydrolysate of concentrations required to cause a 50% inhibition of the enzyme activity) The Diprotin A was used as a reference inhibitor.

3.2.9 Determination of Degree of hydrolysis

The degree of hydrolysis of the whey protein hydrolysates samples at different time intervals and enzyme substrate ratio was analyzed by O-phthalaldehyde (OPA) method Nielsen *et al.,* 2001. In this method, the free amino groups of peptides released during the hydrolysis of proteins reacts with OPA reagent in the presence of beta-mercaptoethanol forming a colored compound detectable at 340 nm in a spectrophotometer.

3.2.9.1 Reagents

(i) Borax / Di-sodium tetraborate dehydrate 100mM

Di-sodium tetraborate dehydrate	3.9 g
Distilled water	100ml

(ii) Sodium dodecyl-sulfate (SDS) 20%

Sodium-dodecyl-sulfate	20g

Distilled water 100ml

(iii) O-phthaldialdehyde (OPA)

O-phthaldialdehyde 80mg

Ethanol 2 mL

(iv) The OPA reagent solution 100 mL

Di-sodium tetraborate dehydrate 50 mL

SDS 5 mL

OPA reagent 2 mL

Beta-mercaptoethanol 200 µL

To be prepared freshly just before use

(v) Standard serine

The serine standard was prepared; 50 mg serine was diluted in 500 mL distilled water (0.9516 meqv/L).

The sample solutions were prepared; X g sample was dissolved in 100 mL distilled water. X is 0.1 to 1.0 g sample containing 8% to 80% protein. The DH of the sample also influences the amount required.

3.2.9.2 Procedure:

The 400 µL of hydrolyzed whey protein solutions and 400 µL standard serine solution was added to a test tube containing 3mL of OPA reagent and mixed well for 5sec. The mixture was incubated at room temperature for exactly 2 min before being measure the absorbance at 340 nm in a spectrophotometer. The 400 µL of distilled water is treated as blank. After reading the absorbance in the spectrophotometer, the following steps were used to calculate the degree of hydrolysis.

At first, the since NH_2 content was determined as given below,

$$Serine - NH2 = \frac{(OD\ of\ sample - OD\ of\ blank}{(OD\ of\ standard - OD\ of\ blank)} \times 0.9516 \frac{meqv}{L} \times 0.1 \times D \times \frac{100}{X} \times \frac{1}{P}$$

Where,

Serine-NH_2 is expressed as meqv of serine NH_2 per gram of protein

X is the gram of sample

P is the protein % in sample

0.1 is the sample volume in liter (L).

D is the dilution factor of the sample

The degree of hydrolysis was calculated using the formula:

$$DH(\%) = \frac{h}{h_{\text{tot}}} \times 100$$

Where,

h = (serine-NH2 - β) / α meqv/g protein, where α and β are specific to the raw materials.

$h_{tot\ is}$ the specific to the raw materials and α, β and h_{tot} are constants. For whey proteins, the values of α, β and h_{tot} are 1.00, 0.40 and 8.8 respectively (Adler-Nissen, 1986).

Chapter-4

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSION

This chapter deals with the outcomes obtained during present investigation. The study included preparation of β-lactoglobulin and α-lactalbumin hydrolysates from *Gir* cow milk using pepsin and trypsin enzymes and evaluation of β-lactoglobulin and α-lactalbumin hydrolysates for dipeptidyl peptidase-IV inhibition (antidiabetic potential).

4.1 Preparation of whey proteins

Whey proteins accounts for approximately 20% of total milk protein in bovine milk (Fox, 2001). The whey was prepared from skim milk of *Gir* cow milk, after separation of cream. The pH of the skim milk was then adjusted to 4.6 (isoelectric point of casein) to precipitate casein, as casein remain insoluble at this pH and thus, whey was also separated from caseins. The yield of whey was ~72.84% from skim milk and details are summarised in Table 4.2.

4.1.1 Fractionation of whey proteins by Ultrafiltration

Whey was fractionated using ultrafiltration membrane to concentrate whey proteins and to remove major impurities like lactose, minerals and salts, along with these impurities some part of water also removed out as permeate. Atra *et al*. (2005) reported that concentration of whey proteins can be achieved through 15-20 kDa ultrafiltration membrane and optimal temperature suggested for ultrafiltration was 50°C owing to low viscosity of whey at this temperature. At temperature above 50°C, denaturtion of the whey proteins was observed which caused fouling of membrane material. The total solid and protein contents of whey, UF retentate and UF permeate are summarized in Table 4.1.

Table 4.1: Total solids and protein content of whey UF retentate and UF permeate from *Gir* cow milk

Parameters (%)	Whey	UF retentate	UF permeate
Protein	0.55±0.07	1.72±0.42	0.09±0.05
T.S	6.45±0.17	6.07±0.12	4.66±0.07

Note: Results are Mean± SD, n=6

4.1.2 Preparation of β-lactoglobulin (β-Lg)

The β-Lactoglobulin was prepared from fractionated and clarified whey using the method described by (Alomirah and Alli, 2004) with some modification as detailed in section 3.2. This method is based on the solubility of β-Lg at low pH in the presence of sodium chloride (NaCl). Most of whey proteins and other impurities were precipitated out at pH 2.0 with addition of NaCl (@7%), except β-Lg which remained in supernatant. Further addition of NaCl (@23%) to supernatant made β-Lg to precipitate out at pH 2.0. The yield and recovery % β-Lg by this method was found to be 1.78 g/L and 50.85% respectively. The results are detailed in Table 4.2.

4.1.3 Preparation of α-lactalbumin (α-La)

The α-lactalbumin was prepared from fractionated and clarified whey by salting out method with tri-chloro-acetic acid (TCA). All the proteins remains in supernatant and only α-La precipitate with TCA. The yield and recovery % of α-La by this method was found to be 1.15 g/L and 76.66% respectively. Data are summarized in Table 4.2.

Table 4.2: Yield and recovery of whey, β-Lg and α-La obtained from milk

Sample	Yield (g/L)	Recovery (%)
Whey	728.4 ± 0.49	72.84
β-Lg	1.78 ± 0.12	50.85
α-La	1.15 ± 0.06	76.66

Note: Results are Mean± SD, n=6

4.1.4 Purification of β-Lg and α-La

Purification of both of β-Lg and α-La fractions was done by using cellulose dialysis membrane of 12kDa molecular cut off. Both fractions were dialyzed against distilled water for 12 hours with intermittent replacement of distilled water with fresh one after 6 hours duration. Then the contents were freeze-dried and protein content in freeze-dried samples of β-Lg and α-La was found to be 76.17±2.06% and 72.91±1.91%, respectively. Composition of purified and freeze dried β-Lg and α-La are showed in Table 4.3.

Table 4.3 Protein and Moisture content of purified β-Lg and α-La

Parameters (%)	β-Lg	α-La
Protein	76.17±2.06	72.91±1.91
Moisture	9.98±0.61	10.94±0.35

The value expressed as Mean ± SD, n=6

4.1.5 Resolution of β-Lg and α-La on SDS-PAGE

Fractionated and purified samples of β-Lg and α-La were resolved on 15% separating and 4% stacking gel. The resolved band pattern of β-Lg and α-La fractions are shown in Fig. 4.1.

