

Cross-Linking of Wheat Gluten Using a Water-Soluble Carbodiimide

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ABSTRACT:

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Wheat gluten was cross-linked using water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC). To enhance cross-linking, *N*-hydroxysuccinimide (NHS) was added to the reaction mixture. The cross-linking efficiency was evaluated by the decrease in the amount of amino groups, the solubility of the protein in aqueous solutions with different pH levels, and by the change in the molecular weight distribution of the cross-linked compounds. Cross-linking was dependent on the reaction time, the molar ratio of added reactants, and the pH level of the reaction mixture. If the reaction was carried out at pH 3, no decrease in the amount

of amino groups or solubility was observed. At pH 5–7, the amount of amino groups decreased from 15 to 10 mmol/100 g of protein. This was accompanied by a large decrease in the water solubility of the protein (<10%, w/v). Finally, reaction at pH 11 decreased the amount of amino groups from 15 to 8 mmol/100 g of protein. However, hardly any decrease in the water solubility was observed. Based on these results and SDS-PAGE experiments, two cross-link mechanisms are suggested: one resulting in inter- and the other resulting in intramolecular cross-links.

Wheat gluten is a protein that is used especially in food and feed applications. Due to very specific physicochemical properties such as elasticity, extensibility, and insolubility in water, it also has a large potential for use in nonfood applications such as adhesives, coatings, and thermoplastic materials. Generally, modifications are applied to render wheat gluten with properties that are required for a specific application. For example, the water sensitivity and the mechanical properties of wheat gluten films and coatings are subjected to improvement. Different methods available to change the functional properties of proteins can be classified as physical (Haschemeyer 1973), chemical (Feeney 1987, Lens et al 1999), and enzymatic (Larré and Schwenke 1996) modifications. For food and feed applications, modifications are restricted to physical and enzymatic methods. However, chemical modifications of proteins are very well suited to improving properties required for nonfood applications. Cross-linking, especially, leads to a decreased susceptibility to swelling or even insolubility in polar solvents and a relatively high wet strength of protein-based products.

Numerous cross-linking agents are known to react with different functional groups (Means and Feeney 1990, Wong 1993) and cross-linking of different proteinaceous materials such as collagen (Weadock et al 1983, Van Wachem et al 1994a,b), bovine serum albumin (Murphy and Howell 1991), and gelatin (Sheehan and Hlavka 1957, Rao and Das 1973, Hayashi et al 1990) have been reported. Most commercial cross-linking agents react with different amino acids. For example, dialdehydes may react with the amino groups of lysine residues, phenol groups of tyrosine, and sulfhydryl groups of cysteine moieties. This makes a mechanistic study on cross-linking of proteins relatively complicated. A good understanding of the processes occurring during cross-linking would increase the usefulness of this tool to improve the properties of wheat gluten. Consequently, in the present study a carbodiimide was used that selectively links carboxylic acid and amino groups together.

Carbodiimides, which are hetero bi-functional coupling reagents, are available in a variety of molecular structures (DeTar and Silverstein 1966a,b). However, by far the most widely used in protein modification studies is the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Lundblad and

Noyes 1984). In combination with *N*-hydroxysuccinimide (NHS), it has been used for peptide synthesis (Bauminger and Wilchek 1980), for the coupling (Beckers et al 1992) and conjugation of proteins (Carlsson et al 1978), for preparing immobilized enzymes and proteins (Kobayashi et al 1989, Valuev et al 1998), for stabilization of proteins and enzymes (Wong and Wong 1992), and for determination of structural and functional aspects of macromolecular assemblies (Kunkel et al 1981).

Cross-linking of proteins with EDC involves the activation of carboxylic acid groups of polypeptide chains to give an *O*-acylisourea group (Hoare and Koshland 1967). Cross-links are formed after reaction with free amino groups (nucleophilic substitution) of the same or another polypeptide chain resulting in intra- or intermolecular cross-linking, respectively (Fig. 1). In absence of amino groups, the active *O*-acylurea undergoes rapid hydrolysis regenerating the original carboxylic acid group and a *N*-substituted urea. Cross-linking is greatly enhanced by the catalytic reagent NHS. Its action is supposed to reduce the possibility of hydrolysis of activated species (Mattson et al 1993) and to suppress side reactions (Staros et al 1986). NHS reacts under mild conditions with carboxyl-containing compounds to give aminoacyl esters. Comparing their rates of reaction with amino groups, the stable active esters hydrolyze slowly in aqueous solutions. Furthermore, formation of the NHS-activated carboxylic acid group reduces the possibility of racemization and formation of *N*-acylureas.

