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Quantitative Analysis of the Reaction between Gliadin and Citric Acid under Weak Acidic and Weak Alkaline Conditions

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by

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Alkaline Conditions

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Gliadin was reacted with citric acid under weak acidic and weak alkaline conditions in both wet and dry states and the reaction mechanism was studied. The low morphological stability in an aqueous environment and inferior mechanical properties have restricted the applications of plant proteins, although these materials possess a unique structure, biocompatibility and biodegradability. Carboxylic acids such as citric acid are inexpensive and nontoxic chemicals and are preferred for crosslinking proteins and cellulose to improve the desired properties of the materials.

In this study, gliadin was chosen as a model of plant proteins because it contained relatively more amine side groups that can react with a carboxylic acid crosslinker than other plant proteins, e.g. zein and soy proteins. In order to avoid using toxic chemicals and experiencing strength loss and/or yellowing of the crosslinked materials, alkaline catalyzed crosslinking of gliadin powders in aqueous citric acid solutions at low temperatures was employed. However, previous research only provided limited evidences, such as improvement in mechanical properties to support the presence of the acylation reaction. To explore the reaction mechanism, titration method was used to investigate the influences of pH, citric acid concentration and reaction temperature on both carboxyl and amine group changes during the reaction. The kinetic parameters of both reaction states have been obtained at different temperatures. Additionally, to further improve the crosslinking degree, a dry state crosslinking of gliadin films with citric acid was also studied. A relationship between the mechanical properties and the crosslinking degree has also been developed.

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CHAPTER 1: INTRODUCTION

1.1 The advantages of plant proteins

To develop green materials by utilizing renewable resources has attracted the world's attention in the effort to replace materials derived from petroleum sources. The wide availability of plant proteins has led to a new interest in industrial applications because of their renewable and abundant resources. [1-7] These proteins can be obtained as byproducts generated in cultivation of agricultural crops and coproducts generated in processing cereal grains for food and biofuels, such as ethanol from wheat (Europe) and corn kernel (US). **Table 1.** shows the world production of raw crops and their protein content availability. [8] Million tons of plant proteins can be obtained annually, leading to inexpensive prices.

Table1. World production of raw crops and protein content availability

Raw Material	World Production (Million tons)	Protein Content (%)
Soybean	206.5	44
Wheat	632.6	13
Corn	724.6	9
Milk	622.3	3
Peanut	30.2	27
Total	2216.2	-

Like common proteins, plant proteins also possess unique properties, such as biocompatibility, biodegradability and affinity to both hydrophilic and hydrophobic materials, as well as the ability to maintain tunable attractions to either positively or negatively charged substances by adjusting the pH conditions of the surrounding environment. Furthermore, proteins

have been showed to have great potential uses in biomedical applications including tissue engineering and drug delivery. [9,10] Currently, attempts have been to develop fibers, films, composites, nano particles using plant proteins, such as soyproteins, zein from corn and wheat gluten. [1-7,11]

1.2 Current problems of plant proteins and potential solutions

The properties of the regenerated plant protein materials developed so far to date do not match the quality of natural protein materials, such as wool and silk in current use. The poor mechanical properties and low water stability of the materials generated from soyproteins, zein, wheat gluten, and gliadin are the two major defeats which impede the further applications of these materials. [12–14] To improve the mechanical properties and water stability of regenerated products, blending two or more proteins and/or synthetic polymers has been proposed. [15-17] The disadvantage of this method is that some properties of blended polymers, such as high hydrophobicity or poor degradability, may cause undesirable changes to the surface properties and the biodegradability of the final products.

Crosslinking is one of the most common approaches used to improve the properties of regenerated protein materials and to make them useful for various applications. Currently, formaldehyde and glutaraldehyde are commonly used to crosslink plant protein materials such as soyprotein, gliadin and wheat gluten. [18-20] Although these agents are efficient in improving the mechanical properties and water stability of protein materials, they are toxic. [21-23] For example, glutaraldehyde irritates the eyes and is difficult to handle during processing. Some non-toxic crosslinking agents like 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)

and N-hydroxysuccinimide (NHS) have also been used but they are either inefficient in enhancing water stability or high in cost. [24-25]

Carboxylic acids such as citric acid are inexpensive and nontoxic chemicals preferred for crosslinking proteins and cellulose. It has been reported that zein fibers have been crosslinked with citric acid and butanetetracarboxylic acid (BTCA).[26] However, carboxylic acid crosslinking is usually done under the dry state and needs phosphorus-containing catalysts and high-temperature curing (150-185 ℃) for the cross-linking reaction to occur. Phosphorus-containing catalysts are toxic to use, and can cause substantial strength loss and/or yellowing of the crosslinked materials, especially when crosslinked at high temperatures. The crosslinking of molecules leads to lower flexibility which also contributes to the strength loss of the cross-linked materials. The yellowing of the fabrics is caused by the dehydration of the carboxylic acids at high temperature, forming an unsaturated acid that changes the color of the cross-linked materials. [27] For example, cotton fabrics crosslinked with BTCA experienced mechanical strength loss ranging from 43 to 88%. [28] Silk fabrics crosslinked with citric acid showed strength loss ranging from 2 to 15% and a decrease in the whiteness index by about 30%. [27] Mechanical strength loss and unwanted changes in the properties of the carboxylic acid crosslinked materials could be avoided if the crosslinking is performed at relatively low temperatures and a high pH. Thus, a new way of wet cross-linking of gliadin fibers using citric acid with alkali catalysts and low temperatures has been reported. [29] For amino acid residues that have an amine in the side group, gliadin, soyprotein, and zein all contain histidin, lysine and arginine that can react with a carboxylic acid crosslinker. Gliadin contains abundant amine in the side groups and was taken as a model of plant proteins to study the reactionn. The content of amino acids with an amine in the side group in gliadin, soyprotein and zein is shown in Table 2.[30]

Table2. The Content of Amino Acids with an Amine in the Side Group in Gliadin, Soyprotein, and Zein Proteins

Amino Acid	Amino Acid Content (%)		
	Gliadin	Soyprotein	Zein
Lysine	0.64	5.40	0.20
Histidine	2.23	2.30	1.07
Arginine	2.72	5.80	1.56

1.3 The mechanism of alkaline catalyzed acylation of gliadin with citric acid

Nevertheless, the chemical processes of the alkaline catalyzed acylation reaction of gliadin with citric acid still remained unknown. A possible mechanism for this reaction is proposed as a hypothesis, as shown in **Scheme 1**. This reaction is a nucleophilic substitution. When carboxylic acid is added, the amine groups take on positive charges under acidic condition. Without addition of alkali, it would be difficult for the positively charged amine in the protein to react with partially positively charged carbonyl carbon in carboxylic acid. In the presence of alkali, the amine groups are less likely to carry positive charges, and therefore they could attack the carbonyl carbon of the carboxyl groups and more readily form amide linkages. When citric acid is provided as the crosslinker, it could be less possible for all of the three carboxyl groups to take part in the substitution reaction. When more than one carboxyl group of a citric acid reacts with amine groups to form amide linkages, the amount of carboxyl groups on the crosslinked materials would increase and the amount of remaining amine groups would decrease. Previous research only provides limited evidences like improvement in mechanical properties to support the presence of the crosslinking reaction. [29]

To explore the mechanism of the reaction, titration method was used to investigate the influences of pH, citric acid concentration and reaction temperature on both carboxyl and amine group changes during the reaction. The kinetics parameters of both crosslinking states have been

obtained at different temperatures. Additionally, to further improve the crosslinking degree of the

final product, a dry state crosslinking of gliadin films with citric acid was studied. A relationship

between the mechanical properties and crosslinking degree has also been developed.

Scheme 1. Possible mechanism for the alkali-catalyzed reaction between citric acid and an amine

group in a protein (P)

1.4 Titration method for amine and carboxyl end group analysis

The titration method, which is inexpensive and has high precision, is suggested to analyze

the amine and carboxyl groups change on the crosslinked materials. Chemical end group analysis

is one of the most widely applied techniques for polymer characterization. Instrumental methods

such as nuclear magnetic resonance (NMR) and infrared spectroscope (IR) are often less

sensitive than chemical functional group analysis. Some good chemical analysis methods have

been developed for nylon 6 and can be applied to polyamides. For nylon, the methods to measure

the remaining amine and carboxyl groups after polymerization have been well established. [31]

Most methods for amine end groups analysis utilize titration with a strong acid in a

predominately nonaqueous solution, in which amine and carboxyl groups can hardly get ionized.