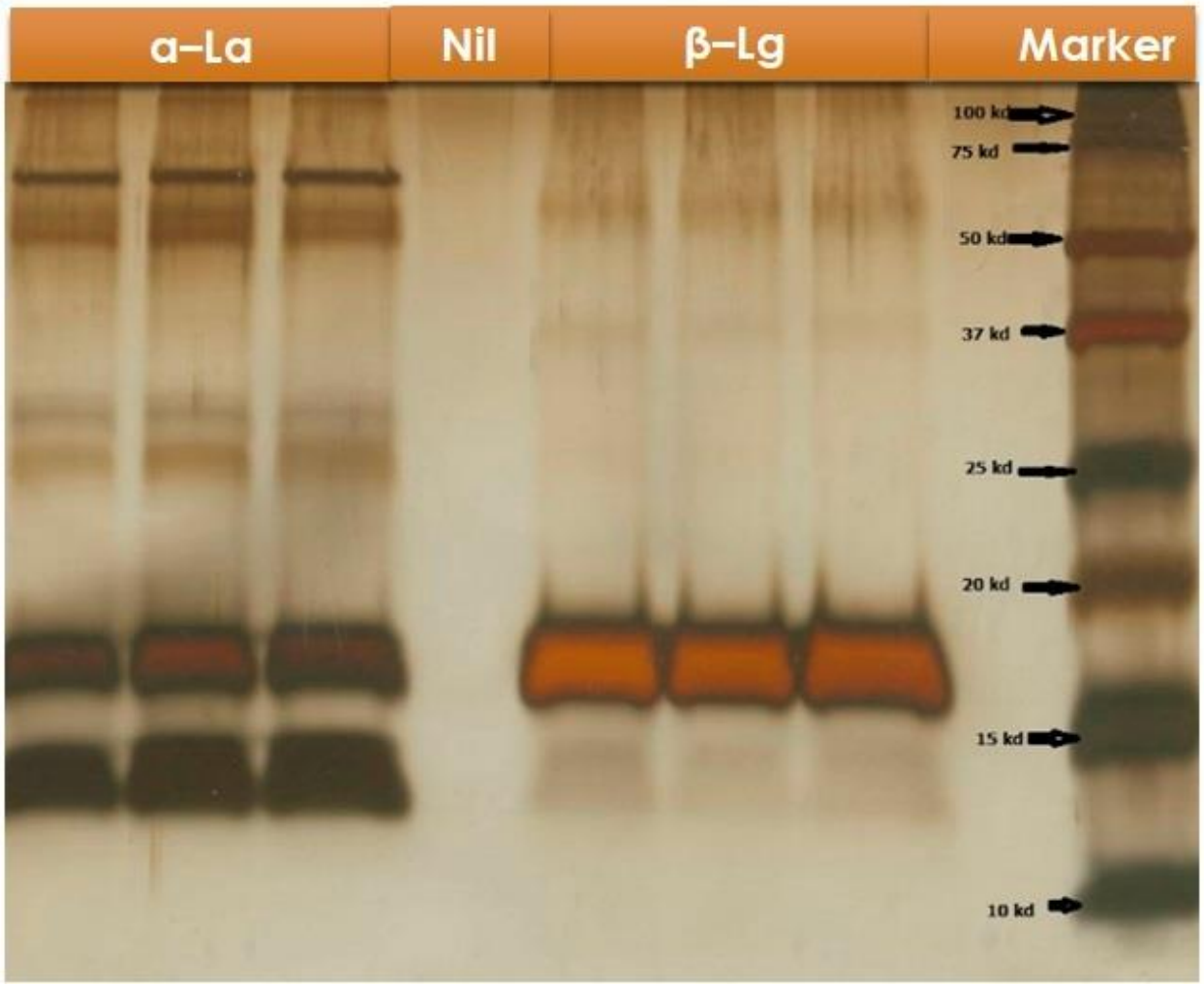

Fig 4.1: SDS-PAGE band pattern of β-Lg and α-La

As depicted in the Fig. 4.1, clear bands of β-Lg are obtained and molecular weight of the β-Lg is confirmed to 18.4kD. Hence the β-Lg fraction is pure and free from casein. The

α-La bands are also clearly visible and confirmed to its molecular weight of 14.2kD, however, α-La fraction also has β-Lg fraction, but, both of these proteins are free from caseins.

4.2 Enzymatic hydrolysis of β-Lg and α-La

Enzymatic hydrolysis of β-Lg and α-La was carried out separately by using pepsin and trypsin enzymes, at three different enzyme to substrate ratio (1:25, 1:50, and 1:100), and for six different durations *viz.,* 2h (minimum), 4h, 6h, 8, 10h, and 12h (maximum). Several studies reported the production of bioactive peptides after enzymatic hydrolysis for a period of 90 min (Devi *et al.,* 2002) to 48h (Pihlanto *et al*., 1998), although a satisfactory release of peptides reached only after hydrolysis of minimum 10h (Maeno *et al*., 1996).

4.2.1 Effect of duration on degree of hydrolysis of β-Lg

The extent of enzymatic hydrolysis of proteins generally referred as %age break down of peptides bonds, which is measured by degree of hydrolysis (%DH). In this study DH was measured by o-phthaldehyde (OPA) method (Nielsen *et al.,* 2001). The OPA method described that absorbance at 340 nm is increases as enzymatic hydrolysis or breakdown increases due to formation of complex between OPA and primary amine group of peptides in the presence of β-marcaptoethanol.

Degree of hydrolysis profile of β-Lg hydrolysates obtained with pepsin and trypsin individually at 1:25 E:S ratio for a period of 12h is shown in Fig. 4.2. An increase in %DHs was observed with respect to time for all the pepsin and trypsin treated hydrolysates of β-Lg. Hydrolysates obtained after pepsin treatment were showed an increase in %DH from 16.4 ± 0.57% (at 2h) to 28.48±1.29% (after 12h), whereas trypsin treated hydrolysates varied from 13.47 ± 0.62 (at 2h) to 22.07 ± 0.95 (after 12h). Hence, higher % DH was observed in pepsin treated β-Lg in compare to trypsin.

Nongonierma and FitzGerald (2013b**)** hydrolysed whey protein isolate (WPI) by using trypsin enzyme at different enzyme concentrations (0.50, 1.25 and 2.00% on protein basis) for different durations (60, 150 and 240 min), and found that % DH varied between 6.98 ± 0.31 to 12.75 ± 0.62%. These values are substantially lower than our findings, and might be due to the lesser duration of hydrolysis and lower enzyme to substrate ratio. Kim *et al.* (2007) hydrolysed heated and native WPC by using pepsin and then followed by

trypsin at different enzyme concentration (0.1, 0.5 and 1% on protein basis) and durations (30, 60, 90 and 120 min.). The %DH was increased with increased in duration and enzyme concentration. Maximum %DH reported in this study was 25.23% with 1% pepsin and followed 1% trypsin for 120min. The heated WPC was more prone to hydrolysis than its native counterparts. The denaturation of proteins at higher temperature favours higher %DH, although lower concentration of enzyme and shorter duration was adapted in their study.

4.2.2 Effect of duration on degree of hydrolysis of α-La

The % degree of hydrolysis profile for hydrolysates obtained from α-La with individual pepsin and trypsin treatments at 1:25 E:S ratio for 12h duration is shown in figure 4.3. The % DH for all hydrolysates obtained with pepsin and with trypsin is increased with respect to

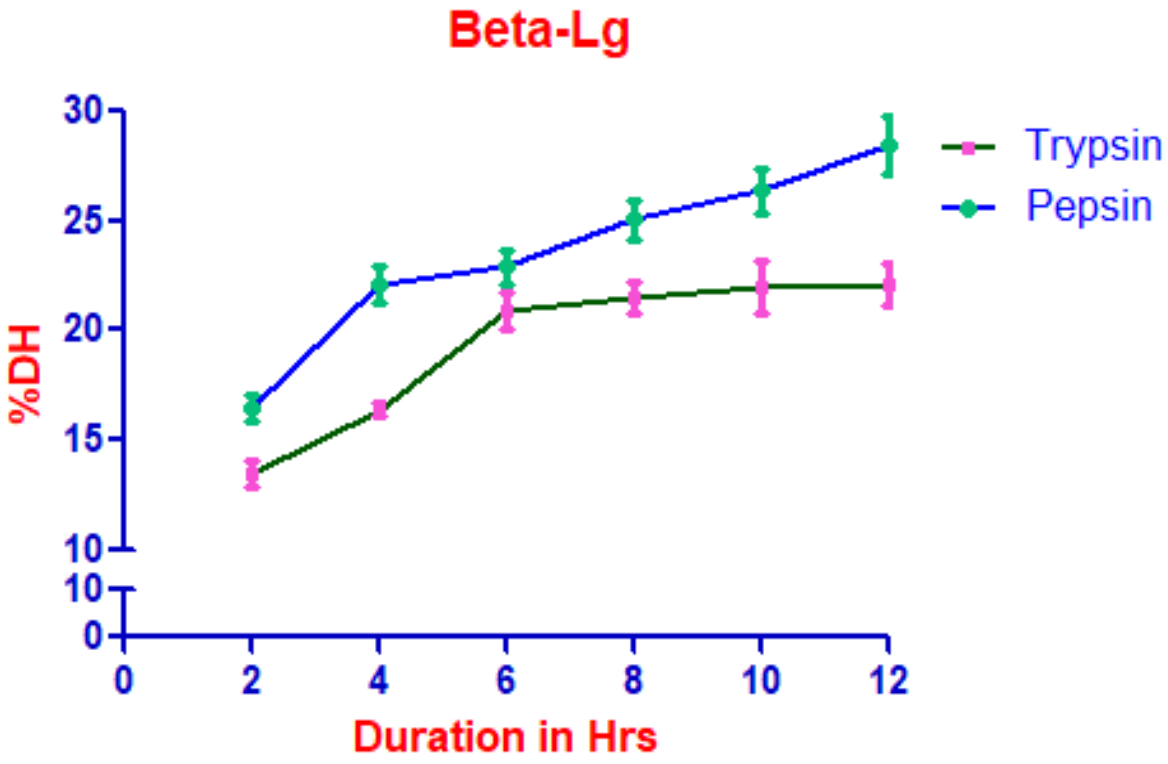

Fig 4.2: Effect of duration on %DH of β-Lg

time. The % DH in α-La increased from 18.47 ± 1.11% to 23.07± 1.61% and from 13.97 ± 0.57% to 22.17 ± 1.31% with pepsin and trypsin, respectively during 12 h hydrolysis. The increase was steeper during initial 6h of hydrolysis while marginal increase was

observed after 6h in both the cases. Pintado *et al*. (1999) hydrolysed whole whey by using protease 2A and trypsin and found maximum of 23%DH with protease 2A after 12h digestion, while in case of trypsin maximum % DH found was approx 8%, and no significant change was observed after 6h of hydrolysis. Hence, it is evident that maximum hydrolysis of α-La occur during the first 6h duration.