The present article reports the aspects of cross-linking wheat gluten proteins with EDC-NHS. Varying parameters such as reaction time, molar ratio of reactants, and pH of the reaction mixture optimized reaction conditions. To estimate the degree of modification and to assess the effectiveness of cross-linking, the amount of residual amino groups in cross-linked samples was quantitatively determined using a trinitrobenzenesulfonic acid (TNBS) assay. Furthermore, data on the solubility of cross-linked gluten in aqueous solutions of different pH levels and the molecular weight distribution of modified proteins were used to obtain more knowledge about the type of cross-links formed during reaction.

MATERIALS AND METHODS

Wheat gluten (76.5% proteins, 11.8% starch, 5.0% lipids, 0.7% ash, 40 mmol of carboxylic acid groups/100 g of protein, all w/w on dry weight basis) was supplied by Amylum (Aalst, Belgium). 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide HCl (EDC), NHS, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (St. Louis, MO). Sulfuric acid (H₂SO₄ 95–98%) and acetic acid were purchased from Riedel-de Haën AG (Seelze, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

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Cross-Linking of Wheat Gluten

In a typical experiment, wheat gluten (3 g, containing ≈ 1.2 mmol of carboxylic acid groups) was dispersed in 90 mL of deionized water by slowly adding the protein through a small sieve under continuous stirring. Subsequently, 2.5 mL of an aqueous solution of NHS (0.69 g, 6 mmol) and 2.5 mL of an aqueous solution of EDC (1.15 g, 6 mmol) were added. The dispersion was adjusted to pH 5.5. During the reaction the pH level was kept constant by adding an aqueous solution of 0.5M sodium hydroxide (NaOH) or 0.1M H₂SO₄. After stirring for 4 hr at room temperature (RT), the reaction was stopped by adding acetic acid to a final acetic acid concentration of $\approx 1M$. The dispersion was extensively dialyzed against deionized water. Subsequently, the dispersion was lyophilized, giving a white solid product with a yield of 2.8 g.

Additionally, the reaction time was varied up to 15 hr. The reaction was carried out at pH 3–11. The influence of the molar ratio of NHS to EDC on the degree of cross-linking was studied by carrying out the reaction using a molar ratio of EDC to COOH of 5:1 and NHS to EDC ratios of 0:5, 1:5, 2:5, 3:5, 4:5, and 5:5. The influence of the molar ratio of EDC to COOH was studied using EDC to COOH ratios of 1:1, 2:1, 3:1, 4:1, and 5:1. Equal molar amounts of EDC and NHS were used during these last experiments. Blanks were obtained by dispersing wheat gluten at similar conditions without the addition of NHS and EDC.

Determination of the Amount of Amino Groups

The free amino group content of protein samples was determined using TNBS (Habeeb 1966, Fields 1972, Adler-Nissen 1979). This method, known for soluble proteins, was adapted for insoluble wheat gluten.

Phosphate buffer (1.0 mL) (0.2M, pH 8.5) and 1.0 mL of a freshly prepared aqueous TNBS solution (0.5% w/v) were added to a gluten sample of ≈ 10 mg. After keeping this mixture for 4 hr at 50°C in the dark, 8 mL of 50% H₂SO₄ was added. This mixture was vigorously stirred at RT in the dark for 1 hr and left standing at 50°C for 30 min to obtain a clear solution. Subsequently, 1 mL of the solution was diluted with 3 mL of 50% H₂SO₄, after which the absorbance was measured at 345 nm. A blank was prepared applying the same procedure without adding wheat gluten. Separately, wheat gluten was treated similarly without the addition of TNBS. The blank value consisted of the addition of the absorbance values of both of the last readings. The free amino group content was calculated using a molar absorption coefficient of 14,600 l.mol⁻¹ cm⁻¹ for trinitrophenyl lysine.

Determination of Protein Solubility by Kjeldahl Analysis

Wheat gluten (0.05 g) was dispersed in 45 g of deionized water. The pH level was adjusted by adding an aqueous solution of 0.5M NaOH or 50% (v/v) acetic acid. The dispersion was stirred at RT for 1 hr using a magnetic stirring device. The weight was adjusted to 50 g and the dispersion was stirred for another 5 min. Subsequently, 25 mL of the dispersion was centrifuged (15 min, 11,500 $\times g$). The supernatant was filtered and the nitrogen content of the supernatant was determined by Kjeldahl analysis.