End points can be determined potentiometrically, conductometrically, or by the use of visual

indicators. In order to reduce the analysis time of potentiometric and conductometric methods, thymol blue, whose transition point is 1.2-2.8, is usually employed as a visual indicator. Although this modification reduces the precision of the method, but good results may still be obtained. When thymol blue is chosen as the visual indicator, the amount of remaining amine groups can be measured directly because the protons from strong acids for titration will change most of the amine groups from the forms of NH_2 to NH_3^+ at pH 1.2-2.8. Therefore, the amount of consumed acids equals the amount of amine end groups. On the other hand, most carboxyl end group analysis is also based on nonaqueous titrimetry with sodium hydroxide. If an appreciable concentration of water exists, the carboxyl titration will yield a double break due to salt formation of excess amine end groups. For nylon 6 and nylon6,6 titration, benzyl alcohol is used as a solvent to a phenolphthalein end point, at which most of carboxyl groups change to unprotonated forms. [31]

1.5 Kinetics for the acylation reaction

During the acylation reaction process, it is important to study the kinetic parameters because kinetics provides the intrinsic properties of a reaction, such as reaction rate, reaction order and activation energy, leading to an understanding of the mechanisms of a reaction. The acylation reaction between citric acid and proteins is shown as equation (1), given as:

$$a \text{ protein} + b \text{ citric acid} \Leftrightarrow c \text{ acylateded protein} + d \text{ H}_2\text{O} \tag{1}$$
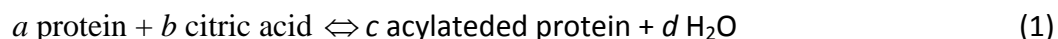
Where a-d are the molar constants at equilibrium

The reaction rate, r, could be represented by equation (2), as:

$$r = \frac{dC_{AP}}{dt} = ka^m C_p^n \tag{2}$$

Where k is the reaction rate constant,

C_{AP} is the concentrations of functional groups in the acylated protein,

C_P is the concentrations of functional groups in the non-reacted protein,

a is the activity of citric acid, and

m and n are the powers related to the reaction mechanism.

The acylation reaction between gliadin and citric acid is substantially a reaction between amine groups of the protein and carboxyl groups of citric acid. Thus equation (2) can be modified as equation (3), given as:

$$r = k_1 [COO^-]^m [NH_2]^n \tag{3}$$

Where k_1 is the reaction rate constant,

[COO⁻] is the concentration of carboxyl groups,

[NH$_2$] is the concentration of amine groups, and

m and n are the powers related to the reaction mechanism.

During the acylation reaction, only a small portion of the total functional groups available in the proteins are reacted, while the amount of carboxyl groups of citric acid added at the beginning of the reaction is much more than that of amine groups of proteins. Therefore, [COO⁻] could be considered as constant, and equation (3) could be rewritten as equation (4), given as:

$$r = k_2 [NH_2]^n \tag{4}$$

Where $k_2 = k_1 [COO^-]^m$, which is the pseudo-reaction rate constant, and

n is the pseudo-rate order.

In order to set up a linear relationship between reaction rate r and amine concentration [NH$_2$], the form of equation (4) is changed to equation (5) by using Log function on both sides of the equation, given as:

$$\log r = \log k_2 + n \log[NH_2] \tag{5}$$

The acylation reaction basically occurred between carboxyl groups and amine groups. Thus carboxyl groups and amine groups can be deemed as the two reactants. Furthermore, one mole of the carboxyl groups with one mole of the amine groups can form one mole amide linkages, which means the molar constants of either reactant equals one. Therefore, the reaction rate r can also be described as equation (6), given as:

$$r = -\frac{d[NH_2]}{dt}$$ (6)

Where t is time.

By combining equations (5) and (6), a relationship between the first derivative of amine concentration to time and the amine group concentration is obtained, which is shown as equation (7). By plotting equation (7), the reaction order n and pseudo-reaction rate constant k_2 can be calculated, as:

$$\log(-\frac{d[NH_2]}{dt}) = \log k_2 + n\log[NH_2]$$ (7)

According to the Arrhenius equation, given as equation (8), a linear relationship between the natural logarithm of the pseudo-reaction rate constant k_2 and the inverse of temperature may be obtained. By plotting equation (8), the activation energy E_a as the slope can be obtained, as:

$$\ln k_2 = -\frac{E_a}{RT} + C$$ (8)

Where Ea is the activation energy of the crosslinking reaction

The specific objectives of this research is (a) to prove the presence of the acylation reaction between gliadin and citric acid, (b) to study the influence of pH, citric acid concentration and temperature on the crosslinking reaction, (c) to obtain the kinetic parameters to understand the

mechanism of the reaction and (d) to develop a relationship between the film tensile strength and the crosslinking degree.

CHAPTER 2: Materials and Methods

2.1 Materials

Gliadin was extracted from commercially available wheat gluten (Whetpro 80). Wheat gluten (Whetpro 80) was obtained from Archer Daniels Midland Company, Decatur, IL. Citric acid, sodium hydroxide, sodium carbonate, hydrochloric acid, thymol blue, alizarin yellow R and ethanol were reagent-grade chemicals purchased from VWR international, Bristol, CT.

2.2 Methods

2.2.1 Extracting gliadin

Gliadin was extracted from the wheat gluten using aqueous ethanol (70%w/w) in a 4:1 (ethanol/gluten) weight ratio stirring overnight at room temperature. The solution was then centrifuged at 10,000 rpm for 30 min. The supernatant was then collected and dried using a vacuum oven at 50 ℃ to obtain the gliadin. All gliadin was grounded into a powder. About 35% gliadin was extracted based on the weight of the wheat gluten used.

2.2.2 Wet State Crosslinking

Five grams gliadin powder was placed into a 250mL conical flask and mixed with the reaction solution following a liquid to solid ratio of 40:1. The mixture then reacted at a particular pH, temperature, and for the required amount of time. Citric acid forms solutions with pH 1.5–2 when dissolved in water. The pH of the crosslinking solution was adjusted from its original pH using sodium hydroxide. Various crosslinking conditions, such as citric acid concentration (0.4 to 1.8 M), temperatures (25–70 ℃), time (0.5–4h), and pH (4.3–9.0) were varied to obtain gliadin with different crosslinking degree. After crosslinking, the powders were washed with vacuum

filtration in distilled water (pH 6.0-6.5) until the water pH changed no more. The wet materials were collected and dried at 40 ℃ over night in a hot air oven. All the crosslinked samples were collected and stored in a refrigerator to prevent hydrolysis. For each treatment condition, at least three blank samples without the presence of citric acid were prepared.

2.2.3 Dry State Crosslinking

Gliadin was dissolved in 70% (w/w) ethanol for film casting. The film forming conditions, such as citric acid concentration (5%-11% of the weight of gliadin), pH of the film forming solution (3.5-7.5) were varied to obtain films. Five grams of gliadin powder was used for each treatment condition and 60 grams of the film forming solution was obtained before casting. Sodium Carbonate instead of sodium hydroxide was used to adjust the pH, in order to reduce the pH change between the final film obtained and the film forming solution. The film forming solution was poured onto Teflon coated glass plates and the ethanol was allowed to evaporate. It took about 6-8 hours for the proteins to dry under ambient conditions (21 ℃ and 65% relative humidity) and form stable films. The films obtained were later annealed at different temperatures conditions (80-170 ℃) for different time (1-4h). The films used for chemical end groups' analysis were next washed with vacuum filtration in distilled water (pH 6.0-6.5) until the water pH changed no more. For each treatment condition, at least three blank samples without the presence of citric acid were prepared.

2.2.4 Amine and Carboxyl End Groups' Analysis

One gram of wet state crosslinked gliadin and about 0.25 gram dry state of crosslinked gliadin were used for titration for each condition. It took about 24 hours for the titration samples

to dissolve in 70% ethanol. Hydrochloric acid was employed for amine groups' analysis with alizarin yellow R as the indicator. According to the amino acid content of gliadin reported previously, the major amine groups were from lysine, arginine and histidine, whose protonated form obtained pK_as of 10.53, 12.48 and 6.00, respectively. Thus, phenolphthalein can not be simply chosen as the indicator, as suggested by the previous research with the nylon amine end groups' analysis. At pH, 7.0, protonated amine groups of lysine and arginine would be the predominant state of ionization. Even a significant fraction of histidine's amine groups are positively charged at pH 7.0. [32] When the crosslinked gliadin samples were washed with distilled water at pH 6.0-6.5, most of the amine groups would be positively charged. The amount of amine groups can be determined by titrating the protons on the amine end groups. Alizarin yellow R is the indictor with highest pH transition range, at which the major part of amine end groups of gliadin could be changed to an uncharged form.

