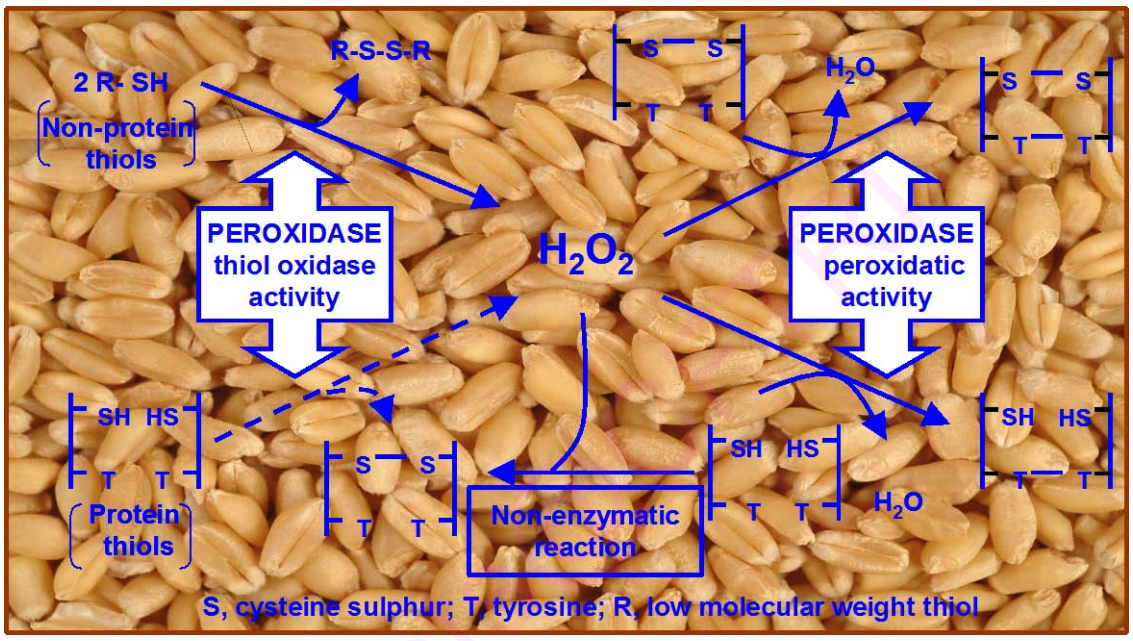
Effect of oxidative enzymes on structure-function of wheat proteins

A thesis

submitted to the

University of Mysore



for the award of the degree of

Doctor of Philosophy

In

BIOCHEMISTRY

By

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October 2008

Declaration

I, B. T. Manu, hereby certify that, the thesis entitled "**Effect of oxidative enzymes on structure-function of wheat proteins**" is the result of research work done by me under the supervision of **Dr. U. J. S. Prasada Rao**, at the **Department of Biochemistry and Nutrition**, **Central Food Technological Research Institute, Mysore, India**. I am submitting this thesis for the award of **Doctor of Philosophy degree** in **Biochemistry** of the **University of Mysore**. I further certify that the results presented in this thesis have not been submitted by me for the award of any other degree or diploma of this or any other University.

Signature of doctoral candidate
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Signature of Guide
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Signature of Head of the Department
with name and official seal

Date

ACKNOWLEDGEMENTS

I take this opportunity to express my deep sense of gratitude to all the people who directly or indirectly extended their help, advice and encouragement throughout this work.

I am sincerely grateful to:

My guide, Dr. U. J. S. Prasada Rao for his excellent guidance, extreme patience and critical review of the work.

Dr. V. Prakash. Director, CFTRI for giving me an opportunity to use the facilities and to submit the results in the form of a thesis.

My mentor, Dr. S. G. Bhat, former head of the department for his constant encouragement, valuable suggestions, keen interest and involvement in my work.

Dr. P. V. Salimath, Head of the department for valuable advice and guidance both in academic and personal matters.

My colleagues in the laboratory Ajila, Anitha and Hemalatha for their help and support at all the time.

My friends in the department for making my stay at CFTRI memorable.

Staff of central instrumentation facility and services for assisting in conducting many experiments.

My heartfelt thanks to my grandparents for their love and blessings. I am grateful to my parents, sisters, wife and all my family members for being with me through thick and thin.

The award of junior and senior research fellowships by the Council of Scientific and Industrial Research is gratefully acknowledged.

(B. T. MANU)

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LIST OF ABBREVIATIONS

$(NH_4)_2SO_4$	Ammonium sulphate
AACC	American Association of Cereal Chemists
CM	Carboxymethyl
cm	centimetre(s)
CTAB	Cetyltrimethylammoniumbromide
C-terminal	Carboxy-terminal
DEAE	Diethylaminoethyl
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EC	Enzyme commission
EDTA	Ethylenediaminetetra acetic acid
g	Gram(s)
GSH	Gluthathione reduced
h	Hour(s)
H_2O_2	Hydrogen peroxide
HMW-GS	High molecular weight glutenin subunits
Hz	Hertz
IAA	Indole acetic acid
kDa	Kilo dalton
kg	Kilo gram
LMW-GS	Low molecular weight glutenin subunits
mg	milligram(s)
min	minute(s)
ml	Millileter(s)
mM	Millimolar
mm/s	millimetre per second

N	Newton
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
nm	Nanomater
nmol/g	Millimole per gram
N-terminal	Amino-terminal
NTSB	Disodium 2-nitro-5-thiosulphobenzoate
SD	Standard deviation
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
sec	Second(s)
SE-HPLC	Size exclusion-high performance liquid chromatography
TEMED	Tetraethylmethylenediamide
TFA	trifluoro acetic acid
TPA	Texture profile analysis
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v/v	volume by volume
V_0	Void volume
Ve	Elution volume
w/v	Weight by volume
x g	times acceleration due to gravity
µg	Microgram(s)
µl	Microleter(s)
µM	Micromolar
µmol/g	Micromole per gram

Introduction

Wheat is an important cereal crop of the world. Food products made from wheat are the staple food for the majority of world population. Wheat is unique among the cereals because of its ability to form visco-elastic dough, when mixed with water. A variety of leavened and unleavened food products are made from wheat dough. The balance between elasticity and viscocity is wheat variety dependent and can differ greatly between varieties. This property has a major influence on the type of food product that can be prepared.

The visco-elastic property of the dough is mainly attributed to the gluten proteins namely gliadins, and glutenin. The polymeric glutenin provides elasticity and the monomeric gliadin provides viscosity to the dough. Glutenin contains subunits namely high molecular weight glutenin subunits and low molecular weight glutenin subunits, cross-linked by disulfide and dityrosine bonds. The composition of glutenin subunits profoundly affects the rheological properties of dough and quality of products made from dough. The wheat protein cross-linking also has an influence on the properties of the dough and the subsequent baked product. Many chemical agents have been used in bakery industry as dough improvers. But there is a paradigm shift towards the use of enzymes because of the toxicity of chemicals used as dough improvers. Many enzymes such as protease, amylase, lipase, oxidative enzymes like glucose oxidase, hexose oxidase, polyphenol oxidase, peroxidase etc., are reported to improve the quality of dough. Wheat also contains a few endogenous oxidative enzymes like peroxidase, polyphenol oxidase, and super

oxide dismutase etc. Peroxidases are ubiquitous enzymes and reported to have various physiological roles and industrial applications. Exact mechanism by which peroxidase enzyme improves the dough quality is not understood. However, peroxidases from different sources have been shown to cross-link several food proteins including bovine serum albumin and β-lactoglobulin. Peroxidase may have a similar role in cross-linking of wheat proteins and dough formation. Therefore, the major focus of the present thesis was to study the role of peroxidase in wheat dough formation.

Thus, the present study has following three objectives:

a) To purify and characterize the enzyme peroxidase from wheat bran
b) To elucidate the role of peroxidase in cross-linking of wheat proteins
c) To study the role of wheat proteins in rheological properties of dough and chapati texture.

An outline of the studies carried out towards this end forms the subject matter of this thesis and is presented chapterwise.

Chapter I. General Introduction

This chapter provides an overview of the current knowledge regarding wheat proteins and oxidative enzymes, with special reference to wheat protein cross-linking. The topics covered in this chapter include a general account of wheat and wheat dough, wheat protein classification, structure and composition of gluten, glutenin and their role in dough functionality. A brief description of enzymes that are reported to have influence on dough and product quality with more emphasis on peroxidase is presented in this chapter. Current literature on protein cross-linking relevant to food systems has been given. This chapter also highlights the objectives and scope of the present investigation.

Chapter II. Materials and Methods

This chapter provides details of various materials and methodologies used to conduct experiments in the present study.

Chapter III. Results and Discussion

This chapter deals with the results obtained during the present investigation, which is organized in three sections followed by a general discussion. Section A deals with the purification and characterization of peroxidase from wheat bran. Section B deals with the role of peroxidase in cross-linking of isolated glutenin subunits. The subject matter of section C is the role of wheat proteins in determining the rheological properties of dough and chapati quality.

Section A: Purification of wheat bran peroxidase and its characterization

Contents of this section are divided into two divisions, a) screening different oxidative enzymes in wheat flour and purification of wheat bran peroxidase and, b) Characterization of purified wheat bran peroxidase.

a) Screening different oxidative enzymes in wheat flour and Purification of wheat bran peroxidase

Different wheat varieties were screened for few oxidative enzymes viz, peroxidase, lipoxygenase, polyphenol oxidase and superoxide dismutase. The varieties screened showed good variation in different enzyme activities. As wheat contains good amount of peroxidase, work was focused on peroxidase. Peroxidase activity in different milled fractions of wheat was determined, and bran was selected as source for purification because it contains good amount of peroxidase, free from gluten proteins that complicate the purification, and it is a cheap byproduct of wheat milling industry. Peroxidase from wheat bran was extracted with water and purified by ammonium sulfate precipitation, anion exchange chromatography, cation exchange chromatography and gel filtration chromatography. Purified enzyme showed a single band on SDS-PAGE. A typical hemoprotein spectrum with a characteristic peak at 280nm for protein and 403nm for heme moiety was observed. When peroxidase in crude extract was assayed in buffer containing 1 mM calcium chloride a phenomenal increase in its activity was observed. Therefore, purification was also carried out by including calcium chloride in all the steps of purification. The specific activity of purified peroxidase was 1026 U/mg with a yield of 12%. Although there was no

change in the overall purification profile, specific activity was 10 fold (10240 U/mg) and yield was 1.4 fold (17%) more when the purification was carried out in presence of calcium.

b) Characterization of peroxidase purified from wheat bran

Purified wheat bran peroxidase was a glycoprotein with a molecular weight of 44 kDa. The pH optimum of purified peroxidase was 4.8. Kinetic study using different substrate concentrations revealed that the peroxidase follows bi bi ping pong type of reaction. The kinetic parameters like Km, both in presence and absence of calcium were determined. The Km values for o-dianisidine were 5 x 10^{-3} M and 10 x 10^{-3} M, in absence and presence of calcium, respectively. The Km values for H_2O_2 were 7.14 x 10^{-3} M and 40 x 10^{-3} M, in absence and presence of calcium respectively. The purified peroxidase exhibited thiol oxidase activity. The thiol oxidase activity utilized cysteine as substrate and generated H_2O_2. Production of H_2O_2 increased as cysteine concentration increased up to about 500 µM, beyond this there was decrease in its production. The pH optimum for thiol oxidase activity was 8. Addition of calcium in the range of 0.5 mM to 5 mM increased the peroxidase activity in a dose dependent manner up to 444%. The activity increase by calcium was biphasic in nature with an initial rapid increase followed by slow increase of activity. The thiol oxidase activity was also increased by 500% in presence of calcium. An attempt was made to explain structural basis for the calcium mediated activity enhancement of peroxidase. Addition of calcium did not

change the tryptophan fluorescence spectra of purified peroxidase but produced a change in soret band absorption at 403nm. Thermal stability studies showed that about 80% activity lost at 40^0C and heating at 60^0C for 25 min destroyed almost all peroxidase activity. Presence of calcium improved the thermal stability of purified peroxidase. In presence of calcium peroxidase retained 50% of activity even after heating for 25 min at 60^0C. Heating of peroxidase at 60^0C for 25 min resulted in the loss of 50% of thiol oxidase activity whereas in presence of calcium 83% of thiol oxidase activity was retained.

Section B: Role of peroxidase in cross-linking of glutenin proteins

The results presented in this section reveal the ability of peroxidase to cross-link isolated glutenin subunits. Isolated glutenin subunits were first treated with different concentrations of H_2O_2 and different units of purified wheat bran peroxidase, separately and in combination. SDS-PAGE of treated subunits under reducing and non-reducing conditions revealed that H_2O_2 was able to cross-link glutenin proteins by disulfide bonds but in combination with peroxidase it also created non-disulfide covalent cross-links. The fluorescence spectrum of cross-linked proteins indicated the presence of dityrosine.

Section C: Fractionation of wheat flour proteins and correlation to flour functionality

The relation of wheat proteins and thiol content with rheologicalcl properties and chapati quality is presented in this section. The high molecular weight glutenin subunit composition of different wheat varieties was determined

and the Glu-1 score was deduced and related to chapati quality. Wheat proteins were fractionated based on their size by gel filtration chromatography on Sephdex G-200 and the correlation between different fractions and rheological/ chapati quality were determined. SE-HPLC was also used to fractionate the wheat proteins. The thiol and disulfide contents in flours of different wheat varieties were determined. The percentage of polymeric protein in flour protein significantly positively correlated to dough hardness and chapati cutting force. The quantity of non-protein thiol showed a negative correlation to the dough hardness and chapati cutting force.

Section D: General discussion

The dough formation is a complex phenomenon involving interaction between various flour components such as proteins, carbohydrates, small molecular weight thiols etc. A number of enzymes including oxidative enzymes are reported to have roles in the process of dough formation. Peroxidase is one such enzyme present in wheat in significant amount. In the present investigation an attempt was made to elucidate the role of peroxidase in dough formation. The results indicated that variation in protein size distribution has a significant effect on the rheological properties of the dough. Peroxidase affects the protein size distribution by two mechanisms. Firstly, by thiol oxidase activity, peroxidase oxidizes small molecular weight thiols and generates H_2O_2. This alters the ratio between oxidized and reduced forms of these compounds and thus makes the thiol-disulfide redox status favorable for the formation of disulfide cross-links

between glutenin molecules. Second mechanism is the direct cross-linking of glutenin subunits by peroxidase. In presence of H_2O_2, peroxidase catalyzes the formation of dityrosine cross-links between glutenin subunits.

Chapter IV: Summary and Conclusion

Different wheat varieties showed good variation in the activities of few oxidative enzymes like peroxidase, polyphenol oxidase, lipoxygenase and superoxide dismutase. Peroxidase enzyme has been purified from wheat bran. Purified peroxidase was a glycoprotein and a hemoprotein. Peroxidase activity was greatly influenced by calcium chloride. The activity increase by calcium was dose dependent and was in a biphasic manner. A method has been developed to purify peroxidase in presence of calcium. This purification method gave good specific activity and better yield of enzyme. In addition to peroxidase activity, purified peroxidase also exhibited thiol oxidase activity. Thiol oxidase activity of peroxidase oxidized cysteine and generated H_2O_2. The thiol oxidase activity was also increased by calcium. Presence of calcium improved the thermal stability of both peroxidase activity and thiol oxidase activity. Peroxidase catalyzed the formation of non-disulfide covalent cross-links (dityrosine) in isolated glutenin subunits. The percentage of polymeric proteins influenced the rheological properties of dough and textural properties of chapati. The results also indicated that flours containing more amount of thiols yielded dough with less hardness, and chapati with soft texture.

The polymeric proteins influence the dough rheological properties. Thiol oxidase activity of peroxidase may have an important role in the *in situ* H_2O_2 generation. Peroxidase by virtue of its dual activity may directly catalyze the formation of covalent cross-links between glutenin subunits and indirectly facilitate the formation of these cross-links through shifting the redox status of dough towards oxidizing environment by means of removing small molecular weight thiols.

After chapter IV a collective bibliography for all the chapters is presented.

CHAPTER I

GENERAL INTRODUCTION

1.1. The Wheat in perspective

1.1.1. General considerations

Wheat is an important cereal crop of the world. Food products made from wheat are the staple food for the majority of world population. Botanically wheat belongs to the family graminae; genus triticum and eighteen species are identified under this genus. Among these 18 species, most commonly cultivated and used are aestivum and durum [Shellenberger, 1978]. *Triticum aestivum* is hexoploid whereas *Triticum durum* is tetraploid wheat. *Triticum aestivum* has 42 chromosomes derived from three different but related genomes viz, A, B, D and each genome contains seven pairs of chromosomes [Payne, 1987].

1.1.2. Characteristics of wheat grown in India

Three species of wheat are being cultivated in India. They are bread wheat (*T. aestivum*), macaroni wheat (*T. durum*) and emmer wheat (*T. dicoccum*). Bread wheat occupies nearly 88% of the total area; a little over 11% is occupied by macaroni wheat, while emmer wheat is grown in very limited area [Rao, 1978]. All the varieties of wheat grown in India, although planted in winter, are essentially of the spring type. In India, about 90% of wheat is consumed as unleavened pan-baked bread, which is called ‘chapati’ [Rao, 1978a].

1.1.3. Structure and composition of wheat grain

Wheat grain, a one seeded fruit also called kernel, strictly speaking a caryopsis, is from 4 to 10 mm long depending on the variety and location. Kernal consists of germ, or embryo, and endosperm enclosed by an epidermis

and a seed coat. A fruit coat, or pericarp, also called testa, surrounds the seed coat.

During milling, seed coat and fruit coat separate as bran. Wheat grain contains 2-3% germ, 13% bran and 83-85% endosperm on whole grain weight basis. The chemical compositions of these three parts differ markedly (Table 1.1). The flour obtained after roller flour milling, which is devoid of bran and germ is called white or refined flour and is mainly used for the preparation of leavened products like bread. When wheat is milled in a chakki mill, all the components of the kernel are retained and the flour is called whole-wheat flour, which is mainly used for the preparation of chapati, unleavened flat bread used in Indian subcontinent.

Table. 1.1. Composition of wheat grain, endosperm, bran and germ [Bushuk, 1986]

Constituent	Grain %	Endosperm %	Bran %	Germ %
Dry matter	100	(82)	(15)	(3)
Carbohydrate	82.7	86.4 (85)	70 (13)	50.6 (2)
Protein (Nx5.7)	12.8	11.2 (72)	16.7 (20)	32.4 (8)
Fat	2.5	1.6 (52)	5.4 (32)	11.9 (16)
Minerals	2	0.8 (34)	7.4 (58)	5.1 (8)

Figures in bracket are % of total in the grain

1.1.4. Applications of wheat

Wheat is unique among all the cereals because, when wheat flour is mixed with water a visco-elastic dough is formed. The balance between elasticity and viscocity is genetically controlled and can differ greatly between varieties. This property has a major influence on the type of food product that can be prepared. Samples of wheat flour vary widely in strength, and this is exploited in selecting grain for specific end use. For example, weak flours are preferred for making cakes and biscuits, and strong flours for breadmaking. Therefore, wheat flour can be processed into a variety of food products like bread, pasta, biscuit, chapati etc. Apart from food uses, wheat is used for the manufacture of biodegradable films. The visco-elastic property of the wheat dough is attributed mainly to the proteins present in the wheat.

1.2. Wheat proteins

1.2.1. General description of wheat proteins

The protein content of the wheat is generally given as 12.8% (Table 1.1). However the total protein content varies depending on the variety of wheat and growing conditions. The range of protein content in wheat is generally found to be 10-13% but protein values as high as 21% and as low as 6.5% have also been reported [Lasztity, 1984; Huebner, 1976]. Endosperm contains about 70% of the total protein of the grain. Total protein of the wheat can be classified into two groups viz, gluten proteins and non-gluten proteins. Gluten proteins are the storage proteins of the seed whereas non-gluten proteins are metabolic (mainly enzymes) or structural proteins. All

water soluble proteins like albumins and globulins are classified under non-gluten proteins. Approximate distribution of these proteins is given in Fig. 1.1 In general, albumins and globulins each account for about 6-10% of the total protein [Schofield and Booth, 1983].

1.2.2. Gluten and its composition

Gluten is a complex material, which can be obtained, in the form of a rubbery mass by gentle washing of flour-water dough in excess water to remove most of the starch and much other soluble material. It was first isolated by Beccari in 1782. On freeze drying, gum gluten gives a light solid, which consists of 75-80% protein, 5-10% lipid and 10-15% carbohydrate [Kasarda et al, 1976]. Gluten represents about 80% of the total protein of the flour. Gluten proteins were extensively studied by Osborne in the beginning of 20th century and his method of isolation and nomenclature are still used. In his method, gluten is first extracted with 70% alcohol to remove gliadin; this is followed by an extraction with dilute acetic acid to obtain glutenin and the residue is called residue protein or insoluble glutenin. The components that constitute wheat flour proteins is given in Fig. 1.1.

The amino acid composition of wheat grain, wheat flour and wheat proteins, viz. albumin, globulin, gliadin, glutenin and residue protein are given in Table 1.2. Gluten proteins are also called prolamins due to the higher content of amino acids, proline and glutamine present in them. Gliadins and glutenins, differ in their polymerization properties also: gliadins are monomeric proteins that form only intra-molecular disulphide bonds, if present, whereas

glutenins are polymeric proteins whose subunits are held together by inter-molecular disulphide bonds, although intra-chain disulphide are also present.

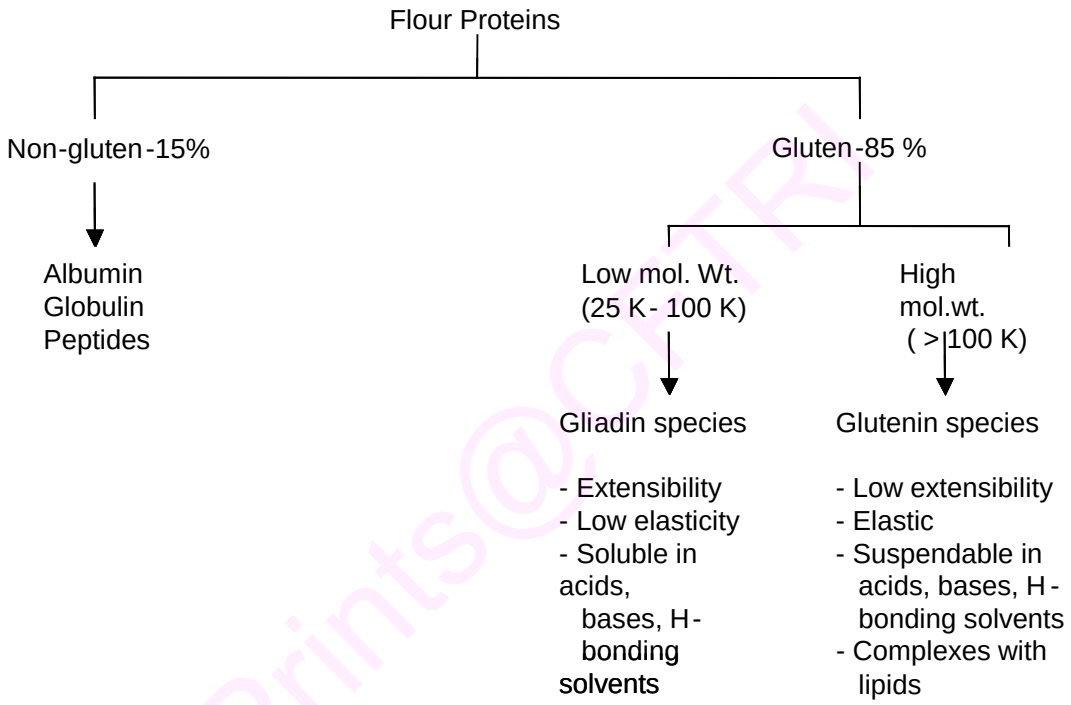


Fig.1.1. A schamatic representation of the protein fractions of wheat flour (Source: Pomeranz, 1971)

1.2.3. Gliadin

Gliadin is highly heterogeneous mixture of proteins and is the 70% ethanol soluble fraction of wheat gluten protein. The amount of gliadin is a varietal characteristic and, in general, its content in gluten ranges from 30-40% [Huebner et al, 1976]. When fractionated by gel electrophoresis at low pH gliadins separate into 4 groups viz, α, β, γ, and ω type gliadins [Jones et al, 1959]. Two-dimensional gel electrophoresis suggests that a single wheat variety contains about 45 gliadin components. Gel filtration chromatography, SDS-PAGE and ultracentrifuge techniques have been used for determining the molecular weights of gliadins. On the basis of molecular weights gliadin can be classified into two groups. They are ω-gliadins having molecular weights in the range of 64,000-80,000 and α, β, γ, -gliadins having molecular weights in the range of 34,000-44,000 [Bietz and Wall, 1972; Charbonnier, 1974]. α–type and γ-type gliadins contain highly conserved cysteine residues and accordingly they are also called sulfur-rich prolamins. In contrast, ω-type gliadins lack cysteine residues and therefore these gliadins are classified separately as sulfur-poor prolamins [Shewry et al, 1986].

Table. 1.2. Amino acid composition of wheat grain, wheat flour and wheat proteins.

Amino acid	Wheat[1] grain	Wheat[1] Flour	Albumin[1]	Globulin[1]	Gluten[2]	Gliadin[1]	Glutenin[1]	Residue Protein[1]
Asp	4.7	3.7	5.8	7.0	2.7	1.9	2.7	4.2
Thr	2.4	2.4	3.1	3.3	2.3	1.5	2.4	2.7
Ser	4.2	4.4	4.5	4.8	3.8	3.8	4.7	4.8
Glu	**30.3**	**34.7**	**22.6**	**15.5**	**38.9**	**41.1**	**34.2**	**31.4**
Pro	**10.1**	**11.8**	**8.9**	**5.0**	**14.4**	**14.3**	**10.7**	**9.3**
Gly	3.8	3.4	3.6	4.9	3.2	1.5	4.2	5.0
Ala	3.1	2.6	4.3	4.9	2.4	1.5	2.3	3.0
Cys	2.8	2.8	6.2	5.4	1.6	2.7	2.2	2.1
Val	3.6	3.4	4.7	4.6	4.8	2.7	3.2	3.6
Met	1.2	1.3	1.8	1.7	1.6	1.0	1.3	1.3
Ile	3.0	3.1	3.0	3.2	4.0	3.2	2.7	2.8
Leu	6.3	6.6	6.8	6.8	7.1	6.1	6.2	6.8
Tyr	2.7	2.8	3.4	2.9	3.3	2.2	3.4	2.8
Phe	4.6	4.8	4.0	3.5	4.8	6.0	4.1	3.8
Try	1.5	1.5	1.1	1.1	1.1	0.7	2.2	2.3
His	2.0	1.9	2.0	2.6	2.1	1.6	1.7	1.8
Lys	2.3	1.9	3.2	5.9	1.2	0.5	1.5	2.4
Arg	4.0	3.1	5.1	8.3	3.2	1.9	3.0	3.2

[Bushuk, [1] 1986; Kasarda et al, [2] 1971]

1.2.4. Structure of gliadins

It is generally believed that gliadins are single polypeptide chains having intramolecular disulfide bridges. Molecular weight determination of gliadins, before and after reduction, have given similar values both by SDS-PAGE and ultracentrifugal studies, thereby indicating the absence of intermolecular disulfide bonds in gliadin molecules [Graveland et al, 1982].

The fact that gliadins are very rich in proline (15-30%) has an effect on the secondary and tertiary structure of gliadins. Proline is believed to provide the turning points in the folding of polypeptides and its very high content in gliadin may be responsible for their folded globular structure. Proline is also known to interrupt the ordered secondary structures like α-helices and β-structures in proteins and may prevent the polypeptide chains of gliadins from assuming extended ordered structures. Glutamine, which is the major amino acid in gliadin, acts as an H-bond donor and acceptor. Therefore, it can participate in intramolecular H-bond formation with other donor and acceptor amino acids as well as itself. It has been observed that a large amount of glutamine facilitates in the formation of β-structures in synthetic polypeptides of glutamine [Krull et al, 1965]. However, only 10% β-structure is present in gliadin. This may be due to the presence of high amounts of proline. Optical and viscosity studies on gliadins have revealed that gliadins may have compact tertiary structures similar to those of globular proteins [Kasarda, 1970].

The α-helical content in gliadin is about 35% in 70% ethanol [Kretschmer, 1958]. Kasarda et al, have shown that the helical conformation of α-gliadin is stable below 35^0C. Above this temperature the helical content

decreases, but even at 90^0C about 25% of the helical structure present at 25^0C remains intact.

1.2.5. Glutenin

Removal of gliadin by extraction with 70% ethanol from gluten leaves behind 60-70% of gluten. About one half of this gluten can be extracted with dilute acetic acid and this acid soluble and alcohol insoluble protein is known as Osborne glutenin. This represents about 30-40% of the total gluten [Huebner et al, 1976]. Glutenin is a mixture of many proteins. Determination of exact molecular weight of glutenin has been very complicated task because of its low solubility and huge size, which is still difficult to resolve by modern protein chemistry techniques. In the early 1960s several groups of workers determined its molecular weight by ultracentrifugal studies. By using the sedimentation equilibrium method, weight average molecular weight of glutenin was found to be 1.5 to 2 million in aluminium lactate-lactic acid (pH 3.1) by Jones et al, (1964) and about 300,000 in 0.03 M NH_4OH containing 1 M urea (pH 10.6) by Nielsen et al (1962).

Subsequently, the molecular weight of glutenin was more often estimated by gel filtration chromatography. Huebner and wall (1976) chromatographed the AUC (0.1 M acetic acid, 3 M urea, 0.01 M CTAB) extracts of several varieties of wheat flour on Sepharose 4B and 2B with 5.5 M guanidine hydrochloride as eluent and obtained four fractions. They designated the first two peaks as glutenin I and glutenin II and estimated their molecular weight as more than 20 million and 100,000 to 5 million, respectively.

Graveland et al, (1985) have chromatographed glutenin both on Sepharose CL-4B and Sephacryl S-400 using 1.5% SDS as eluent. In both cases two peaks were obtained. The molecular weight of these peaks was estimated to be more than 20 x 10^6 and 8 x 10^5 respectively. Very recently, flow field-flow fractionation has been used to fractionate and characterize very small amounts of wheat proteins. Its use for studying larger polymeric wheat proteins may be particularly advantageous, since, unlike electrophoresis and size-exclusion chromatography, resolution is not impeded by an exclusion limit [Stevenson and Preston, 1996].

1.2.6. Glutenin subunits

Glutenin is made up of two types of building blocks viz, high molecular weight glutenin subunits (HMWS-GS) and low molecular weight glutenin subunits (LMW-GS) stabilized by disulphide bonds. More than 20 different HMW-GS were detected so far in wheat varieties [Veraverbeke and Delcour, 2002]. LMW-GS are more heterogeneous and less well characterized than HMW-GS. About 40 different LMW-GS are identified in *Triticum aestivum* and number of different LMW-GS in a single variety varies from 7 to 16 [Gupta and Shepherd, 1990].

1.2.7. High molecular weight glutenin subunits

In *Triticum aestivum*, which is a hexaploid containing A, B and D genomes, high molecular weight glutenin subunits (HMW-GS) are encoded at the loci Glu-A1, Glu-B1 and Glu-D1, on the long arms of group 1 chromosome

[Payne et al, 1982]. At each locus, two types of HMW-GS are encoded, a higher molecular weight y-type and a lower molecular weight x-type. These two types of subunits can be distinguished by their mobility on SDS-PAGE, x-type having greater mobility than y-type [Payne et al, 1981]. Therefore, one would expect six different HMW-GS in *Triticum aestivum* wheat varieties. However, for the reasons unknown at present the genes encoding the x-type HMW-GS at the Glu-A1 locus and the y-type HMW-GS at the Glu-B1 locus are not always expressed, while the gene encoding the y-type HMW-GS at the Glu-A1 locus is generally not expressed. Because of this type of expression pattern the number of different HMW-GS found in *Triticum aestivum* wheat varieties varies from three to five [Payne et al, 1981; Payne and Lawrence, 1983]. However, in rare cases six HMW-GS can occur [Margiotta et al, 1996].