The nitrogen content of the original sample was determined by completely combusting ≈ 30 mg of the protein. Subsequently, the amount of nitrogen was obtained using a rapid-N apparatus, Foss Electric (Hoom, The Netherlands). Protein solubility was calculated:

$$\text{Solubility (\%)} = (N_{\text{sol}}/N_{\text{tot}}) \times 100$$

where N_{tot} = total amount of nitrogen in the starting material and N_{sol} = total amount of nitrogen in the supernatant.

SDS-PAGE

To a SDS-PAGE sample (containing at least 50 mg of wheat gluten), 6 mL of electrophoresis buffer (63 mM Tris, 20% glycerol w/v, 2% SDS w/v, 10% β -mercaptoethanol w/v, and bromophenyl blue) was added and left standing at RT for 2 hr with vortexing every 15 min. Subsequently, the mixture was centrifuged at 8,000 $\times g$ for 10 min. The supernatant was kept at -20°C for later electrophoresis. The pellet was extensively washed with warm water on filter paper. The washed pellets were dried at 40°C for four days.

Before electrophoresis, the supernatant was boiled for 5 min to break the S-S bonds. Subsequently, it was cooled down and applied to a gel prepared from 15% acrylamid. Low molecular weight standards (phosphorylase b 94,000; BSA 67,000; ovalbumin 43,000; carbonic anhydrase 30,000; soybean trypsin inhibitor 20,100; and α -lactalbumin 14,400) from Pharmacia, (Uppsala, Sweden) were used as protein reference. The electrophoresis was conducted using an electrophoresis system (Bio-Rad, Veenendaal, The Netherlands) at a voltage of 150V which was increased to 200V when the protein reached the dividing line between the stacking and the separating gels. Fixation and coloring of the gel was performed by the standard procedure using Coomassie blue. After one night, the gel was flushed with deionized water and put in a destaining liquid. After decoloration, the gel was dried for 1 hr at 60°C.

The extraction efficiency of wheat gluten in the electrophoresis buffer was calculated:

$$\text{Extraction (\%)} = [(P_{\text{tot}} - P_{\text{pellet}})/P_{\text{tot}}] \times 100$$

with P_{tot} = weight of starting material (g), and P_{pellet} = weight of material (g) left on the filter after extraction and washing.

RESULTS AND DISCUSSION

The nature (inter- or intramolecular) and the extent of cross-linking was assessed from changes in the solubility, the amount of amino groups of the protein, and the molecular weight of the soluble fraction of wheat gluten. Solubility was measured by Kjeldahl analysis (Fig. 2). About 50% of wheat gluten was solubilized in aqueous solutions at pH < 5. At higher pH (6–9), the solubility decreased to $\approx 15\%$. Increasing the pH > 9, increased the solubility of wheat gluten, reaching a value of $\approx 70\%$ at pH 11. The curve agreed well with other reported solubility curves of native gluten (Bollecker 1991). At neutral pH, which is around the pI of wheat gluten, the overall electrostatic charges of the molecules are zero promoting aggregation and precipitation of wheat gluten. Both at acid and basic pH, net charge and charge repulsion contributed to greater protein solubility. Initially, the solubility of cross-linked gluten was compared with native gluten at pH 2–11 (Fig. 2). The cross-linked material showed a low water solubility ($\approx 10\%$) over the entire pH range. This decreased solubility was ascribed to an

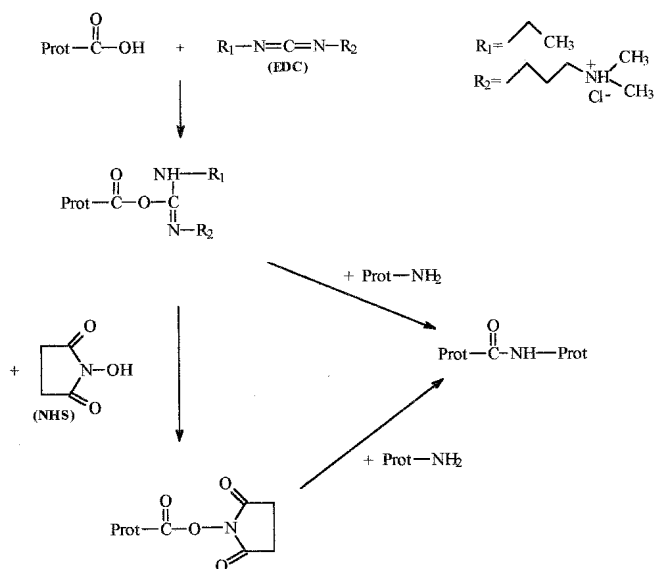


Fig. 1. Cross-linking of proteins with water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) and *N*-hydroxysuccinimide (NHS).

increased average molecular weight of the protein as a result of intermolecular cross-linking (between different protein chains). To estimate the total solubilization curve of (modified) gluten turned out to be very laborious. Consequently, in optimization of the cross-linking reaction, the solubility of cross-linked samples was measured only at pH 3 and pH 11, reflecting the maximum solubility difference between modified and native gluten.