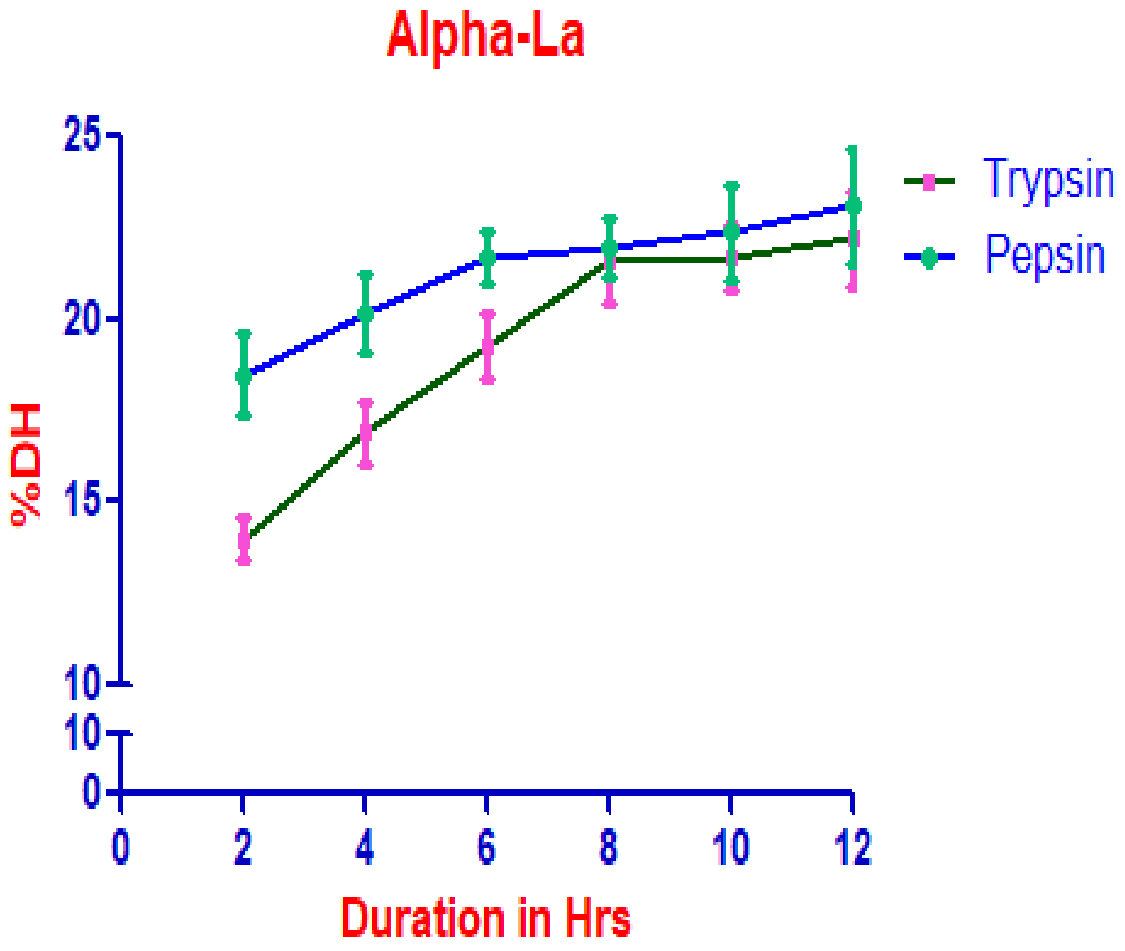

Fig 4.3: Effect of duration on %DH of α-La

4.3 Evaluation of DPP-IV inhibitory potential of β-Lg and α-La hydrolysates

The % DPP-IV inhibition of hydrolysates obtained from β-Lg and α-La was evaluated by using method described by Lacroix and Li-Chan (2013). In this method Diprotin A was used as a positive control and Gly-Pro-pNA as a substrate then absorbance was at 405 nm by using microplate reader with 96- well plate. The detailed procedure is given in section 3.2.8.2.

4.3.1 Effect of E:S ratio and duration on % DPP-IV inhibition of pepsin treated β-Lg

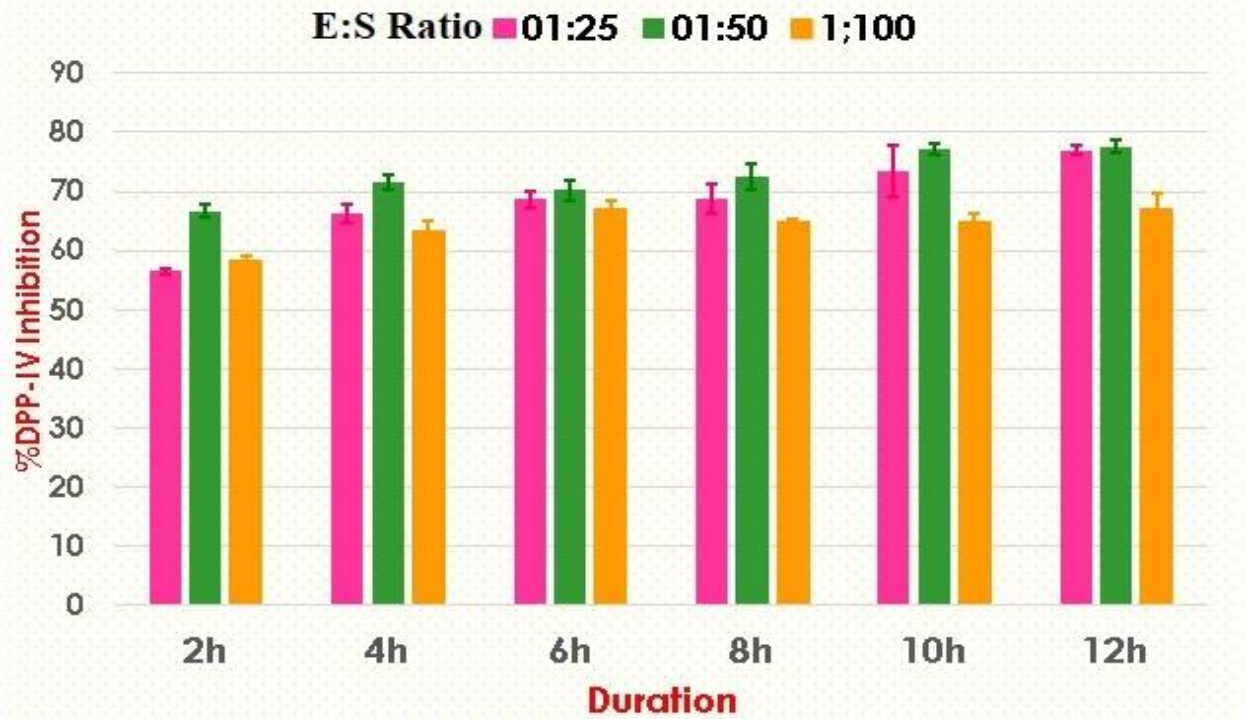

Fig 4.4: Effect of E:S ratio and duration on % DPP-IV inhibition of pepsin treated β-Lg hydrolysates

The % DPP-IV inhibition of pepsin treated β-Lg hydrolysates is shown in Fig 4.4. The different E:S ratio of pepsin to β-Lg viz., P1 (1:25 E:S ratio) , P2 (1:50 E:S ratio) and P_3 (1:100 E:S ratio) were maintained for 2 – 12 h duration. During 2h hydrolysis, no significant difference ($p>0.05$) was observed in % DPP-IV inhibition between P1 (56.29 ± 0.57) and P3(58.37 ± 0.57), whereas P_2 showed maximum (66.73 ± 1.15) inhibition. At 4, 6, 8, 10 and 12h durations also maximum inhibition was observed with P2 (1:50 E:S ratio) followed by P1. The minimal % DPP-IV inhibition was observed in P3. This minimum inhibition was significantly different ($p<0.05$) than others at 8, 10, and 12h duration. However, no significant difference was observed between P1 & P2 at 6, 8, 10 and 12 h durations. Hence, it is evident that P2 (1:50 E:S ratio) is the ideal E:S ratio for production maximum DPP-IV inhibitory peptides from β-Lg with pepsin. The highest potential DPP-IV inhibitory peptides (77.62 ± 0.98 %) were obtained from β-Lg after 12h hydrolysis at 1:50 E:S ratio with pepsin while minimal was obtained after 2h hydrolysis at 1:25 E:S ratio. Although, as the duration of hydrolysis progresses the % DPP-IV inhibition increases in P1 & P2, the raise is significant ($p<0.05$) after 2h and after 10h of duration. However, in case of P3, the rise in DPP-IV inhibition was found to be

significant ($p<0.05$) after 4h from 2h while after 4h the raise in DPP-IV inhibition is non-significant ($p>0.05$).

Lacroix and Li-Chan (2012) hydrolysed WPI (92.4% protein) under similar conditions and found 80% inhibition at 0.5mg/ml after 60 min, which was higher than our study, which might be due to combined effect of all the whey proteins used for hydrolysis initially. Lacroix and Li-Chan (2014) studied the DPP-IV inhibitory potential of pepsin treated WPI, β-Lg and α-La and found the most potent DPP-IV inhibitory fragment with IC_{50} value of 45 and 57 µM respectively.