HMW-GS are named indicating their genetic locus and the type and a number which reflect a ranking according to decreasing molecular weight. In practice this number is determined by the mobility of the peptide on SDS-PAGE e.g., 1Bx7 and 1By8 etc [Payne and Lawrence, 1983]. But after the original ranking, new HMW-GS with mobility intermediate between the previously detected were discovered. However, the original ranking is retained and new numbers are assigned to newly discovered HMW-GS as and when they were reported. Therefore now this numbering does not necessarily represent the molecular weight. Sometimes symbols or decimal numbers (e.g., 2* vs. 2 and 2.1 vs. 2) are added to distinguish HMW-GS with similar mobility on SDS-PAGE. The molecular weight of HMW-GS varies from 80,000 to 120,000 as determined by SDS-PAGE [MacRitchie, 1992].

1.2.8. Structure of HMW-GS

Both x-type and y-type HMWS-GS have a three-domain structure consisting of relatively small N- and C-terminal domains flanking a major repetitive central domain. C-terminal domain has a constant size (42 amino acid residues) and the N-terminal domain varies slightly in length (about 80 to 90 amino acid residues for x-type and 104 amino acid residues for y-type). The length of the central domain is much more variable (about 750 to 850 amino acid residues for x-type and 600 to 700 amino acid residues for y-type HMW-GS). All cysteine residues are located at the ends of the HMW-GS. The secondary structure of N- and C-terminal domains is predominantly α-helix whereas, central domain consists of repetitive sequences that are rich in proline, glutamine and glycine. These repetitive sequences are thought to produce overlapping β-reveres-turns that may form a β-spiral super secondary structure (Fig. 1.2).

These structural features of HMW-GS may be related to their role in determining gluten elasticity. The spiral structure formed by the central repetitive domain contributes to the elastic property by employing non-covalent interactions. This can be compared to the elastic property of the mammalian connective tissue protein, elastin. Elastin has a complex structure, with regions of repeated sequences containing three types of repeats, tetra-, penta- and hexapeptide motifs, which produce β-turns [Urry et al, 1983]. In the case of elastin, stretching probably disrupts an energetically stable state, deforming the peptide backbone and hydrogen bonds and exposing hydrophobic residues to an aqueous environment. Elastic recoil results from the reformation of the

stable state when the stress is removed. The ability of the polypentapeptide to undergo elastic deformation may relate, in part, to the pattern of hydrogen bond formation, which is predominantly intermolecular [Tatham et al, 1985]. The mechanism discussed for elastin could also be applied to the spiral formed by the HMW-GS. Hydrogen bonding could occur via the side chains of glutamine residues, as well as the peptide backbone, with hydrophobic interactions involving the tyrosine residues of the nonapeptide repeats. The stability of the HMW-GS to denaturation is also consistent with this mechanism.

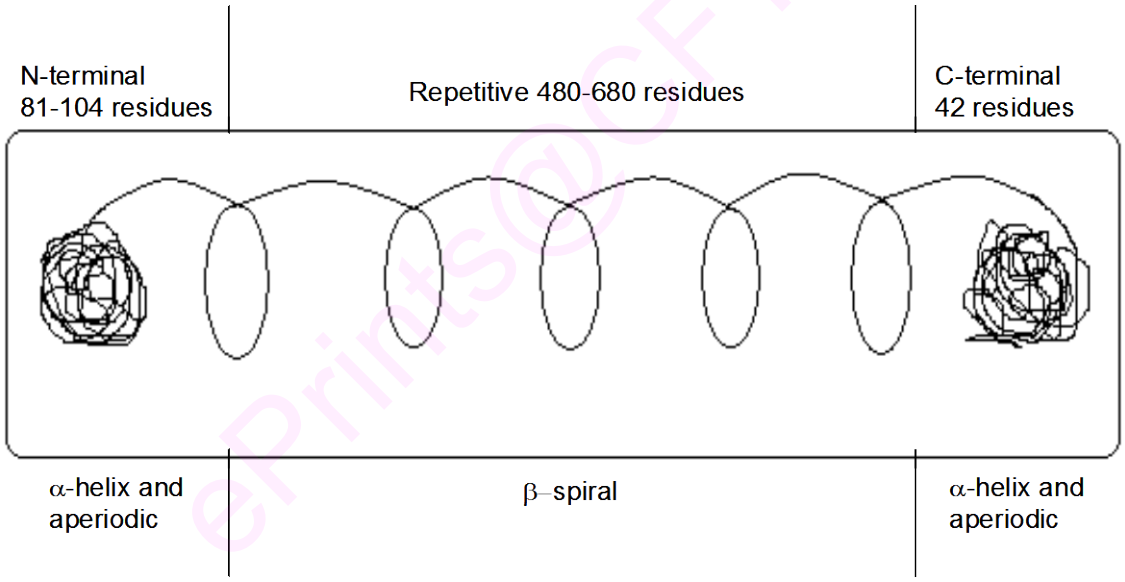


Fig.1.2. A structural model for the HMW-GS, based on predictive, spectroscopic and hydrodynamic studies [Shewry et al, 1992].

The second aspect relevant to elasticity is the degree of cross-linking which is an important factor in determining the bulk elastic properties. The degree of cross-linking is related to the number and distribution of cysteine

residues available to form intermolecular cross-links. The HMW-GS have cysteine residues predominantly on either end of the molecules, allowing deformation/reformation to occur in the central domain. Changes in the number of cross-links would be expected to have major effects on the physical properties of the HMW-GS polymers. With a low degree of cross-linking the extensibility will be high, but with increased cross-linking the material would become more rubber-like. Finally the type of cross-link might be important. Glutenin molecules are cross-linked by disulfide bonds, whereas other proteins which exhibit elastic property have covalent cross-links formed by the condensation of lysine or tyrosine residues, which are unaffected by the addition of reducing agents. The ability of disulfide bonds to exchange and even shear under stress may result in changes in gluten elasticity and viscocity. Although there is high degree of similarity between x-type and y-type HMW-GS, considerable differences exist between them, which are critical for the structure, and functionality of the glutenin polymers. Important among these differences are, molecular weight, length of the central repetitive domain, number and distribution of cysteine residues (Fig 1.3).

1.2.9. Low molecular weight glutenin subunits

Low molecular weight glutenin subunits (LMW-GS) were first identified by gel filtration of extracts of wheat flour as high-molecular-weight gliadins linked by disulfide bonds [Beckwith et al, 1966]. Later, Nielsen et al, (1968) designated this particular fraction as low-molecular-weight glutenin, because their viscosity and electrophoretic mobility differed from those of the gliadin

fraction. Most of the LMW-GS are encoded by genes on the short arm of the group 1 chromosomes, at the complex *gli-I* loci that also encode γ- and ω-gliadins [Payne et al, 1985]. They were originally classified as B, C and D subunits based on mobility on SDS-PAGE [Jackson et al, 1983]. Based on amino acid sequence similarities, both B-type and C-type LMW-GS are classified together with the monomeric α–type and β-type gliadins as sulfur-rich prolamins. D-type LMW-GS are classified together with monomeric ω-type gliadins as sulfur-poor prolamins [Shewry et al, 1986]. The molecular weight of LMW-GS varies from 30,000- 50,000 as determined by SDS-PAGE [Payne and Corfield, 1979].

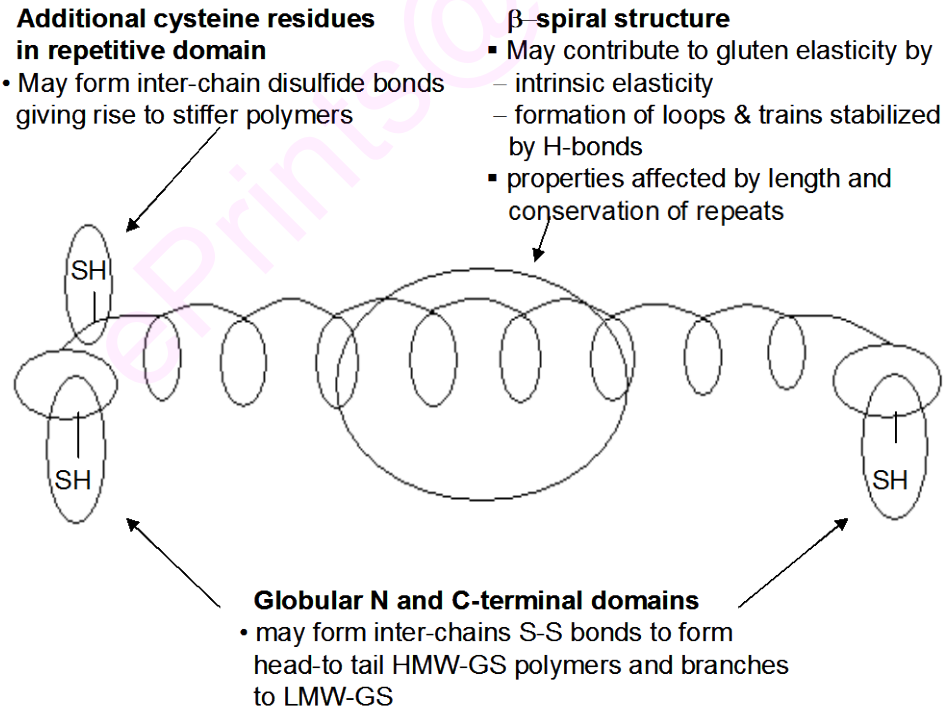


Fig.1.3. Summary of our current knowledge of the structural features of the HMW-GS, [Shewry, et al, 2001].

1.2.10. Structure of LMW-GS

Sulfur-rich prolamins have a two-domain structure. The N-terminal domain contains repetitive sequences rich in proline and glutamine and is predicted to form predominantly β-reverse turns like in HMW-GS. This repetitive domain is preceded by a very short nonrepetitive N-terminal sequence. The C-terminal consists of nonrepetitive α-helical secondary structure. Almost all cysteine residues are located in this domain. The sulfur-poor prolamins contain almost entirely repetitive sequences with very small nonrepetitive N- and C-terminal domains. The repetitive domain is rich in proline, glutamine and phenylalanine and is made up of overlapping β-reverse turns that give a rod like structure to sulfur-poor prolamins [Shewry et al, 1994].

1.2.11. Structure of glutenin

On the weight basis the ratio between HMW-GS and LMW-GS varies from 0.18 to 0.74. This corresponds to a slight to almost six-fold excess of LMW-GS content over the HMW-GS, on the weight basis [Veraverbeke and Delcour, 2002]. Initially glutenin was thought to have a linear polymeric structure consisting of glutenin subunits that are randomly linked head-to-tail by inter chain disulfide bonds. However, partial and controlled reduction studies of glutenin polymers with disulfide reducing agents suggested an ordered assembly of different subunits in glutenin polymers [Kim and Bushuk, 1995; Lindsay and Skerritt, 1998]. Although this type of nonrandom structure hypothesis gathered lot of evidences from different studies, various criticisms raised against it are also worth mentioning. Important among these criticisms is the complex biosynthetic mechanism required for the synthesis of such a

nonrandom structure. A model has been proposed to explain the overall structure of glutenin in which the HMW-GS form an elastic backbone consisting of head to tail polymers with inter-chain disulfide bonds. From this backbone LMW-GS branch out again with inter-chain disulfide bonds. Gliadins interact with glutenin polymers by non-covalent forces, contributing to viscosity of the gluten. Non-covalent interactions between glutenin subunits and polymers also contribute to the elasticity [Shwery et al, 2001].

The experimental results obtained with proteins in the dry or hydrated solid state led to the following conclusions. The proteins are disordered and have little structure when dry. On hydration, proteins gain more mobility and form β-sheet structures. Further hydration leads to further mobility and turn-like structures are formed at the expense of sheet-like structures [Belton et al, 1994; Belton et al, 1995; Wellner et al, 1996; Belton et al, 1998]. The structural behavior of HMW-GS upon hydration of flour to form dough has been explained by “loop and train model” [Belton, 1999]. In the low hydration state there are many protein-protein interactions through hydrogen bonding of glutamine residues. Further increase in hydration lead to the formation of hydrogen bonds between water and glutamine, resulting in the formation of regions in which interchain interactions are broken. Therefore, extension of the dough will result in strain in the network, with the loop becoming stretched and the train regions becoming unzipped at the low extension. As further extension occurs, stiff chains are formed. The formation of extended chains is a mechanism by which elastic energy is stored in the dough. Water plays a critical role in the ratio of

loops to trains. Since the deformation of loops requires less energy than the unzipping of trains, more water will result in a more deformable system.

1.3. Wheat dough

1.3.1. Composition of wheat dough

Dough is a complex mixture formed by the mixing of wheat flour with water. The main components of the dough are proteins and carbohydrates. The most important component is the proteins that include gluten, albumins, globulins and lipoproteins. The most abundant components are the carbohydrates, which include starch, sugars, and soluble and insoluble polysaccharides. The lipids form a small but significant part of the dough. Mixing yields an apparently homogenous mass from the ingredients [Bloksma, 1978].

1.3.2. Dough mixing and microscopic structure

At first when flour is mixed with water a mass of wet lumps with less coherence is formed. Continuation of mixing increases the coherence and the dough develops elastic properties. Further mixing makes the dough more smooth and dough becomes more dry. The acquisition of theses desirable properties is called dough development. When mixing is continued beyond this stage dough eventually loses its elasticity, becomes highly extensible and sticky. This loss of desirable properties is referred to as breakdown. Microscopic structure of dough is far from homogenous. In an unleavened dough three phases like starch, protein and gas cells can be distinguished. In leavened dough, yeast cells constitute a fourth phase [Baker and Mize, 1946].

In dough development, effect of mixing is two fold: the more or less even distribution of ingredients and the development of gluten structure. Testing of the behavior of the dough under deformation is called as dough rheology.

1.3.3. Physical factors that influence dough rheology

Mechanical work and temperature are the two important physical properties that influence the dough rheology. The mechanical work, which in case of dough is in the form of mixing, can cause formation as well as rupture of bonds in the gluten complex. Accordingly, mixing can increase or decrease the resistance of dough to deformation. The dough rheological properties like mixing time, extensibility etc, decrease with increase in temperature. Temperature can also have an indirect effect on dough properties. Chemical reactions in dough proceed faster at a higher temperature [Bloksma, 1978].

1.3.4. Chemical factors that influence dough rheology

Protein is the most important chemical factor that influences the dough properties. Experiments with starch pastes have shown that a minimum of 7.5% of protein in starch-protein mixtures is necessary for dough development [Markley, 1938]. Experiments with flours of various protein contents and flour gluten mixtures showed that the water absorption, dough development time, dough stability, resistance and extensibility, all increase with protein content [Merrit and Stamberg, 1941]. These rheological properties also depend on the quantity and quality of protein fractions such as glutenin and gliadin and the ratio between high and low molecular weight glutenin subunit contents [Smith and Mullen, 1965].

Starch is the most abundant carbohydrate in wheat dough, which gelatinizes during the transformation of dough in to bread [Sandstedt, 1961]. During milling about 5 to 10% of starch is damaged and becomes available for starch-degrading enzymes. This damaged starch also increases the dough water absorption. After enzymatic degradation the water absorbed by the damaged starch is set free, which results in a softening of the dough [Greer and Stewart, 1959]. Pentosans are a small group of carbohydrates, which constitute about 2 to 3% of the flour but have large effect on dough water absorption. They also contribute to the dough strength by forming cross-linked polymers through ferulic acid [Kulp, 1968; Painter and Neukom, 1968].

1.3.5. Role of glutenin in dough functionality

Glutenin quality is related to its degree of cross-linking. The glutenin extractability in different solvents and gel permeation chromatography studies carried out at different laboratories over many years have clearly indicated a direct relation between glutenin proteins and dough strength and/or bread-making performance [MacRitchie et al, 1990; Veraverbeke and Delcour, 2002; Lee et al, 2002]. The subunit composition of glutenin determines its size and ultimately its quality to a large extent. The number and type of subunits present in a particular wheat variety have a great influence on the dough strength [Shwery et al, 1992]. Although the information on LMW-GS is scarce, role of HMW-GS in determining the dough strength is very well established. Certain pairs of HMW-GS like 1Dx5 + 1Dy10 are known to give superior quality whereas other HMW-GS like 1Dx2 + 1Dy12 confer inferior quality to the dough

[Popineau et al, 1994]. The effect of glutenin subunits on its size and quality are due to the number, position and reactivity/accessibility of cysteine residues that are potentially involved in inter-chain disulfide bonds. Thus, properties of glutenin that determine its quality include structure, size distribution and composition. Although variations in these properties are largely dependent on genetics, environment can play a role as well.

1.3.6. Dough improvers

Observations in the bakery industry had revealed that freshly milled flour produced poor quality baked products while flour stored 5-6 weeks after milling produced good quality products. In the late 1940s and into the 1950s, the need for gains in productivity resulted in the introduction of new processes and equipments to mechanize baking process, to reduce production cost and to improve the quality of product. One of those was the introduction of chemical oxidants as dough improvers. The oxidants used included ascorbic acid, azodicarbonamide (ADA), bromates, iodates and calcium peroxide. Although those oxidants gave excellent dough improvement and extensively used by bakers, toxicological research disclosed that some of the chemicals like ADA, bromate etc, are carcinogens. Therefore, in many countries their use is discontinued or banned. There is a great demand for alternative oxidants as dough improvers to replace harmful chemicals.

Enzymes are a very good source of alternate dough improvers because, they are generally regarded as safe, required in very small amounts, and

specific in their action. Due to the prominence of disulfide cross-linkages in glutenin proteins, enzymes that regulate disulfide interchange reactions gained interest. One such enzyme is protein disulfide isomerase, which catalyzes thiol-disulfide exchange, rearranging incorrect disulfide cross-links in a number of proteins in biological systems. In developing wheat kernel, protein disulfide isomerase appears just after anthesis, and almost remains constant throughout the development and dehydration stages. Its time of appearance is consistent with the time of accumulation and aggregation of gluten proteins [Every et al, 2003]. Therefore, protein disulfide isomerase is proposed to play a role in the formation of correct inter as well as intra molecular disulfide bonds in developing wheat kernel. However, to our knowledge, role of protein disulfide isomerase in the dough development is not been investigated. Sulfhydryl oxidase is another enzyme that catalyzes oxidative formation of disulfide bonds from sulfhydryl groups and oxygen. Although its occurrence was reported in animals as well as plants, it is not a well studied enzyme as for as food applications are concerned

Glucose oxidase has been used as good dough improver. Glucose oxidase catalyzes the oxidation of glucose producing H_2O_2 as one of the products. The mechanism of improving action of glucose oxidase is not clearly known. Whether H_2O_2 produced in the reaction directly oxidizes and cross-links glutenin proteins or other enzymes like peroxidase is also involved is still a matter for investigation. Peroxidase and polyphenol oxidase are other two enzymes, which found application in food industries.

1.4. Protein-protein cross-linking

1.4.1. Occurrence of protein cross-links

Protein cross-linking refers to the formation of non-peptide covalent bonds between polypeptide chains within a protein (intramolecular cross links) or between proteins (intermolecular cross links) [Feeney and Whitaker, 1988]. Cross linking results in important changes in chemical, functional, nutritional, and biomedical properties, besides physical properties simply related to molecular size and shape. In biological systems these cross-links are very important for maintaining correct conformation of certain proteins, which is a prerequisite for their biological activity. As biological tissues age, due to various physiological and biochemical reasons further protein cross-links may form that often have deleterious effects and play important role in many conditions of aging [Zarina et al, 2000]. Similar reactions may take place if biological tissues are removed from their natural environment, for example, when harvested as food for processing. Food processing often involves various treatments like high temperature, alteration in pH, exposure to oxidizing conditions and catalysis by either endogenous or exogenous enzymes. These conditions may introduce protein cross-links and result in substantial alteration in the structure of proteins. As a consequence the functional and nutritional properties of the final product changes [Singh, 1991; Friedman, 1999]. The cross-linking of food proteins can influence many properties of food, including texture, viscocity, solubility, emulsification and gelling properties [Motoki and Kumazawa, 2000].

1.4.2. Types of protein cross-links

The side chains of amino acid residues of proteins are known to undergo extensive *in vivo* co- and post-translational modifications. Although only 20 amino acids are incorporated into proteins via translation, some 135 modifications of the amino acid residues are known [Whitaker and Puigserver, 1982]. The reactive amino acid side chains of proteins are capable of participating in many chemical reactions, including cross-linking reactions. However, not all the amino acid side chains participate in cross-linking reactions and those that react, do so with different degrees of reactivity under various conditions. The amino acids, which participate in protein cross-linking, are glutamine, aspartate, serine, tyrosine, cysteine, tryptophan, proline, histidine, arginine and lysine. Protein cross-links are formed either by chemical reactions or by enzyme catalyzed reactions.

1.4.3. Cross-links formed by chemical reactions

Protein cross-linking by chemical reactions occur *in vivo* and *in situ* under pathophysiological conditions and during food processing. Many chemical cross-linking reagents are being used in laboratory to cross-link proteins for various purposes and applications. An important chemical protein cross-linking reaction that often occurs during food processing and many pathophysiological conditions is the Maillard reaction. It is a complex cascade of chemical reactions, initiated by simple condensation of an amine with a carbonyl group. During the course of the maillard reaction, reactive intermediates, such as α-dicarbonyl compounds and deoxysones, are

generated and lead to the production of a wide range of compounds, including polymerized brown pigments called melanoidins, furan derivatives, nitrogenous and heterocyclic compounds [Easa et al, 1996; Fayle and Gerrard, 2002]. Protein cross-links form a subset of the many reaction products of Maillard reaction, and the cross-linking of food proteins by the Maillard reaction during food processing is well established [Mohammed et al, 2000; Gerrard et al, 2002].

Dehydroprotein derived cross-links are another important type formed by chemical reactions. When proteins are treated with alkali, dehydroproteins are formed. Alkali treatment is used in food processing for a number of reasons, such as the removal of toxic constituents and the solubilization of proteins for the preparation of texturized products. Exposure to alkaline conditions coupled with thermal processing, induces racemization of amino acid residues and the formation of covalent cross-links such as dehydroalanine, lysinoalanine and lanthionine [Friedman, 1999]. The resulting intra and intermolecular cross-links are stable. However, food proteins that have been extensively treated with alkali have limited digestibility and reduced nutritional value. Alkali treatment may also produce mutagenic products.

A number of chemical agents have been used for cross-linking of proteins, including glutaraldehyde, formaldehyde and glyceraldehydes. However, the exact mechanism of cross-linking by these reagents is open to speculation. It was suggested that glutaraldehyde specifically acts on lysine residues and creates quaternary pyridinium cross-links [Hermanson, 1996]. Farmaldehyde cross-linking has been attributed to simple methylene bridges

with each cross-link comprising one formaldehyde molecule per two lysines. But other more complex mechanisms are also proposed [Marquie et al, 1997]. Glyceraldehyde has been proposed to undergo Maillard reaction and create malondialdehyde cross-link [Gerrard et al, 2002a].

1.4.4. Cross-links formed by enzyme catalysis

Many enzymes catalyze the protein cross-linking reactions. Some examples are transglutaminase, lipoxygenase, lysyl oxidase, protein disulfide reductase, protein disulfide isomerase, sulfhydryl oxidase, polyphenol oxidase and peroxidase. Transglutaminase catalyze an acyl transfer reaction in which γ-carboxyamide groups of protein-bound glutamine residues are the acyl donors. When ε-amino groups of protein-bound lysine residues are the acceptors, protein cross-linking via ε-(γ-glutamyl)lysine bridges occurs. Transglutaminase has been reported to cross-link several proteins, e.g. casein [Cooke and Holbrook, 1974], soybean protein, β-lactoglobulin [Motoki and Nio, 1983], and collagen [Soria et al, 1975]. Transglutaminase was also reported to cross-link wheat proteins [Gerrard et al, 2001]. Lipoxygenase acts on unsaturated fatty acids to produce fatty acid peroxy free radicals. This reaction requires molecular oxygen. The peroxy free radicals, then abstract a hydrogen from another fatty acid molecule or from other compounds to yield hydroperoxide. The hydroperoxides are decomposed into acids, ketones, and aldehydes (e.g. malondialdehyde). These aldehydes produce protein cross-links via Schiff base formation [Gardner, 1979]. Lysyl oxidase catalyzes the oxidative deamination of lysine to α-amino adipic acid δ-semialdehyde. The aldehyde formed

spontaneously reacts with another aldehyde residue or with lysine and histidine residues to form intra- and inter molecular cross-links [Guay and Lamy, 1979].

Protein disulfide reductase catalyzes the protein disulfide-sulfhydryl exchange reaction using glutathione as one of the substrates. Protein disulfide isomerase catalyzes the rearrangement of random incorrect pairs of half cysteine residues to the native disulfide bonds in several proteins. In the reaction reduced glutathione is converted into oxidized glutathione. Sulfhydryl oxidase catalyzes the *de novo* formation of protein disulfides from protein thiols [Matheis and Whitaker, 1987]. Polyphenol oxidase and peroxidase modify the amino acid side chains of proteins in many ways. In the presence of low molecular weight phenolic compounds, the o-quinones generated by both the enzymes can react with amino, sulfhydryl, thioether, phenolic, indole, and imidazole groups of proteins. In some cases, these reactions eventually lead to protein cross-linking. In the absence of low molecular weight phenolic compounds, tyrosyl groups of proteins can serve as substrate for both the enzymes and lead to protein cross-linking [Feeney and Whitaker, 1988].

1.4.5. Disulfide cross-links

Disulfide bonds are the most common and well-characterized bonds in biological systems. The oxidative coupling of cysteine residues forms them. A suitable oxidant accepts the hydrogen atoms from the thiol groups of the cysteine residues, producing disulfide cross-links. Disulfide bonds provide

structural stability to proteins. In addition to this, reversible formation of disulfide bonds is utilized biologically in enzyme catalytic mechanisms, in the transport of reducing equivalents, in metabolic regulation, and as a cellular defense system [Buchanan, 1980; Sies et al, 1983; Gilbert, 1984; Ziegler, 1985].

Although presence of two cysteine residues either in same protein (intra molecular) or in different proteins (inter molecular) is a prerequisite, the formation of disulfide bond depends both on the thiol-disulfide redox status of the immediate environment and on the kinetic and thermodynamic properties of oxidation and reduction processes [Gilbert, 1984]. Some of the parameters that influence the disulfide bond formation are steric effects, thiol pK_a, entropy etc [Szajewski and Whiteside, 1980; Creighton, 1986]. Disulfide bonds are vital for the heat induced gelling of some food proteins including milk proteins, surimi, soybeans, eggs, meat, wheat gluten and some vegetable proteins [Zayas, 1996]. Through intermolecular cross-links disulfide bonds generate a three-dimensional solid-like network, which provides food with desirable texture [Dickinson, 1997]. Under appropriate conditions thiol-disulfide exchange reactions, transferring reducing equivalents between two thiol-disulfide pairs can occur as follows,

$$RSH + R'SSR' \longleftrightarrow RSSR' + R'SH$$

$$RSH + RSSR' \longleftrightarrow RSSR + R'SH$$

Where, RSH is a protein thiol or a small molecular thiol like cysteine and glutathione.

Wheat glutenin contains substantial amount of disulfide linkages. During dough formation these disulfide bonds are broken and the tightly coiled glutenin becomes uncoiled. As dough development proceeds, new disulfide bonds are formed to maintain the protein network. Small molecular weight thiol compounds assist in this disulfide bond reorganization through thiol-disulfide exchange reactions [Mauritzen and Stewart, 1963].

1.4.6. Tyrosine derived cross-links

Various cross-links formed between two or three tyrosine residues have been found in native proteins and glycoproteins, for example, in plant cell walls [Singh, 1991]. Tyrosine residues are cross-linked to form dityrosine and isodityrosine bonds. Gross and Sizer (1959), first described dityrosine, which they had obtained by oxidation of L-tyrosine with peroxidase and H_2O_2. Subsequently, dityrosine was found to be present in hydrolysates of many structural proteins including resilin, where they are partially responsible for their insoluble and elastic properties [Anderson, 1964; La Bella et al, 1968]. Recently, tyrosine derived bonds are proposed to play an important role in the formation of cross-linked protein network in gluten [Tilley et al, 2001].

1.4.7. Application of enzymes in food processing

Application of enzymes to alter the properties of foods is an area, which has attracted considerable interest, since enzymes are perceived as natural than chemicals. Enzymes are also favored as they require milder conditions, have high specificity, are only required in catalytic quantities, and are less likely to produce toxic products. Thus, enzymes are becoming commonplace in

many industries for improving the functional properties of food proteins [Poutanen, 1997]. The important cross-linking reactions that occur during food processing are summarized in Fig. 1.4.

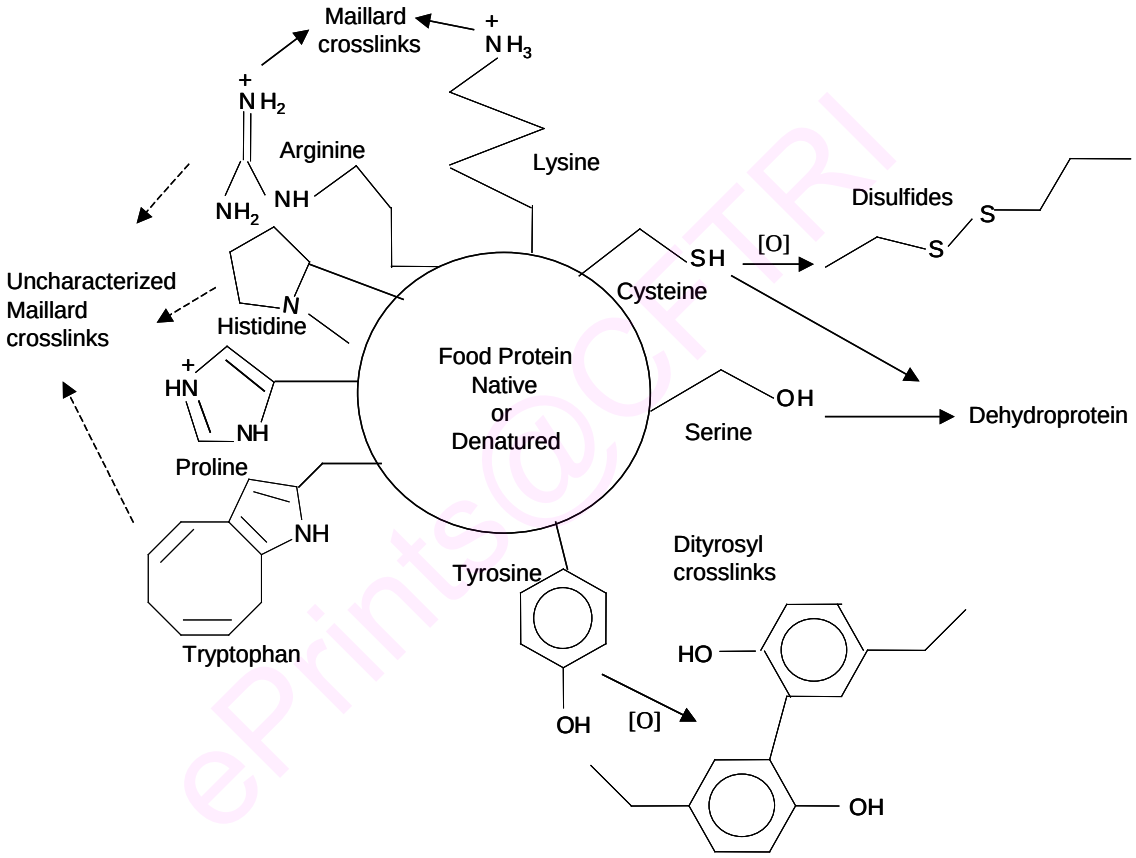


Fig.1.4. A brief account of cross-linking reactions that can occur during food processing. [Feeney and Whitaker, 1988; Friedman, 1999; Singh; 1991].

1.5. Oxidative enzymes

1.5.1. General account

Oxidative enzymes are included under the class 'oxidoreductases'. An oxidoreductase is an enzyme that catalyzes the transfer of electrons from one molecule (the reductant, also called the hydrogen acceptor or electron donor) to another (the oxidant, also called the hydrogen donor or electron acceptor). For example, an enzyme that catalyze this reaction would be an oxidoreductase:

$$\mathbf{A^- + B \rightarrow A + B^-}$$

In this example, A is the reductant (electron donor) and B is the oxidant (electron acceptor). Oxidoreductases are classified as EC 1 in the EC number classification of enzymes. Oxidoreductases can be further classified into twenty two subclasses.

