*Pak. J. Bot.*, 43(1): 459-465, 2011.

# REDUCTION AND CARBOXYMETHYLATION OF SOME OF THE GLIADIN WHEAT PROTEINS

**AHMAD SAEED KHAN[1*] AND IHSAN ILAHI[2]**

*[1]Department of Chemistry[1] and [2]Department of Biological Sciences, Karakoram International University, University Road, Gilgit, Gilgit-Baltistan, Pakistan*
**Correspondence author email: ahmad_kiu@yahoo.com*

**Abstract**

Pure β-Gliadin from Maris Widgeon wheat homogeneous on gel chromatography, disc gel electrophoresis (PAGE) and purified on ion exchange chromatography was subjected to investigation for possible presence of intermolecular disulphide bonds. The protein was reduced by β mercaptoethanol followed by alkylation. Reduced and alkylated β-gliadin was then isolated after chromatography through G-75 and G-100 Sephadex gels. PAGE electrophoresis of the two halves showed two mobility bands. The molecular weight of the proteins after reduction and alkylation was the same as the original β-gliadin before it was subjected to reduction and alkylation. It was concluded that they were not two chains of the same protein but two separate proteins.

## Introduction

The present research was carried out on winter wheat variety Maris Widgeon. Wheat gluten (composed of gliadin and glutenin) is unique among proteins because of its cohesiveness, extensibility, pliability and elasticity when hydrated. It has long been known that disulphide bonds contribute to the unique viscoelastic properties or wheat gluten proteins and enable gluten to hold dough together and retain gas produced by fermentation, thus giving baked goods their characteristic cellular structure (Shewry & Tatham, 1997). Nielsen *et al*., (1962) demonstrated that disulphide bond-breaking agents cause dough or a gluten ball to relax and fall apart whereas agents that oxidize sulphydryl group cause dough or gluten balls to become tougher. Woychik *et al*., (1964) established that reduction cleaves disulphide bonds linking polypeptide chains and thereby fragments the protein into a number of low molecular weight molecules. In the major and 70% (w/w) alcohol soluble fraction of gluten (i.e., gliadin), all the cystine-cysteine sulphur exists as disulphide. However, the number average molecular weight of gliadin was shown by Pence & Olcott (1952) to change only slightly upon reduction. Gliadin disulphide bonds therefore must be predominantly intramolecular. But an increase in viscosity and diminished solubility of gliadin in solution following reduction were also noted by these workers. Based on the additional study of the physical properties of gliadin that occur during reduction, Beckwith *et al*., (1963) concluded that the cleavage of disulphide bonds in acid solutions allows the gliadin molecules to unfold and molecular conformation to change from compact constrained molecules to random coil structures. Most or these studies were carried out on the entire complex of gliadin proteins. Gliadin was shown to consist of several proteins separable by electrophoresis (Jones *et al*., 1959; Woychik *et al*., 1961). After preparing pure γ-gliadin, Woychik & Heubner (1966) found that, upon reduction, the molecular weight did not decrease and the protein contained only single amino and carboxylic end groups. Their results established that γ -gliadin consists of a single polypeptide chain containing intramolecular disulphide bonds. Reduction and starch gel electrophoresis studies of

wheat gliadin and glutenin by Woychik *et al*., (1964) have revealed the presence of some components which are perhaps common to both proteins. There are marked quantitative differences in the distribution of the components. The release of twenty components from the previously unresolved and immobile glutenin fraction is further evidence that extensive intermolecular disulphide bonding is responsible for its high molecular weight. These workers also pointed out the presence of a limited extent of intermolecular bonding in the gliadin fraction. In view of the reports made by Pence & Olcott (1952) and Beckwith *et al*., (1963) classical gliadin was subjected to gel filtration by Beckwith *et al*., (1963). These separations showed that classical gliadin contained a relatively small amount of high molecular weight fraction. The moving boundary electrophoretic pattern of this fraction was quite similar to that of glutenin. It moved as a smear in starch gel electrophoresis whereas glutenin was immobile. The intrinsic viscosity of this fraction was between that of glutenin and that of classical gliadins as was its molecular weight (mol.wt.)**.** Woychik *et al*., (1964), working on reduction and starch gel electrophoresis of gliadin, found that although the electrophoretic pattern of reduced gliadin did not show an apparent increase in number of components , it is possible that the complexity of the fraction may have obscured the results of disulphide cleavage. They were particularly interested in determining whether individual components consisted of more than one polypeptide chain; these components were isolated from the preparative starch gels and reduced. The results obtained were that the mobilities of the reduced components were 15 to 20% less than that of the native proteins.