Effect of Molar Ratio of EDC and NHS to COOH

The molar ratio of EDC and NHS to COOH was varied to increase the efficiency of cross-linking reaction with a minimum use of reagents. Cross-linking reactions were carried out at pH 7 for 4 hr at RT. TNBS analysis showed a decrease of the amount of amino groups by increasing the molar ratio of EDC to COOH

(Fig. 3). At a molar ratio of EDC to COOH of 4:1, the amount of amino groups reached a minimum of 9.5 mmol/100 g of protein (compared with native gluten [protein content 76.5%] 15 mmol/100 g of protein). Simultaneously, the solubility decreased to ≈9% at pH 3 and 18% at pH 11. The trends in the solubility curves at pH 3 and pH 11 were the same, demonstrating that the results were not influenced by the breakage or formation of disulfide bridges at alkaline pH.

Likewise, increasing the ratio of NHS to EDC, the free amino group content decreased to 9.3 mmol/100 g of protein (Fig. 4). When the molar ratio of NHS to EDC was 0.4, the solubility at pH 3 and pH 11 was at a minimum. Consequently, to obtain the lowest amount of free amino groups and the lowest solubility, the optimal molar ratio of EDC to NHS to COOH was 5:2:1.

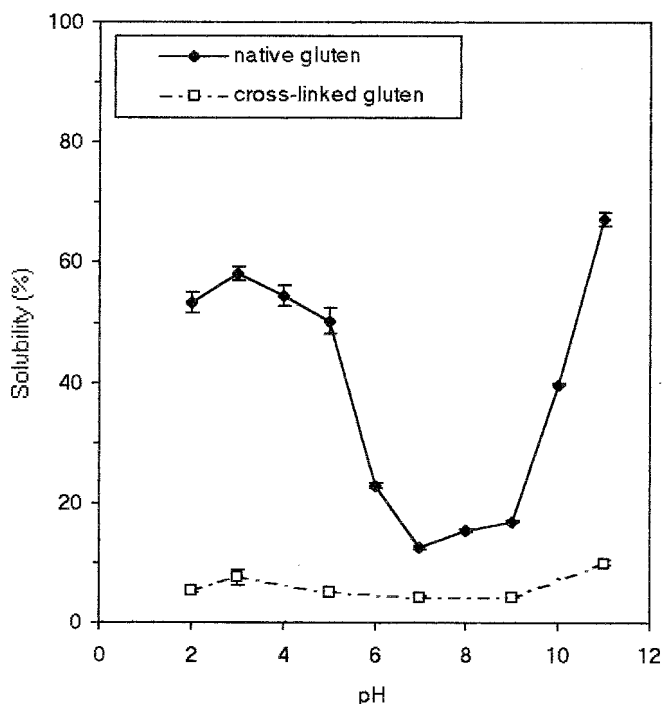


Fig. 2. Solubility of native and modified gluten. Reaction conditions: pH 5.5, reaction time 4 hr, and 5:5:1 ratio of water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and COOH ($n = 3$, \pm standard deviation).

Influence of Reaction Time and pH Level

The effect of reaction time and pH level on the degree of modification was studied by carrying out the reaction for different periods of time and varying pH at room temperature with a molar ratio of EDC to NHS to COOH of 5:5:1. Although EDC to NHS to COOH ratio of 5:2:1 already proved to be the settings, the present ratio was chosen to ensure plateau conditions were maintained. The amount of amino groups and the solubility at pH 3 and pH 11 were determined for each cross-linked sample (Figs. 5–7).

At pH 3, the amount of amino groups did not substantially change up to a reaction time of 15 hr. The solubility of the modified samples was only slightly affected (data not shown). At pH 7, the amount of amino groups decreased with increasing reaction time to reach ≈10 mmol/100 g of protein after a reaction time of 5 hr (Fig. 5). The decrease in amino groups was largest within the first 2 hr of the reaction. Simultaneously, during these first 2 hr, the solubility of modified gluten substantially decreased by ≈60–10%. Consequently, the initial loss of amino groups was accompanied by a large decrease in solubility. The same results were obtained when the reaction was carried out at pH 5.5 (data not shown). At pH 11, the amount of amino groups strongly decreased to reach ≈7 mmol/100 g of protein after 4 hr. In contrast to the reaction at pH 7, the solubility only slightly decreased with increasing reaction time.