4.3.2 Effect of E:S ratio and duration on % DPP-IV inhibition of trypsin treated β-Lg

The % DPP-IV inhibition of trypsin treated β-Lg hydrolysates is shown in figure 4.5. The E:S ratio used are 1:25, 1:50 and 1:100 and abbreviated as T1, T2, and T3 respectively. Maximum DPP-IV inhibition (74.89 ± 2.33%) was observed from T1 hydrolysates after 12h duration, while minimum DPP-IV inhibition (46.95 ± 2.83%) was observed from T3 hydrolysates after 2h hydrolysis. At 1:25 ratio (T1), trypsin enzyme treated hydrolysates exhibited better DPP-IV inhibitory peptides then T2 and T3. Hydrolysates of T3 showed lesser DPP-IV inhibition and DPP-IV inhibition of T3 hydrolysates were found to be significantly ($p<0.05$) different than other groups at 2h, 8h, 10, and 12h. Silveira *et al.* (2013) hydrolyzed whey protein concentrate (WPC) rich in β-Lg with trypsin at 1:20 E:S ratio and found similar result as maximum DPP-IV inhibition of ~75% was observed after 3h hydrolysis. The minimal IC_{50} value found in this experiment was 1.15 mg/ml.

4.3.3 Comparison of β-Lg hydrolysates treated with pepsin and trypsin enzymes

The % DPP-IV inhibition data of β-Lg hydrolysates resulted from pepsin and trypsin enzymes for 2-12h are summarized in Table 4.4. Among all hydrolysates obtained from enzymatic hydrolysis of β-Lg, maximum DPP-IV inhibition (77.62 ± 0.98 %) was exhibited by P_2 after 12h (Fig. 4.6). However, there was no significant difference ($p>0.05$) observed between P_1, P_2 and T_1 at 12h hydrolysis duration. Minimum DPP-IV inhibition was showed by T_3 (46.95 ± 2.83%), after 2h digestion among all the hydrolysates (36 combinations) obtained from enzymatic hydrolysis of β-Lg, which was significantly ($p<0.05$) lower in comparison to other hydrolysates obtained from β-Lg.

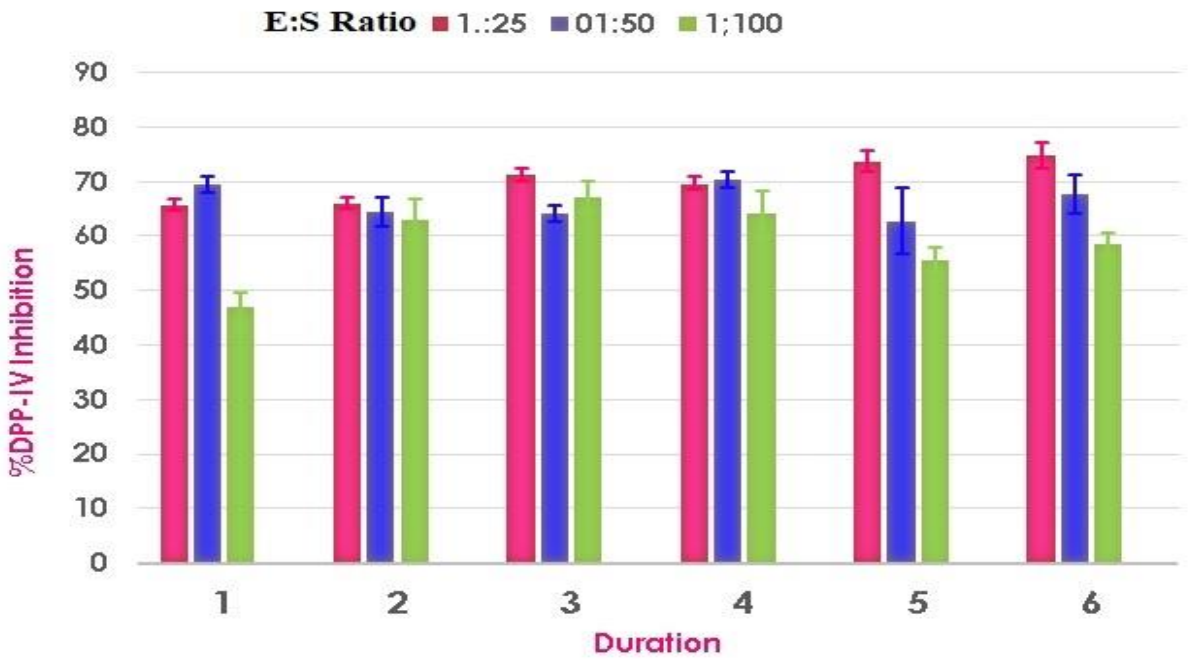

Fig. 4.5: Effect of E:S ratio and duration on % DPP-IV inhibition of trypsin treated β-Lg hydrolysates

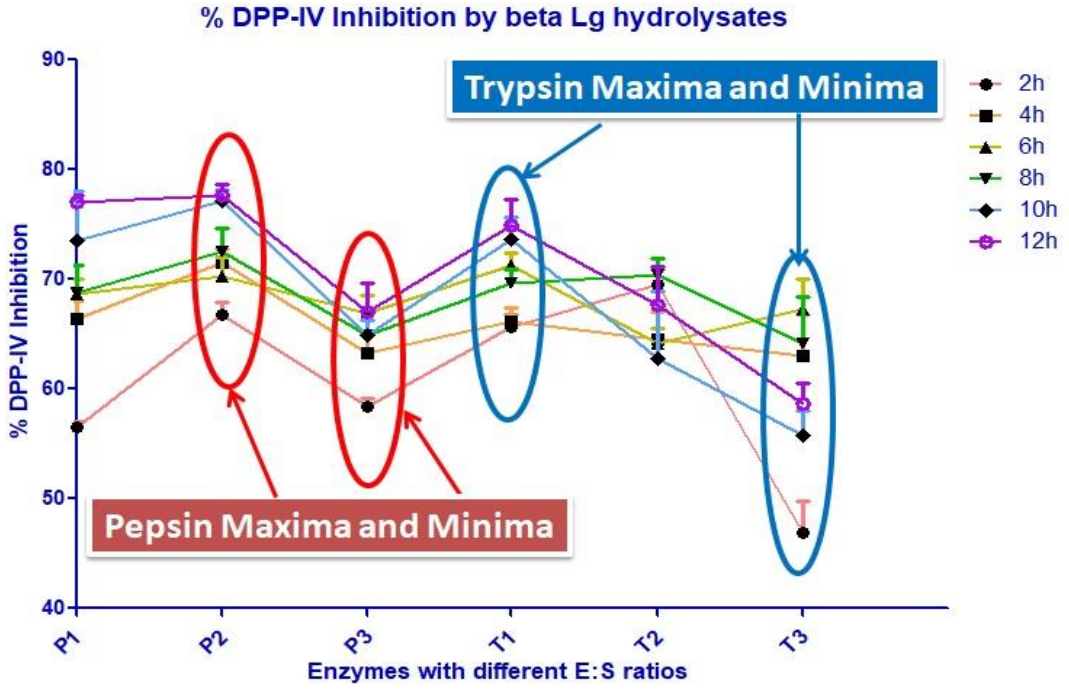

Fig. 4.6: Means of %DPP-IV inhibition by β-Lg hydrolysates treated with different enzymes and ratios for 2-12 h

Recently, Nongonierma *et al*. (2017c) produced DPP-IV inhibitory hydrolysates from camel milk WPC by using trypsin enzyme and interpreted that E:S ratio had significant

effect on DPP-IV inhibitory activity. They found maximum DPP-IV inhibition at 0.0125 E:S ratio and arrived at conclusion that higher E:S would generate more DPP-IV inhibitory peptides. Similar results were found in our study as well. As shown in Fig. 4.6 trypsin hydrolysates showed better DPP-IV inhibition at 1:25 E:S ratio and DPP-IV inhibition reduced to minimal with decrease in E:S ratios viz., in 1:50 and 1:100.

4.3.4 Effect of E:S ratio and duration on % DPP-IV inhibition of pepsin treated α-La

The % DPP-IV inhibition of pepsin treated α-La hydrolysates is shown in figure 4.7.