Three of the gliadin components isolated by Woychik *et al*., (1964), namely $\alpha_1$, $\alpha_2$ and $\gamma$ continued to migrate as single components after reduction. The $\beta_3$ and $\beta_4$ components behaved similarly. These results were indicative of single chain structures containing only intramolecular disulphide bonds. Whereas on the other hand the $\beta_1$ and $\beta_2$ proteins indicated the presence of interchain disulphide bonds each yielding two components with very similar mobilities after reduction.

Studies of Muller & Wieser (1995 and 1997) showed presence of intramolecular disulphide bonds in α-gliadins and γ-glidins. Hence in view of the studies by Woychik *et al.*, (1964) and earlier of Pence & Olcott (1952) and Beckwith *et al*., (1963), the present study was undertaken to determine the presence of types of bonds in the isolated β components (i.e., presence of one polypeptide chain or more than one joined by intermolecular S-S bonds).

## Material and Methods

The following materials were used in this process

i. Sephadex $SP_{25}$ Cation exchanger, purchased from Pharmacia Sweden
ii. Sephadex G-100, purchased from Pharmacia Sweden
iii. Deionised Urea, prepared by passing through mixed bed of ion exchanger
iv. 0.2 M Dimethyl Formamide and 0.01 M Acetic acid solutions in water

β-gliadin (beta-Gliadin), homogenous on gel chromatography and disc gel polyacrylamide electrophoresis (D.P. A.G.E.), isolated from the ion exchange Sephadex $SPC_{25}$ columns (30x1.5 cm) and eluting with various buffers containing different salt concentration and purified by gel filtration chromatography on Sephadex G-100 (90x2.5cm), prepared in 2.0 M DMF + 0.01M acetic acid, in a Sephadex J25/100 type

column as shown in Fig. 1, was subjected to investigation for the presence of intermolecular disulphide bonds .The temperature around the column was adjusted at 25$^{o}$C . The first experiment was modeled on the method described by Woychik *et al*., (1964).This experiment was conducted on a small scale and was qualitative (analytical) in nature. The protein was subjected to electrophoresis on polyacrylamide gels according to method of Reisfeld *et al*., (1962). The second experiment was carried out on a quantitative scale. The procedure used, were described by Crisfield *et al*., (1963), and modified by Hirs (1956). These authors found after amino acid analysis that this procedure was specific for sulphur bonds and that methionine sulphoxide formation was negligible. In this experiment reduction was followed by alkylation and the reduced and alkylated β-gliadin was isolated after chromatography, through G-75 or G-100 Sephadex gels.

**Experiment No. 1: Reduction of β-Gliadin on the small scale (Qualitative):** This experiment was carried out on the qualitative scale. The β-gliadin protein (0.4mg) homogenous electrophoretically and by gel filtration, was taken up in 0.1M phosphate buffer which was 8M in urea. A fifteen fold excess (by weight) of β-mercaptoethanol was added and the reduction was allowed to proceed for 1 hour according to the method of Woychik *et al*., (1964).The pH of the reaction solution was then adjusted to 5 with few drops of 0.1N HCl solution. The protein concentration in this resultant solution was not less than 1% (by weight). 10 μ L of this solution was then transferred to acrylamide gels and electrophoresis was done according to procedure of Cresfield *et al*., (1963) and modified by Hirs (1956).After staining with Coomassie blue and destaining the gels revealed the presence of two bands.