For the reactions carried out at both pH 7 and pH 9, a minimal protein solubility was obtained after 2–4 hr. Although the amount of amino groups continued to decrease with longer reaction time, this was not accompanied by a further decrease in solubility. In contrast to the reaction time, the pH of the reaction seemed to be the most important parameter in the cross-linking of wheat gluten with EDC-NHS.

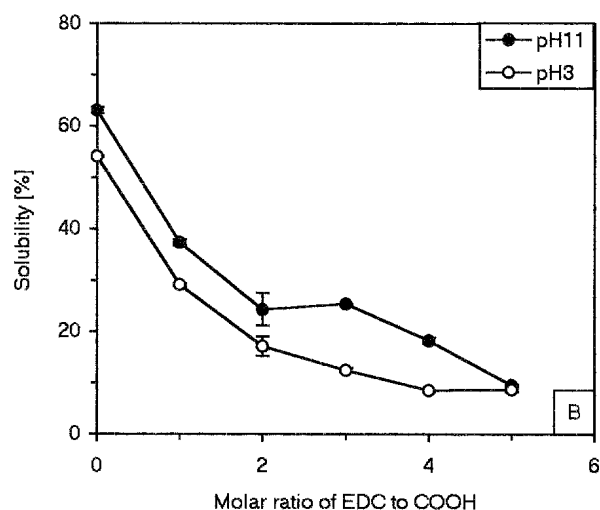
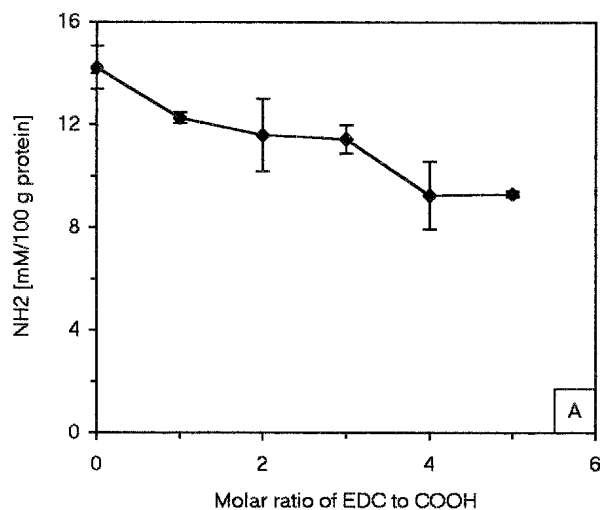


Fig. 3. Amount of free amino groups (A) and solubility curves of cross-linked wheat gluten at pH 3 and 11 (B) as a function of the ratio of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to COOH (pH 7, 4 hr, 1:1 ratio of *N*-hydroxysuccinimide [NHS] to EDC) ($n = 3$, \pm standard deviation).