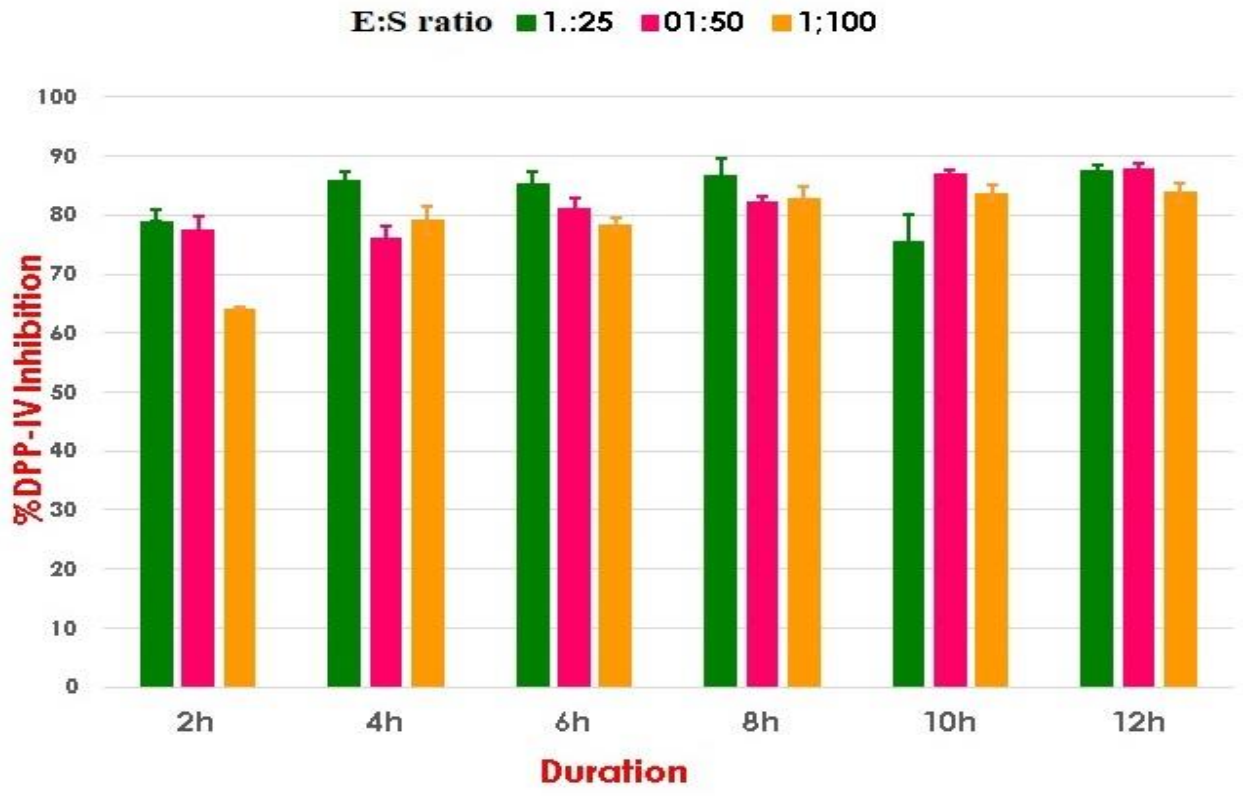

Fig 4.7: Effect of E:S ratio and duration on % DPP-IV inhibition of pepsin treated α-La hydrolysates

As depicted in figure 4.7, the % DPP-IV inhibition for P_1, P_2 and P_3 was varied from 78.86 ± 2.0, 77.45 ± 2.23 and 64.01 ± 0.29 (after 2h) to 87.69 ± 0.82, 87.81 ± 0.84 and 83.87 ± 1.42 (after 12h) respectively. Initially, after 2h, P_1 and P_2 showed significantly ($p<0.05$) higher DPP-IV inhibition than P_3. While after 12h of digestion, no significant difference was observed between DPP-IV inhibitory activity of P_1, P_2 and P_3. The maximum DPP-IV inhibition (87.81 ± 0.84) was observed with 1:50 E:S ratio after 12 h

hydrolysis, however, there was no significant difference ($p>0.05$) was observed between P2 (12h) , P1 (12h), P3 (12h), P1 (8h), P2 (10h). Lacroix and Li-Chan (2014) hydrolysed WPI, α-La, β-Lg, serum albumin and lactoferrin by using pepsin enzyme and found maximal DPP-IV inhibitory activity in the hydrolysates of α-La followed by WPI and least in β-Lg. These results corroborate our findings. In contrast, Tulipano *et al*. (2015) suggested the β -Lg could be a better source for DPP-IV inhibitory peptides than α-La based on *in-silico* hydrolysis of these whey proteins with pepsin and trypsin.

4.3.5 Effect of E:S ratio and duration on % DPP-IV inhibition of trypsin treated α-La

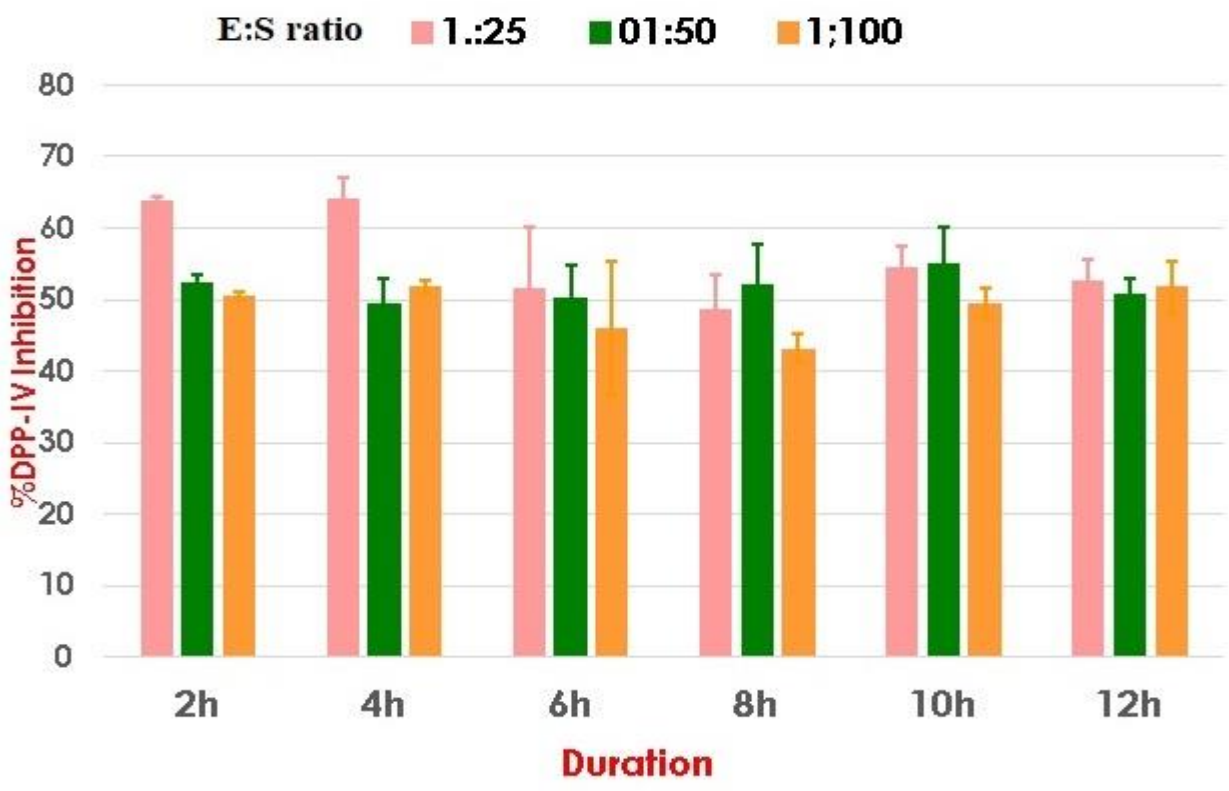

Fig 4.8: Effect of E:S ratio and duration on % DPP-IV inhibition of trypsin treated α-La hydrolysates

The % DPP-IV inhibition of all hydrolysates obtained from trypsin treated α-La showed in figure 4.8. DPP-IV inhibition of T_1 was varied significantly from 63.81 ± 0.70 (after 2h) to 52.79 ± 2.91 (after 12h) while there was no significant difference ($p>0.05$) observed in DPP-IV inhibitory activity of T_2 and T_3 hydrolysates obtained during 2h to 12 h. DPP-IV inhibition of T_2 and T_3 observed was 52.42 ± 1.05 and 50.55 ± 0.60 at 2h and

50.72 ± 2.21 and 51.79 ± 3.62 after 12h. Again T1 hydrolysates exhibited maximum % DPP-IV inhibition activity after 4h of hydrolysis and no significant difference was observed with hydrolysates of T1 after 2h. Hence, trypsin hydrolysates of shorter duration (up to 4h) at 1:25 E:S were able to generate maximum DPP-IV inhibitory peptides.

4.3.6 Comparison of α-La hydrolysates treated with pepsin and trypsin enzymes

The % DPP-IV inhibition data of all α-La hydrolysates obtained after pepsin and trypsin digestion are shown in Table 4.5. Maximum DPP-IV inhibition (87.81 ± 0.84) among all the hydrolysates obtained from enzymatic hydrolysis of α-La hydrolysed by pepsin at 1:50 E:S ratio after 12h hydrolysis. Although there was no significant difference ($p>0.05$) observed between P_1, P_2 and P_3 at 12h. Minimum % DPP-IV inhibition was showed by trypsin hydrolysate at 1:100 E:S ratio after 8h duration among all the hydrolysates obtained from α-La. Again pepsin hydrolysates exhibited better DPP-IV inhibition peptide fractions than trypsin (Fig. 4.9) irrespective of hydrolysis duration. This clearly demonstrates the superiority of pepsin in generating DPP-IV inhibitory peptides than trypsin in α-La.