Both hydrochloric acid and sodium hydroxide were used for carboxyl end groups' determination. An excess amount of hydrochloric acid was first added into the titration sample first. As described before, the amine groups took on a positive charge when washed with distilled water at 6.0-6.5. At the same time, the carboxyl groups were ionized. In the presence of enough hydrochloric acid, most of the carboxyl groups will bind with protons. The excess part of hydrochloric acid was titrated using sodium hydroxide with thymol blue as the indicator. Therefore, the amount of carboxyl groups could be calculated by subtracting the amount of protons sodium hydroxide neutralized from the amount of originally added hydrochloric acid. For each condition, at least three blank samples prepared at the same reaction conditions but without the presence of citric acid were titrated.

2.2.5 Tensile Properties

Films made of dry state crosslinked gliadin with 7-11% citric acid at pH 4.6, 140℃, as well as wet state crosslinked gliadin with citric acid concentration of 0.9M and 1.8M at pH 8.0, 50℃ were used to study the relationship between the tensile properties and the crosslinking degree. For each condition, control samples without the presence of citric acid were also prepared. All samples were conditioned in a standard testing atmosphere of 21 ℃ and 65% relative humidity for at least 24 h before testing. Thickness of the films was measured using a thickness gauge (AMES, Model: LG2600, Waltham, MA) with an accuracy of 1μm. The tensile properties of the gliadin films were determined according to ASTM standard D 882-02 on a MTS tensile tester (MTS Corporation, Model: QTest 10). Testing was done on five samples cut from the cast films. At least 15 specimens (repeats) were tested for each sample.

2.2.6 SDS Electrophoresis

One micro gram of cross-linked and non-crosslinked gliadin samples were dissolved in 100 mL 2x LDS Sample buffer (4x LDS Sample buffer, 5% 2-beta-mercaptoethanol, milliQ water), and then incubated at 70 C for 10 min. About 10 mL Invitrogen SeeBluePlus2 Prestained standard 1x and 10 mL of each sample solution were loaded into individual slot in the NuPage 4-12% Bis-Tris Gel with NuPage 1x MES Running Buffer (5% NuPage 20x MES Running Buffer and MilliQ water). After electrophoresis, the gel was stained with Protica Microwave Blue for 7 min and rinsed in deionized water until a clear background was formed. The molecular weight of the protein standard (Invitrogen SeeBluePlus2 Prestained Standard 1x) ranged from 3 to 188 kDa.

2.2.7 Statistical Analysis

The data were analyzed using one-way analysis of variance with Tukey's pairwise multiple comparison with SAS. The confidence interval was set at 95%. A difference was considered to be statistically significant when the p-value was smaller than 0.05. In the following results, data labeled with the same number were not significantly different from each other.

CHAPTER 3: Results and Discussions

3.1 Wet state crosslinking

3.1.1 The influence of pH

Increasing the pH is favorable for improving the acylation reaction extent between citric acid and gliadin when reacted using 0.9M citric acid solution at 50 ℃ as shown in Figures 1 and 2. Figure 1 shows the influence of pH on the amine groups' change during the reaction. Each condition had achieved balance at 4h. Increasing reaction time up to 4 hours decreased the amount of amine groups left on gliadin, because they had gradually reacted with carboxyl groups to form amide linkages. Therefore, the amount of reacted amine groups, which equals the amount of amide linkages formed, showed an increased trend when increasing the reaction time. At the same time point, more amine groups participated in the reaction at higher pH than at lower pH, indicating a higher reaction extent could be obtained when increasing the pH from 4.3 to 9.0. This is because if the alkali added was not enough and the reaction was taken place at a lower pH, the amine groups were more likely to take on positive charges under acidic condition. Therefore, it would be difficult for the positively charged amine in the protein to react with partially positively charged carbonyl carbon in carboxylic acid. In the presence of enough alkali, the amine groups are less likely to carry positive charges, and therefore they could attack the carbonyl carbon of the carboxyl groups and more readily form amide linkages.
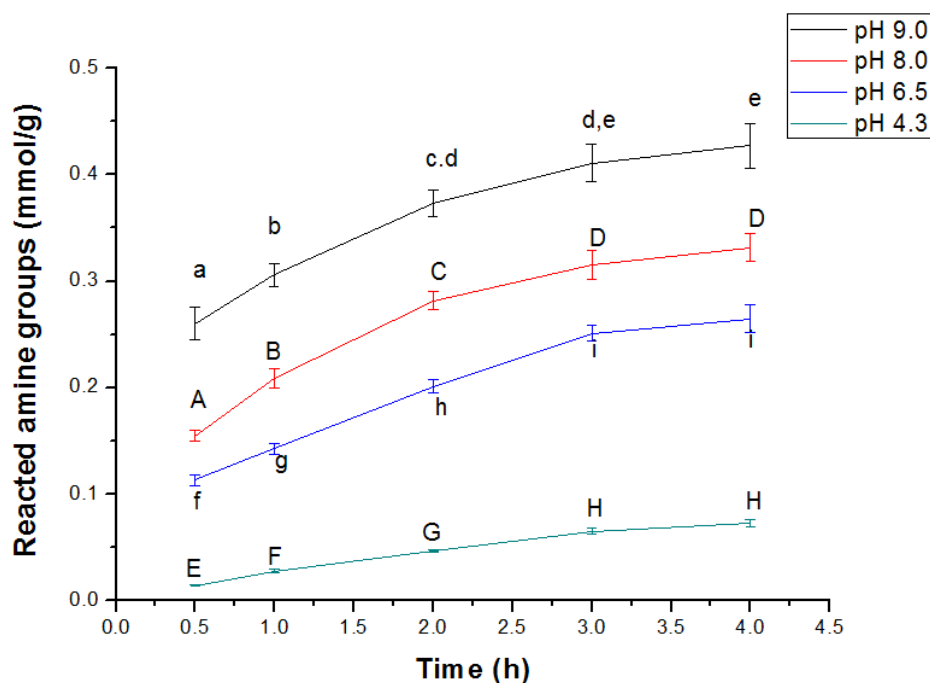
Figure 1. Influence of pH on amine groups' change when reacted with 0.9M citric acid solution

at 50 ℃

Figure 2 shows the influence of pH on the carboxyl groups' change during the reaction.

Each condition had achieved a reaction balance at 4h. Increasing the reaction time increased the

amount of carboxyl groups on crosslinked gliadin. It was less possible for all the three carboxyl

groups of citric acid to take part in the acylation because of the hindrance effect between citric

acid molecules and long chain structure of proteins. When more than one carboxyl group of citric

acid reacted with amine groups on gliadin, connecting the citric acid molecules to polymer

chains, the amount of carboxyl groups on the reacted product would obtain an increase.

Therefore, the amount of carboxyl groups showed an increase trend at different pH conditions.

At the same time point, more carboxyl groups were detected at higher pH than at lower pH,

indicating more citric acid molecules had participated in the reaction when increasing pH from

4.3 to 9.0 and a higher reaction extent could be obtained.
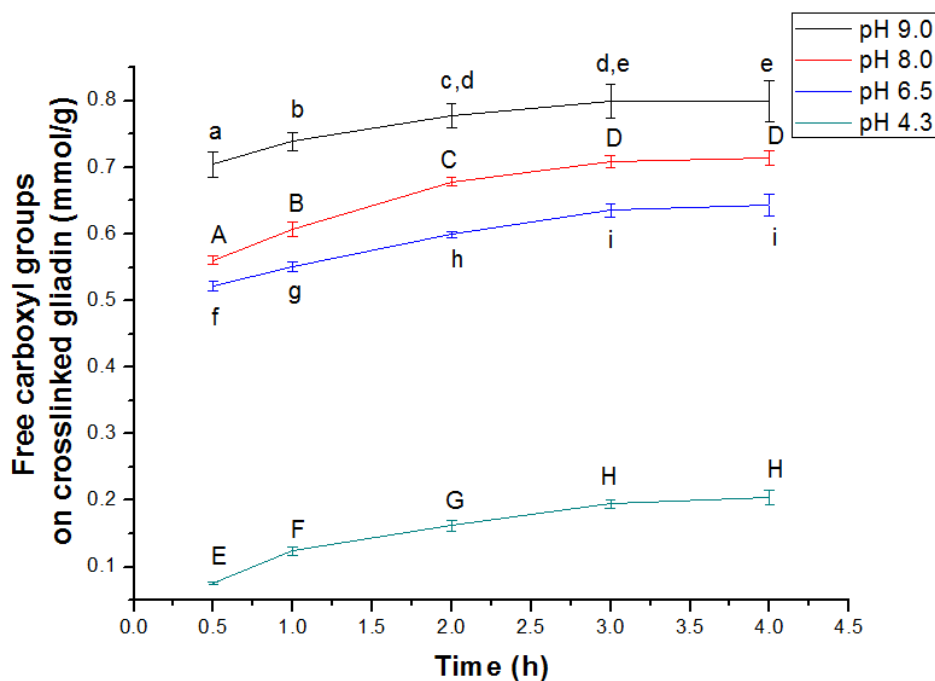
Figure 2. Influence of pH on carboxyl groups' change when reacted with 0.9M citric acid solution at 50 ℃