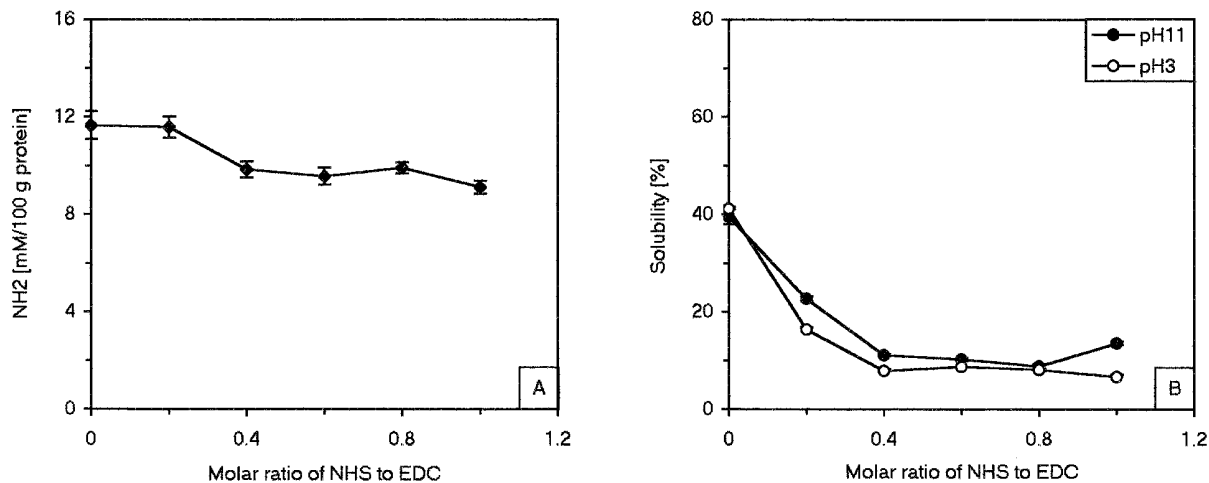


Fig. 4. Amount of free amino groups (A) and solubility curves of cross-linked wheat gluten at pH 3 and 11 (B) as a function of the ratio of *N*-hydroxysuccinimide (NHS) to water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (pH 7, 4 hr, 5:1 ratio of EDC to COOH) ($n = 3$, \pm standard deviation). Reaction performed under the same conditions without EDC and NHS for a blank.

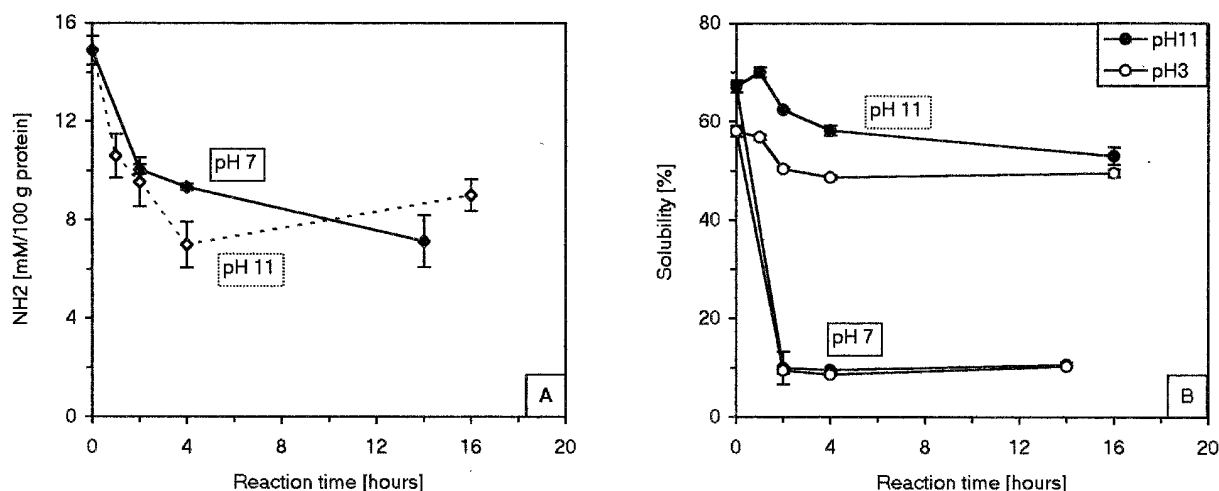


Fig. 5. Amount of free amino groups (A) and solubility curves of cross-linked wheat gluten at pH 3 and 11 (B) as a function of the reaction time (pH 7 and pH 11, room temperature, 5:5:1 ratio of water-soluble 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride [EDC], *N*-hydroxysuccinimide [NHS], and COOH) ($n = 3$, \pm standard deviation). Reaction performed under the same conditions without EDC and NHS for a blank.

The results of varying the pH level are summarized in Fig. 6. By increasing the pH from 3 to 11, the amount of amino groups decreased almost linearly to reach ≈ 7 mmol/100 g of protein at basic pH. The solubility at pH 3 and 11 of wheat gluten that was modified at different pH levels showed similar profiles. If the reaction was carried out at pH 3, the solubility was $\approx 61\%$. This was close to the solubility of native gluten, which was $\approx 58\%$. Carrying out the reaction at pH 5.5 or 7, the solubility reached a minimal value of $\approx 11\%$. Finally, at pH 11, the solubility remained relatively high ($\approx 51\%$).

Combining the results from the determination of the amount of amino groups and the solubility, it is obvious that cross-linking of wheat gluten with EDC-NHS occurred through different mechanisms. To analyze the influence of pH on the cross-linking reaction, the solubility of native protein in the dispersion and the reactivity of the functional groups at different pH levels have to be taken into account. Thus, the following mechanism of wheat gluten cross-linking is suggested (Fig. 7). For schematic reasons, a protein chain represents any protein of the gluten protein mixture (gliadins, glutenins). Consequently, two gluten submits belonging to the same covalent (S-S) association is considered as one protein.