**Experiment No. 2: Reduction and alkylation of β-Gliadin on a quantitative scale:** The following materials were used in this reaction:

1. Urea, deionised
2. Trimethyl amine BDH, 25% solution
3. Mercaptoethanol, was distilled and stored under nitrogen atmosphere.
4. Sodium Hydroxide
5. Iodoacetic acid, was recrystallised from petroleum ether and stored in a desiccator covered with aluminium foil.
6. Acetic ccid
7. Sephadex G-75, purchased from Pharmacia, Sweden
8. Disodium ethylene diamine tera acetate (EDTA)

**Apparatus**

1. Gel filtration chromatography set up with an automatic fraction collector
2. Disc polyacrylamide gel electrophorsis
3. pH-thermostat apparatus

**Procedure:** This experiment was conducted on a quantitative scale in order to isolate the two components visible on polyacrylamide gels, as a result of reduction in the first qualitative experiment.

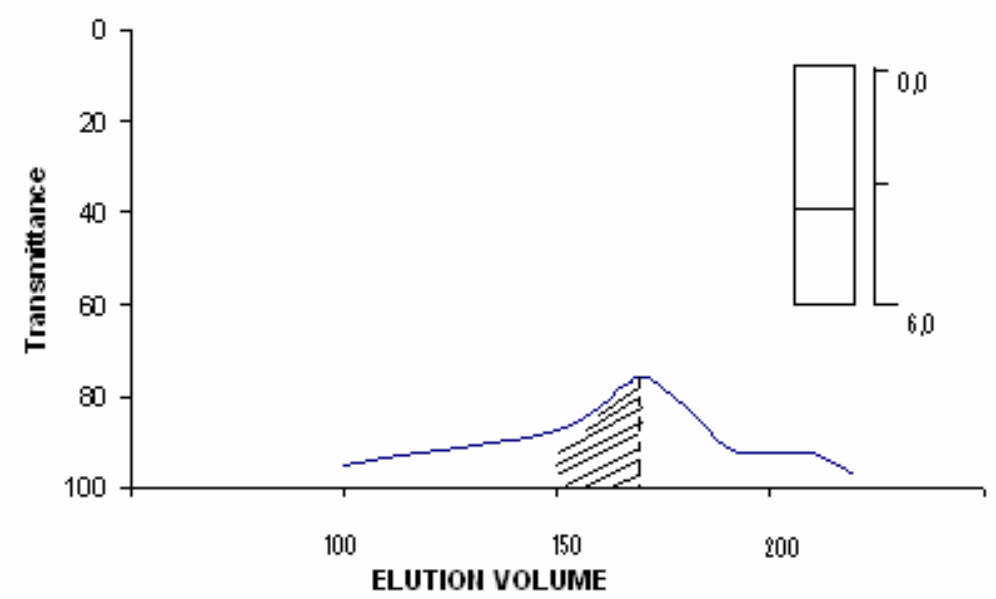


Fig .1. Separations of beta -1 and beta-2 glaidins on Sephadex G-100 column after reduction and alkylation.

3.61g of urea (deionised and recrystallised) and 0.3ml of EDTA solution were mixed with 7ml of distilled water when the resultant solution had a final concentration of 0.2% for EDTA and 8 M urea. This solution was transferred to the filtration vessel of the automatic titrator (Radiometer) with a side arm to pass nitrogen. The solution was thoroughly flushed with nitrogen (free of oxygen) for twenty minutes and the pH of the solution was adjusted to 8.5 with 0.1% aqueous methyl amine solution dispensed with a syringe. The burrette of the automatic titrator was charged with 2M NaOH solution .All the air bubbles were removed before the reaction was started. All the knobs on titrator were set at appropriate positions.

28mg of pure β-gliadin (isolated as described above (Table 1) was dissolved in the aqueous urea (urea and water) and EDTA solution prepared as above; the pH of the solution dropped towards the acid side and this change in pH was adjusted with 1% methylamine solution. The solution in the vessel was continuously stirred and nitrogen was thoroughly flushed during stirring operations. 0.1ml (1.55m moles) of mercaptoethanol was added to the solution. The pH immediately dropped down to 7 and this was brought back automatically to pH 8.5 by the addition of 2M NaOH from the burette. The reduction was allowed to take place for four hours. After this period the reaction vessel was covered with an aluminum foil to cut out the light and the freshly prepared 1.45m molar solution of Iodoacetic acid in 1N NaOH was used to keep pH at 8.0. The alkylation took one hour and its completion was evident by no further release of $H^+$ ions. The solution was acidified to pH 4.0 with the addition of glacial acetic acid. The solution was then directly transferred on to a G-100 (90 x 2.5 cm) column made according to the procedure described in the beginning of materials and method section. The column was covered with aluminum foil to cut out the light. 5 ml fractions were collected. On plotting the reading of UV transmission against the fraction, the graph shown in Fig. 2 was obtained. Reduced and alkylated $\beta_1$(beta-1) and $\beta_2$ (beta-2) gliadins were isolated by dividing the peak in Fig. 2 into two halves, and purified by rechromatography of each half though the same gel column. After freeze drying the materials, the total yield obtained was 90% (Table 2).