Lacroix and Li-Chan (2013) hydrolysed WPI, β-Lg and α-La by using pepsin enzyme at 4% E:S ratio for 60 minutes and found the IC_{50} value of 0.075mg/ml, 1.279mg/ml and 0.036mg/ml respectively, and interpreted that α-La have more potential to inhibit DPP-IV enzyme which is also in agreement with our study. Nongonierma and FitzGerald (2014) evaluated α-La through *in silico* analysis and found that it has highest protein coverage (PC) 43.9% and potency index (PI) of 5.66, while β-Lg has 34% PC and 3.27 PI. In this study PC denotes proportion of amino acid residues participating in DPP-IV inhibitory peptide sequences with IC50 values <2000 μM, whereas PI expressed in μM per gram by taking into account IC50 value of peptide. Lacroix and Li-Chan (2014) identified three most potential DPP-IV inhibitory peptides viz., WCKDDQNPHS, LAHKALCSEK and LCSEKLDQWL with inhibition constant (K_i) values of 76±5, 217±9 and 217±15μM using SPOT technology.

Table 4.4 Effect of E:S ratio and duration on % DPP-IV inhibition of β-Lg hydrolysates

Enzymes		β-lactoglobulin					
		Duration					
		2h	4h	6h	8h	10h	12h
Pepsin	1:25	56.48 ± 0.57^{bA}	66.38 ± 1.57^{aB}	68.66 ± 1.35^{abBC}	68.8 ± 2.43^{abcdBC}	73.52 ± 4.43^{cCD}	77.00 ± 0.69^{cD}
	1:50	66.73 ± 1.15^{cA}	71.52 ± 1.26^{bAB}	70.22 ± 1.74^{bAB}	72.47 ± 2.14^{dBC}	77.20 ± 0.89^{cCD}	77.62 ± 0.98^{cD}
	1:100	58.37 ± 0.80^{bA}	63.24 ± 1.72^{aAB}	66.95 ± 1.60^{abB}	64.89 ± 0.47^{abB}	64.86 ± 1.42^{bB}	67.06 ± 2.59^{bB}
Trypsin	1:25	65.70 ± 1.07^{cA}	66.09 ± 1.027^{aA}	71.28 ± 1.10^{bBC}	69.69 ± 1.16^{bcdAB}	73.66 ± 1.94^{cBC}	74.89 ± 2.33^{cC}
	1:50	69.49 ± 1.37^{cB}	64.47 ± 2.56^{aAB}	64.14 ± 1.35^{aA}	70.39 ± 1.54^{cdB}	62.74 ± 6.14^{bA}	67.66 ± 3.49^{bAB}
	1:100	46.95 ± 2.83^{aA}	63.04 ± 3.77^{aCD}	67.26 ± 2.74^{abD}	64.10 ± 4.266^{aD}	55.7 ± 2.28^{aB}	58.60 ± 1.87^{aBC}

Results are mean values ± SD, n=3. Values with different superscripts (upper case) in a row and with different superscripts (lower case) in a column are significantly different ($P<0.05$)

Table 4.5 Effect of E:S ratio and duration on % DPP-IV inhibition of α-La hydrolysates

Enzymes		α-Lactalbumin					
		Duration					
		2h	4h	6h	8h	10h	12h
Pepsin	1:25	78.86 ± 2.01^{cAB}	85.94 ± 1.46^{dBC}	85.42 ± 1.81^{bBC}	86.91 ± 2.56^{cC}	75.49 ± 4.59^{bA}	87.69 ± 0.82^{bC}
	1:50	77.45 ± 2.23^{cA}	76.27 ± 1.90^{cA}	81.15 ± 1.83^{bAB}	82.36 ± 0.90^{cAB}	86.98 ± 0.58^{cB}	87.81 ± 0.84^{bB}
	1:100	64.01 ± 0.29^{bA}	79.21 ± 2.38^{cdBC}	78.32 ± 1.14^{bBC}	82.86 ± 2.06^{cC}	83.73 ± 1.33^{cC}	83.87 ± 1.42^{bC}
Trypsin	1:25	63.81 ± 0.70^{bB}	64.13 ± 3.02^{bB}	51.50 ± 8.58^{aA}	48.75 ± 4.66^{abA}	54.66 ± 2.92^{aA}	52.79 ± 2.91^{aA}
	1:50	52.42 ± 1.05^{aA}	49.42 ± 3.40^{aA}	50.17 ± 4.50^{aA}	52.17 ± 5.53^{bA}	54.94 ± 5.23^{aA}	50.72 ± 2.21^{aA}
	1:100	50.55 ± 0.60^{aB}	51.74 ± 0.84^{aB}	46.07 ± 9.27^{aAB}	43.17 ± 1.96^{aA}	49.44 ± 2.22^{aAB}	51.79 ± 3.62^{aB}

Results are mean values ± SD, n=3. Values with different superscripts (upper case) in a row and with different superscripts (lower case) in a column are significantly different ($P<0.05$)

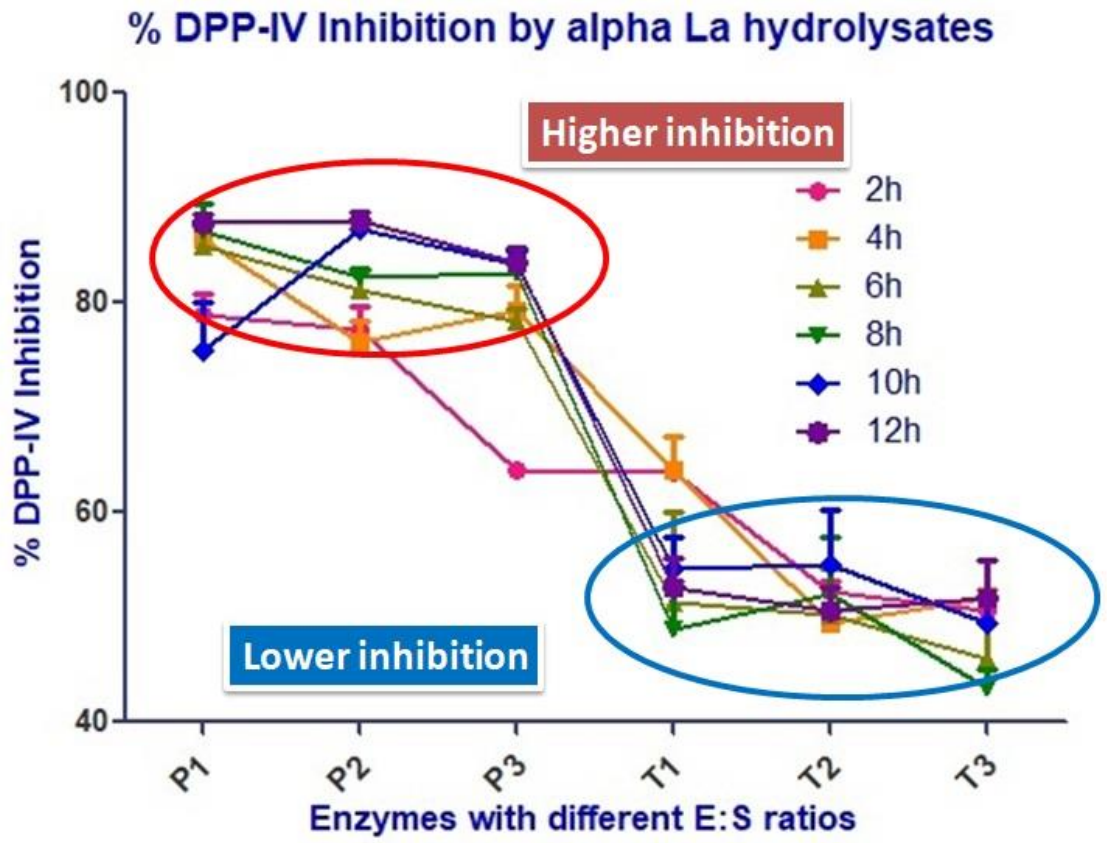

Fig. 4.9: Means of %DPP-IV inhibition by α-La hydrolysates treated with different enzymes and ratios for 2-12 h

4.3.7 IC_{50} values of maximum %DPP-IV inhibitory hydrolysates

IC_{50} value is the concentration of peptides fraction required to exhibit 50% inhibition of DPP-IV enzyme. Hence, lower IC_{50} value has maximum DPP-IV enzyme inhibition potential. The positive control (Diprotein A) has IC_{50} value of 3.4µM (Nongonierma and FitzGerald, 2014). Therefore, to evaluate DPP-IV inhibition potential of α-La and β-Lg hydrolysates, IC_{50} value of maximum DPP-IV inhibitory peptide fractions were evaluated for IC_{50} value in this study. Different concentrations peptides were prepared and % DPP-IV inhibition was measured. From the resultant graph the concentration to inhibit 50% DPP-IV enzyme activity was measured using the equation. The IC_{50} value of Diprotein A was measured and found that 4.22µM (Fig. 4.10). IC_{50} value of pespin treated β-Lg hydrolysates (P2, 12h) was found to be 3.78mg/ml (Fig. 4.11).