At low pH, wheat gluten is relatively soluble. Reaction of the carbodiimide with the protein may lead to derivatives resulting

from modification of carboxylic acid groups. However, amino groups of the lysine residues of the protein are protonated, which eliminates the nucleophilic character of this group and prevents cross-linking. Consequently, the amount of amino groups and the solubility of the protein were not affected. Since electrostatic forces between molecules are at a minimum, wheat gluten aggregates are formed at pH 5–7, resulting in low protein solubility. Under these conditions, reagents may only reach the surface of the protein but penetration inside aggregates may be difficult. Thus, intermolecular cross-linking between carboxylic acid and amino groups of different molecules belonging to different protein associates will be favored. Consequently, the amount of amino groups decreased and, due to an increase of the average molecular weight, the solubility at acidic and basic pH drastically decreased ($\approx 60\%$). Finally, when the reaction is carried out at pH 11, wheat gluten is even more soluble than at pH 3. However, in contrast to the low pH, amino groups of lysine residues are now deprotonated and nucleophilic attack to NHS activated carboxylic acid groups is very efficient (Van Delden et al 1996). Since the protein chains were solubilized, EDC and NHS could penetrate inside the core of protein. Thus, both inter- and intramolecular cross-links may be formed given the fact that a strong decrease in the amount of amino groups was accompanied by only a slight decrease in the

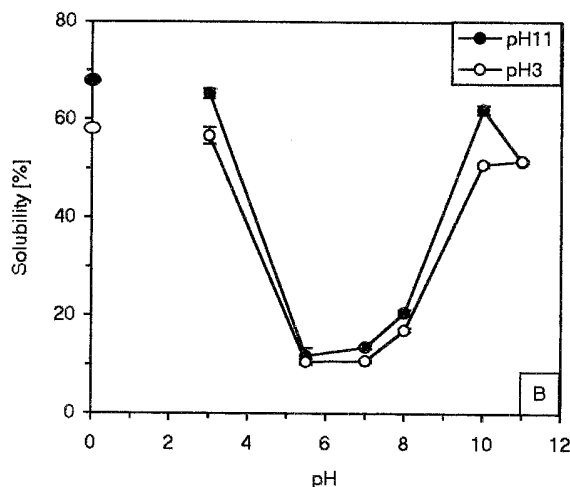
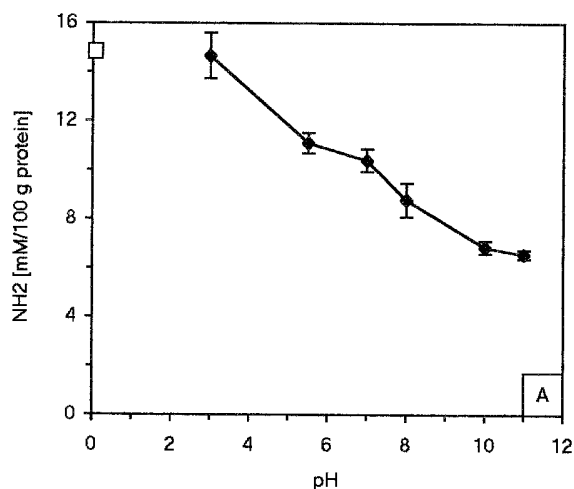


Fig. 6. Amount of free amino groups (A) and solubility curves of cross-linked wheat gluten at pH 3 and 11 (B) as a function of the pH (room temperature, 4 hr, 1:5:5 ratio of COOH, *N*-hydroxysuccinimide [NHS], and water-soluble 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride [EDC]) ($n = 3, \pm$ standard deviation). Amount of amino groups in native gluten is 14.8 mmol/100 g of protein. Solubility of native gluten is 58% at pH 3 and 67% at pH 11.

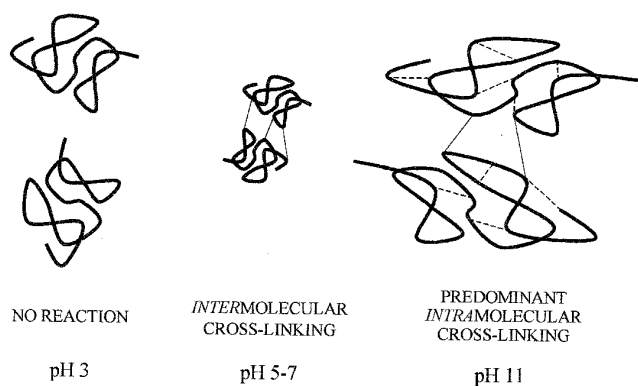


Fig. 7. Proposed mechanism of cross-linking of wheat gluten with water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) and *N*-hydroxysuccinimide (NHS) at different pH levels.