1.5.2. Peroxidases

Peroxidases (EC number 1.11.1.7; donor: hydrogen peroxide oxidoreductase) are enzymes that catalyzed the oxidation of a wide variety of substrates using H_2O_2. They are ubiquitous in nature; occur in animals, plants and microorganisms. They have diverse physiological functions [Dunford, 1999]. Peroxidases are classified as plant peroxidase super family and animal peroxidase super family. Plant peroxidase super family contains three classes. Class I comprises prokaryotic peroxidases, Class II contains peroxidases from fungi and secretory peroxidases from plants and Class III includes extra cellular peroxidases [Veitch, 2004]. On the basis of prosthetic group present they can be divided into two main classes (1) iron containing peroxidases and

(2) flavoprotein peroxidases. The iron containing peroxidases can be subdivided into two groups: ferriprotoporphyrin peroxidases and verdoperoxidases. The former contain ferriprotophorphyrin III (Hematin) prosthetic group and are brown in pure form and occur mainly in higher plants, animals and microorganisms. The verdoperoxidases also contain an iron protophorphyrin but different from ferriprotophorphyrin III and green in pure form. They are present in animal organs and in milk. The flavoprotein peroxidases contain flavin-adenine-dinucleotide as prosthetic group and occur in microorganisms and animal tissues [Whitaker, 1995].

1.5.3. Reaction mechanism of peroxidase

Peroxidases are able to catalyze at least four types of reactions, peroxidatic, oxidatic, catalytic and hydroxylation. Peroxidatic reactions occur when phenolic compounds or amines are the hydrogen donors. These reactions require hydrogen peroxide. With phenolic compounds reaction products are either quinones, compounds of the *o,o'*-diphenol type (e.g. dityrosine) or other polymerized products. Oxidatic reactions occur with certain hydrogen donors e.g., dihydroxyfumaric acid or ascorbic acid, and they require molecular oxygen. In the absence of a hydrogen donor peroxidase slowly converts H_2O_2 to water and oxygen in a catalase-type reaction. In the presence of certain hydrogen donors, particularly dihydroxyfumaric acid, peroxidase can hydroxylate various aromatic compounds including tyrosine and *p*-cresol. This hydroxylation reaction requires molecular oxygen [Whitaker, 1972]. The mechanism of peroxidatic type of reaction is presented here in brief.

In general terms, the majority of reactions catalyzed by the classical peroxidases can be represented as follows

$$H_2O_2 + 2RH \longrightarrow 2H_2O + 2R^{\cdot}$$

Where RH and $R^{\cdot}$ represent a reducing substrate and its oxidized radical product, respectively. The actual catalytical mechanism of the enzyme can be expressed by the following set of equations.

$$E(Fe^{III}, P) + H_2O_2 \xrightarrow{k1} E(Fe^{IV}{=}O, P^{\cdot+}) + H_2O$$

Resting state — Compound I

$$E(Fe^{IV}{=}O, P^{\cdot+}) + RH \xrightarrow{k2} E(Fe^{IV}{=}O, P) + R^{\cdot}$$

Compound II

$$E(Fe^{IV}{=}O, P) + RH \xrightarrow{k3} E(Fe^{III}, P) + R^{\cdot} + H_2O$$

Resting state

Where, E represents a class III plant peroxidase for which the oxidation state of the heme iron atom and the status of the porphyrin (P) are indicated in parenthesis. RH and $R^{\cdot}$ represent a reducing substrate and its oxidized radical product, respectively. The rate constants k_1, k_2, and k_3 refer to the rate of compound I formation, rate of compound I reduction and rate of compound II reduction, respectively. In the first step of this cycle resting enzyme reacts with hydrogen peroxide and generates the high oxidation state green colored intermediate known as compound I, which is considered to be two oxidizing equivalents above the Fe(III) resting state of the enzyme. This intermediate contains an iron(IV) oxyferryl center and a porphyrin-based cation radical. In the first of subsequent two one-electron reduction steps, compound I is reduced to red colored intermediate known as compound II and the reducing substrate

reduction step, one more molecule of reducing substrate is oxidized to radical product and the enzyme returns to its resting state [Veitch, 2004].

1.5.4. Physiological role of plant peroxidases

Plant peroxidases exist in different isoforms; some of them are constitutively expressed while some are developmentally regulated or induced by environmental stresses. Individual peroxidase isoenzymes have been isolated from a number of plants and characterized. Generally these differ in molecular weight, amino acid and sugar composition, kinetic properties and substrate specificities. The higher number of isozymes and their remarkable catalytic versatility allowed them to be involved in a broad range of physiological and developmental process all along the plant life cycle [Passardi et al, 2006].

Peroxidases have been implicated in many physiological processes in plants. For example peroxidases have been suggested or shown to have roles in oxidation and polymerization of soluble phenolics [Srivastava and vanHuystee, 1977], formation of H_2O_2 [Madder et al, 1982] suberization [Espelie & Kolattukudy, 1985], lignification (Lagrimini et al, 1987), auxin degradation (Lagrimini et al, 1997), etc. Cellular metabolic pathways may generate cytotoxic oxygenated byproducts, such as peroxides and the super oxide anion that produce oxidative stress. Peroxidase along with superoxide dismutase and catalase, provides defense against such oxidative stress [Cowan, 1997]. It is difficult to assign a definite physiological role to a specific

peroxidase isozyme because peroxidases are capable of oxidizing a wide variety of substrates.

1.5.5. Structure of peroxidase

The plant peroxidases contain a single polypeptide chain of approximately 300 amino acid residues, iron(III) protoporphyrinIX, and two calcium ions. The unit comprising protoporphyrinIX and the associated ferric metal ion that are attached together through four coordination bonds are usually referred to as heme. Other structural features are four disulfide linkages and a buried salt bridge. The cysteine residues participating in these disulfide bridges and aspartate and arginine residues participating in salt bridge are highly conserved throughout plant peroxidases. Most plant peroxidases have several *N*-linked glycans, and the total carbohydrate content can be as high as 25% [Schuller et al, 1996; Henriksen et al, 1998]. The molecule contains two domains and the heme group is situated between the distal (above the heme) and the proximal (below the heme) domains. The secondary structure is predominantly made up of α-helices. A number of amino acid residues are involved in the formation of heme pocket, which is the catalytic site of the peroxidases. For example, in peanut peroxidase histidine 169, aspartate 238 on the proximal side and arginine 38, histidine 42, and asparagine 70 are the most important amino acid residues as for as structure-function relationship is concerned. These residues are either invariant or highly conserved in all class III plant peroxidases [Veitch, 2004]. The fifth ligand of the heme iron is histidyl imidazole contributed by proximal invariant or highly conserved histidine

residue. The sixth coordination position is not clear but in some cases it has been suggested to be occupied by a water molecule [Poulos and Kraut, 1980].

1.5.6. Effect of pH and temperature on peroxidase

The pH optima of peroxidase activity vary with the enzyme source, the isozyme composition, the donor substrate, and the buffer used. The pH optima of peroxidases from different sources vary from 4 to 7. The broad pH optima observed with peroxidase from some sources are due to the presence of isozymes of different pH optima. Activity decreases at low and high pH values. The loss of activity observed on acidification was attributed to the change in protein conformation from native state to the reversible denatured state due to the detachment of heme [Lopez-Molina et al, 2003]. Plant peroxidases exhibit remarkable heat stability. The high thermal stability of peroxidases was attributed to the presence of sugar and calcium ions in their structure [Mellon, 1991; O'Donnel and van Huystee, 1992]. However, the thermal stability also depends on the enzyme source and isozyme composition. The proposed mechanisms of thermal inactivation of peroxidases are, 1) the dissociation of the prosthetic group from the holoenzyme; 2) conformation change in the apoenzyme and, 3) the modification and the degradation of the prosthetic group [Tamura and Morita, 1975].

1.5.7. Wheat peroxidase

The horseradish peroxidase isoenzyme C is the most widely studied peroxidase among plant peroxidases. It has important commercial applications, for example, as a component of clinical diagnostic kits and for

immunoassays. Its amino acid sequence has been determined and three-dimensional structure was solved by using X-ray crystallography. Isolation and characterization of peroxidase isoenzymes have been reported also from several dicots such as potato, tomato, tobacco and to a lesser extent from monocots. However, very less information is available on wheat peroxidases. This is probably because of the difficulty in isolating a pure protein due to a large number of isoenzymes encoded by hexaploid genome [Veitch, 2004]. A cell wall bound peroxidase isoenzyme was purified and characterized from wheat seedlings. This peroxidase isoenzyme exhibited indole acetic acid (IAA) oxidase activity and produced H_2O_2 in presence of Mn^{2+} ions, a phenolic cofactor and IAA [Zmrhal and Machackova, 1978]. Three cationic peroxidase isoenzymes differing in their catalytic behavior and substrate specificities were purified from wheat germ [Converso and Fernandez, 1995]. A basic non-glycosylated peroxidase isoenzyme was purified from wheat kernel and shown to have antifungal activity [Caruso et al, 2001].

1.6. Objectives and scope of the investigation

Seed storage protein in wheat namely gluten proteins, that give unique visco-elastic property to the wheat dough, are made up of cross-linked subunits. Dough formation is a complex process involving interaction between many components of the flour like proteins, carbohydrates, small molecular weight thiol containing compounds etc. During dough formation, polymeric gluten protein network is developed. The quantity and the size of gluten polymer are the two major factors that determine the dough quality. Development of gluten polymer involves the formation of non-peptide covalent bonds like disulfide and dityrosine cross-links. Although disulfide bond is formed when two -SH groups are nearby, the rate of this reaction depends on many other factors. In baking industry many chemical compounds such as bromate and iodate are used as dough improvers and their dough improving action is mainly due to the thiol oxidation. Thiol oxidation and disulfide bond formation are also catalyzed by enzymes like sulfhydryl oxidase, protein disulfide isomerase etc. Dityrosine bond formation is a free radical mediated reaction and is catalyzed by enzymes.

In recent years chemical dough improvers are avoided due to their toxicity. On the other hand enzymes are being used as they are generally regarded as safe and perceived by consumers as natural. Many enzymes such as protease, amylase, lipase, oxidative enzymes like glucose oxidase, hexose oxidase, polyphenol oxidase, etc., are reported to improve the quality of dough. Peroxidase is one such enzyme which was reported to improve the dough but exact mechanism is not known. The investigations were carried out by the

addition of peroxidase from other sources like horseradish peroxidase. However, wheat has abundant endogenous peroxidase and the role of endogenous peroxidase in gluten protein cross-linking and ultimately in dough formation is not investigated. Therefore major objectives of the present investigation are:

1) To purify and study the properties of endogenous wheat peroxidase
2) To study the ability of wheat peroxidase to catalyze the cross-linking of the gluten proteins
3) To elucidate the role of peroxidase in dough formation

An investigation on the role of peroxidase in protein cross-linking has two major benefits. In particular, it will help the plant breeders to select good wheat germ lines for breeding programmes aimed to develop novel wheat varieties. In general, due to the ubiquitous nature of peroxidase, its role in protein cross-linking has many applications in medical research and biotechnology industry.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Wheat procurement

Eight Indian wheat varieties (*Triticum aestivum*) used in this study namely, GW322, HD2189, HD2781, HD2501, K9644, MACS2496, NIAW34, and NI5439 were procured from the Agharkar Research Institute, Pune, India. Wheat bran was collected from a commercial roller flour mill of 20 TPD (Buhler mill, Buhler AG, uzwill, Switzerland).

2.1.2. Chemicals

Catechol, *o*-dianisidine, TEMED, ammonium persulfate, coomassie brilliant blue G-250, Trizma base, β-mercaptoethanol, linoleic acid, glycine, acrylamide, bis acrylmide, sodium dodecyl sulfate, trifluroacetic acid, 5,5'-dithio-bis-(2-nitrobenzoic acid), and protein molecular weight standards were obtained from Sigma Chemical Co., St. Louis, USA. H_2O_2 was obtained from Qualigens Fine Chemicals Mumbai, India. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Extraction and assay of oxidative enzymes in wheat flour

The oxidative enzymes from different wheat flours were extracted by stirring 1g flour in 10 ml of 50 mM sodium acetate buffer, pH 5.7 at 4^{o}C for one hour and centrifuged at 10000g for 10 min at 4^{o}C. Different oxidative enzymes were then assayed as described below.

Peroxidase

Peroxidase activity was determined according to the method described by Aparicio-Cuesta et al, (1992) using 100µl of 1% hydrogen peroxide (292 mM) and 100µl of 8 mM *o*-dianisidine as substrates and varying amounts of appropriately diluted flour extract in 1 ml of reaction mixture. Remaining volume was made up with 50 mM sodium acetate buffer (pH 5.7). One unit of peroxidase activity was defined as the amount of enzyme, which produced an increase of absorbance of 1.0 per minute at 460 nm.

Polyphenol oxidase

Polyphenol oxidase activity was assayed according to the method described by Coseteng and Lee, (1987), using catechol as substrate. The 1 ml reaction mixture contained varying amounts of appropriately diluted enzyme, 100 µl of 0.5 M catechol and the remaining volume was made up with 50 mM sodium acetate buffer (pH 6.0). One unit of polyphenol oxidase activity was defined as the amount of enzyme that caused increase in absorbance of 1.0 per minute at 420 nm.

Lipoxygenase

The lipoxygenase activity was measured according to the method of Shiiba et al, (1991). The 1 ml reaction mixture contained 30 µl of 7.5 mM linoleic acid in appropriate volume of 50 mM sodium acetate buffer (pH 5.5). Reaction was started by adding varying amounts of appropriately diluted flour extract and the hydroperoxide produced was measured at 234 nm. The hydroperoxide formed was estimated by using extinction coefficient value of 25000. One unit of activity was defined as amount of enzyme that produced one micromole of hydroperoxide per minute.

Superoxide dismutase

The superoxide dismutase activity assay was carried out based on the inhibition of auto oxidation of epinephrine by the enzyme according to the method described by Misra and Fridovich, (1972). The 3 ml reaction mixture contained varying amounts of appropriately diluted flour extract and an appropriate volume of 50 mM sodium bicarbonate buffer (pH 10.2). Reaction was started by the addition of 100 µl of 600µM epinephrine and the absorbance was recorded at 425 nm. One unit of enzyme was defined as amount of enzyme that caused 50% inhibition in the auto oxidation of epinephrine.

Thiol oxidase

Thiol oxidase activity of peroxidase was assayed by measuring the consumption of cysteine. The incubation mixture (3 ml) contained homovallinic acid (10 mM) and cysteine at different concentrations in a borate buffered solution (0.01 M, pH 8.5). The reaction was started by the addition of various

amounts of peroxidase. Aliquots of the incubation solution were removed every 2 min to determine the cysteine consumption. One unit of activity was defined as the amount of enzyme required to oxidize 1µM of cysteine per minute.

2.2.2. Cysteine measurement

Cysteine concentration was measured according to the method of Ellman et al., with minor modifications. DTNB was dissolved in methanol containing 5% NaOH (0.1M). The amount of 2-nitro-5-mercapto-benzoicacid was determined photometrically at 412 nm using extinction coefficient value of 13600. Aliquots of the incubation solution were removed at required intervals to determine the consumption of cysteine.

2.2.3. Hydrogen peroxide estimation

Hydrogen peroxide generation was measured by the fluorimetric method according to Guilbault et al., (1968). Incubation mixtures (3ml) contained homovanillic acid (10mM) and cysteine at different concentrations in a borate buffered solution (0.01 M, pH 8.5). The reaction was started by addition of various amounts of wheat bran peroxidase. Fluorescence readings were recorded with an excitation wavelength of 315 nm and emission wavelength of 418 nm. Standard curves of the fluorescence development were prepared under the same conditions without cysteine using H_2O_2 as the substrate for the peroxidase-linked phenol oxidation (Fig. 2.1.)

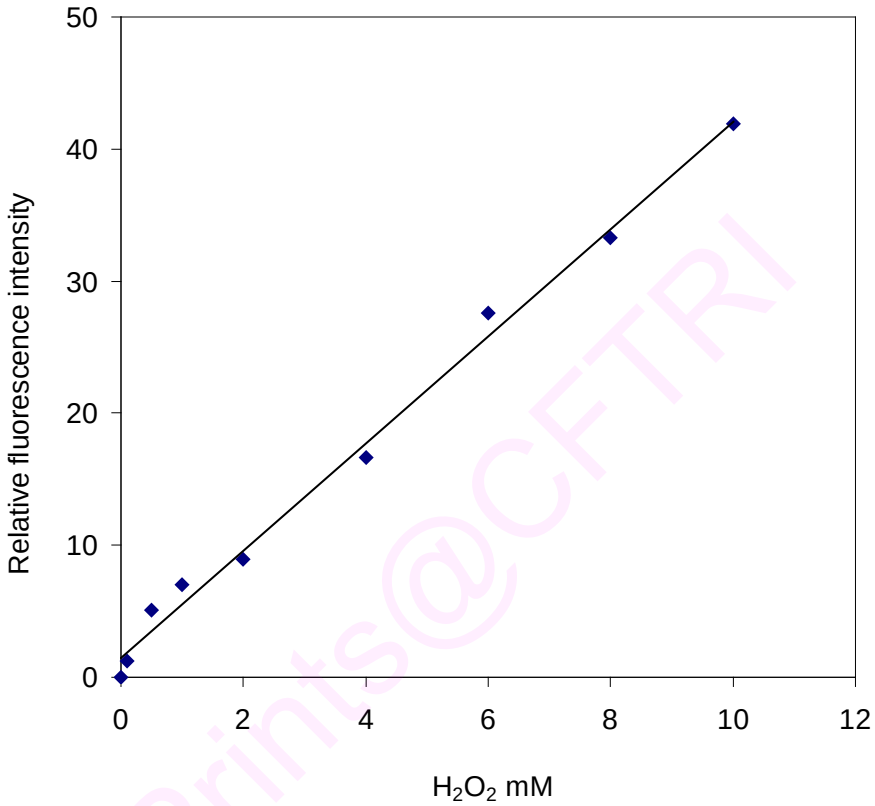


Fig. 2.1. Calibration curve for the estimation of H_2O_2 generated by the thiol oxidase activity of wheat bran peroxidase.

2.2.4. Purification of peroxidase from wheat bran

Wheat bran (10 g) was extracted with water (60 ml) by stirring for 1 h at 4^oC and centrifuged for 10 min at 10000 g. The supernatant (48 ml) obtained was precipitated with 0-30%, 30-50%, and 50-70% $(NH_4)_2SO_4$. Each addition of $(NH_4)_2SO_4$ was followed by a 4 h equilibration at 4^0C and centrifugation at 10,000 g at 4^0C. The 50-70% precipitate dissolved in 4 ml of 20 mM Tris buffer (pH 7.4) and dialyzed extensively with the same buffer. Dialyzed sample (4.5 ml) was loaded on to DEAE-cellulose column that was equilibrated with 20 mM Tris buffer, pH 7.4. More than 75% peroxidase activity was found in the cationic fraction that eluted with the same buffer. This was dialyzed against 20 mM sodium acetate buffer pH 5.6 and chromatographed on CM-cellulose column equilibrated with the same buffer. The peroxidase was eluted with a linear gradient of 0-0.6 M NaCl in the same buffer. This was further purified by gel filtration chromatography on Sephadex G-100 column equilibrated with 20 mM sodium acetate buffer pH 5.6 and eluted with the same buffer. All buffers in all the steps starting from the initial extraction contained 1 mM calcium chloride when bran peroxidase was purified in presence of Ca^{2+}. Fractions showing peroxidase activity were pooled, lyophilized and used for further studies.

2.2.5. Protein estimation

Protein content was determined according to the method of Lowry et al, (1951). Bovine serum albumin was used as a protein standard (Fig 2.2). Total protein contents in wheat flours were determined by micro-kjeldhal method according to the standard AACC methods (1995).

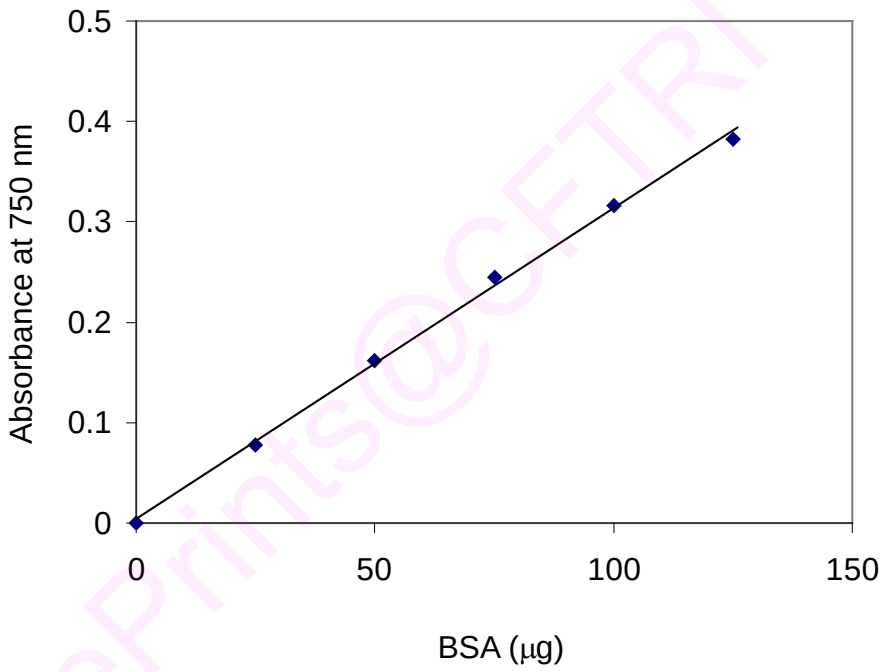


Fig. 2.2. Calibration curve for protein estimation

2.2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight determination

SDS-PAGE was performed according to the method of Laemmli et al, (1970), by using 12% separation gel and 4% stacking gel, in presence of 0.1% sodium dodecyl sulfate. Samples were prepared in Tris-HCl buffer containing 1% SDS, 5% mercaptoethanol, 10% glycerol and boiled for 5 min. The pH of the running buffer was 8.5 and a constant voltage of 50 V was employed. Following the run, the proteins were stained with 0.1 % Coomassie brilliant blue for 5 h and destained by a mixture of 10% acetic acid and 50% methanol in water. The molecular weight of the purified peroxidase was determined by using a mixture containing following protein standards: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), soybean trypsin inhibitor (20.1kDa) and α-lactalbumin (14.2 kDa). Molecular weight was determined using the calibration curve shown in Fig. 2.3.

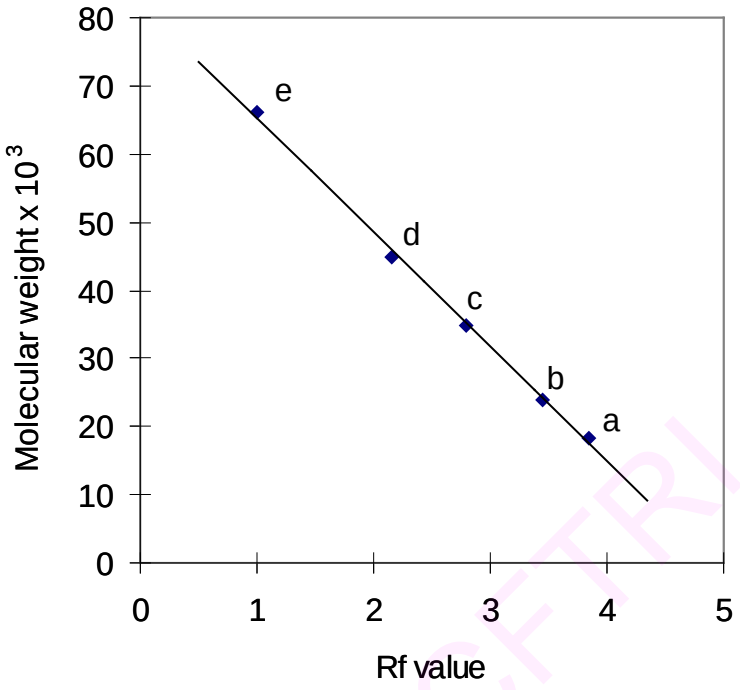


Fig. 2.3. Calibration curve for molecular weight determination using SDS-PAGE
a. α-lactalbumin (14.2 kDa); b. soybean trypsin inhibitor (20.1kDa);
c. glyceraldehyde-3-phosphate dehydrogenase (36kDa);
d. egg albumin (45 kDa); e. bovine serum albumin (66 kDa)

2.2.7. Glycoprotein staining and estimation of carbohydrate content

Glycoprotein nature of purified peroxidase was determined by performing periodic Acid-Schiff staining [Zacharius and Zell, 1969], after the SDS-PAGE. The carbohydrate content in purified peroxidase was estimated by phenol-sulfuric acid method (Fig. 2.4) using glucose as standard [Dubois et al, 1956].

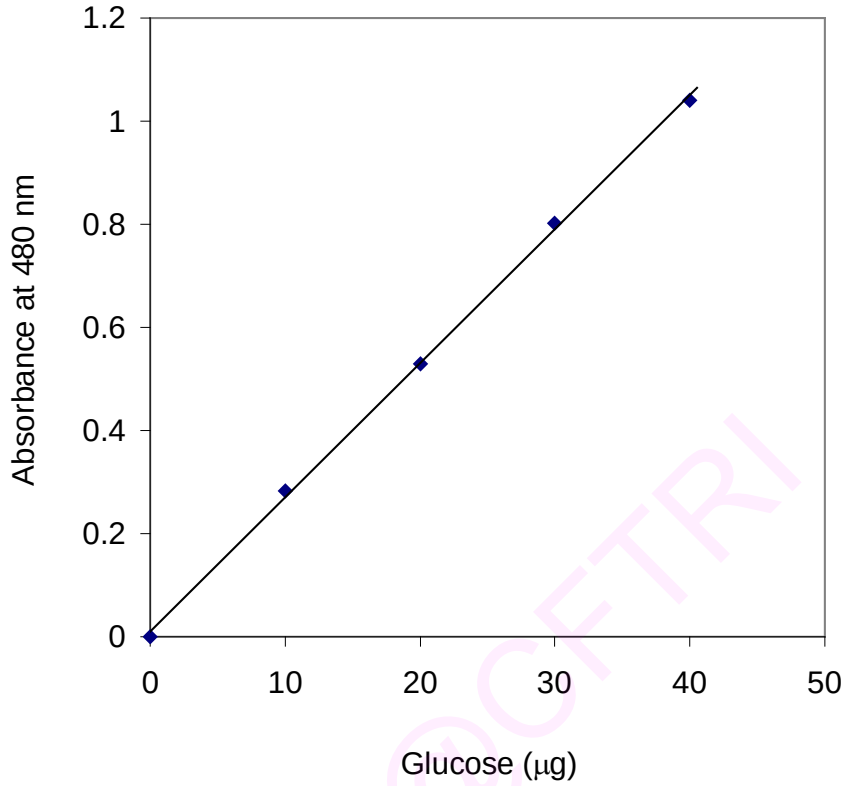


Fig. 2.4. Calibration curve for the estimation of carbohydrate content by phenol-sulfuric acid method

2.2.8. Molecular weight determination by gel filtration chromatography

In order to determine the molecular weight of purified wheat bran peroxidase by using Sephadex G-100, a calibration run was made. The void volume (V_0) was determined using blue dextran (2000 kDa). The log molecular weight of each standard was plotted against its Ve/ Vo (Ve-elution volume of particular standard) as shown in Fig. 2.5. Following molecular weight standards were used for the column calibration: carbonic anhydrase (29 kDa), bovine

serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200kDa).

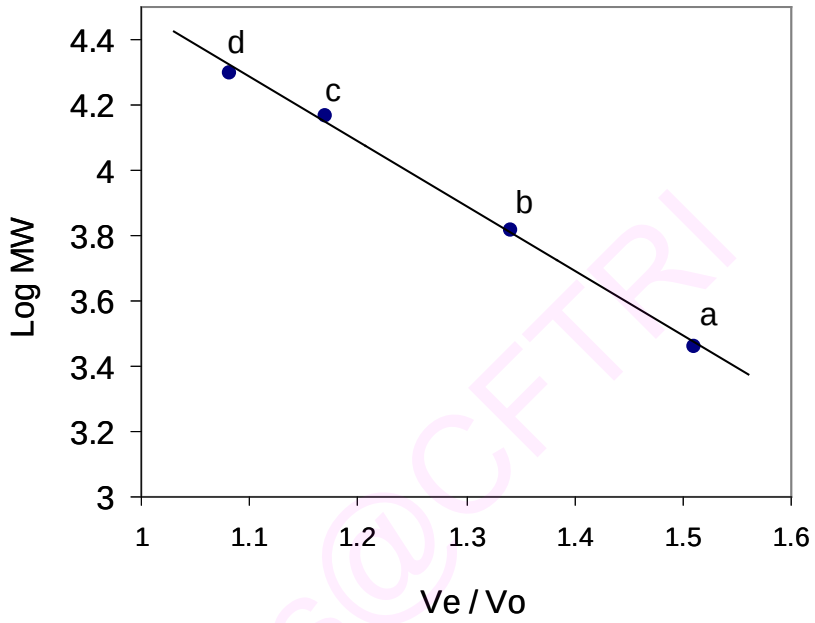


Fig 2.5. Calibration curve for molecular weight determination using Sephadex G-200 gel filtration chromatography. a. carbonic anhydrase (29000); b. bovine serum albumin (66000); c. alcohol dehydrogenase (150000); d. β-amylase (200000).

2.2.9. Determination of optimum pH

The pH optima for the peroxidase and thiol oxidase activities were determined by assaying the activities at pH values ranging from 4 to 6 (0.05 M sodium acetate buffer), 6 to 8 (0.05 M sodium phosphate buffer) and 8 to 10

(0.05 M borate buffer). The enzyme assays were carried out as described earlier (2.2.1).

2.2.10. Effect of substrate concentrations on peroxidase activity

The enzyme activities, at varying concentrations of o-dianisidine (1 mM to 40 mM) at fixed concentration of H_2O_2 (29.2 mM) and varying concentrations of H_2O_2 (5 to 60 mM) at fixed concentration of o-dianisidine (790 µM), both in presence and absence of calcium chloride in the assay buffer were determined. The enzyme activities were also determined at varying ratios between o-dianisidine and H_2O_2 concentrations. All other assay conditions were maintained as described earlier.

2.2.11. Spectral analysis

Fluorescence measurements were carried out using a HITACHI F 2000 spectrofluorimeter. The emission spectra were recorded using 0.71µg/µl peroxidase (purified without calcium) in presence (3 mM) and absence of Ca^{2+} in 20 mM sodium acetate buffer, pH 4.8 with an excitation wavelength of 295 nm. Heme absorption measurements were recorded using Ultraspec 4300 pro UV-Visible spectrophotometer. Peroxidase (0.71µg/µl) purified without calcium was supplemented with 0.2 mM and 0.5 mM Ca^{2+} in 20 mM sodium acetate buffer, pH 4.8 and the absorbance was recorded at 403 nm.

2.2.12. Thermal stability study of peroxidase

Heat inactivation studies were conducted at temperatures of 40, 50 and 60^0C using pyrex glass tubes. Tubes containing 2 ml of 50 mM sodium acetate buffer, pH 4.8, were preheated to the required temperature in a water bath. For thermal stability study in presence of calcium, 1 mM calcium chloride was included in the buffer. Once the buffer solution reached the specific temperature, a 0.15 ml aliquot of purified enzyme (0.7 mg/ml) was added to each tube, vortexed and immersed again into the water bath. After heating for a designated time at the fixed temperature, the tubes were cooled in an ice-water bath and immediately assayed for peroxidase activity at 25^0C as described earlier. Thermal stability of thiol oxidase activity was studied by incubating the enzyme sample at 60^0C for 25 min in borate buffer (0.01M, pH 8.5). Thiol oxidase activity was assayed as described earlier (2.2.1).

2.2.13. Disulfide and sulfhydryl content estimation

The disulfide and sulfhydryl content in the flours was estimated by solid phase assay using $NTSB^{2-}$ according to the method of Chan & Wasserman, (1993). Non-protein thiol content in the flours was estimated after precipitating the flour proteins with perchloric acid according to the method described by Jocelyn (1987).