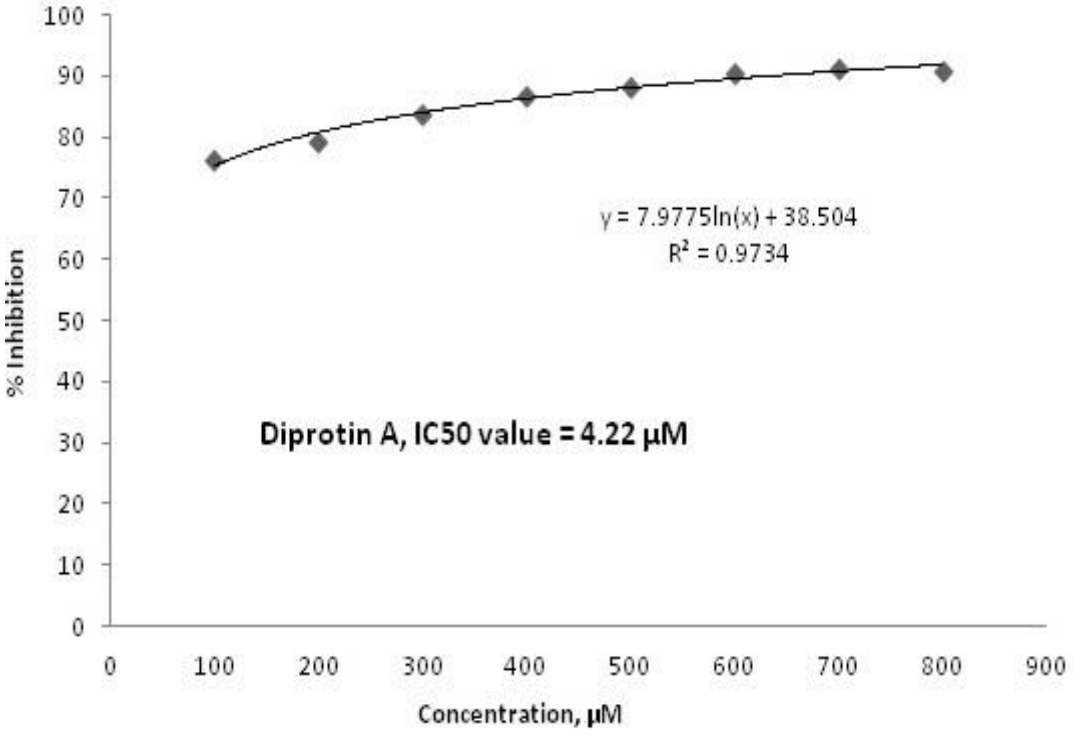

Fig. 4.10: IC_{50} value of Diprotein A

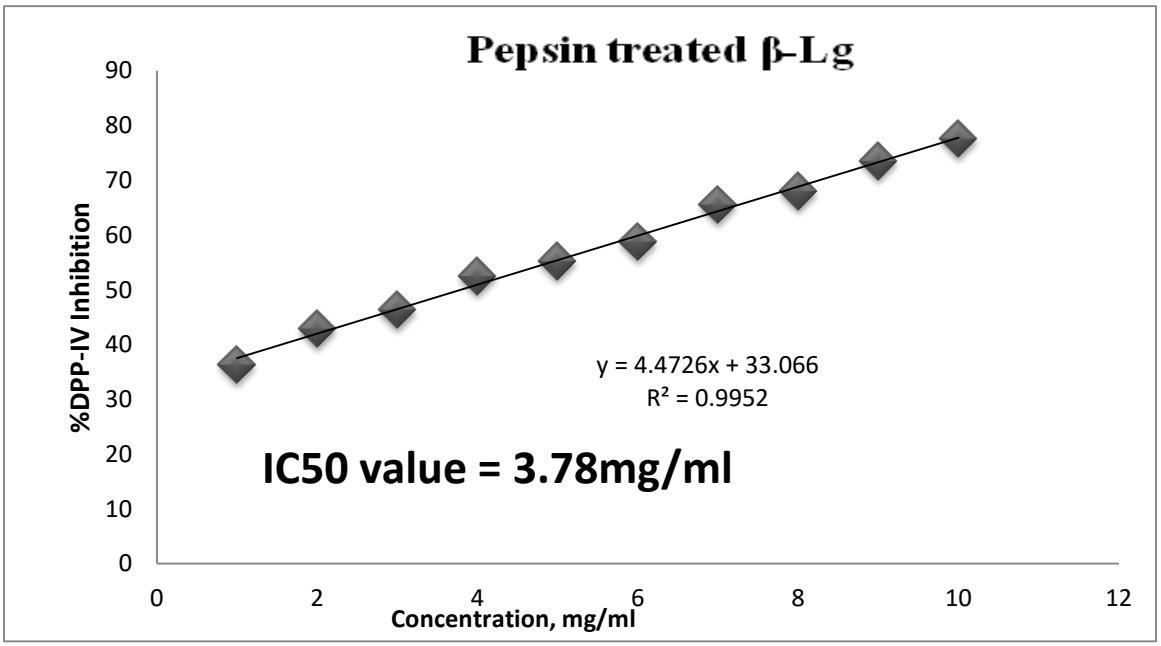

Fig. 4.11: IC_{50} value of pepsin treated β-Lg

Nongonierma and FitzGerald (2013b) reported DPP-IV inhibitory potential of WPH obtained after pancreatic digestion. The IC_{50} value obtained for WPH was 1.34 ± 0.11 mg/ml. The IC_{50} value obtained for most potent trypsin treated β-Lg hydrolysates was 5.03 mg/ml (shown in figure 4.12).

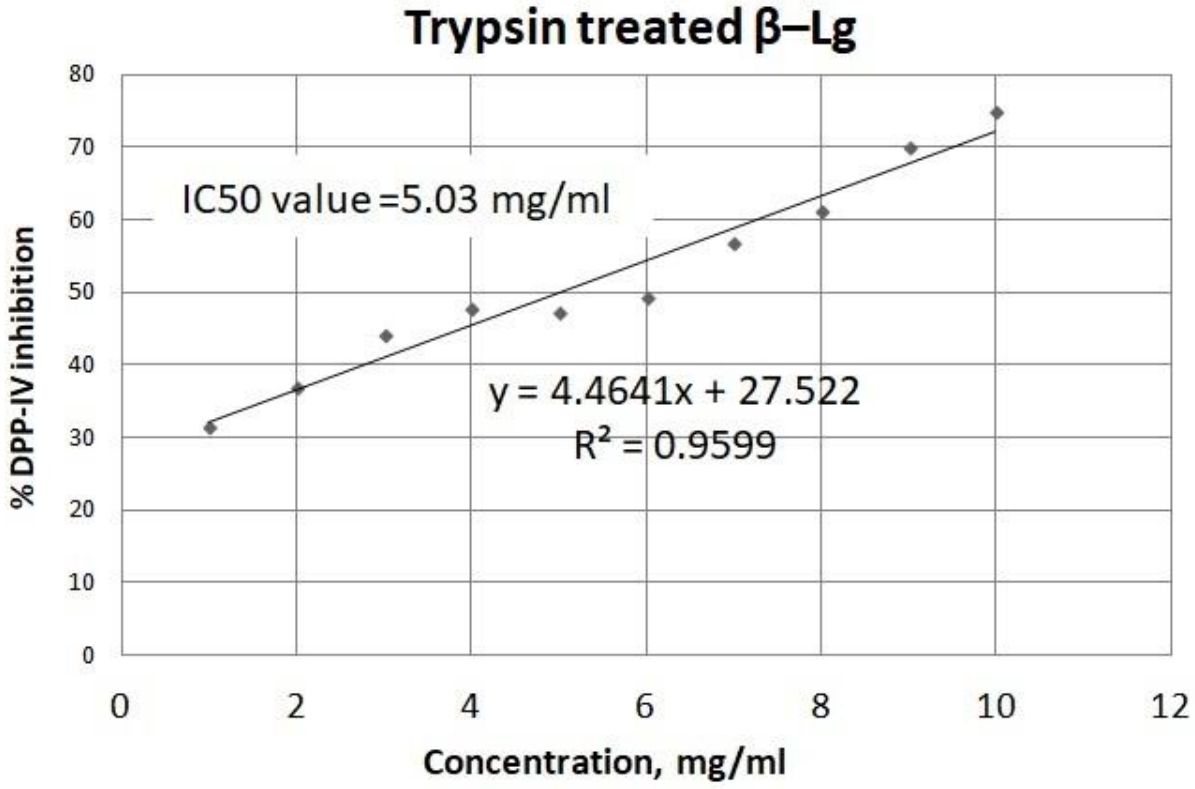

Fig. 4.12: IC_{50} value of trypsin treated β-Lg

The IC_{50} value of trypsin treated β-Lg hydrolysates fractions was examined and lowest IC_{50} value (44.7μM) was reported by Silveira *et al*. (2013). The lower IC_{50} value is for purified fraction of β-Lg fraction (f(78-82) separated using HPLC-MS/MS. Uchida *et al*. (2011) reported that trypsin treated β-Lg with an IC_{50} value of 210μM or 3.9mg/ml. Power *et al*. (2014) digested β-Lg by using trypsin enzyme and studied the effect of ultrafiltration on DPP-IV inhibition, they found that IC_{50} value was decreased after ultrafiltration from 1.6 ± 0.31 to 0.53 ± 0.05 mg/ml in 1 kDa permeate. Nongonierma *et al*. (2017b) hydrolysed bovine milk protein isolate by using trypsin and found that the IC_{50} value ranged from 0.68 ± 0.06 to

1.59 ± 0.11 mg/ml. The IC_{50} value of α-La was found to be 0.78mg/ml and this is the lowest among all (Fig. 4.13) at 1:50 E:S ratio and after 12h duration.