The portion of the three carboxyl groups of citric acid participated in the reaction was used to represent the crosslinking degree. If this portion equals 1/3, indicating only one carboxyl group in the three was connected to a polymer chain on average; then the majority of the protein chains were not crosslinked and the increase in molecular weight would be limited. The wet crosslinking of gliadin with 0.9M citric acid solution at pH 9.0 finally achieved a portion of 0.348, which means 34.8% carboxyl groups in a single citric acid molecule took part in the reaction. It indicates that the gliadin was crosslinked, but the crosslinking degree was not high. This was possibly because the reaction occurred in an aqueous atmosphere, the distance between each two protein molecules was large and the possibility for the citric acid molecules to connect polymer chains was limited.

3.1.2 The influence of citric acid concentration

Increasing the citric acid concentration was found to be favorable for improving the acylation reaction extent between citric acid and gliadin when reacted using pH 8.0 at 50 ℃ as shown in Figures 3 and 4. Figure 3 shows the influence of citric acid concentration on the amine groups' change during the reaction. Each condition had achieved balance at 4h. Increasing reaction time up to 4 hours decreased the amount of amine groups left on gliadin. Thus, the amount of reacted amine groups showed an increased trend when increasing the reaction time. At the same time point, more amine groups participated in the reaction at higher citric acid concentration than at lower concentration, indicating higher reaction extent could be obtained when increasing the concentration of citric acid.
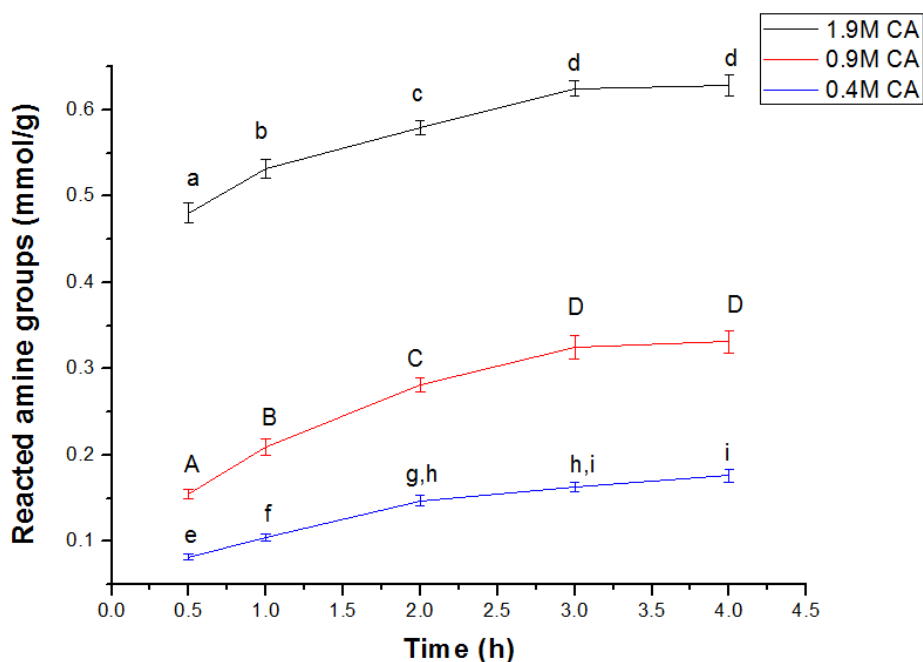
Figure 3. Influence of citric acid concentration on amine groups' change when reacted at pH 8.0, 50 ℃

Figure 4 shows the influence of citric acid concentration on the carboxyl groups' change during the reaction. Each condition had achieved a reaction balance at 4h. Increasing the reaction

time increased the amount of carboxyl groups on acylated gliadin. Therefore, the amount of carboxyl groups showed an increased trend at different citric acid concentrations. At the same time point, more carboxyl groups were detected at higher citric acid concentration than at lower concentration, indicating more citric acid molecules had taken part in the reaction when increasing citric acid concentration from 0.4M to 1.9M and higher reaction extent could be obtained.

The portion of carboxyl groups participated in the reaction achieved 39.3% when reacted using 1.9M citric acid at pH 8.0, 50 ℃. It means 1.2 of the three carboxyl groups in a single citric acid molecule had reacted with amine groups and the gliadin powders were crosslinked. This is the highest crosslinking degree which can be achieved in the wet state reaction. Furthermore, the crosslinking degree increased with the increase of citric acid concentration. This is because the first step of the crosslinking reaction is one side acylation between a citric acid molecule and a gliadin polymer chain. When the concentration of the citric acid molecules attached to the polymer chains was low, it would be difficult for the second carboxyl groups in these citric acid molecules to connect with another polymer chain. When more citric acid molecules had attached to the polymer chains, the possibility for the second carboxyl groups in these citric acid molecules to form crosslinkages with another polymer chain also increased. Therefore, increasing the citric acid concentration is favorable for improving the crosslinking degree.
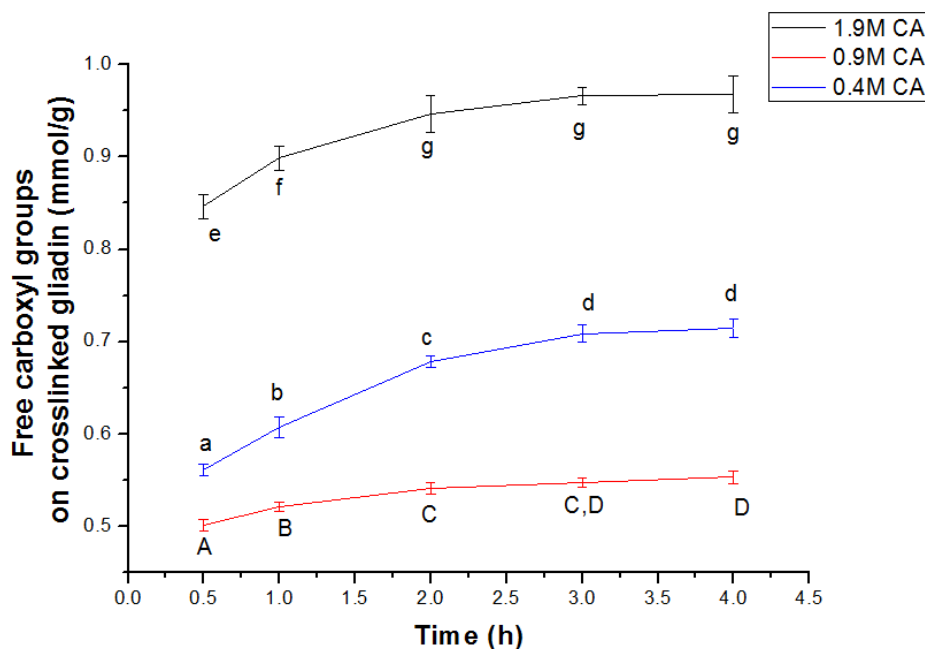
Figure 4. Influence of citric acid concentration on carboxyl groups' change when reacted at pH 8.0, 50 ℃