2.2.14. Isolation of glutenin subunits

The glutenin subunits were isolated from wheat flour according to the method described by Verbruggen et al, (1998). 100 g flour was defatted by extracting twice with 500 ml chloroform, filtered and air dried. 30 g defatted flour was then extracted twice for 30 min with 150 ml of propan-1-ol (50% v/v) and centrifuged for 15 min at 2500g. The residue was extracted with 150 ml of propan-1-ol (50% v/v) containing 1% dithiothreitol (w/v) for 30 min and centrifuged for 20 min at 10000g. The supernatant was adjusted to 60% propan-1-ol containing 1% DTT and allowed to stand for 1h at 7^0C to precipitate high molecular weight glutenin subunits. HMW-GS precipitate was removed by centrifugation for 30 min at 10000g. The supernatant was adjusted to 85% propan-1-ol by adding propan-1-ol without DTT to precipitate low molecular weight glutenin subunits. The precipitated LMW-GS was removed by centrifugation for 30 min at 10000g.

2.2.15. Effect of peroxidase and H_2O_2 on isolated glutenin subunits

One ml reaction mixture containing 980 µg of HMW-GS or 490 µg of LMW-GS or a mixture containing 490 µg each of HMW-GS and LMW-GS in sodium acetate buffer (50 mM, pH 4.8) was incubated with different units of wheat bran peroxidase or horseradish peroxidase in presence of 0.01% H_2O_2 at 37^0C for 1h. To study the effect of H_2O_2 alone, the glutenin subunits were incubated with different concentrations of H_2O_2 without peroxidase as described above. The peroxidase and H_2O_2 treated samples were then analyzed by SDS-PAGE under both reducing and non-reducing conditions.

2.2.16. Determination of high molecular weight glutenin subunit composition of different wheat varieties

High molecular weight glutenin subunit composition of eight wheat varieties was determined by subjecting the flour extract to SDS-PAGE followed by coomassie blue staining as described in 2.2.4. 100 mg of flour was extracted with 100 µl of SDS-PAGE sample buffer containing 1% SDS, 5% mercaptoethanol, 10% glycerol in Tris-HCl buffer (pH 6.8), and loaded on to a 10% polyacrylamide gel. After destaining, subunit composition was determined by comparing with standard wheat varieties. The presence of 1BL/1RS wheat-rye translocation was determined by non-reducing SDS-PAGE analysis. Glu-1 quality score and Glu-1 score adjusted for the presence of the 1BL/1RS translocation was calculated according to the procedure described by Payne et al. (1997).

2.2.17. Gel filtration chromatography of wheat flour proteins

Whole wheat flour (1g) was extracted with 10 ml of 0.1 M acetic acid for 1 h and centrifuged at 10,000g for 20 min at 4^{o}C and chromatographed on Sephadex G-200 using 0.1 M acetic acid as eluent as described by Prasada Rao et al, (2002). Protein elution was monitored by measuring the absorbance at 280 nm.

2.2.18. Size exclusion high performance liquid chromatography

Proteins from the flours were extracted with a two-step extraction procedure according to Gupta et al., (1993) with modification. In the first step, flour samples (11 mg) were stirred for 30 min in 5 ml of sodium phosphate buffer (pH 6.9) containing 0.5% sodium dodecyl sulphate (SDS). Extractions were followed by centrifugation for 10 min at 10000g. In the second step, residues were resuspended in the same buffer (2.5 ml) and sonicated for 90 sec at a frequency of 50 Hz using a Julabo bath type ultrasonic device (model USR-1). Sonication was followed by centrifugation for 10 min at 10000g. The supernatant of the first step contains SDS-extractable proteins (proteins soluble in sodium dodecyl sulphate – buffer solution) while the supernatant from the second step contains SDS-unextractable proteins (proteins soluble only after sonication).

SE-HPLC was performed using LC 10 A Shimadzu HPLC system and a Biosep–SEC–S–4000 Phenomenex column (300 x 7.8 mm, 5 micron). All the samples were filtered through a 0.45 μm filter (Millipore, Type HA). Volume of 15 μl each was injected to the column and the protein eluted was monitored at 280 nm. The mobile phase used was 50% acetonitrile containing 0.1% TFA with a flow rate of 0.5 ml/min. Column was calibrated using the following standard proteins: β-amylase (200,000 Da), alcohol dehydrogenase (150,000 Da), bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da) and cytochrome c (12,400 Da). The percentage of large extractable polymeric protein (LEPP), large unextractable polymeric protein (LUPP) and total

polymeric protein (TPP) were calculated using the following equations as described by Kuktait et al, (2004):

$$\% \text{ of LEPP in flour protein} = \frac{\text{SDS extractable (FI) x 100}}{\text{Flour protein}}$$

$$\% \text{ of LUPP in flour protein} = \frac{\text{SDS unextractable (FI) x 100}}{\text{Flour protein}}$$

$$\textbf{\%} \text{ TPP in flour protein} = \frac{[\text{SDS extractable (FI +FII)+ SDS unextractable (FI +FII)}] \text{ x 100}}{\text{Flour protein}}$$

$$\% \text{ of LEPP in TPP} = \frac{\text{SDS extractable (FI) x 100}}{[\text{SDS extractable (FI +FII) + SDS unextractable (FI +FII)}]}$$

$$\% \text{ of LUPP in TPP} = \frac{\text{SDS unextractable (F1) x 100}}{[\text{SDS extractable (FI +FII) + SDS unextractable (FI +FII)}]}$$

F I, F II, F III are large polymeric proteins, small polymeric proteins, and monomeric proteins as reported in Fig. 3.34, page no 115.

2.2.19. Texture profile analysis of chapati dough

Doughs were prepared from whole-wheat flour by adding adequate quantity of water (equal to Farinograph water absorption value) and were analyzed for texture profile analysis using a Universal Texture Measuring

System (Lloyds, LR5K, UK). Dough was cut into cylindrical pieces of 2.2 cm diameter and 2 cm height and measured for dough hardness (peak force during the first compression cycle or first bite), cohesiveness (ratio of the positive force area during the second compression to that of the first compression) and adhesiveness (negative force area for the first bite). Triplicate measurements were taken for each variety. TPA was measured with cross head speed of 50 mm/ min, load cell of 5 kg, compression of 50% of sample height and probe diameter of 3.5 cm.

2.2.20. Texture (cutting force) measurement of chapati

Cutting force of chapati was determined to measure objectively the texture of chapatis. It was evaluated using the texture analyzer (Stable Micro System; Model TA-Hdi, UK) using the Warner Bratzler blade (HDP/BSW). Chapatis, which were packed in polypropylene pouches, were removed and three strips measuring 4 cm x 2 cm were cut from each chapati. One strip at a time was placed on the sample holder and the blade was allowed to cut the strip. The force (N) required to cut the chapati strip into two pieces was recorded. The speed was maintained at 1.70 mm/s. Measurement for four chapatis were recorded and average values of these were reported.

CHAPTER III

RESULTS AND DISCUSSION

Section A: Purification of wheat bran peroxidase and its characterization

3.1.1. Purification of peroxidase from wheat bran

3.1.1.1. Screening of wheat flour of different varieties for oxidative enzyme activities

Eight Indian wheat varieties were screened for the presence of four oxidative enzymes namely, peroxidase, polyphenol oxidase, lipoxygenase and superoxide dismutase. Activities and specific activities of these enzymes in the flours of different wheat varieties are shown in Table 3.1 and Table. 3.2, respectively. There are large variations in the activities of these oxidative enzymes in different wheat varieties. The specific activities of these enzymes also differ in different varieties and these differences are consistent with enzyme activities. Therefore, the differences in these enzyme activities in different varieties are not simply because of differences in protein contents but due to differential amounts of enzymes synthesized and deposited during wheat kernel development. As these oxidative enzymes are involved in many physiological and defense processes in plants, their expression may be influenced by environmental factors and external challenges like pathogen attack [Miller et al, 1989].

Among eight varieties, K9644 had the highest peroxidase activity (5803 U/g) as well as the highest polyphenol oxidase activity (10.5 U/g), whereas NIAW34 had the lowest peroxidase activity (981 U/g) and MACS2496 had the lowest polyphenol oxidase activity (3.6 U/g). Among the seven varieties screened, both Lipoxygenase and superoxide dismutase activities were the

highest in the variety GW322 and the lowest in the variety NIAW34. The distribution of peroxidase activity in different milled fractions of GW322 variety is given in the table 3.3. Coarse bran had the highest peroxidase activity followed by whole-wheat flour. Maida (refined flour) had the least enzyme activity. This differential distribution is expected as peroxidase isozymes are located in different tissues of the wheat kernel and there is quantitative difference in the isozymes, which are located in more than one tissue.

Table. 3.1. Oxidative enzyme activities in the flour of different wheat varieties

Wheat variety	Enzyme activities (U/g)			
	Peroxidase	Polyphenol oxidase	Lipoxygenase	Super oxide dismutase
GW322	3542 ± 37	2.3 ± 0.2	4.33 ± 0.04	430 ± 15
K9644	5803 ± 125	10.5 ± 0.26	ND	365 ± 20
MACS2496	4330 ± 79	3.6 ± 0.16	0.47 ± 0.01	195 ± 08
HD2189	3435 ± 55	8.8 ± 0.37	0.77 ± 0.01	323 ± 15
HD2781	3058 ±120	8.0 ± 0.34	0.62 ± 0.01	ND
HD2501	1791 ± 36	9.3 ± 0.56	0.60 ± 0.07	199 ± 10
NI5439	1587 ± 54	8. 0 ± 0.36	1.04 ± 0.03	319 ± 08
NIAW34	981 ± 45	10.3 ± 0.35	0.19 ± 0.01	163 ± 03

Values are average of at least 3 assays ± SD

TABLE. 3.2. Specific activities of oxidative enzymes in different wheat varieties

Wheat variety	Specific activities (U/ mg protein)			
	Peroxidase	Polyphenol oxidase	Lipoxygenase	Superoxide dismutase
GW322	211	0.14	0.26	26
K9644	ND	ND	ND	ND
MACS2496	221	0.18	0.02	10
HD2189	195	0.50	0.04	18
HD2781	196	0.51	0.04	ND
HD2501	107	0.55	0.03	12
NI5439	90	0.45	0.06	18
NIAW34	53	0.56	0.01	09

Protein content was estimated according to the method of Lowry et al, (1951)

Table. 3.3. Distribution of peroxidase enzyme in different milled fractions of wheat.

Fraction	Enzyme activity (U/g)	Protein content (mg/g)	Specific activity (U/mg protein)
Whole wheat flour	1960	15	134
Coarse bran	2245	18	128
Fine bran	1430	19	76
Maida	1020	16	68

Protein content was estimated according to the method of Lowry et al, (1951)

As can be seen from Table 3.3, bran has got good peroxidase activity and therefore it serves as a good source of starting material for the purification of peroxidase. Although whole-wheat flour showed more peroxidase specific activity compared to the bran, presence of gluten proteins makes the purification of peroxidase complicated from the former. Kent and Evers (1969) reported that gluten-forming proteins are concentrated towards center of the kernel. Kernel center is occupied by the endosperm, which contributes significantly to the whole-wheat flour. Therefore, bran was selected as the starting material for the purification of peroxidase in this study.

3.1.1.2. Crude extract

The peroxidase activity in crude extract was ten fold more, when calcium chloride was present in the extraction buffer. The peroxidase specific activity was also 23 fold more when calcium chloride was present in the extraction

buffer. In contrast, Presence of calcium chloride decreased the protein content in the crude extract by 3 fold (Table 3.4). This may be due to the decrease in the solubility of some other proteins without affecting the solubility and extraction of peroxidase. Thus, calcium chloride eliminated some of the unwanted proteins from the crude extract and first level of purification was achieved simply by including calcium chloride in the extraction buffer.

3.1.1.3. Ammonium sulphate fractionation

The initial step attempted to purify wheat bran peroxidase was ammonium sulfate precipitation. About 60% proteins were precipitated by 0-30% and 30-50% ammonium sulfate concentration. The recovery of total peroxidase activity in the 50-70% ammonium sulfate precipitate was 45%. Calcium did not show any effect on the recovery and fold purification of peroxidase (Table 3.4). This may be due to very low concentration of calcium chloride (1 mM) used compared to the concentration of ammonium sulfate (around 1-1.3 M) used for fractionation.

3.1.1.4. Ion exchange chromatography

Anion exchange chromatography by DEAE-cellulose was employed as the second step in the purification protocol. Earlier Converso and Fernandez (1995) reported that cationic peroxidase activity represents more than 85% of the total peroxidase activity of the crude extract. Therefore, in this study purification protocol was designed to purify major cationic peroxidase. DEAE-cellulose chromatography was employed to remove most of the unwanted

acidic proteins. In the present purification procedure, about 75% of the peroxidase loaded on to DEAE-cellulose eluted unbound. The fold purification of the enzyme was less (4) when Ca^{2+} was included in the buffer, compared to that achieved without Ca^{2+} in the buffer (12). However, the percentage yield of enzyme was more when Ca^{2+} was present in the buffer (Table 3.4).

The unbound fraction from the DEAE-cellulose was subjected to cation exchange chromatography on CM-cellulose. The gradient elution with 0-600 mM NaCl gave five protein peaks as monitored by absorbance at 280 nm. Among these five peaks, three peaks namely PI, PII, and PIII had peroxidase activity as measured by using H_2O_2 and *o*-dianisidine as substrates (Fig. 3.1). PII and PIII had very less activity and therefore they were not analyzed further. In this step also the fold purification of PI was more (51) when Ca^{2+} was not present in the buffer compared to fold purification (11) when Ca^{2+} was included in the buffer. However, the percentage yield of enzyme was similar in both the cases (Table 3.4).

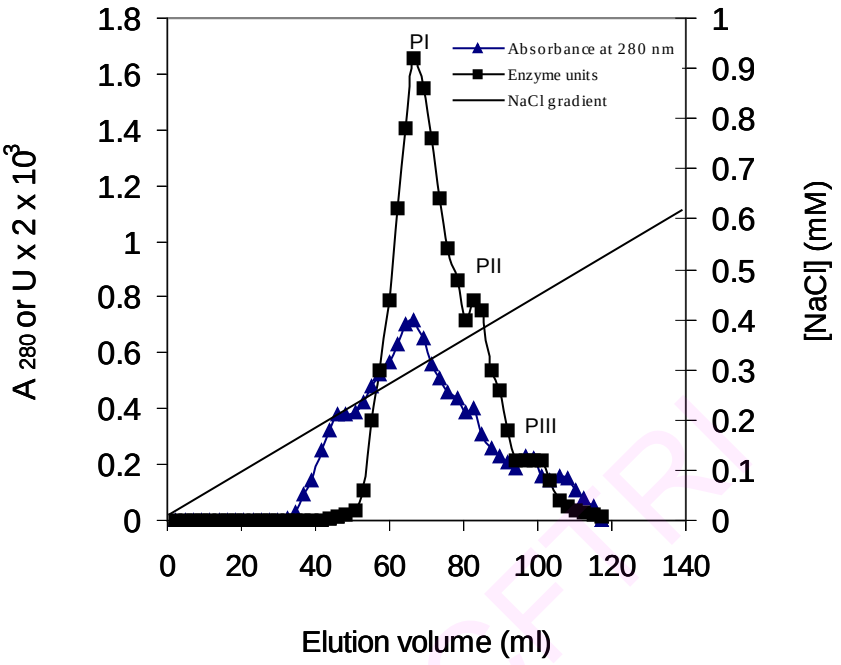


Fig. 3.1. Cation exchange chromatography of DEAE-cellulose unbound fraction on CM-cellulose.

3.1.1.5. Gel filtration chromatography

Gel filtration chromatography of Peak I on Sephadex G-100 gave two protein peaks as monitored by absorbance at 280 nm. Of the two, the early-eluted peak contained peroxidase activity (Fig. 3.2). The purity of this peak was checked by performing SDS-PAGE and subsequent coomassie staining of the lyophilized powder from this peak. A single band was obtained by SDS-PAGE (Fig. 3.3). In this step also there was a difference in the fold purification of the enzyme with and without Ca^{2+} in the buffer. Percentage yield was found to be better if Ca^{2+} was present in the buffer (Table 3.4).

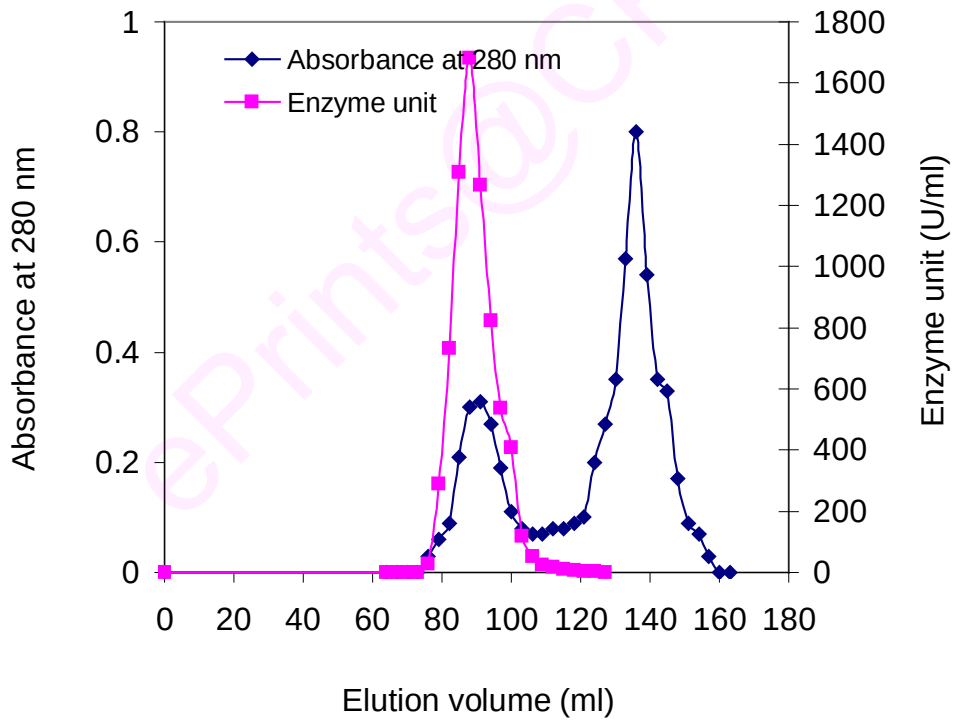


Fig. 3.2. Gel filtration chromatography of CM-cellulose PI on Sephadex G-100.

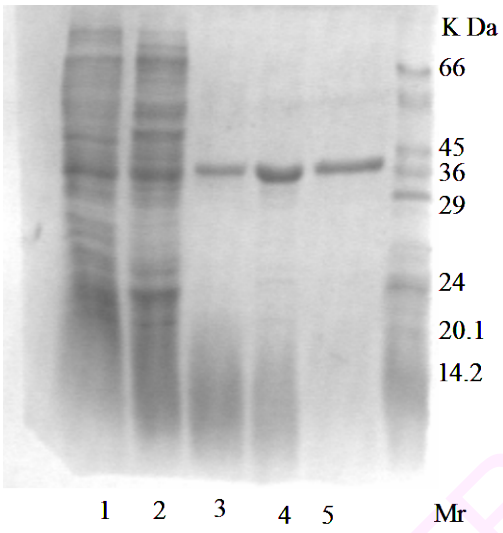


Fig. 3.3. SDS-PAGE of wheat bran peroxidase at different steps of purification, crude extract (lane 1), $(NH_4)_2SO_4$ precipitate (lane 2), DEAE-cellulose peak (lane 3), CM-cellulose peak I (lane 4), Gel filtration peak (lane 5). M_r - Molecular weight marker

As can be seen from the Table 3.4, both the fold purification and percentage yield of peroxidase was influenced by Ca^{2+}. The less fold purification achieved in all the steps when Ca^{2+} was present in the buffer is due to the fact that inclusion of Ca^{2+} in extraction buffer selectively retained peroxidase, eliminating many unwanted proteins. The specific activity of purified bran peroxidase was 10 fold more when it was purified in presence of Ca^{2+} (Table 3.4). But the increase in specific activity by the addition of Ca^{2+} to the bran peroxidase that was purified in absence of Ca^{2+} was only 4 fold. Therefore, it can be concluded that Ca^{2+} is playing a stabilizing role during

purification. The stabilizing effect may be due to the improved thermal stability of the enzyme in presence of Ca^{2+}.

Table. 3.4. Purification of wheat bran peroxidase

Step	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (Fold)	Yield (%)
Crude extract	383.5 (912)	468 (19)	179520 (17376)	-	(100)
$(NH_4)_2SO_4$ Fractionation	56.4 (130)	1428 (60)	80512 (7840)	3 (3)	45 (45)
DEAE-cellulose	29.7 (19.7)	1912 (225)	56772 (4438)	4 (12)	32 (26)
CM-cellulose Peak I	7.05 (3.96)	1912 (824)	36656 (3264)	11 (51)	20 (19)
Gel filtration	3 (1.95)	10240 (1026)	30720 (2000)	22 (54)	17 (12)

Figures in brackets are for the purification without calcium chloride

3.1.2. Characterization of peroxidase purified from wheat bran

3.1.2.1. Molecular weight determination

The molecular weight of the purified wheat bran peroxidase was found to be 44 kDa by SDS-PAGE and 40 kDa by gel filtration chromatography on Sephadex G-200. Peroxidase isozymes from different parts of the wheat kernel have been reported to differ in their molecular weights in the range of 35 to 36.5 kDa. The molecular weights of three isozymes purified from wheat germ were 35 kDa, 36 kDa, and 36.5 kDa [Converso and Fernandez, 1995]. The molecular weight of a basic isozyme from wheat kernel was 36 kDa [Caruso et al, 2001]. Higher molecular weight of bran peroxidase in our study may be due to the higher carbohydrate content associated with it.

3.1.2.2. Peroxidase spectrum and glycoprotein nature

The UV-visible absorption spectrum of the purified peroxidase exhibited a characteristic heme absorption peak at 403 nm (Fig. 3.4). This indicated that wheat bran peroxidase is a hemoprotein. The periodic acid Schiff staining of the purified bran peroxidase after SDS-PAGE gave a pink band indicating the presence of carbohydrates associated with the enzyme. The content of carbohydrate on bran peroxidase was found to be 13.6% as estimated by phenol-sulfuric acid method. The occurrence of sugars on peroxidases has been known for a long time [van Huystee, 1987]. Although the exact role of glycosylation is not known, it has been demonstrated that glycosylation is important in cell-to-cell recognition when proteins occur on cell surface [Drickamar and Taylor, 1998]. Glycosylation gives resistance against

degradation by protease and improved thermal stability to many proteins [van Huystee and McManus, 1998]. Peroxidases from different sources and different isozymes from the same source differ in their carbohydrate content. A basic peroxidase from wheat kernel was shown to be devoid of carbohydrates where as other three isozymes from wheat germ were shown to contain 7.5%, 7.9%, and 4.1% carbohydrate [Caruso et al, 2001; Converso and Fernandez, 1995]. However, some plant peroxidases contain as high as 25% carbohydrates [Henriksen et al, 1998].

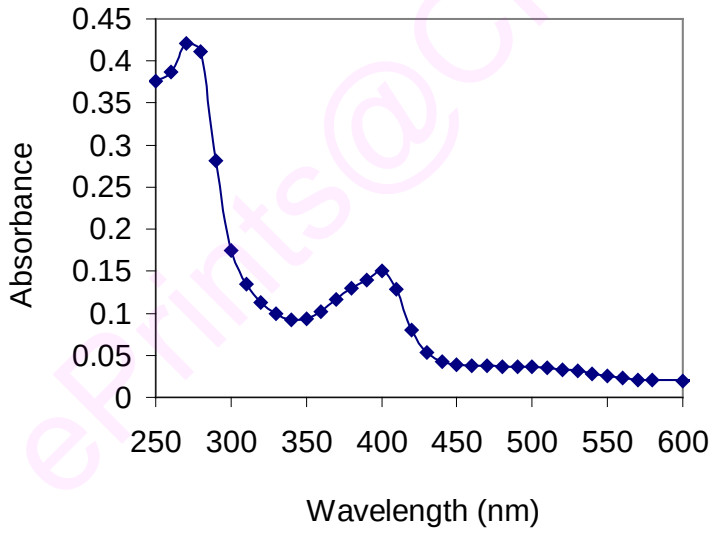


Fig. 3.4. UV-visible absorption spectrum of wheat bran peroxidase

3.1.2.3 pH optimum

The maximum peroxidase activity was obtained at pH 4.8. Peroxidase activity decreased below and above this value. Below pH 2.5 peroxidase activity lost completely whereas about 20% of activity remained even at pH 8(Fig 3.5).

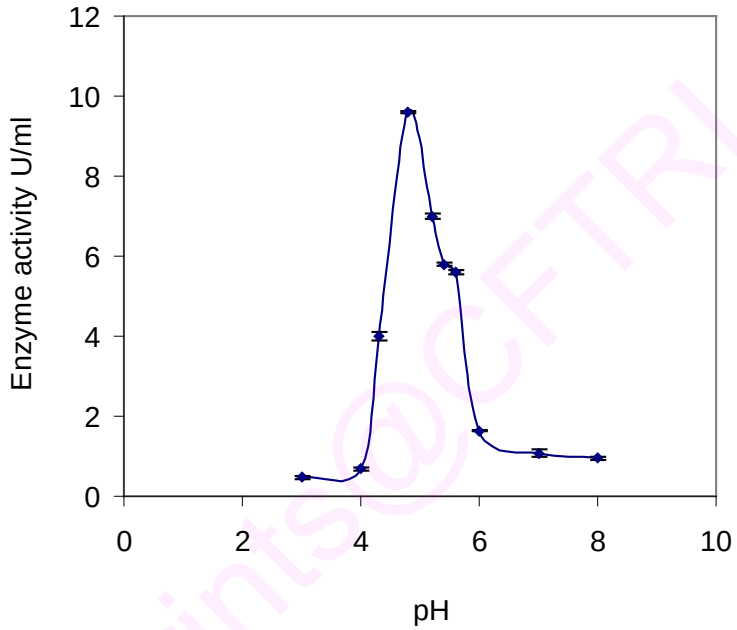


Fig. 3.5. Effect of pH on wheat bran peroxidase activity. Value at each point is average of three experiments ± SD

3.1.2.4. Effect of substrate concentration

The effect of substrate concentrations (H_2O_2 and *o*-dianisidine) on purified bran peroxidase was studied by varying the concentration of one substrate at a time, while the concentration of other substrate was kept constant. This was done both in presence and absence of calcium. Peroxidase activity increased with increase in the concentration of o-dianisidine up to 5 mM

and 20 mM in presence and absence of calcium, respectively. Beyond these concentrations there was a slight decrease in the peroxidase activity (Figs. 3.6. and 3.7. Inset). The Km values calculated from Lineweaver-Burk plot were 5 x 10^{-3} M and 10 x 10^{-3} M in absence and presence of calcium, respectively. Peroxidase activity also increased with the increase in H_2O_2 concentration up to 20 mM and 50 mM in the absence and presence of calcium respectively (Figs. 3.8. and 3.9. Inset). As observed with o-dianisidine, beyond these concentrations there was a slight decrease in the activity. The Km values were 7.14 x 10^{-3} M and 40 x 10^{-3} M in absence and presence of calcium, respectively.

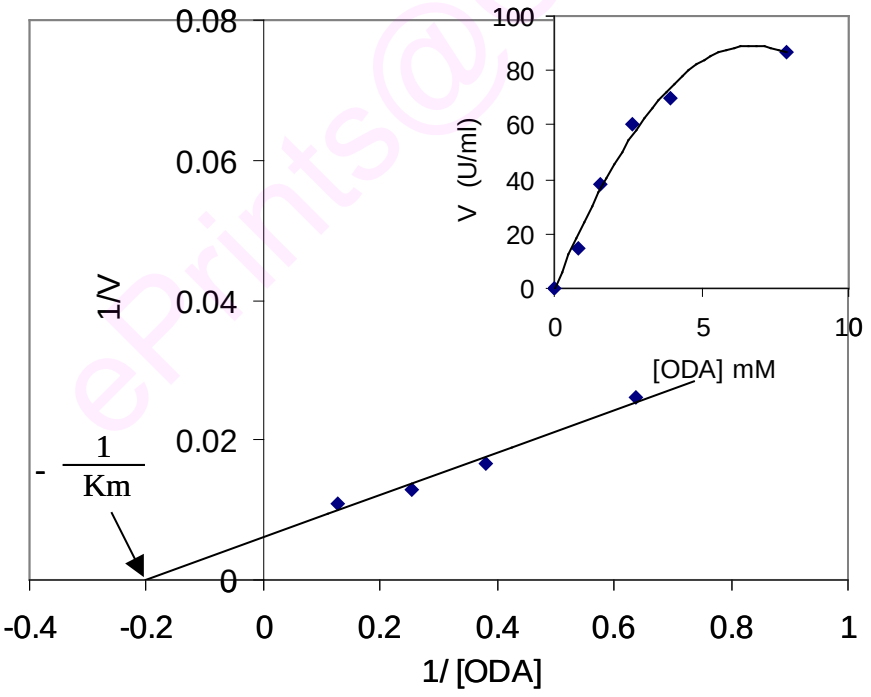


Fig. 3.6. Double reciprocal plot of the effect of concentration of o-dianisidine on the initial velocity of wheat bran peroxidase.
Inset: Effect of o-dianisidine concentration on peroxidase activity

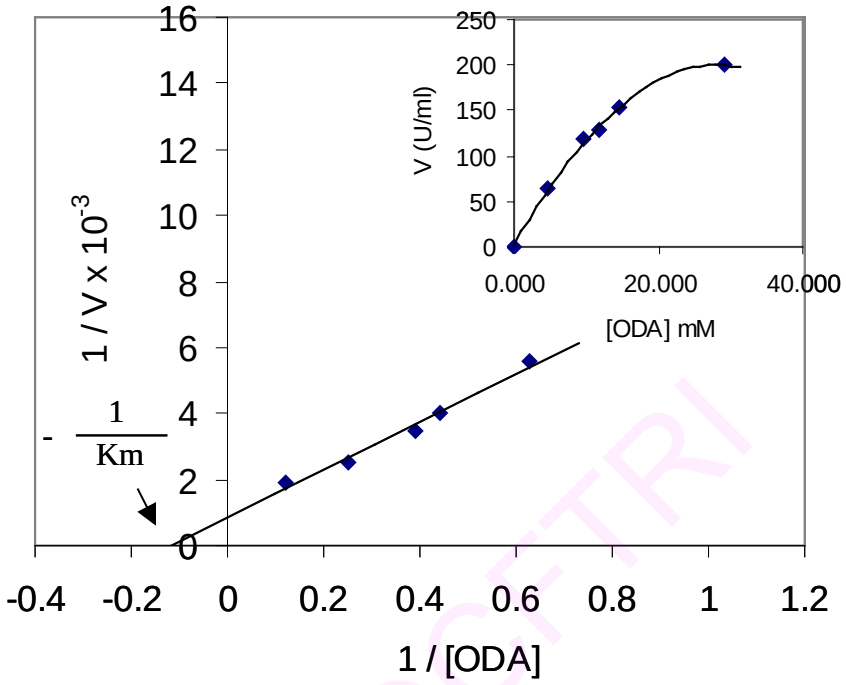


Fig. 3.7. Double reciprocal plot of the effect of concentration of o-dianisidine on the initial velocity of wheat bran peroxidase. Enzyme assays were performed with 1 mM calcium chloride in assay buffer.
Inset: Effect of o-dianisidine concentration on peroxidase activity in presence of 1 mM calcium chloride