**Table 1. The total quantity of beta-1 and beta- 2 mixture isolated from crude beta- gliadin mixture.**

| Crude beta-gliadin mixture used | Purified quantity of beta-1 and beta- 2 gliadin mixture isolated |
|---|---|
| 500 mg | 135 mg |

**Table 2. The quantities of beta -1 and beta-2 gliadins obtained from beta -1 and beta-2 gliadin mixture after reduction and carboxymethylation**

| beta-1 and beta-2 gliadin mixture used | After reduction and Carboxymethylation beta-1 and beta-2 gliadins obtained | |
|---|---|---|
| 28 mg | Beta-1 | Beta-2 |
| | 14.1 mg | 11.4 mg |

## Results and Discussion

By the comparison of the elution profile of the reduced and alkylated β-gliadins (Fig. 1) with those of unmodified β-gliadins mixture on Sephadex G-100 column (Fig. 2), we noticed that reduced and alkylated β-gliadin is eluted slightly earlier from the Sephadex G-100 column (elution volume 220 ml) than the corresponding unmodified β-gliadin mixture (elution volume 240ml). Two inferences can be drawn from this comparison. Firstly, because the reduced and alkylated β-gliadins were eluted at a similar, though smaller elution volume to the corresponding unmodified β-gliadins, smaller molecular weight entities were not obtained. Therefore the possibility of two chains being obtained as a result of reduction and alkylation is ruled out. Secondly, since the reduced and carboxymethylated β-gliadins were eluted slightly earlier from G-100 than the unmodified and native β-gliadins, their molecular weight must be slightly higher. From the calibration graph, Fig.3, they should have a molecular weight of approximately 8,000 units more than unmodified β–gliadins, but this molecular difference is too high to be accounted for by the chemical reaction taking place in this process; namely, the reduction of S- S bonds to S–H bonds and their subsequent alkylation with iodoacetic acid. This apparently anomalous behavior can be explained on the assumption of the presence of intramolecular disulphide bonds in the structure of β–gliadins and the unfolding of the structure when they are cleaved .In the native and unmodified state, the molecules of the β-gliadin proteins are closely coiled due to the disulphide formed across the molecule, but when the disulphide bridges are reduced and alkylated, the molecules get unfolded. Consequently this unfolded molecule will occupy more volume in solution and, on passing down the gel column, will not be able to enter into the smaller pores of the gel; it will be less retarded and eluted earlier than the corresponding unmodified β-gliadin molecules. This observation provides more evidence for the existing hypothesis of the presence of intra-molecular disulphide bonds in gliadin proteins, first put forward by Woychik *et al*., (1964) as a result of the studies on the electrophoretic behavior of the native and reduced and alkylated gliadins on starch gels.

## Conclusions

Electrophoresis of the material of the two halves showed two different mobility bands. The mol. wt. determination from a calibrated G-100 column demonstrated that they had almost the same mol. wt. as that of the original β –gliadin before its subjection to reduction and alkylation. Therefore it is concluded that they are not two chains of the same protein but two separate proteins.