3.1.3 The influence of temperature

Increasing temperature from 25 up to 70 ℃ increases the energy of the system, resulting in a greater reaction extent as seen from Figures 5 and 6. Figure 5 shows the influence of temperature on the amine groups' change during the reaction. Each condition had achieved balance at 4h. Increasing reaction time up to 4 hours decreased the amount of amine groups left on gliadin, resulting in an increased trend of reacted amine groups. At the same time point, more amine groups was reacted at higher temperature than at lower temperature, indicating higher reaction extent could be obtained when increasing temperature from 25 to 70 ℃. A conclusion could be made that the wet crosslinking of gliadin using citric acid was an endothermic reaction.
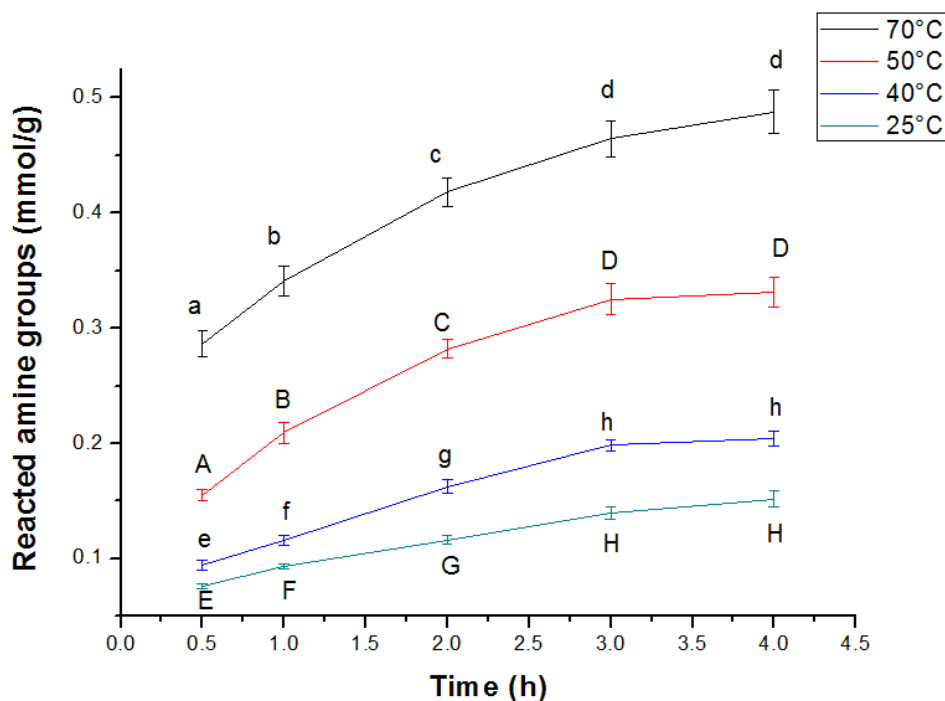
Figure 5. Influence of temperature on amine groups' change when reacted with 0.9M citric acid

solution at pH 8.0

Figure 6 shows the influence of temperature on the carboxyl groups' change during the

reaction. Each condition had achieved a reaction balance at 4h. Increasing the reaction time

increased the amount of carboxyl groups on crosslinked gliadin, resulting in an increased trend

of carboxyl groups when reacted at different temperatures. At the same time point, more

carboxyl groups were detected at higher temperature than at lower temperature, indicating more

citric acid molecules were connected to the polymer chains when increasing temperature from 25

to 70 ℃ and higher reaction extent could be obtained.

The portion of carboxyl groups participated in the reaction achieved 37.6% when

crosslinked using 0.9M citric acid at pH 8.0, 70 ℃. It means 1.1 of the three carboxyl groups in a

single citric acid molecule had reacted with amine groups and the gliadin powders were

crosslinked. This crosslinking degree result was quite close to that obtained using 1.9M citric

acid at pH 8.0 and 50 ℃.
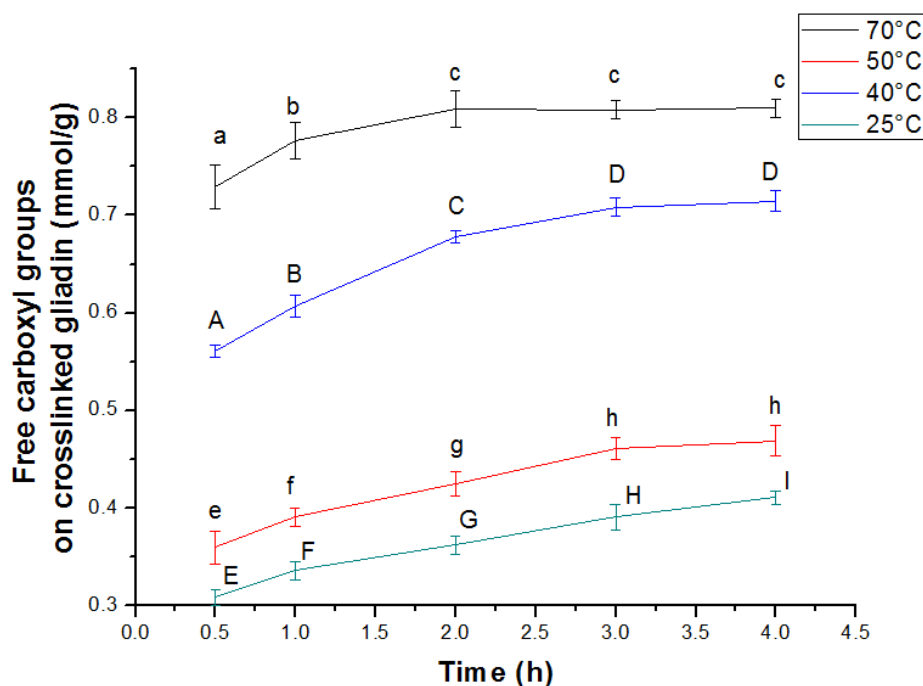
Figure 6. Influence of temperature on carboxyl groups' change when reacted with 0.9M citric

acid solution at pH 8.0

3.1.4 Reaction order and pseudo-reaction rate constant

The plot shown in Figure 7 was obtained according to equations (5) and (7), showing the

effect of reaction rate on amine groups' activity using 0.9 M citric acid solution at pH 8.0 and

50 ℃. As indicated by equation (5), the slope of the plot represented the reaction order; while the

intercept stood for the log function of pseudo-reaction rate constant k_2. Therefore, the result

gives a reaction order of 1.2 in Figure 7 when reacted using 0.9 M citric acid solution at pH 8.0

and 50 ℃. A pseudo-reaction rate constant of 0.22 $mol^{-0.2}$ $L^{0.2}$ s^{-1} was also obtained, showing the

speed of the reaction.

Similarly, the effect of reaction rate on amine groups' activity using 0.9 M citric acid solution at pH 8.0 from 25-70 ℃ can also be plotted, respectively, as shown in Figure 8-10. The average reaction order of 1.2 was obtained. The comparison between the reaction orders obtained from both wet and dry state crosslinking will be discussed later.
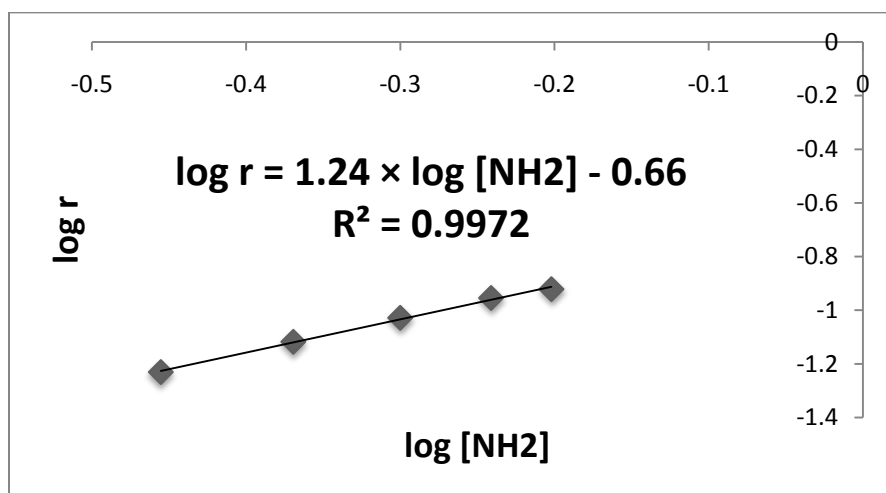