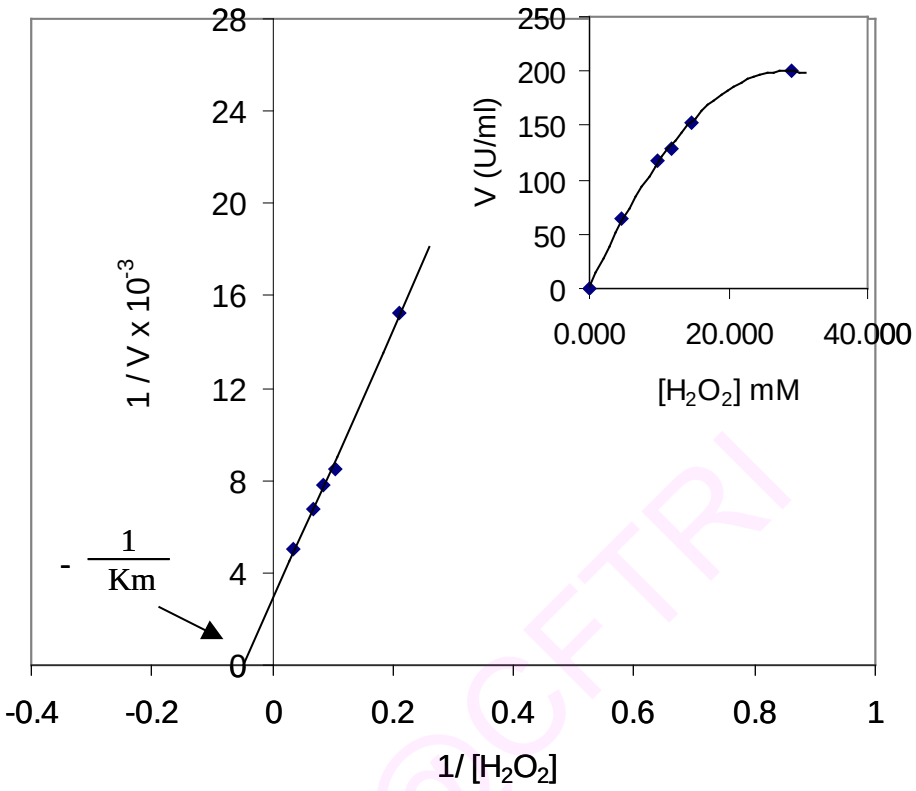


Fig. 3.8. Double reciprocal plot of the effect of concentration of H_2O_2 on the initial velocity of wheat bran peroxidase.
Inset: Effect of H_2O_2 concentration on peroxidase activity

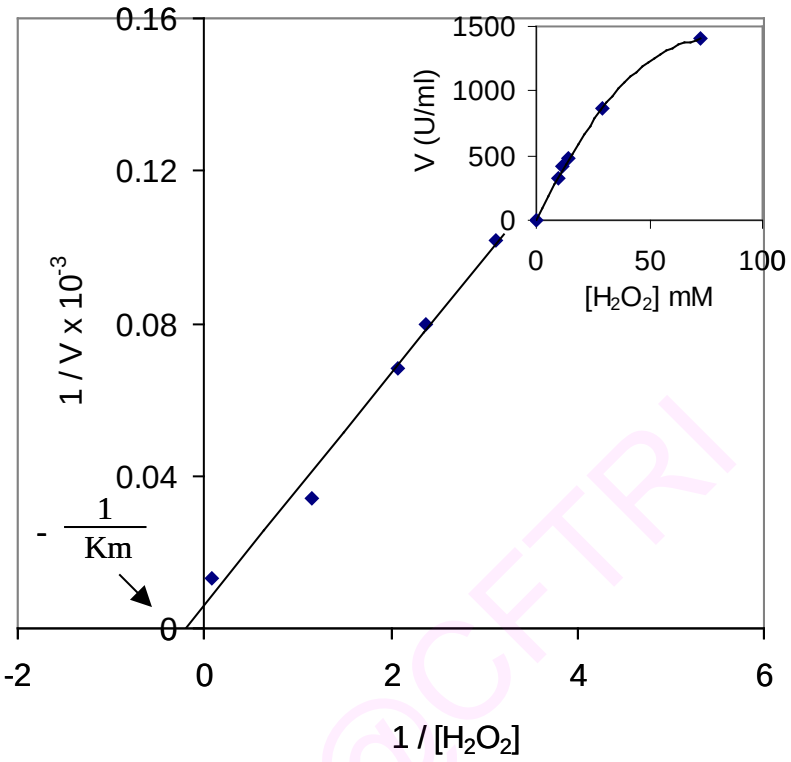
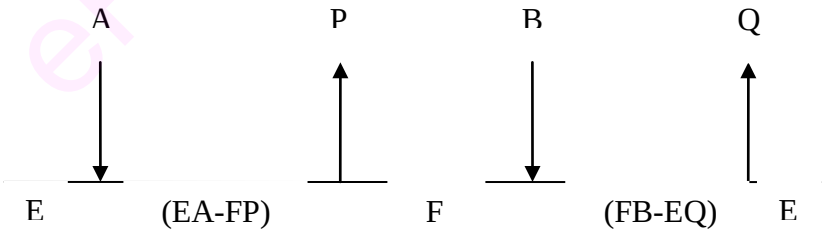


Fig. 3.9. Double reciprocal plot of the effect of concentration of H_2O_2 on the initial velocity of wheat bran peroxidase. Enzyme assays were performed with 1 mM calcium chloride in assay buffer.
Inset: Effect of H_2O_2 concentration on peroxidase activity in presence of 1 mM calcium chloride

3.1.2.5. Kinetic mechanism

The double reciprocal plot of initial velocity versus different H_2O_2 concentrations at three fixed o-dianisidine concentrations gave a series of parallel lines (Fig. 3.10.). The kinetic mechanism of an enzyme catalyzed reaction can be deduced by comparing the kinetic data with the predicted kinetics of possible mechanisms. Reaction catalyzed by peroxidase is a bireactant reaction. This kind of reactions where more than one substrate is involved can be divided into two groups. Theses are sequential reactions and ping pong reactions. In sequential reactions, all reactants combine with enzyme, before reaction takes place and any product is released. In ping pong reactions, one or more products are released before all substrate combine with the enzyme. In ping pong mechanism enzyme oscillates between one or more stable forms. The ping pong bi bi reaction can be described pictorially by using Cleland shorthand notation [Cleland, 1963] as follows.



Where, A and B are substrate, P and Q are products, E is the free enzyme, (EA-FP) and (FB-EQ) are stable enzyme intermediates.

The parallel lines obtained in the double reciprocal plot of initial velocity versus H_2O_2 concentration at different fixed concentrations of *o*-dianisidine

clearly indicate that this peroxidase is following ping pong bi bi mechanism. Earlier, Ping Pong mechanism was proposed for cationic artichoke peroxidase also (Hiner et al., 2004). Double reciprocal plot of initial velocity versus different H_2O_2 concentrations at three-fixed ratio of *o*-dianisidine to H_2O_2, produced a common 1/y-axis intercept (Fig.3.11) A common intercept on ordinate in the double reciprocal plot of initial velocity versus H_2O_2 concentration when the ratio between H_2O_2 and *o*-dianisidine was kept constant confirms the Ping Pong Bi Bi nature of catalysis.

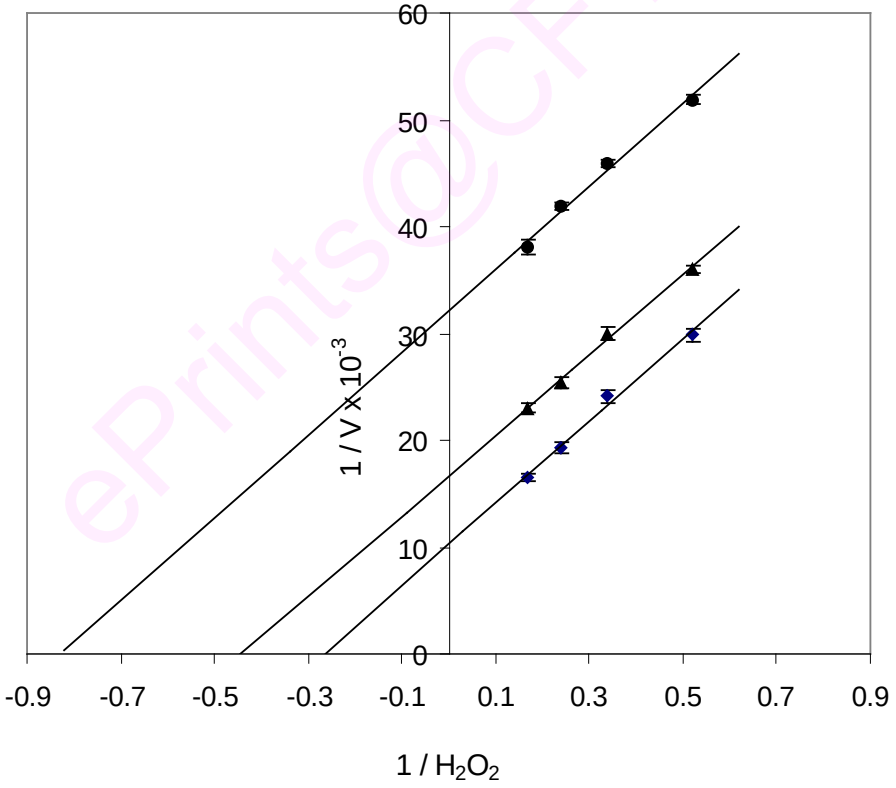


Fig. 3.10. Double reciprocal plot of effect of concentration of H_2O_2 on the initial velocity at three different fixed concentrations of o-dianisidine (▲ 158 µM, ♦ 316 µM, ▪ 790 µM).

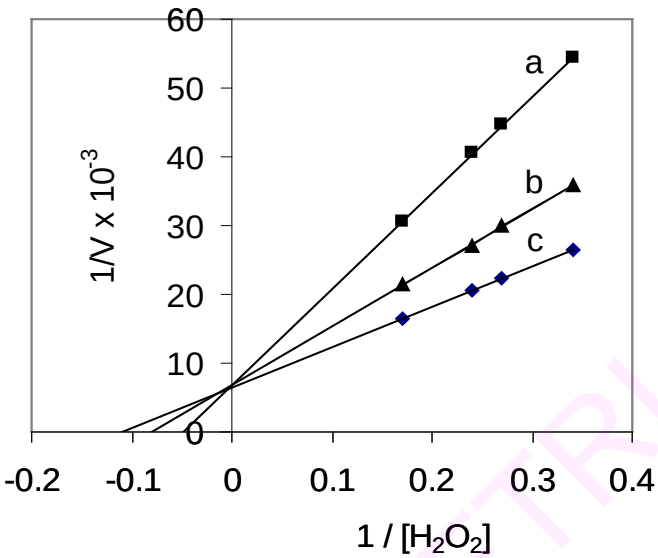


Fig. 3.11. Double reciprocal plot of effect of concentration of H_2O_2 on the initial velocity at three different ratio (x) between o-dianisidine and H_2O_2 was kept constant (a. x = 27; b. x = 90; c. x = 136).

3.1.2.6. Thiol oxidase activity of peroxidase

The purified wheat bran peroxidase exhibited thiol oxidase activity. At pH value above 6.5 the peroxidase was able to oxidize cysteine as measured by the disappearance of cysteine using Ellman method. The rate of oxidation of cysteine was peroxidase concentration dependent (Fig. 3.12). The thiol oxidase function of peroxidase also generated H_2O_2. The oxidation

of cysteine by catalytic quantities of transition metal ions is known [Albro et al, 1986]. In the absence of metal ions or in the presence of convenient chelators the auto-oxidation of cysteine is undetectably slow [Jocelyn, 1972]. Horseradish peroxidase is also able to oxidize the cysteine and this gives rise to thyil free radical. Oxygen is consumed in this reaction and it is reduced to superoxide radical [Harman et al, 1986]. The superoxide radical can dismutates spontaneously to form hydrogen peroxide. The dismutation of superoxide radical into H_2O_2 is also catalyzed by the enzyme superoxide dismutase.

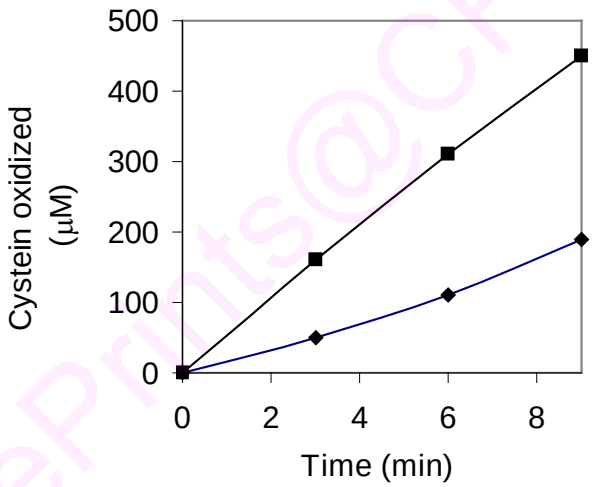


Fig. 3.12. Cysteine oxidation by thiol oxidase activity of wheat bran peroxidase. ♦, 100 U and ■, 200 U of peroxidase.

For thiol oxidase activity of horseradish peroxidase a free radical mechanism has been proposed. According to this proposed mechanism, free radical chain reaction is initialized via the oxidation of cysteine by the haem moiety and the release of thyil free radicals from the reaction center of the

enzyme. The ferric iron of the haem is reduced by the thiol to ferrous iron which will add molecular oxygen to form compound III. This intermediary compound then reacts with cysteine again to yield hydrogen peroxide, thyil free radicals and regenerated enzyme [Pichorner et al, 1992]. The proposed mechanism is outlined in the following equations.

$$E\text{-}Fe^{3+} + RSH \longrightarrow E\text{-}Fe^{2+} + RS^{\cdot} + H^{+}$$

$$E\text{-}Fe^{2+} + O_2 \longrightarrow E\text{-}Fe^{2+}O^{2} = CIII$$

$$E\text{-}Fe^{2+} + O^{2} \longrightarrow E\text{-}Fe^{3+}\,O_2^{\cdot -}$$

$$E\text{-}Fe^{3+}\,O_2^{\cdot -} + RSH \longrightarrow E\text{-}Fe^{3+} + HO_2^{-} + RS^{\cdot}$$

After the initiation of the free radical chain reaction, further H_2O_2 generation is independent of the enzyme and is propagated by the reaction of the thyil radical with oxygen forming superoxide radical and consequently producing H_2O_2. The proposed propagation steps are outlined in the following equations.

$$RS^{\cdot} + O_2 \longrightarrow RSOO^{\cdot}$$

$$RSOO^{\cdot} \longrightarrow RS^{+} + O_2^{\cdot -}$$

$$RS^{\cdot} + RS^{-} \longrightarrow RSS^{\cdot -}R$$

$$RSS^{\cdot -}R + O_2 \longrightarrow RSSR + O_2^{\cdot -}$$

$$2H^{+} + O_2^{\cdot -} \longrightarrow H_2O_2 + O_2$$

3.1.2.7. Generation of H_2O_2 by thiol oxidase function of peroxidase

Oxidation of cysteine by thiol oxidase activity of peroxidase generated H_2O_2. The production of H_2O_2 was dependent on cysteine concentration. An increase in cysteine concentration increased the production of H_2O_2 and reached maximum (12 µM) at cysteine concentration of 500 µM. Beyond this cysteine concentration there was decrease in the generation of H_2O_2 (Fig. 3.13). This reaction appears to be independent of the enzyme. The super oxide formed in the propagation step described earlier can also react with thiolate anion leading to the generation of disulfide and water. Involvement of an intermediate containing a three-electron bond and sulphinyl radical in this reaction was proposed [Pichorner et al, 1992]. The proposed reactions are outlined in the following equations.

$$O_2^{\cdot -} + RS^- \longrightarrow [RS.^{\cdot}\ .\ O\text{-}O^-]^-$$

$$[RS.^{\cdot}\ .\ O\text{-}O^-]^- + H_2O \longrightarrow [RS.^{\cdot}\ .\ OOH]^- + OH^-$$

$$[RS.^{\cdot}\ .\ OOH]^- \longrightarrow RSO^{\cdot} + OH^-$$

$$RSO^{\cdot} + RS^- \longrightarrow RSO^- + RS^{\cdot}$$

$$RSO^- + RS^- + H_2O \longrightarrow RSSR + 2OH^-$$

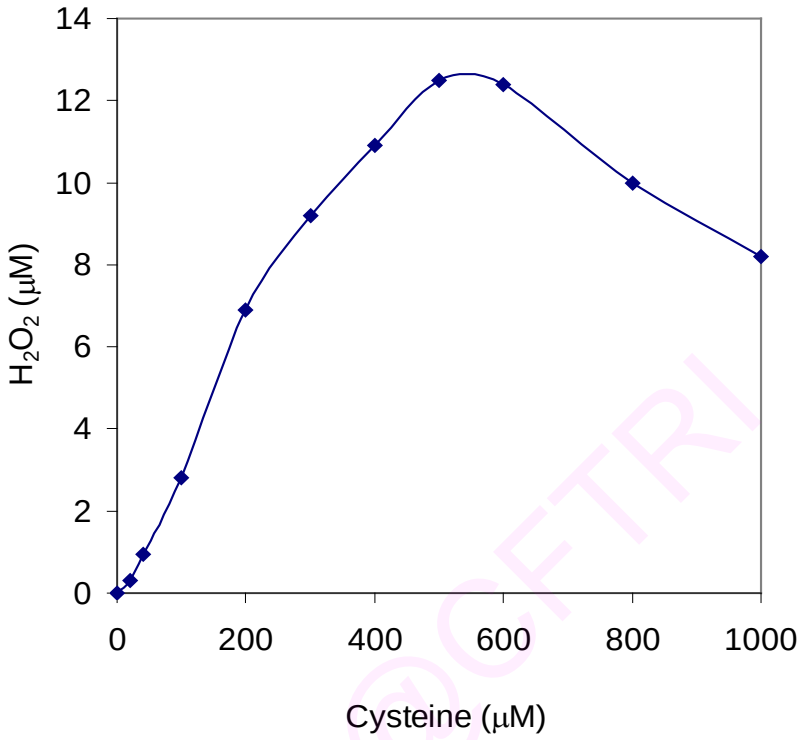


Fig. 3.13. Effect of cysteine concentration on the generation of H_2O_2 by thiol oxidase activity of wheat bran peroxidase

A high concentration of superoxide radical preferably produces H_2O_2, high concentration of thiolate preferably forms water. Different ratio of superoxide radical to thiolate is responsible for the different ratio of H_2O_2 to water [Pichorner et al, 1992].

3.1.2.8. Effect of pH on thiol oxidase activity

Peroxidase exhibited thiol oxidase activity over a narrow range of pH values, 6.5 to 10 (Fig. 3.14). The thiol oxidase activity was maximum at pH 8.

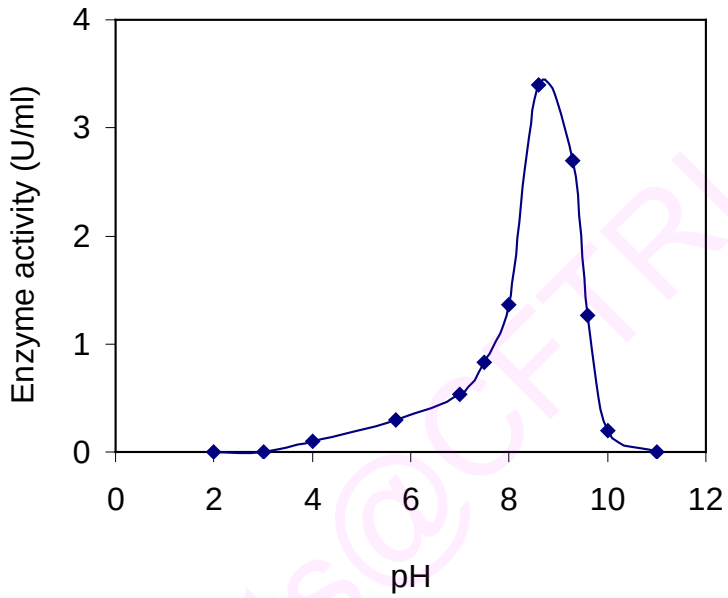


Fig. 3.14. Effect of pH on thiol oxidase activity of wheat bran peroxidase

3.1.2.9. Effect of calcium on activity of wheat bran peroxidase

The wheat bran peroxidase purified in absence of calcium showed an increase of activity up to 444% when assayed in presence of different concentrations of calcium in the range of 0.5 mM to 3 mM (Fig. 3.15). However, two distinct phases were observed, an initial rapid increase of activity followed by a slow phase of activity increase. Peroxidase purified in presence of calcium in all the steps, exhibited loss of enzyme activity when assayed in presence of different concentrations of EDTA in the range of 1 to 10 mM (Fig. 3.16). In this

case also there were two phases, a rapid loss of activity followed by a slow decrease in the enzyme activity. Thiol oxidase activity of bran peroxidase was also influenced by calcium. There was a five-fold increase in thiol oxidase activity when 1 mM calcium was present in the assay buffer.

Calcium was long known for its role in the improvement of the thermal stability of plant peroxidases. But recent studies of calcium and peroxidase interactions are shedding light on the new role of calcium. For the first time Rasmussen et al, (1995), observed the reaction rate enhancing effect of exogenously added calcium in barley peroxidase. Later, a peroxidase isozyme isolated from wheat germ was also reported to exhibit increased activity in presence of exogenously added calcium. It was observed that during purification loosely bound calcium was removed resulting in decrease of enzyme activity. Endogenously added calcium reconstituted the removed calcium and the enzyme activity was recovered [Converso and Fernandez, 1996]. Although calcium is known to increase the activity of plant peroxidases, not all peroxidases exhibit an increase in activity in presence of calcium and activity enhancement is not to the same extent for those peroxidases, which show increased activity in its presence. Shiro et al, (1986) observed a slight change in the rate constant for the reaction with H_2O_2 by removal of calcium in horse radish peroxidase-C, but calcium had no effect on the oxidation of pyrogallal, ascorbic acid and indole acetic acid by wheat germ peroxidase C3 and horse radish peroxidase (type VI) [Converso et al, 1996]. In contrast to this, artichoke cationic peroxidase showed huge change in the rate constant [Hiner et al, 2003].

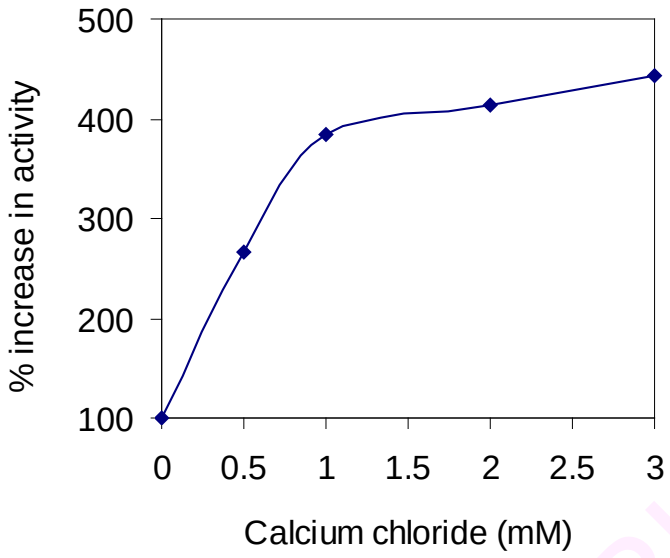


Fig. 3.15. Effect of calcium concentration on the activity of wheat bran peroxidase

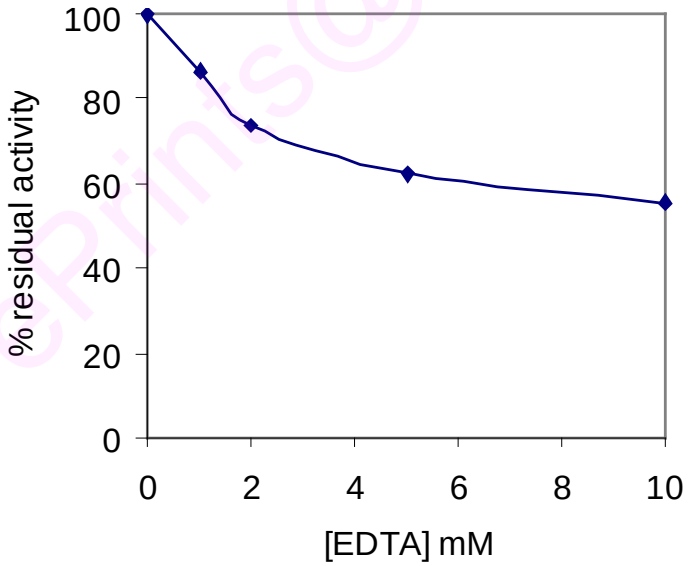


Fig. 3.16. Effect of EDTA concentration on the activity of wheat bran peroxidase

Two distinct phases observed in the activity of wheat bran peroxidase (Fig. 3.15 and 3.16) may be due to two reasons. There may be two species of bran peroxidase of which one is more sensitive to the effect of calcium. This is possible because existence of plant peroxidase in two different forms was reported earlier. Raman resonance and calcium dissociation studies by Hiner et al, (2004) and Lopez-Molina et al, (2003) revealed that artichoke cationic peroxidase can exist in two forms. These are, hexa coordinated high spin 6-aquo (6cHS) and penta coordinated high spin (5cHS). Among these two, 5cHS is more active. These two coordination states are modulated by calcium and in presence of calcium peroxidase existed exclusively in 5cHS form. The second reason may be the differential affinity of calcium to binding sites on peroxidase. Plant peroxidases generally contain two binding sites for calcium [Haschke and Friedhoff, 1978].

3.1.2.10. Effect of calcium on structure of wheat bran peroxidase

The fluorescence spectra of peroxidase purified without calcium and after the addition of 3 mM calcium to the purified peroxidase showed common emission maxima at 335 nm. There was no change in the emission maximum upon the addition of calcium up to 3 mM and fluorescence intensity also remained unchanged (Fig 3.17). However, the soret band absorption at 403 nm due to the heme moiety, increased, when peroxidase was supplemented with calcium. As the concentration of calcium increased the extent of absorbance change also increased (Fig. 3.18). Intrinsic fluorescence property of tryptophan residues was effectively used to study the structural perturbations in

peroxidases. In plant peroxidase, at an excitation wavelength of 295 nm only tryptophan gives fluorescence [Chattopadhyay and Mazumdar, 2000]. The observation that addition of calcium has no effect on the fluorescence emission suggests, there is no alteration in the microenvironment of the tryptophan. However, change in the soret absorption produced by the addition of calcium indicates a change in the microenvironment of heme moiety. Therefore, it is clear that the increase in wheat bran peroxidase activity by calcium is due to a change in the heme environment rather than change in overall protein conformation.

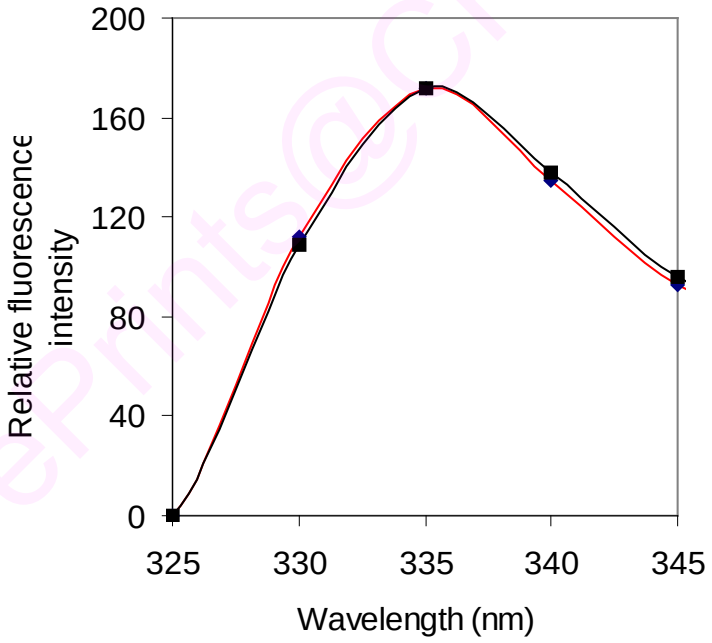


Fig. 3.17. Fluorescence spectra of wheat bran peroxidase purified without calcium (♦) and after the addition of 3 mM calcium (■)

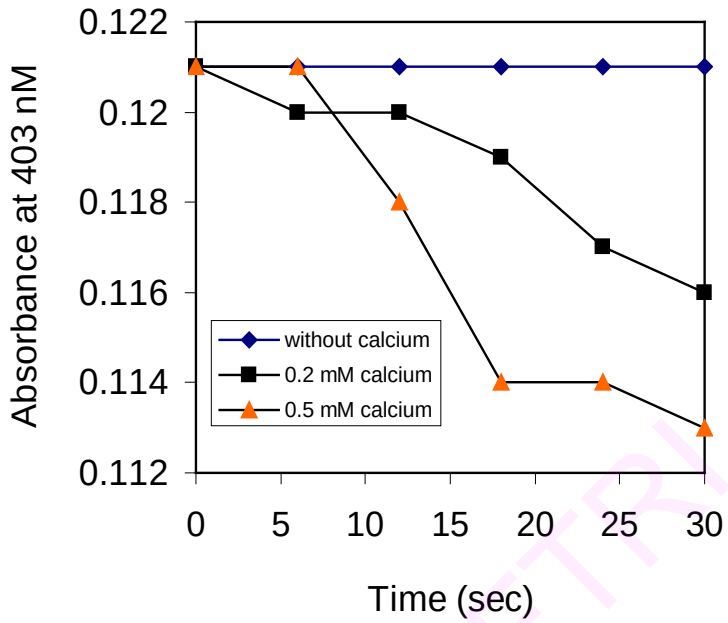


Fig. 3.18. Change in wheat bran peroxidase absorption at 403 nm at different concentrations of calcium chloride.

3.1.2.11. Thermal stability of wheat bran peroxidase

Heating of wheat bran peroxidase at 40^0C for 5 min sharply decreased peroxidase activity. About 80% activity was lost at 40^0C and only slight decrease of activity was found on further heating. At 60^0C heating for 25 min destroyed almost all activity. The enzyme retained about 50% of activity even after heating for 25 min at 60^0C in presence of calcium (Fig. 3.19). The thiol oxidase activity of peroxidase was also temperature sensitive. Heating the peroxidase at 60^0C for 25 min resulted in loss of 50% thiol oxidase activity. But presence of calcium provided stability against thermal inactivation of thiol oxidase activity. Wheat bran peroxidase retained 83% of thiol oxidase activity at

60^0C for 25 min when 1 mM calcium chloride was present in the incubation buffer (Fig. 3.20). Plant peroxidases from different sources exhibit differential thermal stability. A peroxidase from Australian carrot was completely inactivated by heating at 80^0C for 4 min, whereas a turnip anionic peroxidase retained good activity (~ 21%) even after 25 min heating at 80^0C [Vora et al, 1999; Duarte-Vazqez et al, 2003]. Different isozymes of okra lost about 65% of their activity when heated foe 5 min at 65^0C [Yemenicioglu et al, 1998]. Compared to these reported values, wheat bran peroxidase is more heat labile.

Calcium improved the thermal stability of wheat bran peroxidase. This is in consistent with a previous report of the effect of calcium on the thermal stability of artichoke peroxidase C [Sidarch et al, 2006]. The improved thermal stability of artichoke peroxidase by calcium was proposed to be due to the existence of peroxidase exclusively in 5cHS form in presence of calcium. They observed a change in the soret absorption as the temperature was increased which was consistent with the soret band change during the loss of 6-aquo ligand. The loss of 6-aquo ligand resulted in a fall in soret absorption [Hiner et al, 2003]. In wheat bran peroxidase also, addition of calcium resulted in a decrease in soret band absorption indicating possible shift in the equilibrium towards the formation of 5cHS species.