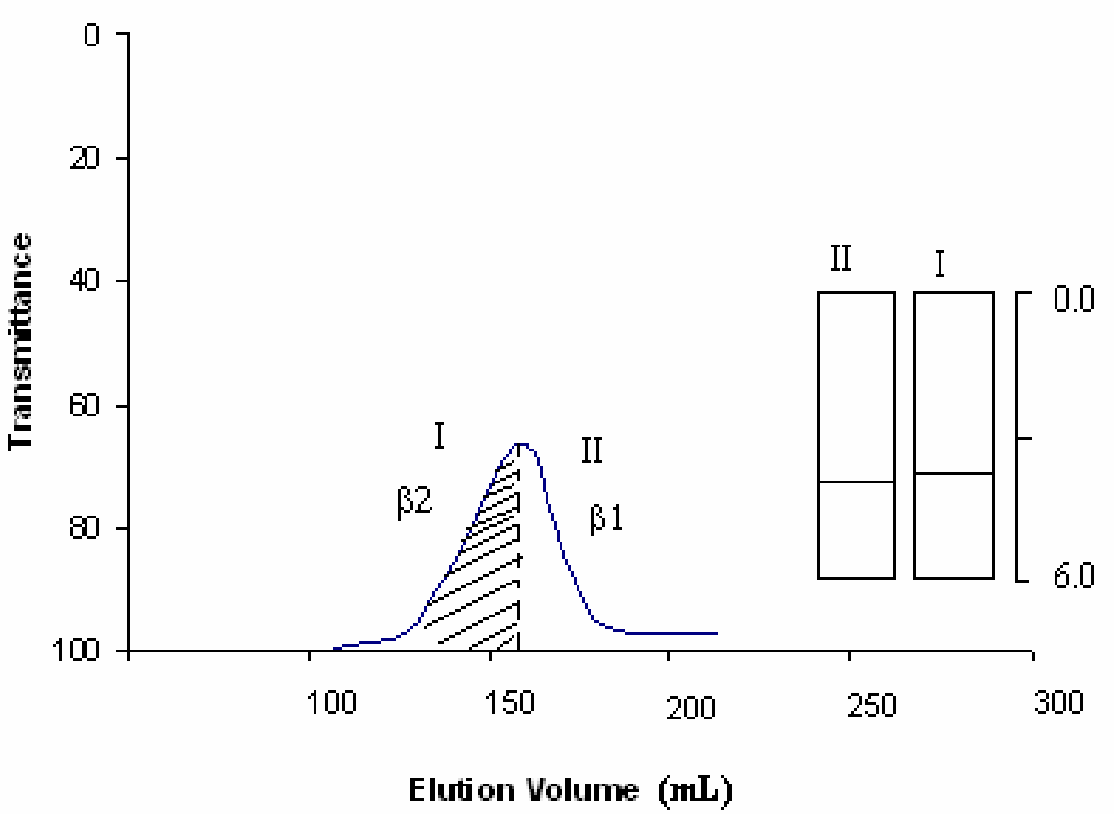


Fig. 2. Separations and purification of beta -1 and beta-2 glaidins (β1 and $\beta_2$) on Sephadex G-100 column.