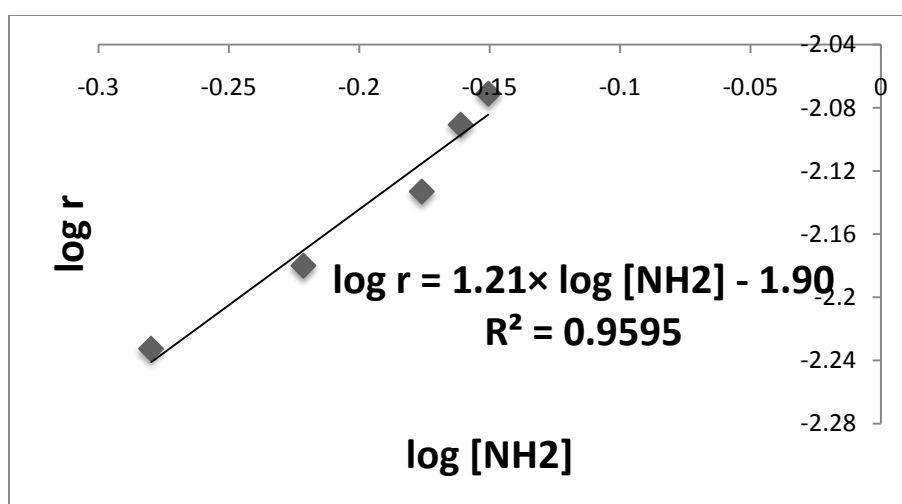Figure 7. Effect of reaction rate on amine group activity using 0.9 M citric acid solution at pH 8.0 and 50 ℃

Figure 8. Effect of reaction rate on amine group activity using 0.9 M citric acid solution at pH 8.0 and 25 ℃
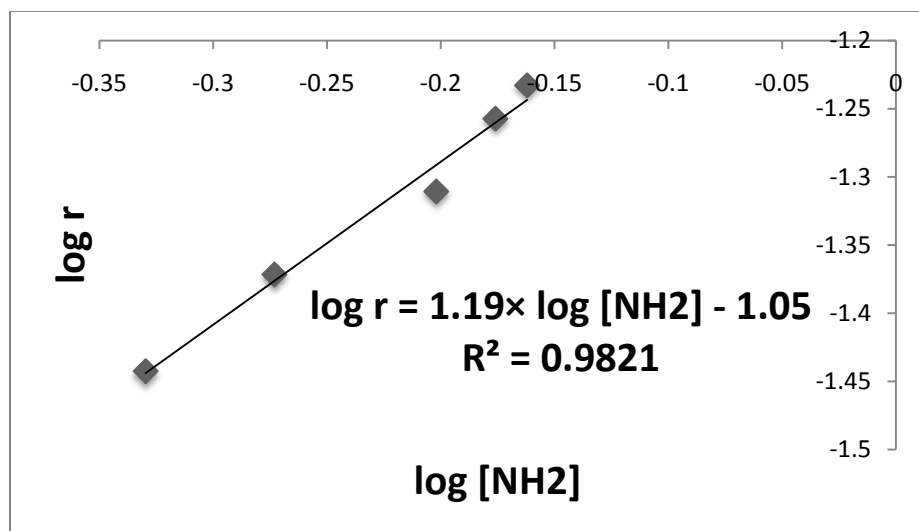
Figure 9. Effect of reaction rate on amine group activity using 0.9 M citric acid solution at pH
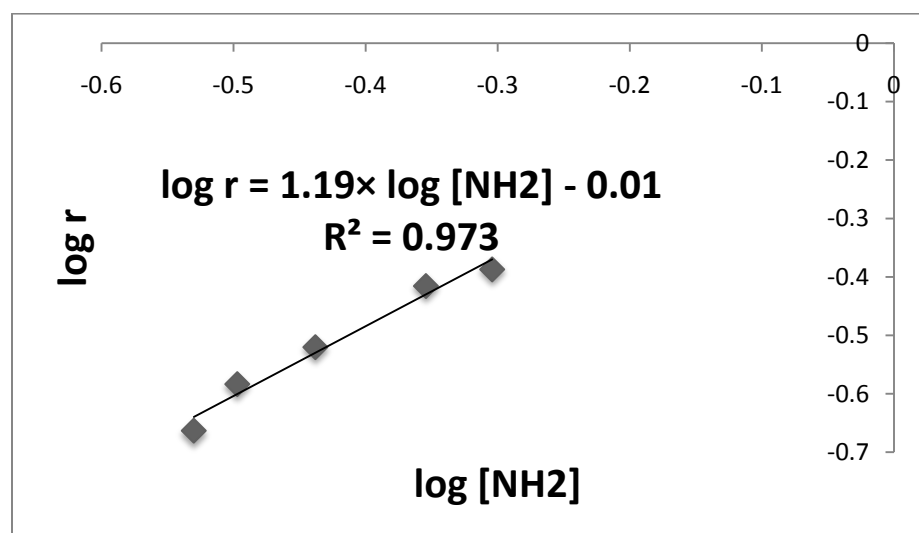
8.0 and 40 ℃

Figure 10. Effect of reaction rate on amine group activity using 0.9 M citric acid solution at pH

8.0 and 70 ℃

3.1.5 Activation energy

According to equation (8), the natural logarithm of k_2 (ln k_2) has an inverse linear relationship with temperature. The slope in Figure 11 represents the value of $-\dfrac{E_a}{R}$ when reacted at pH 8.0, where R is the molar gas constant. Therefore, the average E_a of 81.8 kJ mol^{-1} was obtained, indicating the energy required for the reaction to occur. The comparison between the activation energy obtained from both wet and dry state crosslinking will be discussed later.
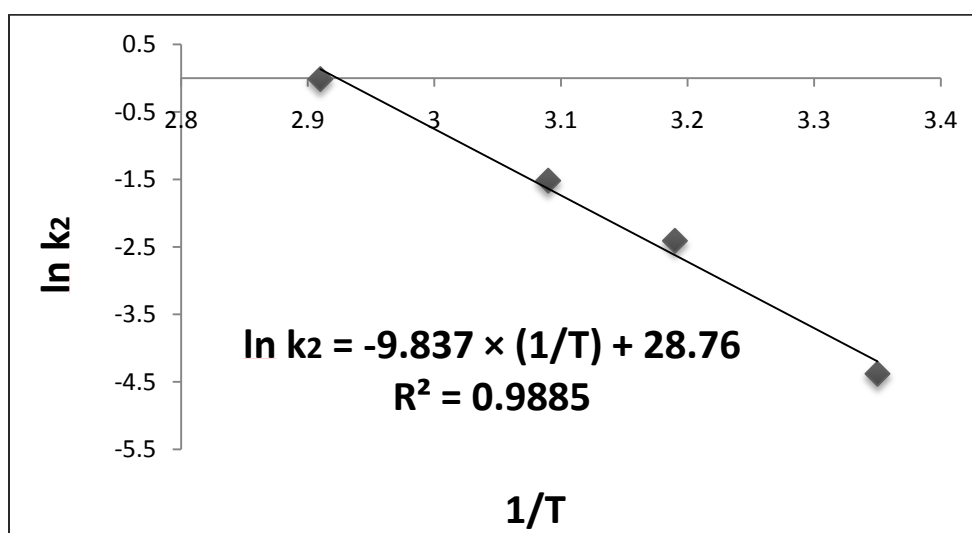
Figure 11. Effect of the inverse of temperature (1/T) on ln k_2 studied using 0.9 M citric acid solution at temperatures from 25 to 70 ℃ at pH 8.0

3.2 Dry state crosslinking

3.2.1 The influence of pH

Increasing the pH is favorable for improving the acylation reaction extent between citric acid and gliadin when reacted at 140 ℃, as shown in Figures 12 and 13. Figure 12 shows the influence of pH on the amine groups' change during the reaction. Increasing reaction time up to 4 hours decreased the amount of amine groups left on gliadin, because the rest had gradually