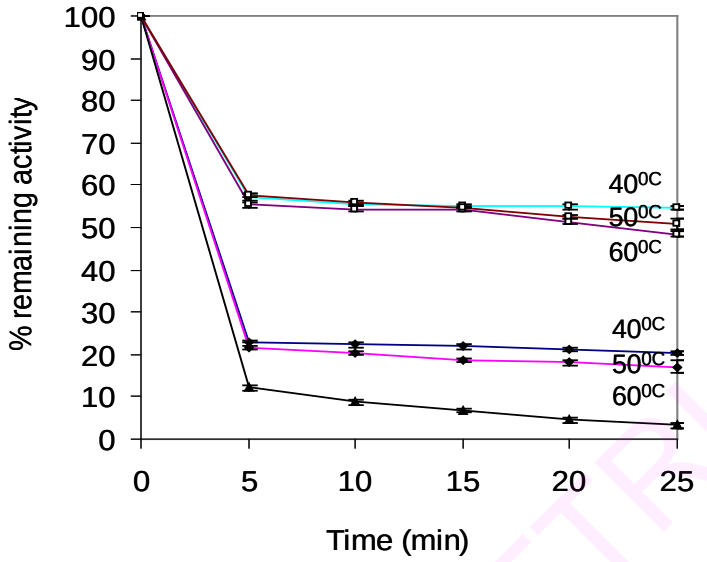


Fig. 3.19. Thermal stability of wheat bran peroxidase purified without calcium in absence (▲) and presence (□, 1 mM) of calcium chloride. Value at each point is average of three experiments ± SD

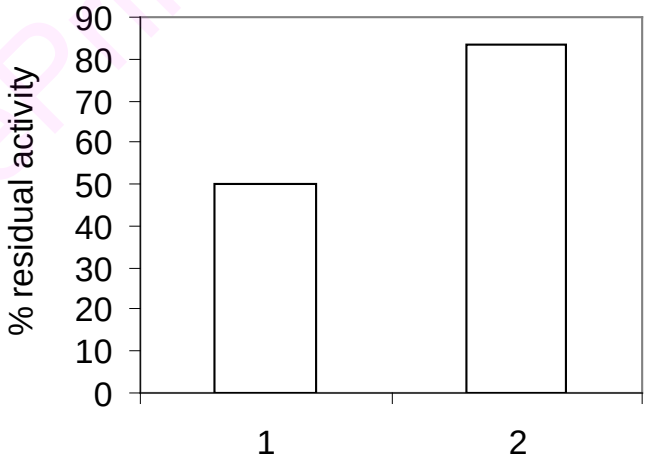


Fig. 3.20. Thermal stability of thiol oxidase activity of wheat bran peroxidase in absence of calcium (1) and in presence of 1mM calcium chloride (2)

Section B: Role of peroxidase in cross-linking of glutenin proteins

3.2.1. Cross-linking of mixture of High molecular weight and low molecular weight glutenin subunits by peroxidase

3.2.1.1. Cross-linking of subunits by wheat bran peroxidase

The mixture of isolated high molecular weight glutenin subunits and low molecular weight glutenin subunits were treated with different concentrations of wheat bran peroxidase purified in presence and absence of calcium. SDS-PAGE of enzyme treated subunits under reducing and non-reducing conditions (Figures 3.21 and 3.22) revealed the formation of higher molecular weight proteins. As can be seen in lanes 2-4 of Fig. 3.21, under non-reducing conditions, new protein bands were seen at the top (origin) of the separating gel as well as stacking gel in peroxidase (purified in absence of calcium) treated samples and no such high molecular weight bands were seen in control (untreated) samples. Therefore, peroxidase/H_2O_2 system produces cross-linked high molecular weight proteins from glutenin subunits.

The Coomassie stain intensity of cross-linked protein bands increased with increase in peroxidase concentration (fig. 3.21, lanes 6-8). The extent of cross-linking of glutenin subunits by peroxidase/H_2O_2 system was peroxidase concentration dependent. There was clear difference in the intensity of cross-linked proteins between non-reducing and reducing conditions. The higher intensity of cross-linked proteins under non-reducing condition reveals that the extent of cross-linking was more in this case. The results suggest that the

cross-linked high molecular weight proteins observed in non-reducing condition may be due to both disulfide bonds and other non-disulfide covalent bonds. As disulfide bonds are cleaved when reducing condition is applied the cross-linked high molecular weight bands in this case (lanes 6-8) may be due to the cross-linking of subunits by non-disulfide covalent bonds.

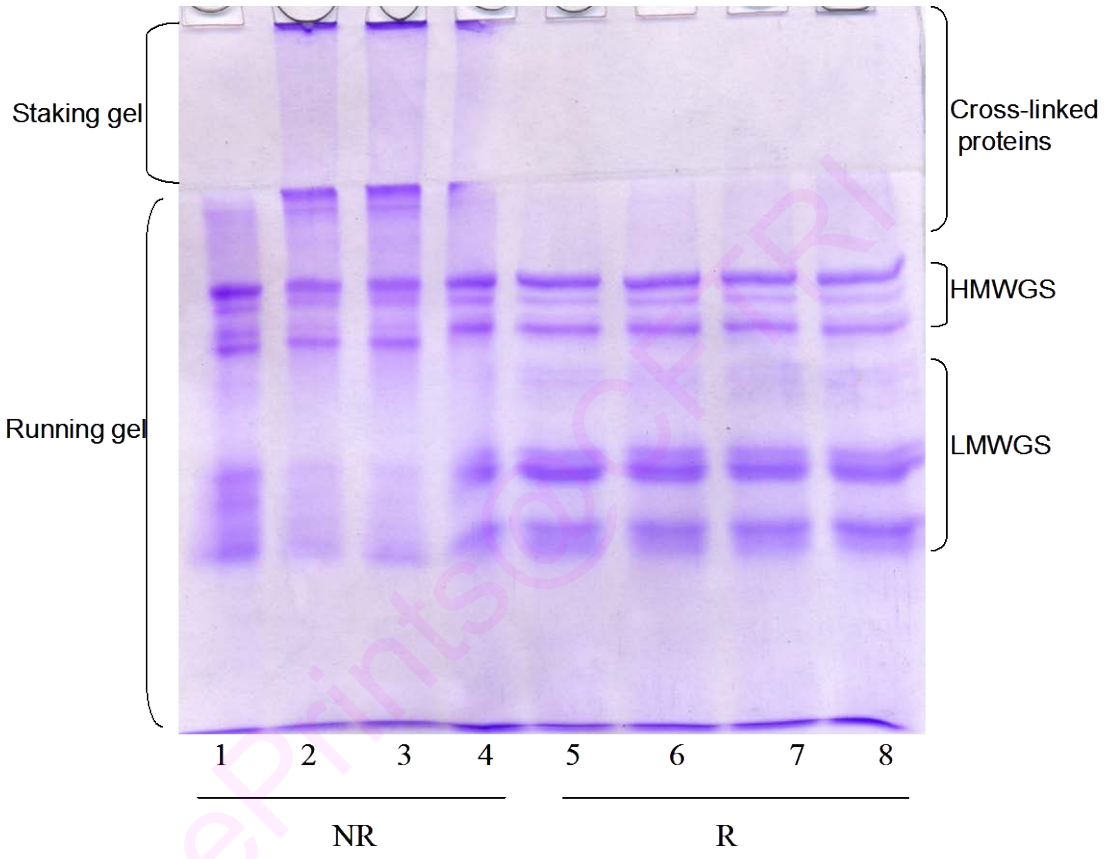


Fig. 3.21: Cross-linking of protein mixture containing equal quantities of high molecular weight glutenin subunits and low molecular weight glutenin subunits by wheat bran peroxidase purified in absence of calcium. Lanes 1 and 5, control; lanes 2 and 6, 50 U enzyme; Lanes 3 and 7, 100 U enzyme; Lanes 4 and 8, 200 U enzyme. 0.1 % H_2O_2 was used as co- substrate for peroxidase. NR, non-reducing condition; R, reducing condition.

When glutenin subunits were treated with peroxidase purified in presence of calcium similar results were observed (Fig 3.22). Therefore, further cross-linking studies were carried out only with peroxidase purified in presence of calcium.

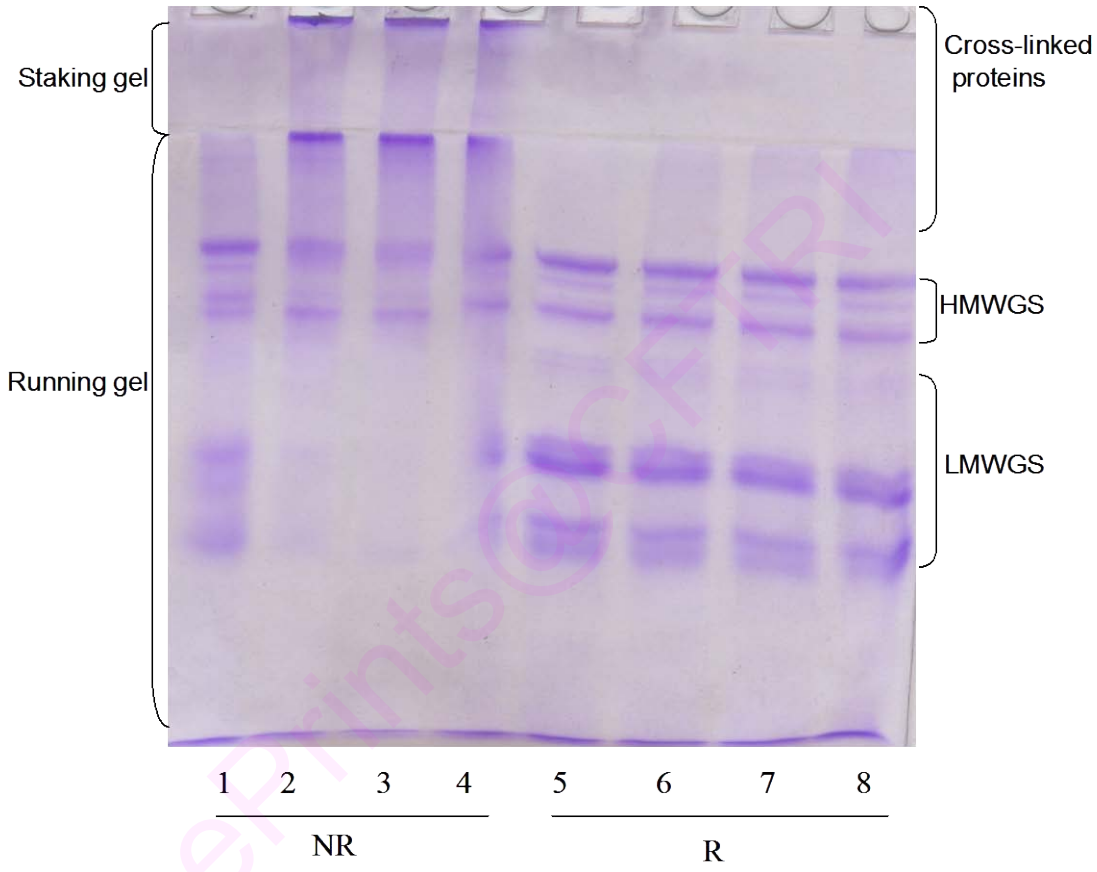


Fig. 3.22: Cross-linking of protein mixture containing equal quantities of high molecular weight glutenin subunits and low molecular weight glutenin subunits by wheat bran peroxidase purified in presence of calcium. Lanes 1and 5, control; lanes 2 and 6, 50 U enzyme; Lanes 3 and 7, 100 U enzyme; Lanes 4 and 8, 200 U enzyme. 0.1 % H_2O_2 was used as co-substrate for peroxidase. NR, non-reducing condition; R, reducing condition.

3.2.1.2. Cross-linking of glutenin subunits by horseradish peroxidase

The mixture of isolated glutenin subunits containing equal quantities of high molecular weight and low molecular weight glutenin subunits gave similar results (as in 3.2.1.1), when treated with different concentrations of horseradish peroxidase. SDS-PAGE of horseradish peroxidase treated subunits under reducing and non-reducing conditions revealed the formation of higher molecular weight proteins (Fig. 3.23). Therefore, the treatment of glutenin subunits with horseradish peroxidase also resulted in cross-linking of the subunits and extent of cross-linking was peroxidase concentration dependent. With the concentration of glutenin subunits used, 1000 U of horseradish peroxidase produced cross-linking of almost all glutenin subunits under non-reducing condition (Fig. 3.23, Lane 5).

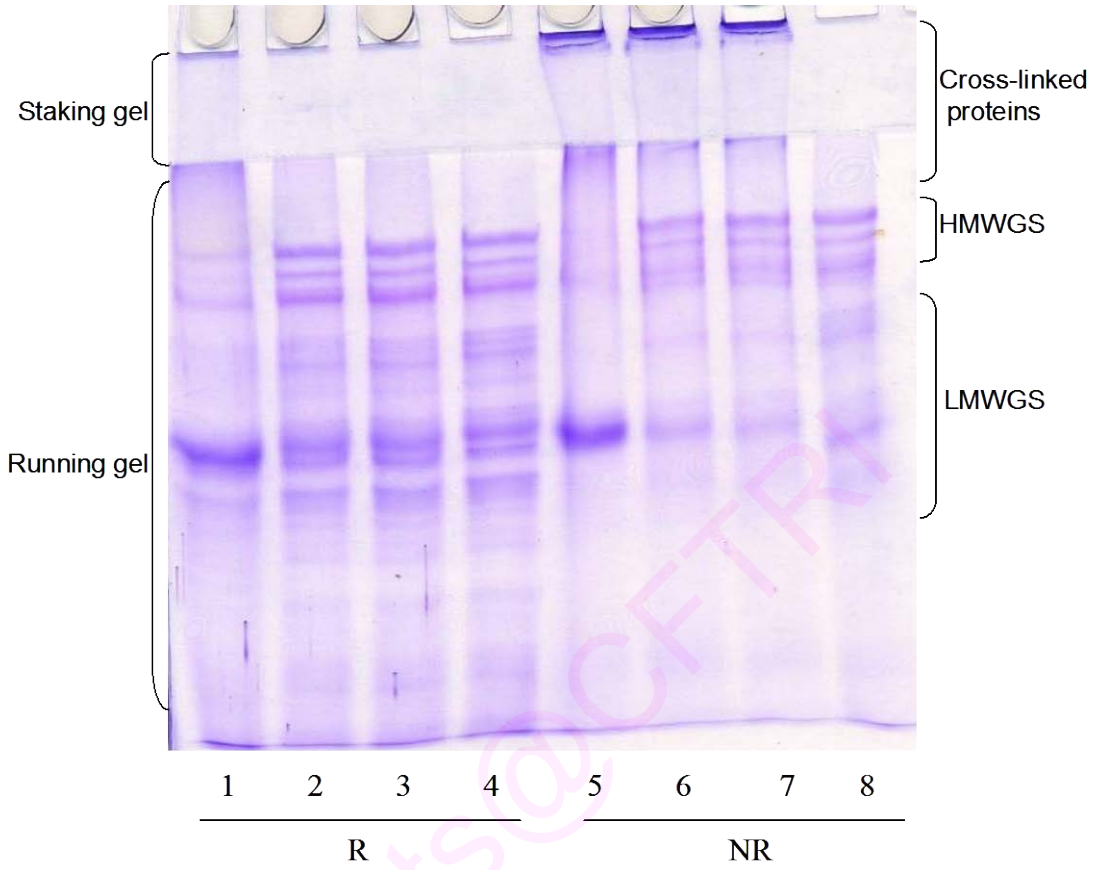


Fig 3.23: Cross-linking of mixture of protein containing equal quantities of high molecular weight glutenin subunits and low molecular weight glutenin subunits by different concentrations of horseradish peroxidase. Lane 4,8 control samples, Lane 3,7 samples treated with 50 U peroxidase, Lane 2,6 Samples treated with 100 U peroxidase, Lane 1,5 Samples treated with 1000 U peroxidase. 0.1 % H_2O_2 was used as co-substrate for peroxidase. NR, non-reducing condition; R, reducing condition.

3.2.2. Cross-linking of individual groups of high molecular weight and low molecular weight glutenin subunits by peroxidase

3.2.2.1. Cross-linking of low molecular weight glutenin subunits by wheat bran peroxidase

The wheat bran peroxidase treatment of isolated low molecular weight glutenin subunits produced cross-linked high molecular weight proteins as revealed by SDS-PAGE (Fig. 3.24). The intensity of cross-linked protein bands was more under non-reducing condition (Fig. 3.24, lanes 2-4) compared to that of reducing condition (Fig. 3.24, lanes 6-8). In both the cases the cross-linked protein band intensity increased with increase in peroxidase concentration. Therefore, the extent of cross-linking was peroxidase concentration dependent. A clear difference in the intensity of cross-linked high molecular weight protein bands between non-reducing and reducing conditions indicated that cross-linking was through both disulfide and non-disulfide covalent bonds.

3.2.2.2. Cross-linking of low molecular weight glutenin subunits by horseradish peroxidase

The horseradish peroxidase treatment of isolated LMW glutenin subunits also produced cross-linked high molecular weight proteins as revealed by SDS-PAGE (Fig. 3.25). Like cross-linking of low molecular weight glutenin subunits by wheat bran peroxidase, in this case also extent of cross-linking was peroxidase concentration dependent and was more under non-reducing conditions. Therefore, similar to wheat bran peroxidase, horseradish

peroxidase treatment produced both disulfide and non-disulfide cross-links in low molecular weight glutenin subunits.

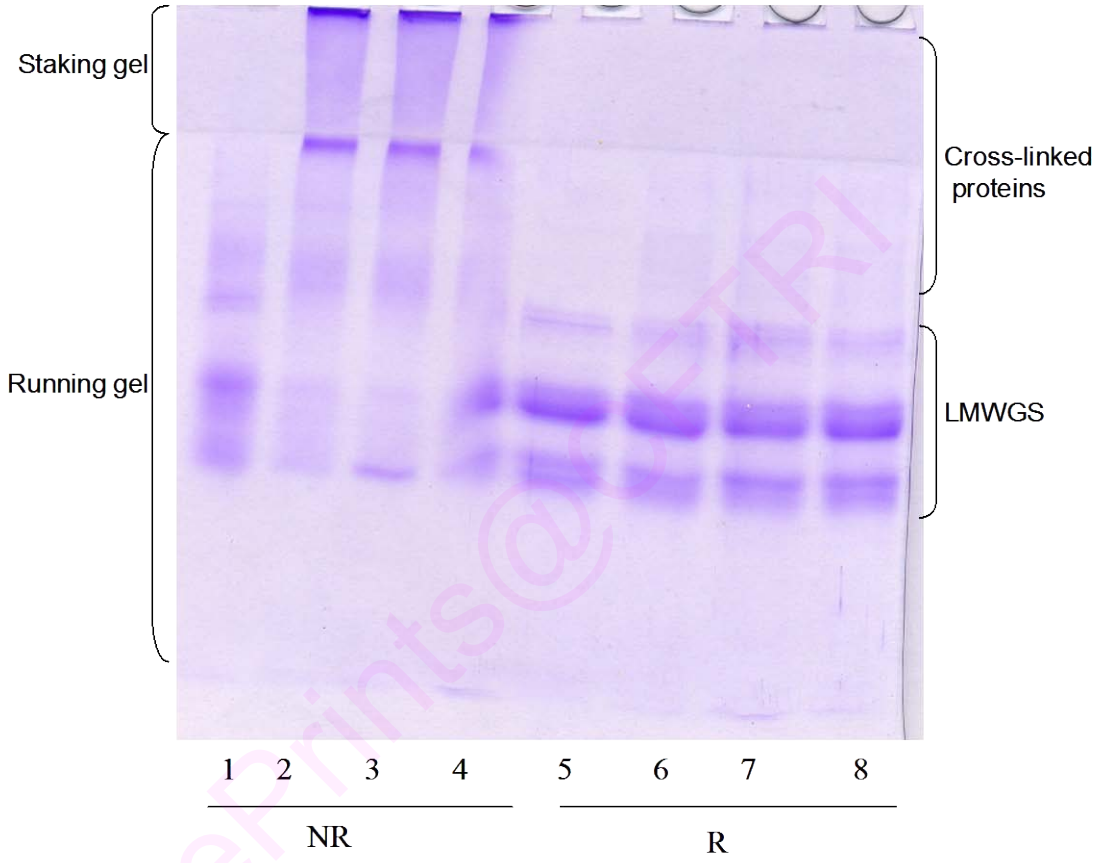


Fig. 3.24: Cross-linking of LMW glutenin subunits by wheat bran peroxidase purified in absence of calcium. Lanes 1 and 5 control; lanes 2 and 6, 50 U enzyme; Lanes 3 and 7, 100 U enzyme; Lanes 4 and 8, 200 U enzyme. NR, non-reducing condition; R, reducing condition.

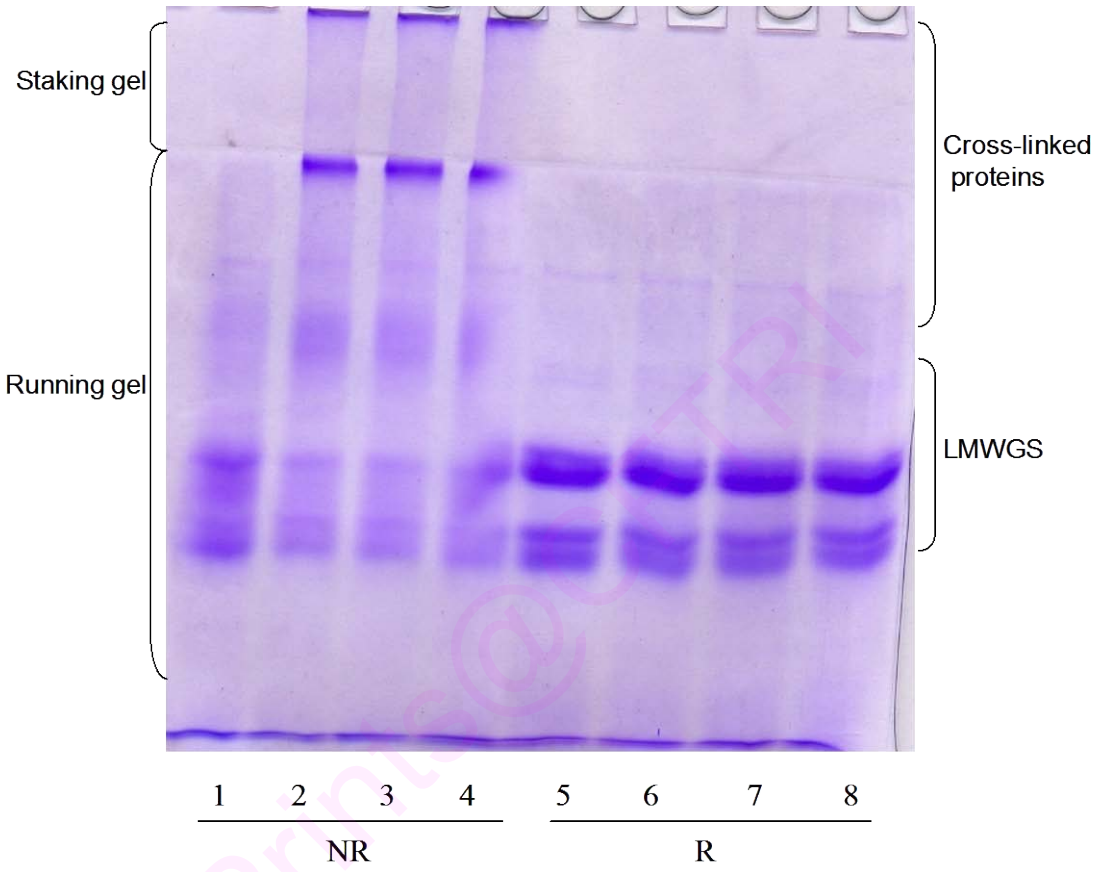


Fig.3.25: Cross-linking of low molecular weight glutenin subunits by horseradish peroxidase. Lane 1 and 5 control, lane 2 and 6- 50 U enzyme, Lane 3 and 7- 100 U enzyme, Lane 4 and 8- 200 U enzyme. NR, non-reducing condition; R, reducing condition.

3.2.2.3. Cross-linking of high molecular weight glutenin subunits by wheat bran peroxidase

The formation of cross-linked high molecular weight proteins were observed by SDS-PAGE of wheat bran peroxidase treated isolated high molecular weight glutenin subunits also (Fig. 3.26). Like previous results the extent of cross-linking was peroxidase concentration dependent. However, compared to low molecular weight glutenin subunits, the extent of cross-linking was less in high molecular weight glutenin subunits. In the former, 200 U of peroxidase cross-linked almost all protein (Fig. 3.24). Comparisons of cross-linked protein bands under non-reducing and reducing conditions reveal that cross-linking may be through both disulfide and non-disulfide covalent bonds.

3.2.2.4. Cross-linking of high molecular weight glutenin subunits by horseradish peroxidase

Treatment of isolated high molecular weight glutenin subunits with horseradish peroxidase also produced cross-linked high molecular weight proteins (Fig. 3.27). The results were similar to that of wheat bran peroxidase. In this case also the extent of cross-linking was peroxidase concentration dependent and less compared to that of low molecular weight glutenin subunits. Therefore, horseradish peroxidase also produces disulfide and non-disulfide covalent bonds in high molecular weight glutenin subunits.

The results indicated that the peroxidase treatment produced cross-linking in both low and high molecular weight glutenin subunits. However, the extent of cross-linking was more in low molecular weight glutenin subunits.

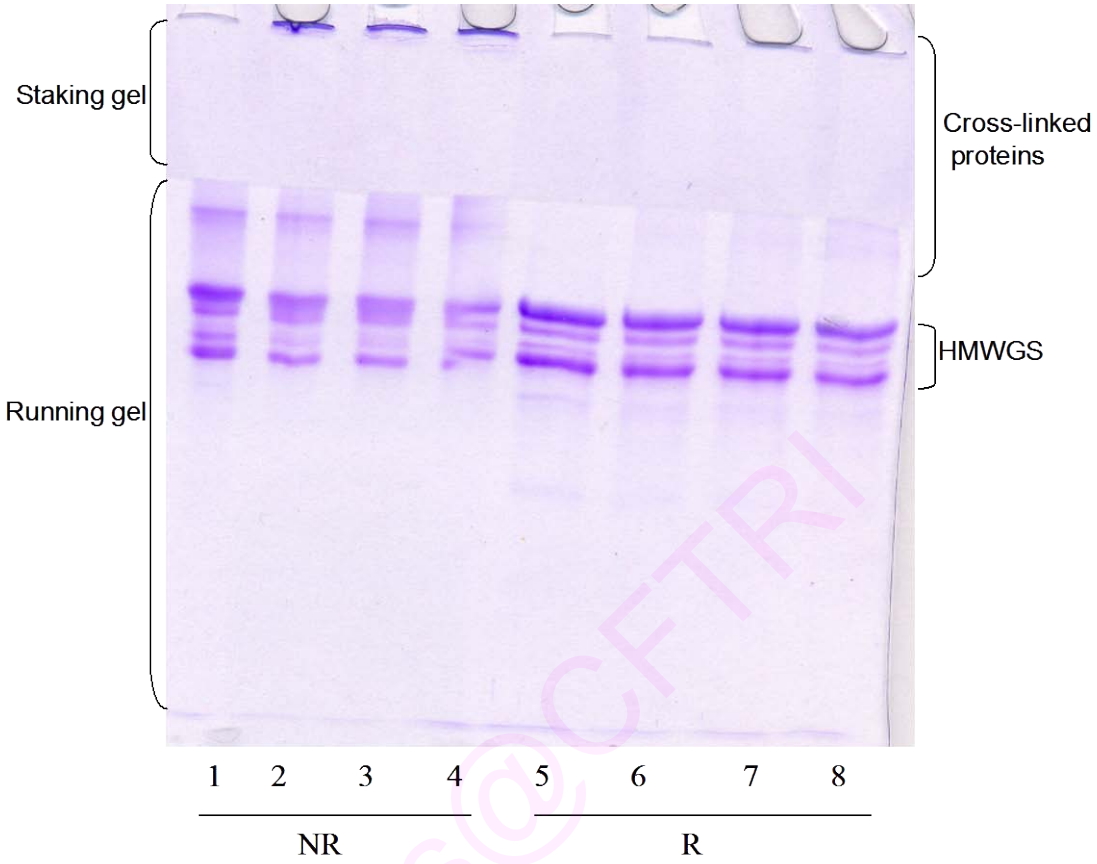


Fig.3.26. Cross-linking of high molecular weight glutenin subunits by wheat bran peroxidase purified in presence of calcium. Lanes 1 and 5 control, lanes 2 and 6- 50 U enzyme, Lanes 3 and 7- 100 U enzyme, Lanes 4 and 8- 200 U enzyme NR, non-reducing condition; R, reducing condition.

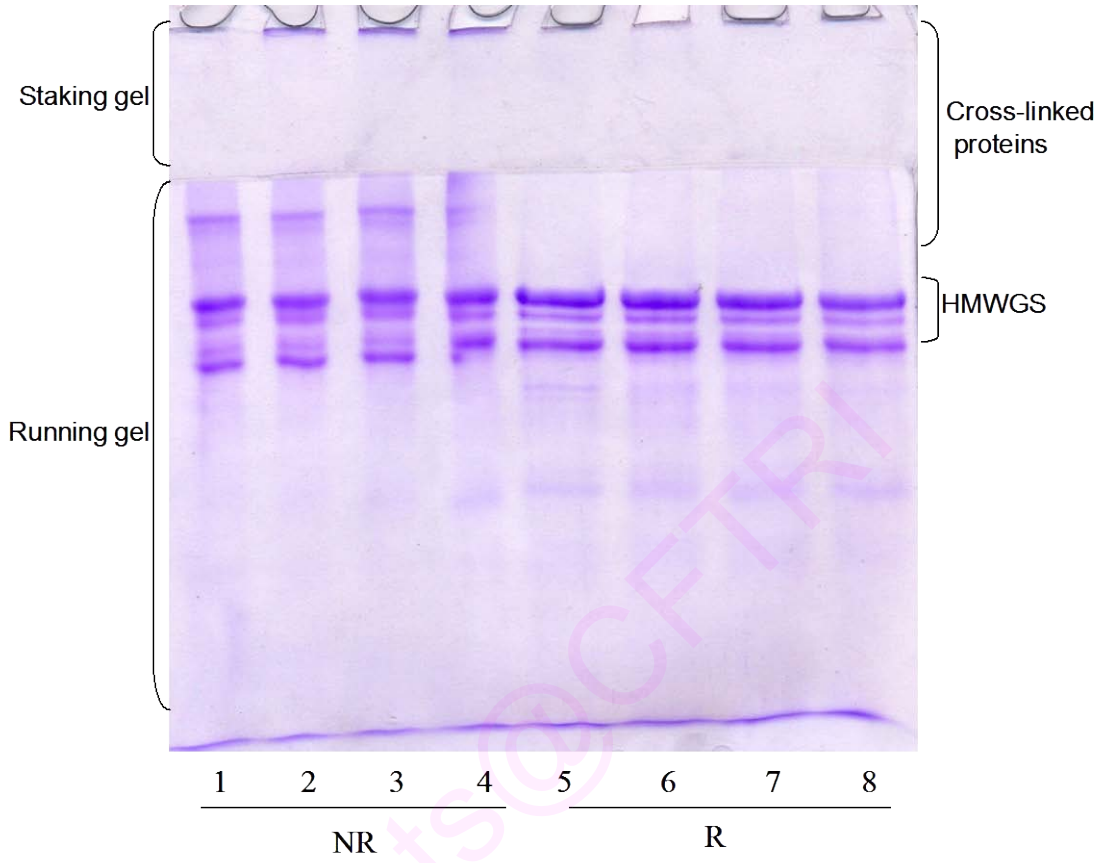


Fig.3.27. Cross-linking of high molecular weight glutenin subunits by horseradish peroxidase. Lane 1 and 5 control, lane 2 and 6- 50 U enzyme, Lane 3 and 7- 100 U enzyme, Lane 4 and 8- 200 U enzyme NR, non-reducing condition; R, reducing condition.

3.2.3. Cross-linking of glutenin subunits by hydrogen peroxide

3.2.3.1. Cross-linking of mixture of High molecular weight and low molecular weight glutenin subunits by hydrogen peroxide

Treatment of mixture of proteins containing equal quantities of low and high molecular weight glutenin subunits with H_2O_2 produced cross-linked high molecular weight proteins (Fig. 3.28). These high molecular weight cross-linked protein bands were observed only under non-reducing conditions (Fig. 3.28, lanes 5-7). When reducing condition was applied no such cross-linked protein bands were observed (Fig. 3.28, lanes 2-4) Therefore, H_2O_2 produced only disulfide bonds in glutenin subunits. The extent of cross-linking was H_2O_2 concentration dependent.