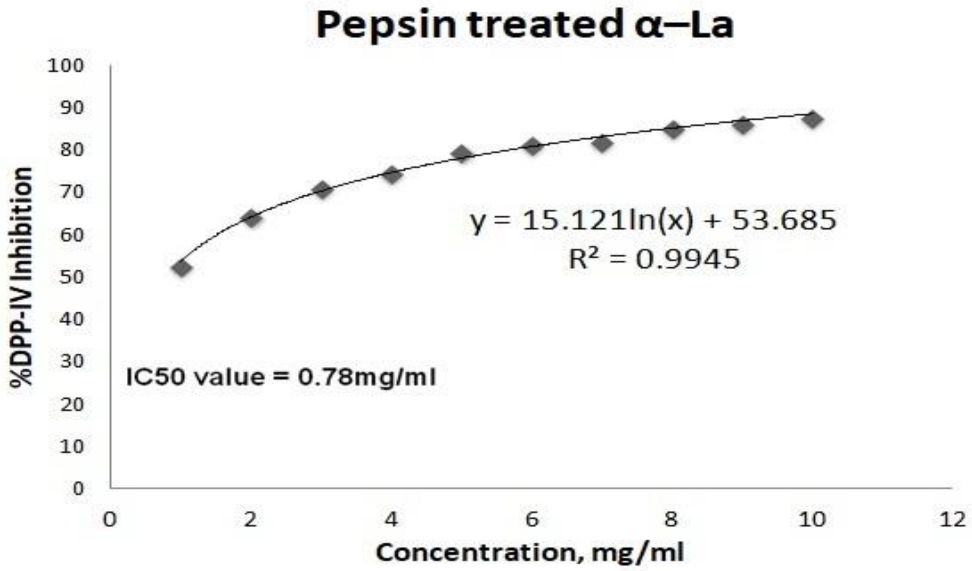

Fig. 4.13: IC_{50} value of pepsin treated α-La

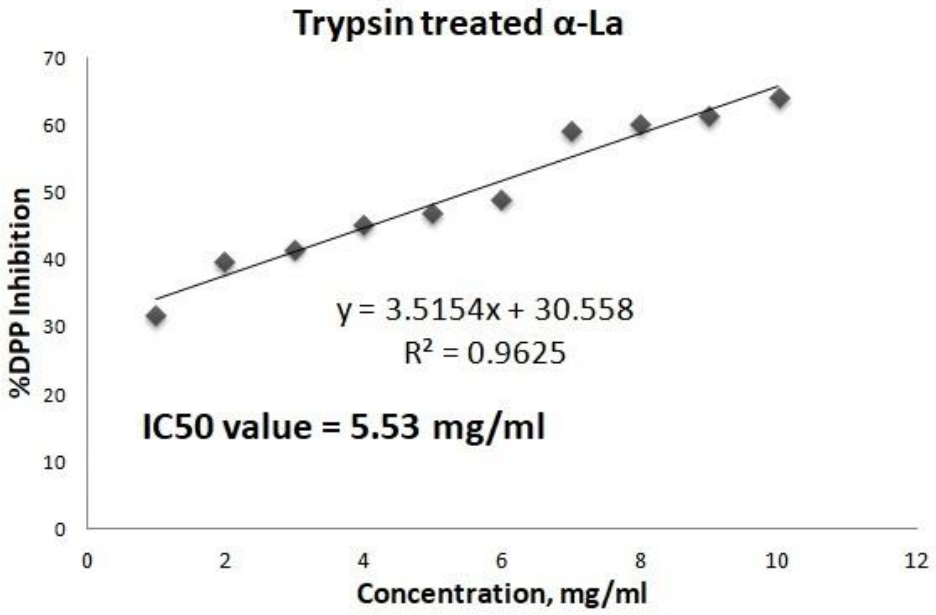

Figure 4.14 IC_{50} value for trypsin treated α-La

Nongonierma *et al*. (2016) used *in silico* approach to study DPP-IV inhibitory potential of α-La after hydrolysis with elastase enzyme, and observed 75.8 ±3.7% inhibition by peptides at 3.1 mg/ml concentration. Similar result found in this study as well, the % DPP-IV inhibition by pepsin treated α-La hydrolysates was found 71% at 3mg/ml.

Among the hydrolysates pepsin treated (1:50 E:S ratio for 12 hours) α-La hydrolysates showed maximum DPP-IV inhibition and lowest IC_{50} value. Also, it is proved that pepsin is the better option than trypsin to produce DPP-IV inhibitory peptides from β-Lg and/or α-La. However, in this study peptides which are responsible for DPP-IV inhibition was not done, and hence, further investigation is required to identify the responsible peptide(s) for DPP-IV inhibition activity.

Chapter-5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

5.1 Summary

The objective of the present study was to evaluate the DPP-IV inhibitory potential of hydrolysates obtained after enzymatic hydrolysis of β-Lg and α-La, isolated from *Gir* cow milk. Detail findings and inferences acquired during fractionation and hydrolysis of β-Lg and α-La and their evaluation for DPP-IV inhibition are summarized below:

- The β-Lg and α-La were fractionated from *Gir* cow milk using ultrafiltration technique followed by salting out method, yield and percent recovery of β-Lg and α-La are found to be 1.78 g/L, 50.85% and 1.15 g/L, 76.66%, respectively.
- Purification of both of β-Lg and α-La fractions was done by using cellulose dialysis membrane, the protein content in purified and freeze-dried samples of β-Lg and α-La was found to be 76.17±2.06% and 72.91±1.91%, respectively.
- Molecular weight of purified β-Lg and α-La samples were confirmed through SDS-PAGE.
- Enzymatic hydrolysis of β-Lg and α-La was done by using pepsin and trypsin enzymes, at different E:S ratio and duration. The degree of hydrolysis was found to increase with duration, Maximum %DH (28.48 ± 1.29) was showed by pepsin treated β-Lg after 12h at 1:25 E:S ratio.
- Trypsin treated β-Lg showed max. 22.07 ± 0.95 %DH by 1:25 E:S ratio after 12h duration, however, after 6h of hydrolysis only marginal increase in %DH was observed.
- Maximum %DH observed in α-La treated with pepsin and trypsin was 23.07 ± 1.61 and 22.17 ± 1.31% respectively.
- Rapid increase in hydrolysis was seen up to 6h in both pepsin and trypsin of α-La afterwards very marginal increase was observed.
- Hydrolysates obtained after pepsin and trypsin treatment of β-Lg and α-La were evaluated for their DPP-IV inhibitory potential
- Pepsin treated α-La showed maximum 87.81 ± 0.84 % DPP-IV inhibition with IC_{50} value of 0.78mg/ml, at 12h duration and 1:50 E:S ratio, and no significant difference (P> 0.05) was observed between 1:25, 1:50 and 1:100 at this duration.

- Trypsin treated α-La showed maximum 64.13±3.02 % DPP-IV inhibition with IC_{50} of 5.53mg/ml at 4h duration and 1:25 E:S ratio, which is significantly higher than 1:50 and 1:100 ratio at this duration
- Maximum DPP-IV inhibition by pepsin treated β-Lg was 77.62±0.98% at 1:50 E:S ratio and 12h duration with IC_{50} of 3.78mg/ml, which was significantly higher than DPP-IV inhibition of 1:100 and no significant difference was observed with 1:25 ES ratio at this duration.
- Trypsin treated β-Lg showed maximum 74.89±2.33% DPP-IV inhibition with IC_{50} of 5.03mg/ml at 12h duration and 1:25 E:S ratio, which was significantly higher than 1:50 and 1:100 ratio at this duration.

5.2 Conclusions

The ultrafiltration and salting out technique followed in this study are able to obtain pure β-Lg. In case of α-La, the SDS-PAGE resolution showed the presence of β-Lg along with α-La. This could be due to cross linking of denatured β-Lg with α-La during milk pasteurization.

The α-La hydrolysates treated with pepsin has shown more DPP-IV inhibition than trypsin hydrolysates, therefore, α-La has better DPP-IV inhibition potential than β-Lg. The pepsin treated hydrolysates showed better DPP-IV inhibition activity in both α-La and β-Lg.

E:S ratio of 1:25 was found to be better for maximum DPP-IV inhibition, might be to availability of maximum substrate to enzyme. E:S ratio of 1:50 also found to be on-par (as there was no significant differences) with 1:25, but E:S ratio of 1:100 was found to not optimal for production of DPP-IV inhibitory peptides.

The peptides with better DPP-IV inhibition were found only after 10h of hydrolysis, therefore, it can be conclude that at least 10h hydrolysis time is required at 1:25 E:S ratio to generate maximum DPP-IV inhibitory peptides using α-La and β-Lg.

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