reacted with carboxyl groups to form amide linkages. Therefore the amount of reacted amine groups, which equals the amount of amide linkages formed, showed an increased trend when increasing the reaction time. At the same time point, more amine groups participated in the reaction at higher pH than at lower pH, indicating higher reaction extent could be obtained when increasing pH from 3.5 to 7.5. This is because if the alkali added was not enough and the reaction was taken place at a lower pH, the amine groups were more likely to take on positive charges under acidic condition. Therefore, it would be difficult for the positively charged amine in the protein to react with partially positively charged carbonyl carbon in carboxylic acid. In the presence of enough alkali, the amine groups are less likely to carry positive charges, and therefore they could attack the carbonyl carbon of the carboxyl groups and more readily form amide linkages.
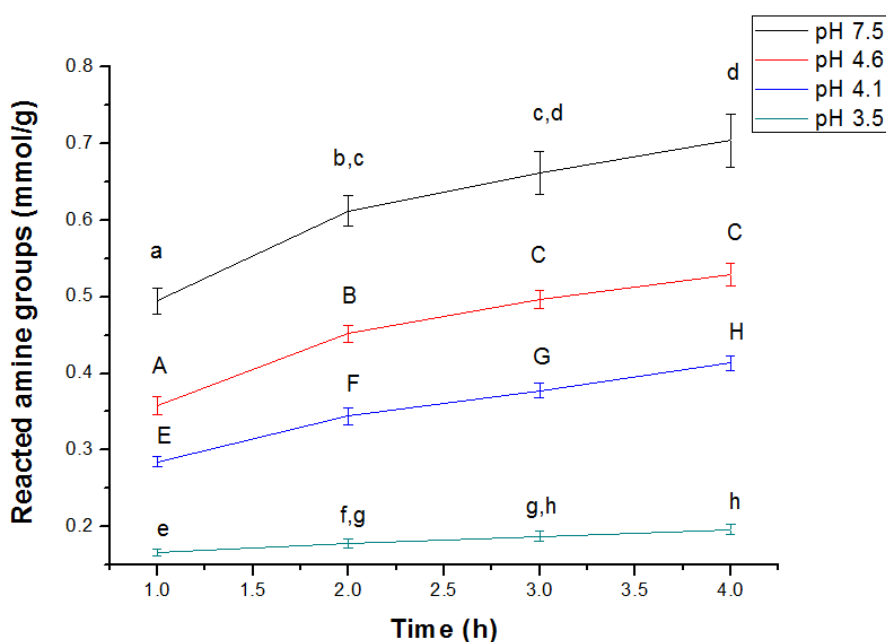

Figure 12. Influence of pH on amine groups' change when reacted with 9% citric acid at 140 ℃

Figure 13 shows the influence of pH on the carboxyl groups' change during the reaction. Increasing the reaction time increased the amount of carboxyl groups on crosslinked gliadin. The

amount of carboxyl groups showed an increased trend at different pH conditions. At the same time point, more carboxyl groups were detected at higher pH than at lower pH, indicating more citric acid molecules had participated in the reaction when increasing pH from 3.5 to 7.5 and higher reaction extent could be obtained.
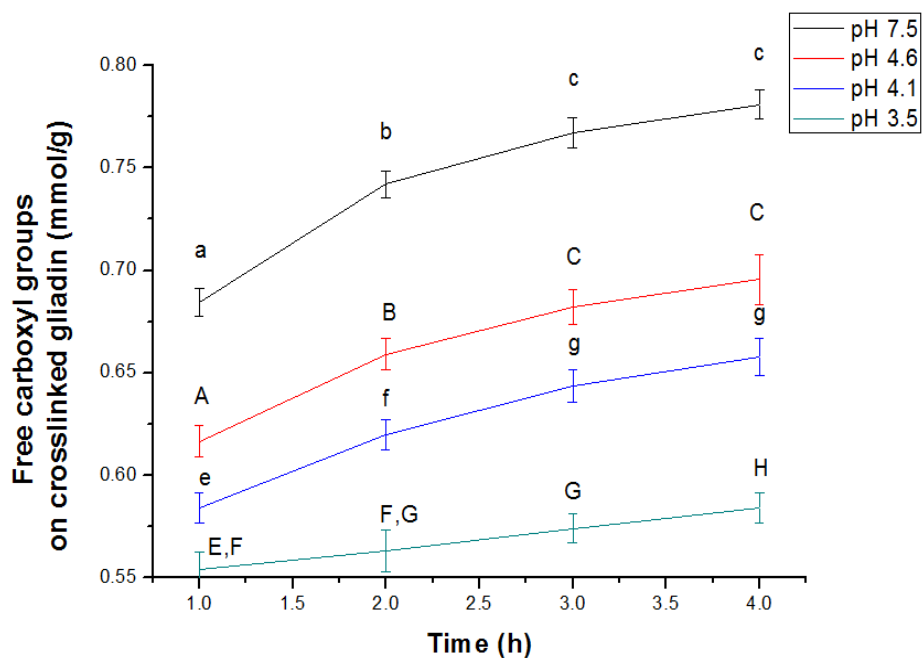

Figure 13. Influence of pH on carboxyl groups' change when reacted with 9% citric acid at

140 ℃

The portion of carboxyl groups participated in the reaction achieved 47.4% when crosslinked using 9% citric acid at pH 7.5, 140 ℃. It means 1.4 of the three carboxyl groups in a single citric acid molecule had reacted with amine groups and the gliadin powders were crosslinked. This is the second highest crosslinking degree that could be achieved in the dry state crosslinking.

3.2.2 The influence of citric acid concentration

Increasing citric acid concentration is also favorable for improving the acylation reaction

extent between citric acid and gliadin when reacted using pH 4.6 at 140 ℃, as shown in Figures

14 and 15. Figure 14 shows the influence of citric acid concentration on the amine groups'

change during the reaction. Each condition had achieved balance at 4h. Increasing reaction time

up to 4 hours decreased the amount of amine groups left on gliadin. Thus, the amount of reacted

amine groups showed an increased trend when increasing the reaction time. At the same time

point, more amine groups participated in the reaction at higher citric acid concentration than at

lower concentration. This indicates a higher reaction extent could be obtained when increasing
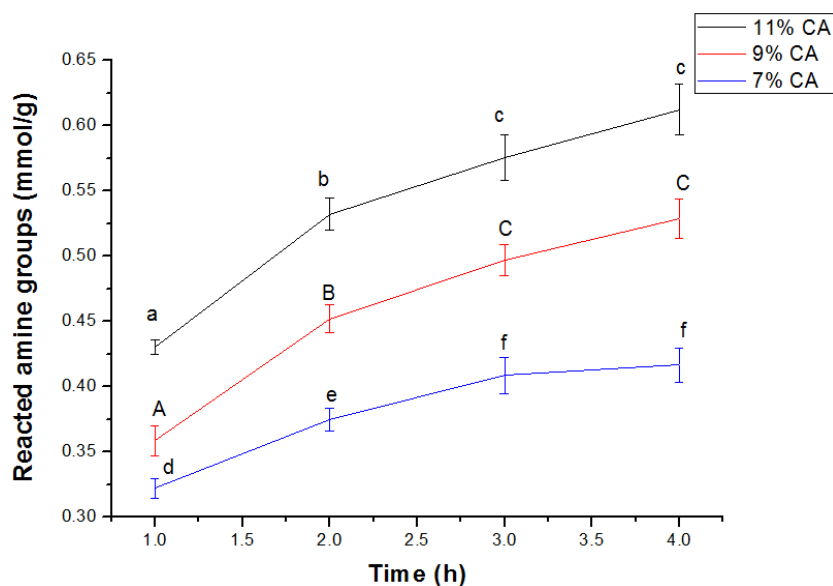
the concentration of citric acid.

Figure 14. Influence of citric acid concentration on amine groups' change when reacted at pH 4.6

and 140 ℃

Figure 15 shows the influence of citric acid concentration on the carboxyl groups' change

during the reaction. Each condition had achieved a reaction balance at 4h. Increasing the reaction

time increased the amount of carboxyl groups on acylated gliadin. Therefore, the amount of

carboxyl groups showed an increase trend at different citric acid concentrations. At the same time point, more carboxyl groups were detected at higher citric acid concentration than at lower concentration, indicating more citric acid molecules had take part in the reaction when increasing citric acid concentration from 7% to 11% and higher reaction extent could be obtained.
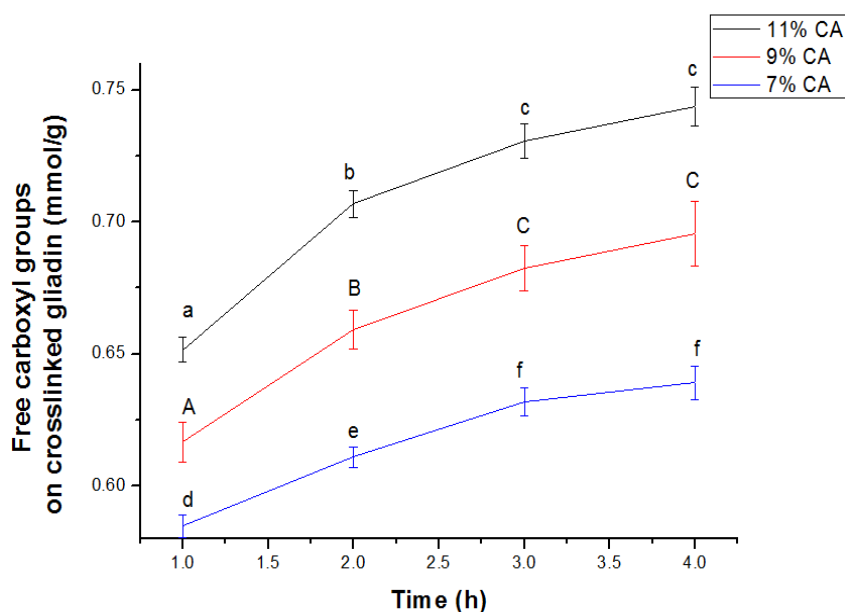