3.2.3.2. Cross-linking of individual groups of High molecular weight and low molecular weight glutenin subunits by hydrogen peroxide

Treatment of individual groups of low and high molecular weight glutenin subunits with 0.1% H_2O_2 produced cross-linked high molecular weight proteins as revealed by SDS-PAGE under non-reducing condition (Fig. 3.29, lanes 2 and 3). Under the reducing condition there was no such cross-linked proteins in both the cases (Fig.3.29, lanes 6 and 8). Therefore, H_2O_2 produced cross-linking in glutenin subunits only through disulfide bonds.

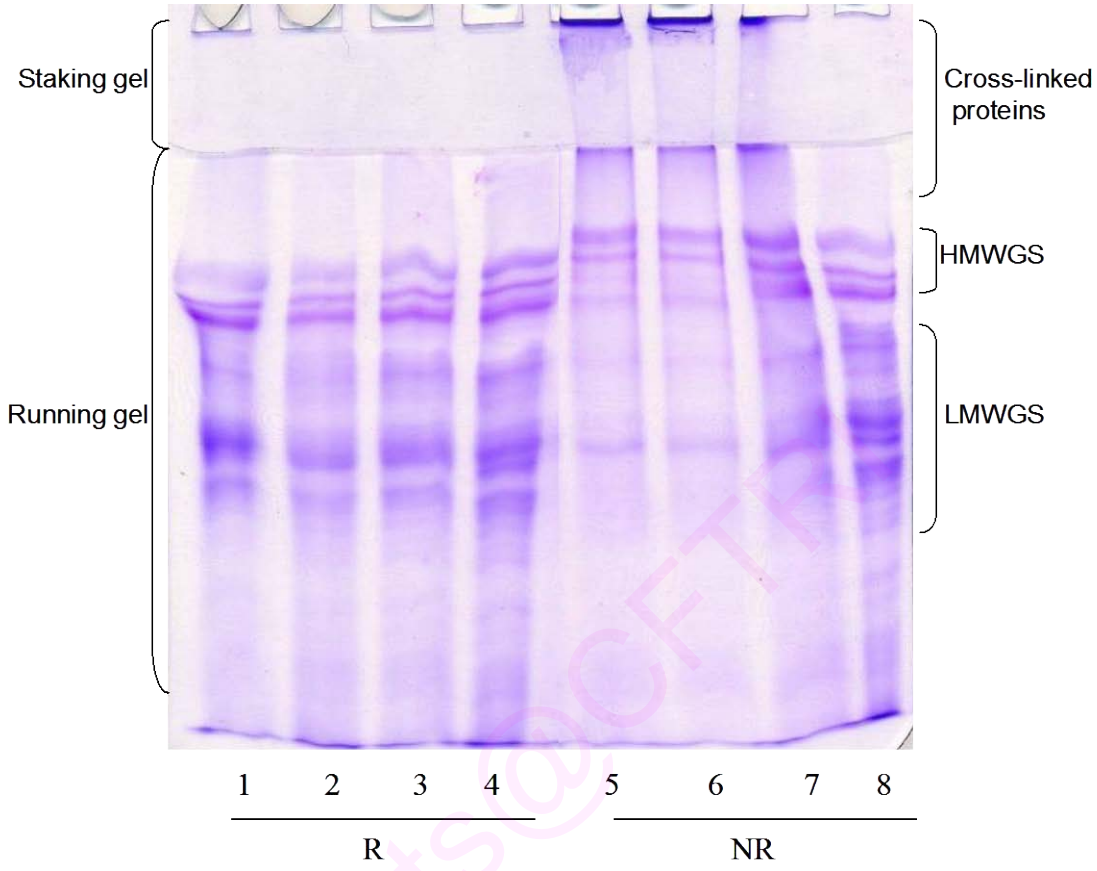


Fig 3.28: Cross-linking of mixture of glutenin subunits containing equal quantities of high molecular weight glutenin subunits and low molecular weight glutenin subunits by different concentration of H_2O_2. Lane 1,8 control samples; Lane 2,7 samples treated with 0.1 % H_2O_2, Lane 3,6 Samples treated with 1% H_2O_2, Lane 4,5 samples treated with 10% H_2O_2. NR, non-reducing condition; R, reducing condition.

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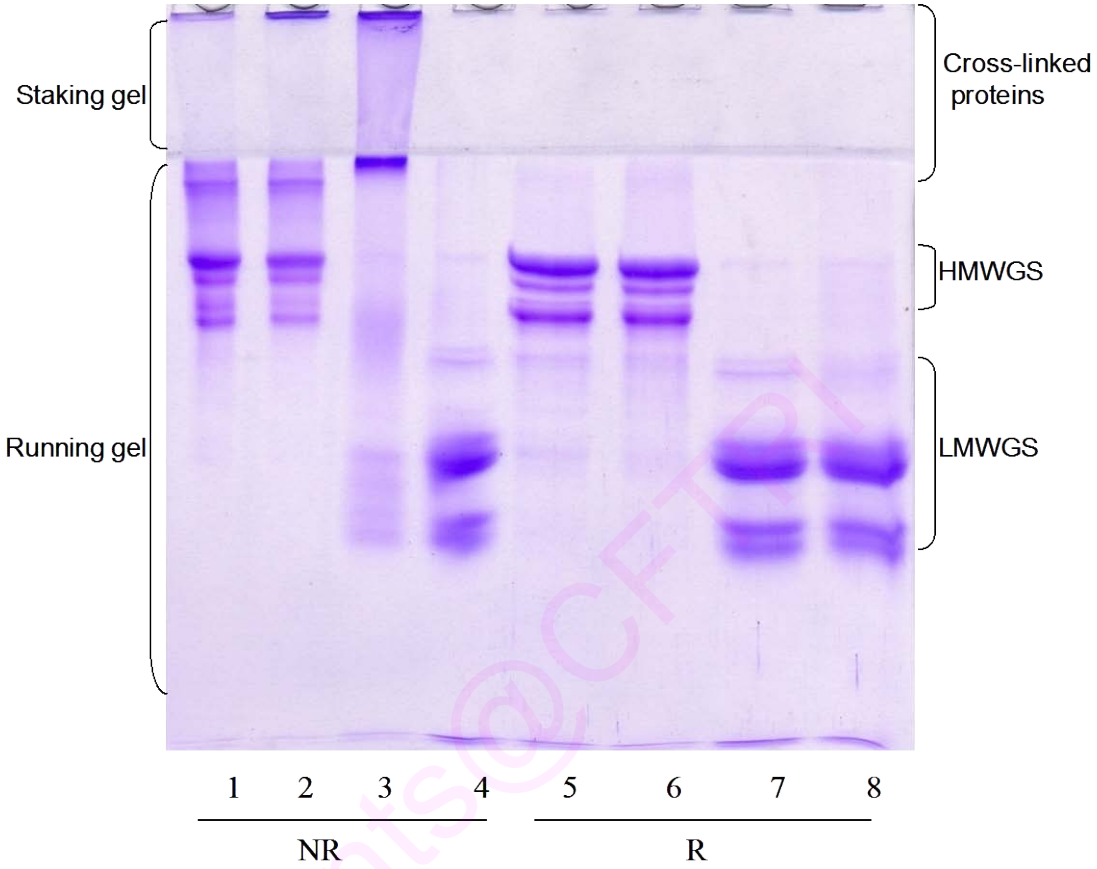

Fig.3.29: Cross-linking of high molecular weight glutenin subunits and low molecular weight glutenin subunits by 0.1% H_2O_2. Lane 1,2 5 and 6 high molecular weight glutenin subunit, Lane 3,4,7 and 8 low molecular weight glutenin subunits. Lane 1,4,5 and 7 control samples, Lane 2,3,6 and 8 H_2O_2 treated samples.
NR, non-reducing condition; R, reducing condition.

3.2.3.3. Characterization of cross-linked protein

The fluorescence emission spectrum of cross-inked proteins with an excitation wavelength of 318 nm gave an emission maximum at 380 nm (Fig. 3.30). A similar fluorescence emission spectrum was obtained for peroxidase catalyzed cross-linked gliadin molecules, with an excitation wavelength of 318 nm. The emission maximum at 380 nm was attributed to the dityrosine molecule [Michon et al, 1999]. Therefore, the non-disulfide covalent bonds observed in the present study are due to dityrosine cross-links.

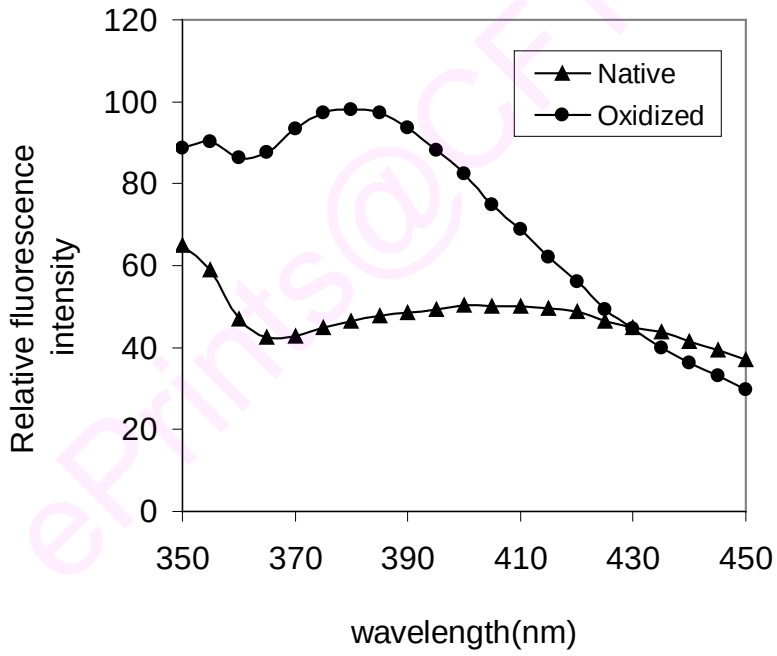

Fig. 3.30. Fluorescence emission spectrum of glutenin subunits.

Transglutaminase is one of the enzymes used extensively for the cross-linking of different proteins. It catalyses the formation of ε-(γ-glutamyl) lysine cross-links in proteins. Studies on cross-linking of gluten proteins by transglutaminase indicated that low molecular weight glutenin subunits were less reactive compared to high molecular weight glutenin subunits (Larre et al., 2000; Tseng and Lai, 2002). In the present study it was found that low molecular weight glutenin subunits were more reactive to form cross-links in presence of peroxidase. This difference may be attributed to the amino acid side chain participating in the cross-linking. Peroxidase used cysteine and tyrosine residues as substrates whereas transglutaminase uses glutamine and lysine residues as substrates.

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Section C: Role of wheat proteins in flour functionality

3.3.1. Wheat flour protein fractionation

3.3.1.1. High molecular weight glutenin subunit composition

Whole-wheat flour extracts of different varieties were subjected to SDS-PAGE on 10% gel. The results showed a clear difference among the varieties with respect to subunit composition (Fig.3.30). NI5439 and K9644 wheat varieties had similar high molecular weight glutenin subunit composition at Glu 1B and Glu 1D loci. Subunits at Glu 1A locus were absent in these two varieties, indicating that they carried null allele. The variety MACS2496 was different from other varieties in having subunits 5+10 at Glu 1D and subunit 1 at Glu 1A. The Glu 1 score, which reflects the synergistic contribution of glutenin subunits to the quality of flour, was also different in the wheat varieties studied. The Glu 1 score of 8 varieties ranged from 6 to 8 (Table. 3.5). The Glu 1 scores showed significant positive correlation to cutting force ($r = 0.70$, $p<0.05$), which is one of the important quality parameters of chapati. Out of 8 varieties studied, only MACS2496 possessed the 1BL/1RS chromosome. Though other varieties did not possess 1BL/1RS chromosome, the overall quality scores of chapatis prepared from these varieties did not show significant difference. Thus, presence of 1BL/1RS chromosome does not seem to influence the quality of chapati.

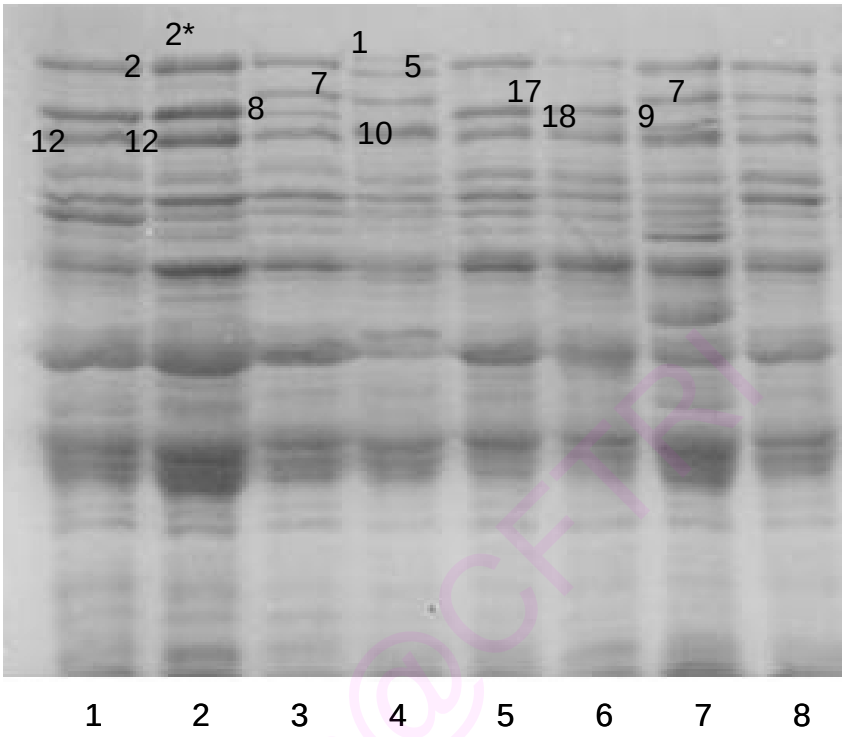

Fig 3.31. SDS-PAGE of whole-wheat flour proteins on 10% gel. Lane 1, HD 2189; Lane 2, HD2781; Lane 3, HD2501; Lane 4, MACS 2496; Lane 5, K9644, Lane 6, NI5439; Lane 7, NIAW34; Lane 8, GW322.

Table 3.5. High molecular weight glutenin subunit composition of different wheat varieties

Wheat variety	High molecular weight glutenin subunits and Glu-1 score				
	Glu 1A	Glu 1B	Glu 1D	Presence of 1BL/RS	Glu-1 score
GW 322	2*	7 + 8	2 + 12	-	8
MACS 2496	1	7 + 9	5 + 10	+	6
HD 2501	2*	7 + 8	2 + 12	-	8
NI 5439	Null	17 + 18	2 + 12	-	6
K 9644	Null	17 + 18	2 + 12	-	6
HD 2189	2*	17 + 18	2 + 12	-	8
HD 2781	2*	17 + 18	2 + 12	-	8
NIAW 34	2*	7 + 9	2 + 12	-	7

The Glu-1 scores were assigned for individual high molecular weight glutenin subunits encoded by Glu 1A locus, but in the case of 1D alleles and some 1B alleles scores were assigned to subunit pairs rather than individual subunits because of the close linkage between the genes for x- and y- type subunits. As for as bread-making quality is concerned, the best quality subunits are the allelic pairs 1Dx5+1Dy10. Only slightly lower in quality are 1Ax subunits 1 and 2* compared with the null allele and the 1B subunit pairs 1Bx17+1By18 and 1Bx7+1By8 [Payne et al, 1979; Payne, 1987]. Qualitative effects on the functionality may result from differences between allelic subunits either in their structures or in their abilities to form polymers with other high molecular weight

subunits and low molecular weight subunits [Flavel et al, 1989; Greene et al, 1988]. Although many reports are available on the relation between high molecular weight glutenin subunit composition and bread making quality, only few reports are available on the chapati making quality. Recently, Anjum et al (2000) have reported that subunits 2+12 and 5+10, encoded by Glu 1D locus have no influence on chapati quality. However, Srivastava et al, (2003) observed that varieties having HMW glutenin subunits 5+12 encode by Glu 1D locus yielded excellent chapatis.

3.3.1.2. Fractionation of wheat flour proteins by gel filtration chromatography and effect of protein fractions on chapati quality

Proteins extracted from whole-wheat flour were fractionated on Sephadex G-200 column. They were separated into four peaks namely, peak I, peak II, peak III, and peak IV with the peak molecular weights >200, 70, 35, 20 kDa, respectively (Fig. 3.31). The yields of peaks I to IV varied from 7 to 13, 13 to 23, 18 to 25 and 28 to 43%, respectively (Table. 3.6). Even though there were differences in the contents of peaks I to III among the varieties, they did not correlate to chapati quality. However, quantity of peak IV was significantly correlated to overall quality score of chapati ($r = 0.78$, $p < 0.05$). The results indicated that varieties containing higher amount of 20 kDa protein fraction (peak IV) yielded good quality chapati. The molecular weight of majority of albumins and globulins is reported to be around 20 kDa, and hence will be present in peak IV. Bread quality was reported to be positively correlated to contents of glutenin (residue protein) and glutenin I fractions [Hamada et al,

1982], while low-molecular-weight fraction like albumin and globulin contents either do not influence the bread quality or negatively correlated [Hoseney et al, 1969; MacRitchie, 1978].

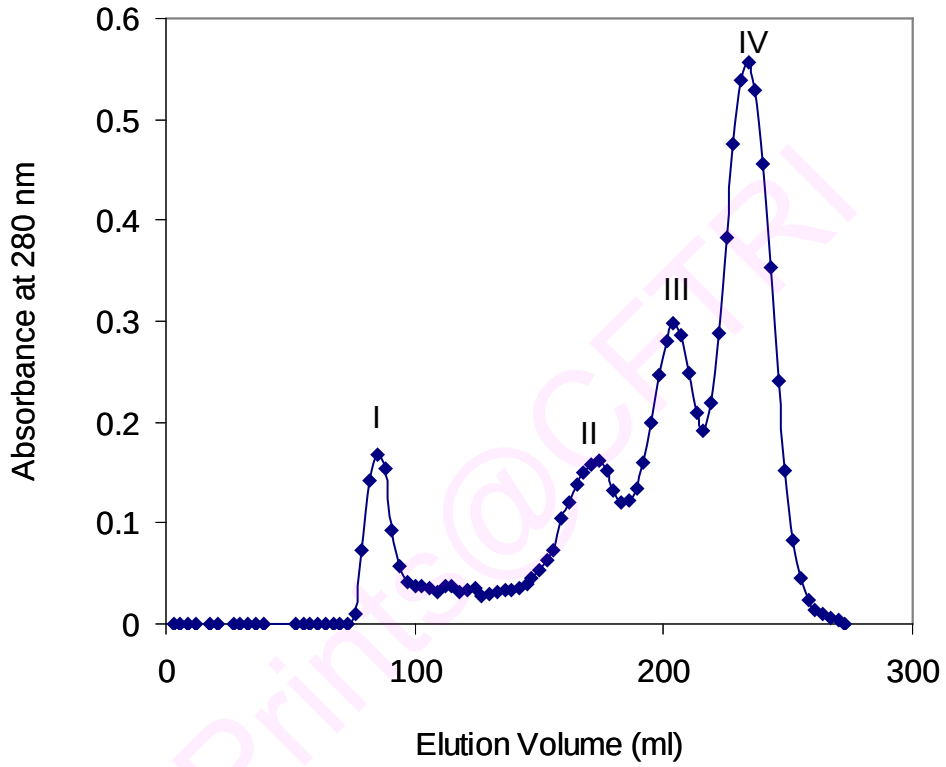

Fig. 3.32. Representative gel filtration chromatogram of whole-wheat flour proteins on Sephadex G-200 using 0.1 M acetic acid as eluent.

Table. 3.6. Percentage distribution of different protein fractions in flours of different wheat varieties obtained from gel filtration chromatography

Wheat variety	Peak I (200 kDa)	Peak II (55 kDa)	Peak III (35 kDa)	Peak IV (20 kDa)	Overall quality score of chapati
GW322	7.0 ± 0.5	19.2 ± 0.9	25.0 ± 1.0	41.0 ± 2.0	41.7
K9644	6.3 ± 0.5	12.5 ± 0.4	18.0 ± 0.7	41.0 ± 1.8	38.2
NI5439	7.9 ± 0.6	14.0 ± 0.4	23.5 ± 1.0	42.6 ± 1.6	40.0
NIAW34	7.0 ± 0.4	15.0 ± 0.8	23.5 ± 0.9	39.8 ± 1.8	39.0
HD2501	12.3 ± 0.5	22.9 ± 1.0	22.0 ± 0.8	28.0 ± 1.0	37.4
HD2189	13.0 ± 0.6	17.5 ± 0.7	20.0 ± 0.8	32.5 ± 1.5	36.0
HD2781	8.0 ± 0.7	21.0 ± 0.9	24.0 ± 0.8	28.0 ± 1.5	37.2
MACS2496	7.8 ± 0.5	18.3 ± 0.8	18.5 ± 1.1	29.0 ± 1.8	35.2

3.3.1.3. Extraction of flour proteins with SDS containing buffer

Proteins from the whole-wheat flours of different varieties were solubilized in SDS containing buffer in two-step extraction procedure. In the first step, flours were extracted in SDS buffer, and in the second step, the unextracted solid was resuspended in SDS buffer and sonicated. Figure 3.32, shows the percentage of protein extracted on the basis of total flour protein. About 70 to 92% was extracted by SDS buffer and about 10% of proteins from the remaining residue were solubilized by sonication.

The main constraint in studying the properties of wheat proteins is the difficulty in completely extracting them without disturbing native state. Dilute

acetic acid and aluminum lactate buffers have been used to solubilize the proteins, but their extractability was very less [Danno et al, 1974]. Subsequently, Gupta et al, (1993) used 0.5% SDS containing buffer and sonication to achieve maximum solubility of wheat flour proteins. Further, by using this procedure they could extract maximum amount of glutenin polymeric proteins. However, protein extractability depends on nature of wheat milling also. About 61 to 78% protein extractability was reported by Singh et al, (1990) from different varieties of bread wheat flour (refined wheat flour) using 2% SDS. Changes in protein extractability can occur due to variations in their molecular size and conformation [Phillips et al, 1994]. The whole wheat flour used in this study may exhibit different protein characteristic and functional properties compared to refined flour as the former contains bran and germ which contribute significant amount of protein to the total protein [MacMaster et al, 1978].

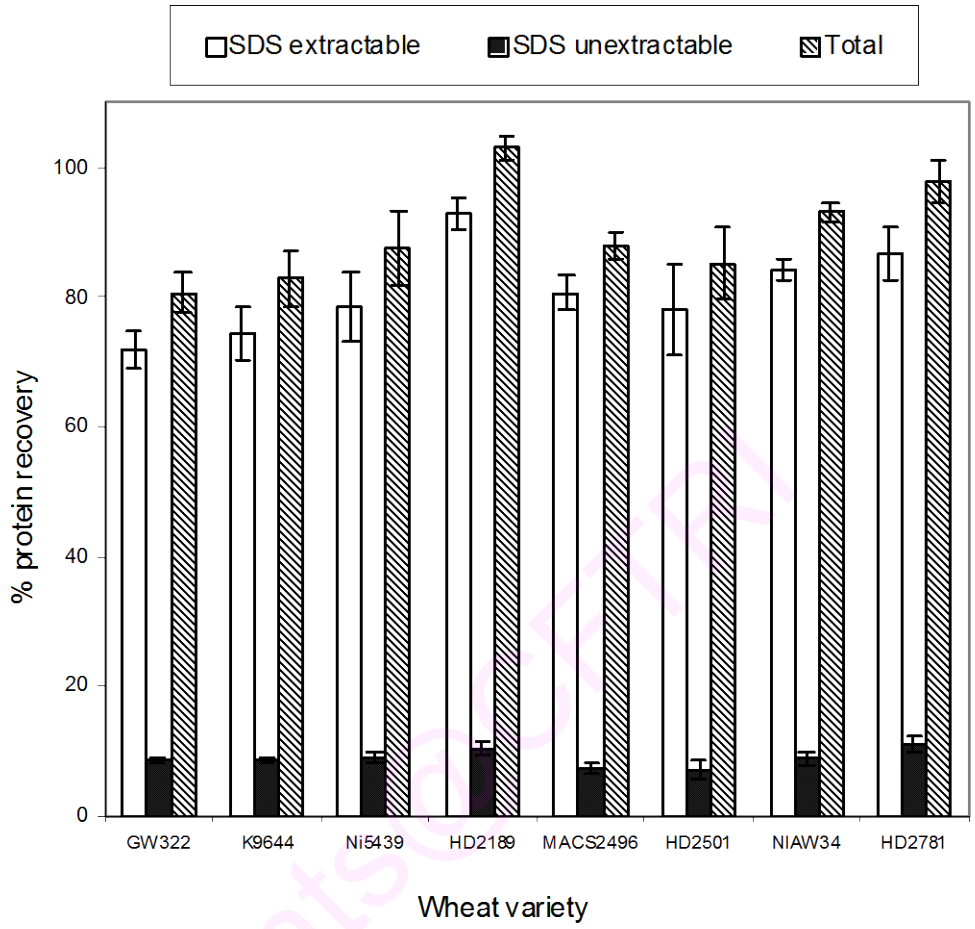

Fig.3.33. Extractabilities of flour proteins (based on Lowry protein estimation) in SDS containing phosphate buffer from eight flours without and with sonication. Values are average of three experiments ± SD.

3.3.1.4. Fractionation of proteins by SE-HPLC

The proteins extracted with SDS buffer as well as solubilized by sonication were fractionated based on their size by SE-HPLC. The protein profiles obtained were similar for both extracts. However, there was difference in the quantitative distribution of these fractions between the two extracts. The chromatograms can be divided into four parts, fractions I, II, III and IV in the decreasing order of their molecular weight (Fig.3.33). Fraction I had proteins of molecular weight >130 kDa (designated as large polymeric proteins), fraction II had proteins of molecular weight between 80 and 130 kDa (designated as small polymeric protein), fraction III had proteins between 10 and 80 kDa (designated as monomeric proteins). Fraction IV had very low molecular weight components (<5 kDa). This fraction may be due to some peptides or phenolic compounds that absorb at 280 nm. This low molecular weight fraction is expected as, whole-wheat flour contains bran and germ which are rich in polyphenols, pigments, peptides and other low molecular weight compounds [Fortmann and Joiner, 1978; Liyana-Pathirana and Shahidi, 2006; Hemalatha et al, 2007].

The percentage recovery of these protein fractions for different varieties is given in Table. 3.7. In most cases the percentage yield of large polymeric protein fraction extracted by SDS buffer is much higher (26-52%) than that of small polymeric protein fraction (16-35%). Extraction of whole wheat flour proteins with dilute acetic acid and subsequent gel filtration chromatography yielded a high molecular weight fraction content of about 10% [Hemalatha et al, 2007]. The difference in these two studies could be attributed to the solvent

used for solubilization of proteins. Mecham et al, (1962), as well as Danno et al, (1974), reported that acetic acid could extract only 62% of total proteins. In the present study, SDS buffer extracted as high as 90% of total proteins.

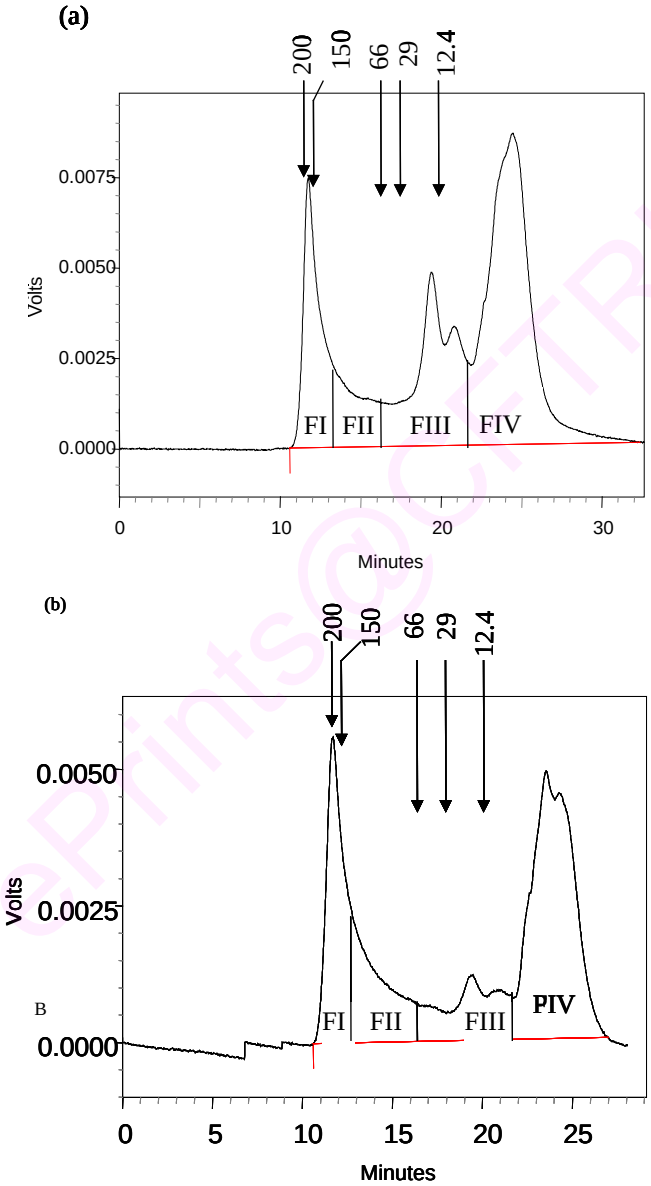

Fig.3.34. Representative SE-HPLC chromatograms; (a) SDS extractable proteins; (b) SDS unextractable proteins; FI large polymeric proteins; FII small polymeric proteins; FIII monomeric proteins.

Table 3.7. Area percent distribution of different protein fractions in flours of different wheat varieties obtained from SE-HPLC

Wheat varieties	Total protein (%)	SDS-extractable			SDS-unextractable		
		FI	FII	FIII	FI	FII	FIII
GW322	13.3±0.05	34.4±2.8	18.5±0.8	47.1±3.5	46.7±2.4	27.4±3.5	25.9±1.4
K9644	13.3±0.16	32.3±1.3	18.8±2.3	48.9±2.2	37.5±4.1	24.0±0.9	30.9±2.1
NI5439	13.5±0.13	47.8±1.3	15.9±4.3	33.8±1.0	16.4±2.6	33.8±2.2	49.8±2.3
HD2189	12.0±0.27	51.8±3.6	21.0±3.5	27.2±3.2	14.9±1.5	49.2±1.5	35.9±3.0
MACS2496	14.6±0.16	42.5±2.9	19.2±2.7	43.0±0.6	42.8±1.5	17.6±2.5	39.6±1.0
HD2501	12.7±0.23	36.5±3.8	16.5±1.4	47.0±2.8	36.9±1.8	26.1±2.3	37.0±1.0
NIAW34	13.7±0.08	32.5±0.8	15.6±2.2	52.9±2.5	14.6±2.4	38.5±2.6	46.8±3.5
HD2781	11.6±0.18	25.7±2.6	34.5±3.8	39.8±1.3	42.2±3.0	21.1±1.9	36.7±1.5

FI, FII, FIII represent peak areas shown in Fig. 3.33.
Data expressed as mean ± SD of three experiments

3.3.1.5. Dough characteristics of different wheat varieties

Flour quality can be determined by the rheological properties of dough and recently, Instron universal testing machine has been used for the determination of rheological properties of bread and biscuit doughs [Gujral et al, 2003; Angioloni and Rosa, 2007; Sudha et al, 2007]. The chapati dough characteristics of different wheat varieties are shown in Table. 3.8. The results indicated that dough hardness varied from 5.6 to 7.5 N and was the highest for HD2189 followed by HD2781 and it was the lowest for MACS2496. Cohesiveness of the dough varied from 0.19 to 0.31 while adhesiveness varied from 0.66 to 1.46 N.

Both the values were higher for the dough obtained from HD2501, HD2189, and HD2781 varieties. However, dough made from MACS2496 had the lowest adhesiveness.