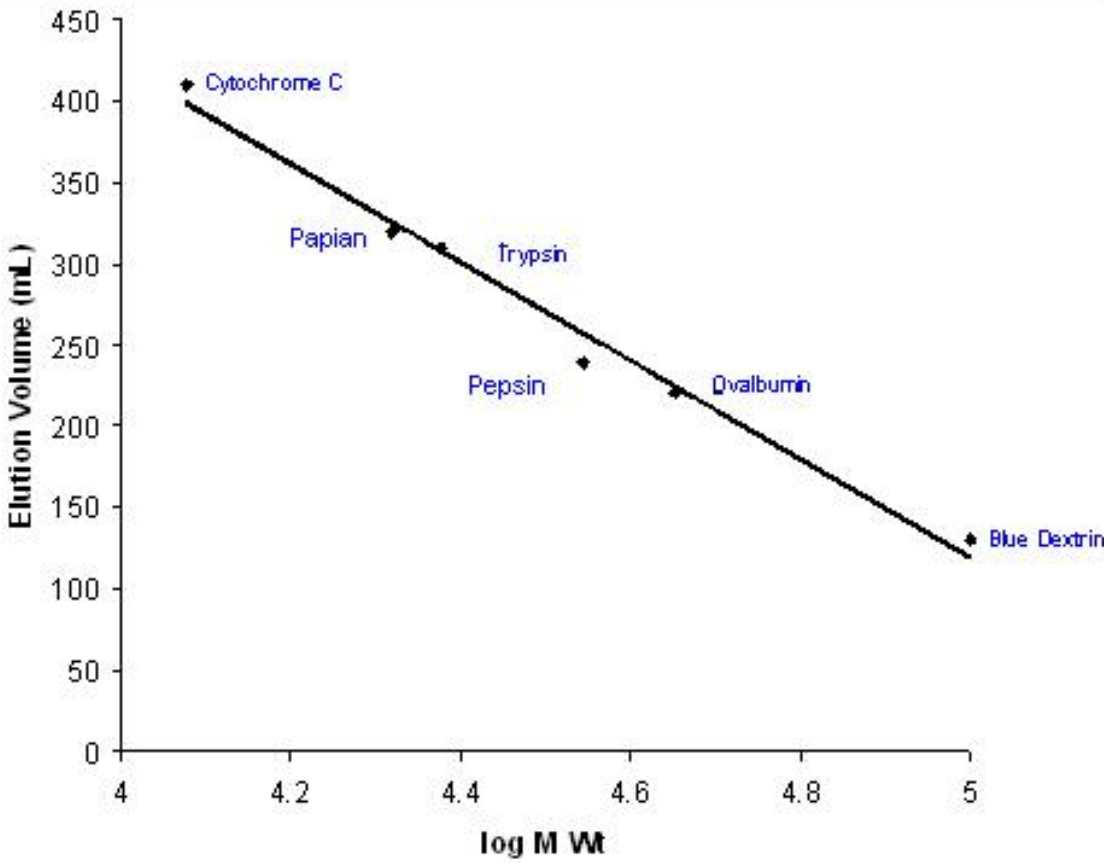


Fig. 3. Calibration of G-100 Sephadex Column using various proteins.

**References**

Beckwith, A.C., H.C. Nielsen, J.S. Wall and F.R Huebner. 1966. Isolation and characterization of a high-molecular-weight protein from wheat gliadin. *Cereal Chem*., 43: 14-28.

Beckwith, A.C., J.S. Wall and R.J. Dimler. 1963. Amide groups as interaction sites in wheat gluten proteins: effects of amide-ester conversion. *Arch Biochem. Biophys*., 103: 319-30.

Cresfield, A.M., G.S. Moore and W.H. Stein. 1963. The preparation and enzymatic hydrolysis of reduced and S- carboxymethylated proteins. *J. Biol. Chem*., 238: 618-621.

Hirs, C.H.W. 1956. The oxidation of Ribonuclease with performic acid. *J. Bio. Chem*., 219: 611-621.

Jones, R.W., N.W. Taylor and F.R. Senti.1959. Electrophoresis and fractionation of wheat gluten. *Arch. Biochem. Biophy*., 84: 363-376.

Muller, S. and H. Wieser. 1995. The location of disulphide bonds in α- type glaidins. *Journal of Cereal Science*, 22: 21-27.

Muller, S. and H. Wieser. 1997. The location of disulphide bonds in monomeric γ- type glaidins. *Journal of Cereal Science*, 26: 169-176.

Nielsen, H.C., G.E. Babcock and F.R. Senti. 1962. Molecular weight studies on glutenin before and after disulfide-bond splitting. *Arch. Biochem, Biophys*., 96: 252-258.

Pence, J.W. and H.S. Olcott. 1952 . Effect of reducing agents on gluten proteins. *Cereal Chem*., 29: 292-298.

Reisfeld, R.A., V.A. Lewis and D.E. Williams. 1962. Disc Electrophoreses of basic proteins and peptides on polyacrylamide gels. *Nature*, 195: 281-283.

Shewry P.R. and A.S. Tatham. 1997. Disulphide bonds in wheat gluten proteins. *Journal of Cereal Science*, 25: 207-227.

Woychik, J.H. and F.R. Heubner. 1966. Isolation and partial characterization of wheat gamma-gliadin. *Biochem. Biophys. Acta*., 127: 88-93.

Woychik, J.H., F.R. Huebner and R.J Dimler. 1964. Reduction and starch-gel electrophoresis of wheat gliadin and glutenin. *Arch. Biochem*., 105: 151-155.

Woychik, J.H., J.A. Boundy and R.J. Dimler. 1961. Starch Gel Electrophoresis of wheat gluten proteins with concentrated urea. *Arch. Biochem. Biophy*., 94: 477-482.

(Received for publication 24 February 2010)