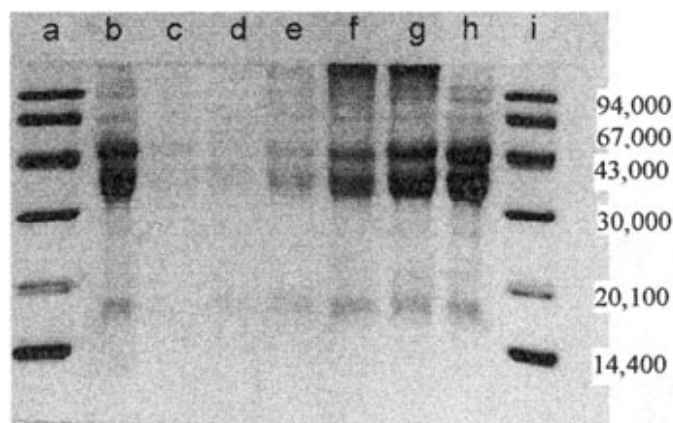


Fig. 8. SDS-PAGE results for standard (a); reactions carried out at pH 3 (b), pH 5 (c), pH 7 (d), pH 8 (e), pH 10 (f), and pH 11 (g); native gluten (h); standard (i).

solubility ($\approx 20\%$). Preferentially intramolecular cross-linking of protein occurred which could increase the conformation stability. For this reason, these conditions were also used to introduce additional tertiary structure into proteins (Blotnick and Muhrad 1994). Consequently, by changing reaction conditions, intermolecular cross-linking (resulting in a decreased protein solubility) or intramolecular cross-linking (mainly leading to conformation stabilization) can be achieved, thereby controlling the specific properties of the final product.

SDS-PAGE Analysis of Native and Cross-Linked Gluten

SDS-PAGE was conducted to confirm the hypothesis of an intra- or intermolecular cross-linking (Fig. 8). The patterns were different for reactions carried out at different pH levels. For reactions at pH 3, the bands were identical to those of untreated protein. Bands at pH 5 and 7 showed a very weak intensity, partly reflecting the low solubility of cross-linked protein in electrophoretic buffer (Table I), which was also depicted in Fig. 6. Furthermore, high molecular weight (protein aggregates) molecules could not penetrate the gel, resulting in the absence of bands. At pH 10 and 11, the protein pattern and intensity were almost the same as native gluten. Additionally, a large band was present on the top of the gel (MW > 94,000). Thus, the average molecular weight was slightly increased by the reaction carried out at high pH, indicating that next to intramolecular cross-linking at pH 11 some intermolec-

TABLE I
Solubility of (Modified) Wheat Gluten in Electrophoresis Buffer

Sample	Solubility %
Native gluten	70 \pm 2
Cross-linked at	
pH 3	65 \pm 2
pH 5	1 \pm 1
pH 7	3 \pm 2
pH 8	15 \pm 3
pH 10	41 \pm 4
pH 11	50 \pm 2

^a $n = 3, \pm$ standard deviation.

ular cross-links were formed. These SDS-PAGE results provide circumstantial evidence at least for the proposed cross-linking mechanism in which changes in gluten structure with varying pH determine the extent and the nature of the cross-linking reaction.

CONCLUSIONS

In this study, the optimal conditions have been defined for EDC-NHS mediated cross-linking of wheat gluten. The extent and character of cross-linking were determined by measuring the amount of amino groups and the solubility in water of different pH levels. It appeared that reaction conditions largely determined the

formation of linkage, inter- or intramolecular cross-links at high pH levels. Intramolecular cross-linking was accompanied by a decrease in the amount of amino groups (up to 60%), while the solubility was hardly affected (decrease of 20%). On the other hand, at neutral pH, intermolecular cross-linking resulted in both a decrease in the amount of amino groups (only 30%) and a decrease of solubility (60%). Thus, depending on the properties or the application required, conditions of the reaction could be adapted. The modifications could be useful to adapt gluten properties to some nonfood uses. The functional properties of wheat gluten films cross-linked with EDC-NHS will be presented in a separate article (V. Tropini et al, *unpublished*). However, it should be noted that the present method is only suitable for laboratory experiments, as was intended. For actual industrial applications, other chemicals that are more effective, much cheaper, and readily available are to be preferred. Nevertheless, the results presented in the present article may be equally valuable.

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