Table. 3.8. Texture profile of dough and chapati cutting force

Wheat variety	Dough hardness (N)	Cohesiveness	Adhesiveness (N)	Chapati cutting force (N)*
GW322	5.96 ± 0.14	0.19 ± 0.01	0.89 ± 0.04	5.10 ± 0.41
K9644	6.08 ± 0.39	0.22 ± 0.02	0.93 ± 0.04	4.22 ± 0.51
NI5439	6.45 ± 0.29	0.24 ± 0.01	1.22 ± 0.04	5.49 ± 0.20
HD2189	7.52 ± 0.09	0.26 ± 0.04	1.42 ± 0.21	6.67 ± 0.15
MACS2496	5.6 0 ± 0.25	0.24 ± 0.02	0.66 ± 0.11	4.51 ± 0.58
HD2501	6.11 ± 0.35	0.20 ± 0.02	1.46 ± 0.06	5.59± 0.18
NIAW34	6.87 ± 0.35	0.31 ± 0.02	1.07 ± 0.11	5.49 ± 0.18
HD2781	7.38 ± 0.33	0.22 ± 0.01	1.34 ± 0.05	6.96 ± 0.21

Mean ± SD of three experiments.
*Hemalatha et al, (2007)

3.3.1.6. Thiol and disulfide contents in flour and their relation to chapati texture

Baking quality of wheat is governed by total protein content, type of proteins, and thiol (SH) and disulphide (SS) content in flour (Pomeranz, 1978). In the present study, protein thiol and disulphide, and non-protein thiol content in different wheat varieties were determined (Table 3.9). The protein SH content varied from 11.75 to 14.52 µmole/g flour, while SS content varied from 3.00 to 9.16 µmole/g flour. Though there are few reports on SS and SH contents in refined wheat flours, no report is available on whole-wheat flour

(chapati flour) with regard to SS and SH contents. The SS and SH contents in different refined wheat flours were reported to range from 8.5 to 16.9 µmole/g and 1.0 to 2.65 µmole/g flour, respectively (Tsen & Bushuk, 1968; Beveridge, et al, 1974). However, results in the present study indicated that SH content is very high in whole wheat flours while SS content is lower compared to refined wheat flours, which are used for bread making.

Wheat based products like chapati and bread are prepared after mixing the flour with water to form the dough. Interchange of thiols and protein disulphide bonds of the gluten proteins has been demonstrated during the dough mixing (Kasarda et al, 1978). In the present study, protein thiol content in the flour negatively correlated to chapati texture ($r = -0.77$ at $p<0.05$) (Table 3.10). Wheat flour also contains non-protein, small molecular weight thiol compounds or free thiols like glutathione and cysteine. Non-protein thiol content in the eight flours studied varied from 0.15 to 0.24 µmole/g (150-240 nmol/g) (Table. 3.9.). Although the free thiol contents are low, they have been considered to play an important role in redox reactions in flour. Content of free thiols like GSH and cysteine was reported to influence the rheological and bread properties of flour (Tkachuk, 1970; Grosch, 1986; Sarwin et al, 1993; Lambert and Kokini, 2001). It has been observed that most SH and SS interchange reactions between gluten proteins and low molecular weight SH compounds takes place during dough mixing resulting in depolymerization of gluten proteins (Dong and Hoseney, 1995). Thus, an analysis of correlation between thiol-disulfide content of different flours and the chapati texture has revealed that high free thiol content in flour decreases the toughness of chapati.

The non-protein thiol and protein disulfide content in flour also influenced the content of polymeric proteins. The SS content in flour was positively correlated to large extractable polymeric protein and total polymeric protein content while free thiol content was significantly negatively correlated to total polymeric protein content in flour. The content of both GSH and cysteine in six wheat flours were reported to be between 16 and 46 nmol/g (Sarwin et al, 1993). While in the present study it ranged from 150 to 240 nmol/g whole wheat flour. Thus, the high content of free thiols may change the polymeric protein content and therefore, the product quality. Earlier studies also reported that the levels of reducing agents in wheat flour are important as these compounds modify the structure and functional properties of gluten proteins, which affects the product quality (Lavelli et al, 1996; Li and Lee, 1996).

Table 3.9. Thiol and disulfide contents in flour

Wheat variety	Protein disulfide (µMol/g)	Protein thiol (µMol/g)	Non-protein thiol (µMol /g)
GW322	4.66 ±0.30	12.59 ±0.12	0.16 ±0.01
K9644	3.00 ±0.14	14.52 ±0.15	0.20 ±0.01
NI5439	4.66 ±0.30	11.75 ±0.11	0.15 ±0.03
HD2189	6.50 ±0.15	12.04 ±0.13	0.19 ±0.01
MACS2496	4.66 ±0.20	13.29 ±0.17	0.24 ±0.01
HD2501	5.00 ±0.22	11.99 ±0.15	0.21 ±0.02
NIAW34	9.16 ±0.21	13.06 ±0.12	0.16 ±0.01
HD2781	6.66 ±0.12	11.91 ±0.10	0.15 ±0.01

Data expressed as Mean ± SD of three experiments.

Table 3.10. Correlation between physical properties, rheological properties, thiol-disulfide and polymeric protein contents of flour.

Parameters	Protein disulfide content	Protein thiol content	Non-protein thiol content	Dough hardness	Chapati texture
Dough hardness	0.66	- 0.49	- 0.59	-	**0.89**
Chapati texture	0.58	**- 0.77**	- 0.53	**0.89**	-
% of large extractable polymeric protein in flour protein	**0.72**	- 0.43	- 0.66	**0.89**	**0.70**
% of large unextractable polymeric protein in flour protein	- 0.55	0.10	- 0.16	-0.33	-0.24
%Total polymeric protein in flour protein	**0.71**	- 0.27	**- 0.74**	**0.71**	0.58
% of large extractable polymeric protein in total polymeric protein	0.52	- 0.49	- 0.63	**0.81**	0.64
% of large unextractable polymeric protein in total polymeric protein	- 0.69	0.17	- 0.23	-0.45	-0.38

Figures in bold are significant at $p < 0.05$.

3.3.1.7. Effect of dough characteristics on the texture of chapati

Earlier studies indicated that texture of chapati could be predicted objectively by determining the cutting force (Sidhu et al, 1988; Haridas Rao, 1993). As shown in Table 3.10, dough hardness was positively correlated with chapati cutting force ($r=0.89$, $p<0.05$) indicating that higher the hardness of the dough tougher will be the chapati texture. Among these varieties HD2189 and HD2781 had higher dough hardness and higher chapati cutting force while GW322, K9644 and MACS 2496 had lower dough hardness and lower cutting force. Cutting force reflects the texture of the chapatis and it simulates the biting action of human teeth on chapatis (Sidhu et al, 1988). Earlier, Sidhu et al, (1988) determined various sensory properties like appearance and hand feel, mouth feel, flexibility and over all scores of chapatis prepared from whole wheat flours of different quality, and reported that cutting force was negatively but significantly correlated with the overall sensory properties of chapatis.

3.3.1.8. Interrelationship between molecular weight of different fractions, dough rheology and chapati quality

The correlation between relative quantities of these protein fractions and quality attributes are given in Table 3.10. The percentage of large extractable polymeric protein in flour protein was significantly positively correlated with dough hardness ($r=0.89$, $p<0.05$) and chapati texture ($r=0.70$, $p<0.05$). Earlier, Gupta et al, (1993) have shown that percentage of unextractable polymeric proteins were significantly correlated to dough strength and Kuktaite et al, (2004) have shown that strong flours contained higher percentage of large

unextractable polymeric proteins. In the present study no significant correlation was observed between unextrctable polymeric proteins and dough rheology as well as chapati texture. This paradox may be due to the difference in the type of milling employed to get the flour for a particular product. Chapati dough is prepared from whole-wheat flour and the quality requirements of flour for chapati may vary from that of refined flour, which is used for bread. However, quantitative distribution of polymeric proteins of different sizes influenced the strength of the dough made from whole-wheat flour. This is in accordance with the observations made by Gupta et al, (1993) and Kuktaite et al, (2004), for bread flour.

General Discussion

A number of studies on wheat proteins established a relationship between protein content and the dough rheology and product quality. Further research in this field revealed the relation between dough rheology/product quality and the qualitative and quantitative difference in the type of protein [MacRitchie et al, 1990; Veraverbeke and Delcour, 2002; Lee et al, 2002]. Improvements in the protein separation and analysis techniques allowed the separation of proteins present in the classical Osborne fractions into various classes like, high molecular weight glutenin subunits, low molecular weight glutenin subunits, and different gliadin proteins such as α, β, γ, ω, etc. [Jones et al, 1959]. These developments culminated in proving the key role played by high molecular weight glutenin subunits in dough rheology.

Different models were proposed to explain the overall structure of glutenin and its contribution to dough rheology. All these models highlighted three points, 1) the cross-linking of glutenin subunits to form a protein matrix, 2) the plasticizing role of gliadin proteins and 3) thiol-disulfide exchange reactions during dough development [Belton, 1999; Shwery et al, 2001]. Following model has been proposed to explain the formation of protein cross-links during the dough development. According to this model during the dough development disulfide bonds are broken either by reduction or due to the mechanical energy imparted in the form of mixing. This results in the uncoiling of the glutenin molecule and the dough becomes free to rise or increase in volume. To maintain this new expanded structure new disulfide bonds, each with a new partner are formed by oxidation [Tieckelmann and Steele, 1991].

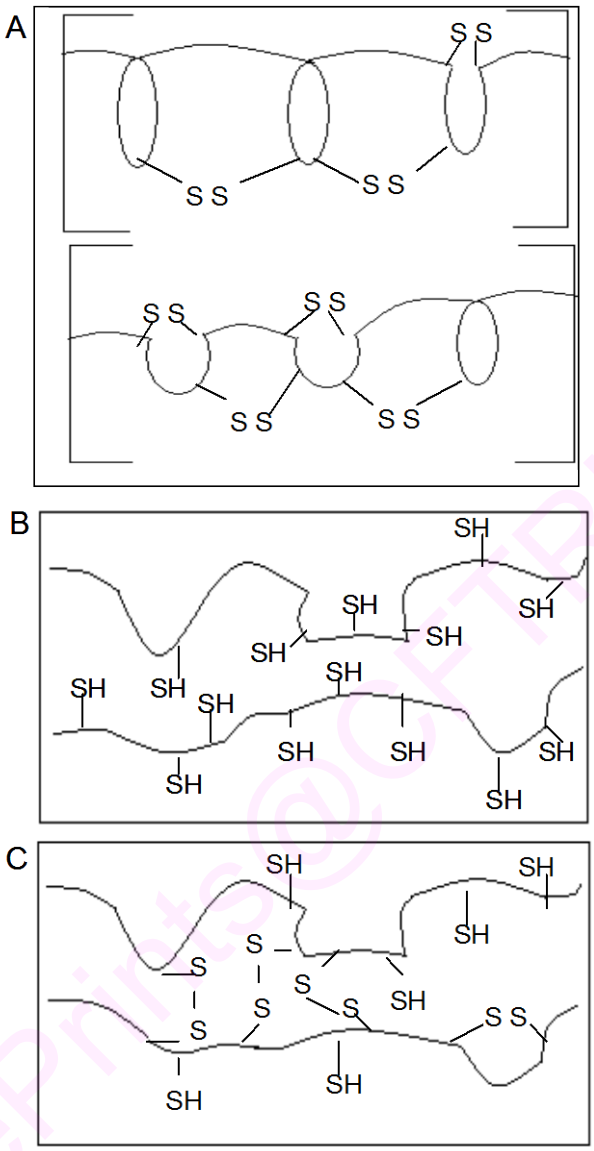

Fig. 3.35. Proposed model for the reduction-oxidation of glutenin during dough development A. Proposed structure of glutenin molecule, B. Reduction of disulfide bonds, C. Reoxidation of sulfhydryl groups. Source [Tieckelmann and Steele, 1991]

Wheat protein fractionation on the basis of size or molecular weight by SE-HPLC and correlation analysis with functionality clearly demonstrated the importance of polymeric proteins. Both dough hardness and chapati cutting force are significantly positively correlated to polymeric proteins above molecular weight more than or equal to 200 kDa (Fig. 3.36). Proteins for SE-HPLC were directly extracted from the flour using buffer containing 0.5% SDS. At this SDS concentration chances of enzymes acting and altering the glutenin proteins is remote because SDS is an enzyme denaturing agent. Therefore, amount of various protein fractions given in table 3.7, represent the protein size distribution pattern in the native state, i.e., in flour. However, the pattern of protein size distribution changes significantly during dough development.

Several workers demonstrated the changes taking place in the protein size distribution during dough development. Gel filtration chromatography of proteins from different stages of dough development by Lee et al, (2002) had revealed that native flour had the most small molecular size proteins, followed by nondeveloped, partially developed, and then developed doughs. In the same study they reported a higher content of –SH groups in partially developed dough compared to native flour, nondeveloped and developed doughs. An opposite trend was observed for S-S content. These reports suggest the occurrence of disulfide-sulfhydryl exchange reactions during dough development. Because of the complexity of dough it is very difficult to assess the role of different factors in the alteration of protein size distribution during dough formation. In the present study we have obtained

data, which corroborate the involvement of peroxidase in the alteration of size distribution of proteins during dough development.

Peroxidase affects the protein size distribution by two separate but coupled mechanisms. The first one is production of H_2O_2 and removal of thiol compounds by thiol oxidase activity of peroxidase. Glutenin proteins are cross-linked by H_2O_2 through the formation of disulfide bonds between glutenin subunits. Wheat flour contains many small molecular weight thiol compounds like free cysteine, glutathione, thioredoxin etc, [Kobrehel et al, 1992; Koehler, 2003]. The small molecular weight thiol compounds are rheologically active because they can alter the glutenin polymer size and ultimately the dough strength. At high concentration these thiol compounds cause a net reduction of protein disulfide bonds through two consecutive sulfhydryl-disulfide interchange reactions. In this reaction thiols become oxidized to corresponding disulfides. At lower concentrations, a single sulfhydryl-disulfide interchange may cleave a protein disulfide resulting in the formation of mixed disulfide comprising the protein and low molecular weight sulfhydryl compound and a free protein sulfhydryl group.

Formation of mixed disulfides can inhibit the sulfhydryl-disulfide interchange reactions between protein molecules. Li et al, (2004), reported a negative correlation between amount of polymeric protein in flour and the amount of protein bound glutathione. We have also shown that non protein thiol content negatively correlates to the amount of polymeric proteins in the flour (Table. 3.10). Therefore, removal of small molecular weight thiols favors thiol-disulfide exchange reactions during dough formation. Thus endogenous

peroxidase has an important role in modulating the concentrations of small molecular weight thiols.

Similar mechanism has been shown to occur when ascorbic acid is used as a flour improver. The improver effect of ascorbic acid is attributed to its action on glutathione. Ascorbic acid added to dough is oxidized rapidly to dehydroascorbic acid by ascorbate oxidase, which is present in wheat flour. Dehydroascorbic acid is reduced back to ascorbic acid by accepting hydrogen from glutathione. Glutathione is oxidized in this reaction. This reaction is catalyzed by glutathione dehydrogenase that is present in wheat [Koehler, 2003].

The ratio of oxidized and reduced forms of small molecular weight thiol compounds is one of the factors that determine the redox status of the dough. Formation of disulfide bonds in proteins under specific biological conditions depends on thiol-disulfide redox status of the immediate environment [Gilbert, 1984]. Thiol oxidase activity of wheat bran peroxidase utilizes cysteine as substrate and oxidizes cysteine into cystine. The hydrogen peroxide generated in this reaction is also an oxidizing agent and it can oxidize cysteine into cysteic acid. A similar study with horseradish peroxidase had shown that thiol oxidase activity of horseradish peroxidase could oxidize glutathione when minute quantity of H_2O_2 was present for initiation [Harman et al, 1986]. Thus the removal of small molecular weight thiols by thiol oxidase activity of wheat bran peroxidase may shift the redox status of the dough towards formation of disulfide bonds.

The second mechanism is the direct catalysis of the cross-linking reactions by peroxidase. The SDS-PAGE analysis of isolated glutenin subunits treated with peroxidase and H_2O_2, under both reducing and nonreducing conditions in the present study demonstrated the formation of nondisulfide covalent linkages. The fluorescence spectrum of cross-linked proteins was similar to that of dityrosine. In glutenin proteins tyrosine mediated bonds may produce additional cross-links, reinforcing the disulfide bonds and may influence functional properties of dough. Presence of dityrosine in wheat proteins and an increase in its content during dough development was discovered very recently [Tilley et al, 2001]. It was long known that tyrosine containing proteins produce tyrosine mediated cross-links like dityrosine and isodityrosine when treated with peroxidase and H_2O_2. But in wheat dough although formation of dityrosine was reported there was no report on *in situ* generation of H_2O_2. The thiol oxidase activity of wheat bran peroxidase and the consequent production of H_2O_2, can explain the formation of tyrosine mediated cross-links catalyzed by peroxidase.

Calcium peroxide and oxidative enzymes like glucose oxidase, L-amino acid oxidase etc, are used as flour improvers. The flour improving action of these additives was attributed to the generation of H_2O_2. Calcium peroxide quickly releases its H_2O_2 upon addition of water. The glucose oxidase catalyzed reaction of glucose oxidation releases H_2O_2, as one of the products. L-amino acid oxidase oxidizes amino acids like L-arginine and produces H_2O_2 in that reaction [Vemulapalliet al, 1998]. Peroxidase may be a better flour improver because it can both generate and utilize H_2O_2 to produce protein-protein cross-linking.

Hydrogen peroxide is produced by a number of enzymatic and non-enzymatic reactions. The following equations represent some of these reactions.

$$2\ \text{L-ascorbate} + O_2 \xrightarrow[\text{Ascorbic acid oxidase}]{} 2\ \text{dehydroascorbate} + 2\ \mathbf{H_2O_2}$$

$$2\ \text{glutathione} + \text{dehydroascorbate} \xrightarrow[\text{Glutathione dehydroascorbate reductase}]{} \text{glutathione disulfide} + \text{ascorbate}$$

$$2O_2^{\cdot -} + 2H^{+} \xrightarrow[\text{Superoxide dismutase}]{} \mathbf{H_2O_2} + O_2$$

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow[\text{Glucose oxidase}]{} \text{D-gluconic acid} + \mathbf{H_2O_2}$$

$$CaO_2 + 2\ H_2O \longrightarrow Ca(OH)_2 + \mathbf{H_2O_2}$$

Ascorbic acid reductase and superoxide dismutase have been reported to present in wheat flour. Glucose oxidase and calcium peroxide are being used as dough additives to improve the dough quality.

In a nutshell, the role of peroxides in the formation of protein network during the dough development is represented in the figure 3.35.

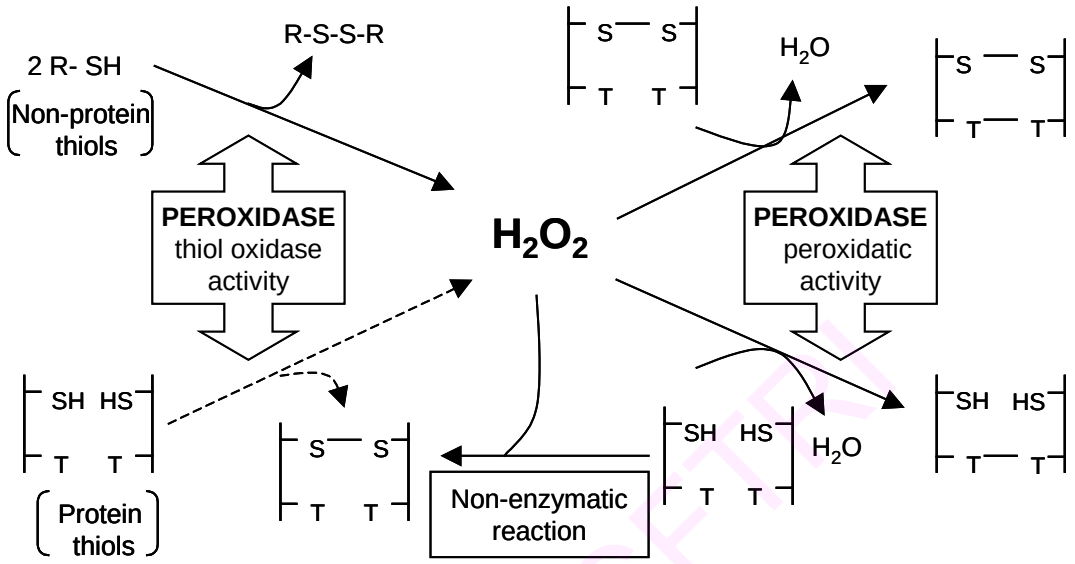

Fig. 3.36. Proposed peroxidase mediated reactions in wheat flour during dough formation. Reactions shown with solid arrows (-) are experimentally demonstrated; dashed arrows (--) not experimentally established.

Thiol oxidase activity of peroxidase oxidizes small molecular weight thiols and produces H_2O_2. The sulfhydryl groups of glutenin proteins are oxidized nonenzymatically to disulfides by H_2O_2. Thiol oxidase activity may also use protein sulfhydryls as substrates and produce disulfide bonds (dashed arrow). The glutenin protein network is further strengthened by the formation of dityrosine bonds, which is catalyzed by peroxidase using H_2O_2 produced by thiol oxidase activity.

SECTION A

WHEAT BRAN PEROXIDASE AND ITS CHARACTERIZATION

4.1. Summary

Wheat dough formation is a complex process, which involves interaction between many components like proteins, carbohydrates, enzymes etc. Proteins play an important role in determining the visco-elastic properties of the dough and quality of baked products. The visco-elastic property of wheat flour dough is mainly attributed to the gluten proteins namely gliadins, and glutenin. The polymeric glutenin provides elasticity and the monomeric gliadin provides viscosity to the dough. The polymeric nature of glutenin is due to the cross-linking of several subunits.

Many chemical agents have been used in bakery industry as dough improvers. But there is a paradigm shift towards the use of enzymes because chemicals used as dough improvers were found to be toxic and enzymes are generally regarded as safe. Many enzymes such as protease, amylase, lipase, oxidative enzymes like glucose oxidase, hexose oxidase, polyphenol oxidase, peroxidase etc., are reported to improve the quality of dough.

Wheat also contains few endogenous oxidative enzymes like peroxidase, polyphenol oxidase, super oxide dismutase etc. Exact mechanism by which peroxidase enzyme improves the dough quality is not understood. However, peroxidase has been shown to cross-link several food proteins including bovine serum albumin and β-lactoglobulin. Peroxidase may have a similar role in cross-linking the glutenin proteins and dough formation. Therefore, in the present investigation peroxidase was purified

from wheat bran and role of the peroxidase in cross-linking of gluten proteins was studied. The results of these investigations are summarized below.

1. Eight Indian wheat varieties were screened for four oxidative enzymes, namely, peroxidase, polyphenol oxidase, lipoxygenase and superoxide dismutase. The varieties screened showed variation in the activities and specific activities of these enzymes.

2. Among different milled fractions of wheat, coarse bran had highest peroxidase activity followed by whole-wheat flour, fine bran and maida. Peroxidase specific activity was more in whole-wheat flour.

3. Calcium chloride had a tremendous effect on the extraction of peroxidase. In presence of calcium chloride both the activity and specific activity of peroxidase in crude extract increased by 10 fold and 26 fold respectively. So simple addition of calcium chloride in the extraction buffer resulted in elimination of some unwanted proteins and achieved first level of purification.

4. Purification of peroxidase was carried out by ammonium sulphate fractionation, cation exchange chromatography on DEAE-cellulose, anion exchange chromatography on CM-cellulose and gel filtration chromatography. About 75% of the peroxidase activity eluted without binding to DEAE-cellulose.

5. Anion exchange chromatography of the unbound fraction with 0-600 mM NaCl gradient gave five protein peaks. Among these peaks, three peaks namely PI, PII and PIII had peroxidase activity. The PI, which showed more activity compared to other two peroxidase peaks was further, purified by gel filtration chromatography on Sephadex G-100. Gel filtration chromatography gave two protein peaks and the first peak

contained peroxidase activity. SDS-PAGE of this peak showed a single protein band.

6. Calcium chloride improved the specific activity and percentage yield of the peroxidase. In cation exchange step specific activity increased by 8.5 fold and percentage yield increased by 1.2 fold. In gel filtration chromatography specific activity increased by 10 fold and percentage yield increased by 1.5 fold.

7. Purified peroxidase had a molecular weight of 44 kDa and was a monomer. It was a glycoprotein and a hemoprotein with characteristic heme absorption at 403 nm. The carbohydrate content was found to be 13.6%. The optimum pH was 4.8, and below pH 2.5 peroxidase lost all the activity whereas at pH 8 it retained 20% of activity.

8. Purified peroxidase followed Michaelis-Menten type of catalysis. Peroxidase activity increased as concentration of both the substrates (o-dianisidine and H_2O_2) increased. Plots of substrate concentrations versus enzyme activity gave hyperbolic curves. The Km values calculated from Lineweaver-burk plots were, 5×10^{-3} M, 10×10^{-3} M for o-dianisidine, 7.14×10^{-3} M, 40×10^{-3} M for H_2O_2 in absence and presence of calcium respectively.

9. Double reciprocal plot of initial velocity versus different H_2O_2 concentrations at three fixed o-dianisidine concentrations gave a series of parallel lines. Double reciprocal plot of initial velocity versus different H_2O_2 concentrations at three fixed ratio of o-dianisidine to H_2O_2, produced a common 1/y axis intercept. This kind of kinetic behaviour is an indication that peroxidase is following ping pong bi bi type of reaction.

10. Peroxidase exhibited thiol oxidase activity. By thiol oxidase activity it oxidized cysteine and the oxidation was peroxidase dose dependent. The

thiol oxidase function of peroxidase generated H_2O_2. Peroxidase displayed thiol oxidase activity over a narrow pH range (6.5 to 10).

11. The generation of H_2O_2 by thiol oxidase activity was cysteine concentration dependent. As cysteine concentration increased H_2O_2 generation also increased, reached maximum value (12 µM) at cysteine concentration of 500 µM. Beyond this cysteine concentration H_2O_2 generation decreased.

12. The activity of purified peroxidase was greatly enhanced by calcium chloride. Activity increased by 4.4 fold when assayed in presence of 3mM calcium chloride. The activity enhancement was calcium dose dependent and biphasic in nature, consisting of a rapid phase of activity increase followed by a slow phase of activity increase.

13. Thiol oxidase function of peroxidase also showed calcium dependent activity enhancement. Presence of 1 mM calcium chloride increased thiol oxidase activity by five-fold.

14. The tryptophan fluorescence emission spectra of purified peroxidase before and after the addition of calcium chloride did not show any change. On the other hand, intensity of 403 nm absorption peak due to the heme moiety increased when peroxidase was supplemented with calcium chloride. Therefore, the activity enhancement by calcium was due to a structural perturbation around the heme moiety.

15. The thermal stability of purified peroxidase was improved by calcium chloride. Heating of peroxidase at 60^0C for 25 min destroyed almost all activity, where as in presence of calcium chloride the same treatment resulted in loss of only 50% of activity.

16. The thiol oxidase function was also improved by calcium chloride. There was 50% loss in thiol oxidase function when peroxidase was heated at 60^0C for 25 min, but loss of activity was only 17%, when calcium chloride was present.

17. Isolated glutenin subunits were treated with different concentrations of H_2O_2 and different units of wheat bran peroxidase, separately and in combination. SDS-PAGE of treated subunits under reducing and non-reducing conditions revealed that H_2O_2 was able to cross-link glutenin subunits by disulfide bonds but in combination with peroxidase it also created non-disulfide covalent cross-links. The fluorescence spectrum of cross-linked proteins indicated the presence of dityrosine.

18. The high molecular weigh subunit composition of eight Indian wheat varieties were determined by SDS-PAGE, and the Glu-1 score, which reflects the synergistic contribution of glutenin subunits to the quality of flour for these varieties were calculated. The Glu-1 scores showed significant positive correlation to cutting force ($r = 0.70$, $p<0.05$), which is one of the important quality parameters of chapati.

19. Gel filtration chromatography of whole-wheat flour extract on Sephadex G-200, gave four protein peaks, namely PI, PII, PIII and PIV. There was variation in protein quantity of these peaks in different wheat varieties. Wheat varieties with quantity of peak IV, which had proteins of molecular weight of around 20 kDa, yielded good quality chapati.

20. About 70 to 92% of protein was extracted from whole-wheat flour of different wheat varieties by using SDS containing buffer. From the remaining residue about 10% protein could be extracted by subjecting it to sonication.

21. Size exclusion high performance liquid chromatography of whole-wheat flour extract in SDS containing buffer gave four protein fractions, namely, FI, FII, FIII and FIV. Fraction I had proteins of molecular weight > 130 kDa (designated as large polymeric proteins), fraction II had proteins of molecular weight between 80 and 130 kDa (designated as small polymeric protein), fraction III had proteins between 10 and 80 kDa (designated as monomeric proteins). Fraction IV had very low molecular weight components (<5 kDa) indicating that there may be some peptides or phenolic compounds that absorb at 280 nm. There was variation in the protein quantity of theses peaks in different wheat varieties.

22. The percentage of polymeric protein in flour protein significantly positively correlated to dough hardness and chapati cutting force. The quantity of non-protein thiol showed a negative correlation to the dough hardness and chapati cutting force.

4.2. Conclusion

Wheat varieties studied have good amount of peroxidase activity and showed good variation in the peroxidase activity. Peroxidase purified from wheat bran showed thiol oxidase activity in addition to peroxidase activity. By thiol oxidase activity, peroxidase catalyzed the oxidation of cysteine and generated H_2O_2. Classical peroxidatic activity utilizes H_2O_2 and oxidizes amines and phenolic compounds. Depending on the conditions peroxidase can act as antioxidant and prooxidant. Both peroxidatic and thiol oxidizing activities of purified peroxidase were enhanced by calcium chloride. Although calcium was known to play a vital role in the stability of plant peroxidases, no attempt was made earlier to purify peroxidase in the presence of calcium. Our results clearly demonstrated that inclusion of the calcium in the buffer during purification of peroxidase gives better yield and higher specific activity of the enzyme. Thermal stability of purified peroxidase was also improved by calcium chloride. Thus, wheat bran, which is a by-product of wheat milling, could be a potential source of peroxidase for various applications.

The relation between wheat protein size distribution and quality of leavened product like bread is known. In this thesis, for the first time an attempt was made to understand the effect of protein size distribution on quality of unleavened product like chapati. Both the dough hardness and chapati texture were found to depend on the content of polymeric protein and the quantitative distribution of these proteins in the flour. As consumer acceptance of any food product is based on the texture of the product, the knowledge of relation between polymeric protein and texture will help to

select the suitable variety for a particular product and also develop new varieties by plant breeding and genetic engineering.

Exogenously added peroxidases like horseradish peroxidase were reported to improve the dough properties and ultimately the product texture. Wheat bran peroxidase purified in this study catalyzed the formation of cross-links between isolated glutenin subunits. Therefore, endogenous peroxidase may have similar role in cross-linking of glutenin subunits *in situ*, which is an important process in the dough development. Interchain disulfide cross-links were also formed by H_2O_2 in these subunits. A number of reactions are known to generate H_2O_2 *in situ,* thiol oxidase function of peroxidase also generated H_2O_2. Further, removal of small molecular weight thiol compounds by thiol oxidase activity may shift the redox status of the dough towards oxidizing environment and may favour the formation of disulfide cross-links in glutenin proteins. In addition, formation of non-disulfide covalent linkage mainly dityrosine linkage catalyzed by peroxidase is also demonstrated in the present study, which may be required to impart additional strength to dough. Due to its dual activity, peroxidase may play an important role in the formation of visco-elastic dough. Thus, it is concluded that peroxidase may play an important role in dough development mainly through the formation of cross-links in glutenin proteins.

SECTION B

ROLE OF PEROXIDASE IN CROSS-LINKING OF GLUTENIN PROTEINS

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SECTION C

ROLE OF WHEAT PROTEINS IN FLOUR FUNCTIONALITY

Papers published/ manuscripts under preparation

1. **B. T. Manu**, and U. J. S. Prasada Rao (2008) ‘ Influence of size distribution of proteins, thiol and disulfide content in whole wheat flour on rheological and chapati texture of Indian wheat varieties’ **Food Chemistry**, 110:88-95 (Impact factor 3.05)

2. **B. T. Manu**, and U. J. S. Prasada Rao (2008) ‘ Calcium modulated activity enhancement and thermal stability study of a cationic peroxidase purified from wheat bran’ **Food Chemistry (In press)**

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