Figure 15. Influence of citric acid concentration on carboxyl groups' change when reacted at pH 4.6 and 140 ℃

The portion of carboxyl groups that participated in the reaction achieved 45.2% when crosslinked using 11% citric acid at pH 4.6, 140 ℃. It means 1.3 of the three carboxyl groups in a single citric acid molecule had reacted with amine groups and the gliadin powders were crosslinked. Furthermore, the crosslinking degree increased with the increase of citric acid concentration. This is because the first step of the crosslinking reaction is one side acylation between a citric acid molecule and a gliadin polymer chain. When the concentration of the citric acid molecules attached to the polymer chains was low, it would be difficult for the second carboxyl groups in these citric acid molecules to connect with another polymer chain. When more citric acid molecules had attached to the polymer chains, the possibility for the second

carboxyl groups in these citric acid molecules to form crosslinkages with another polymer chain also increased. Therefore, increasing the citric acid concentration is favorable for improving the crosslinking degree.

3.2.3 The influence of temperature

Increasing temperature from 80 up to 170 ℃ increased the energy of the system, resulting in a greater extent of crosslinking as seen from Figures 16 and 17. Figure 16 shows the influence of temperature on the amine groups' change during the reaction. Each condition had achieved balance at 4h. Increasing reaction time up to 4 hours decreased the amount of amine groups left on gliadin, resulting in an increased trend of reacted amine groups. At the same time point, more amine groups was reacted at higher temperature than at lower temperature, indicating a higher reaction extent could be obtained when increasing temperature from 80 to 170 ℃. A conclusion could be made that the dry crosslinking of gliadin using citric acid was an endothermic reaction.
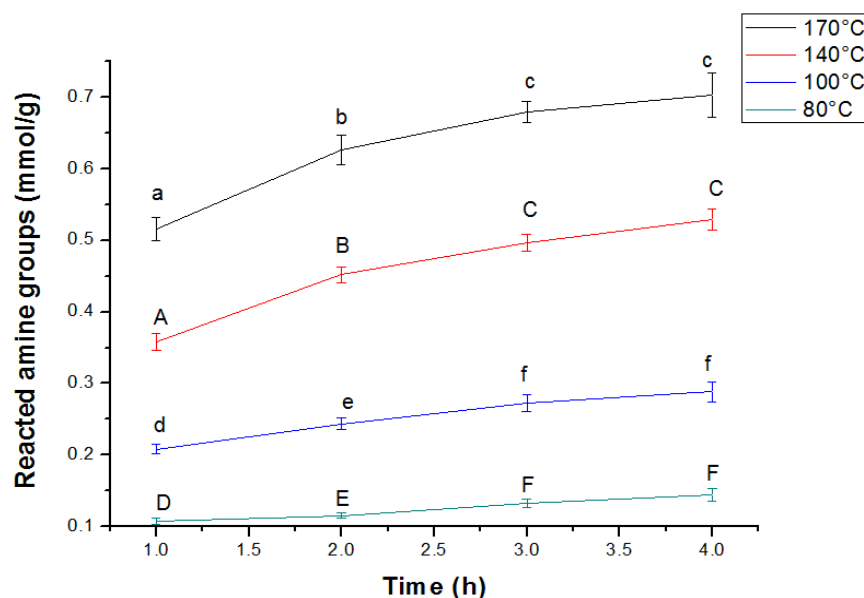

Figure 16. Influence of temperature on amine groups' change when reacted using 9% citric acid at pH 4.6

Figure 17 shows the influence of temperature on the carboxyl groups' change during the reaction. Each condition had achieved a reaction balance at 4h. Increasing the reaction time increased the amount of carboxyl groups on crosslinked gliadin, resulting in an increased trend of carboxyl groups when reacted at different temperatures. At the same time point, more carboxyl groups were detected at higher temperature than at lower temperature, indicating more citric acid molecules were connected to the polymer chains when increasing the temperature from 80 to 170 °C and a higher reaction extent could be obtained.
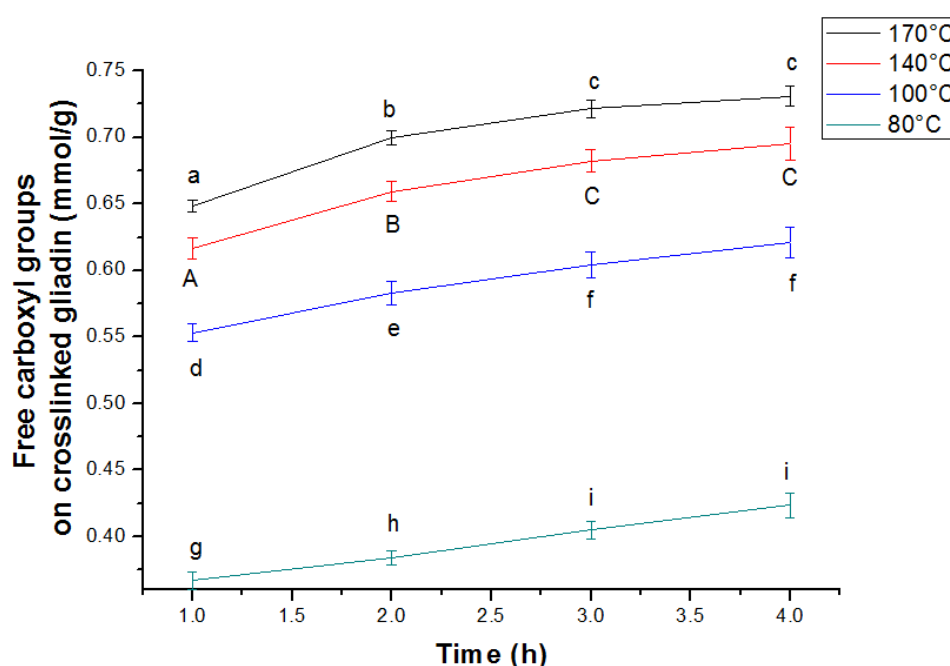

Figure 17. Influence of temperature on carboxyl groups' change when reacted using 9% citric acid at pH 4.6

The portion of carboxyl groups participated in the reaction finally achieved 49.0% when crosslinked using 7% citric acid at pH 4.6 and 170 °C. This means that 1.5 of the three carboxyl groups in a single citric acid molecule had reacted with amine groups and the gliadin powders were crosslinked. This is the highest crosslinking degree which could be achieved in the dry state reaction.

3.2.4 Reaction order and pseudo-reaction rate constant

The plot shown in Figure 18 was obtained according to equations (5) and (7), showing the effect of reaction rate on amine groups' activity using 0.9 M citric acid solution at pH 8.0 and 50 °C. The slope of the plot represents the reaction order; while the intercept stood for the pseudo-reaction rate constant k_2. Therefore, a reaction order of 1.2 was obtained when reacted using 9% citric acid at pH 4.6 and 100 °C. A pseudo-reaction rate constant of 3.55×10^{-3} mol$^{-0.2}$ L$^{0.2}$ s^{-1} was also obtained, showing the speed of the reaction. This value was smaller than that obtained in the wet state reaction, indicating a slower reaction speed. This is possibly because proteins could obtain more sufficient molecular extension in the aqueous atmosphere than in the dry state. Therefore, the wet state reaction was more accessible than the dry state reaction, leading to a faster reaction speed.

The effect of reaction rate on amine groups' activity using 9% citric acid at pH 4.6 from 80-170 °C were also plotted, respectively, as shown in Figure 19-21. The average reaction order found was 1.2, which is the same as that of the wet state reaction. Therefore, both wet and dry state reaction obtained the same reaction mechanism. Because of this, it would be less possible for the carboxyl groups of citric acid to react with the hydroxyl groups on the proteins to form ester bonds in the dry state reaction with the presence of high temperatures.
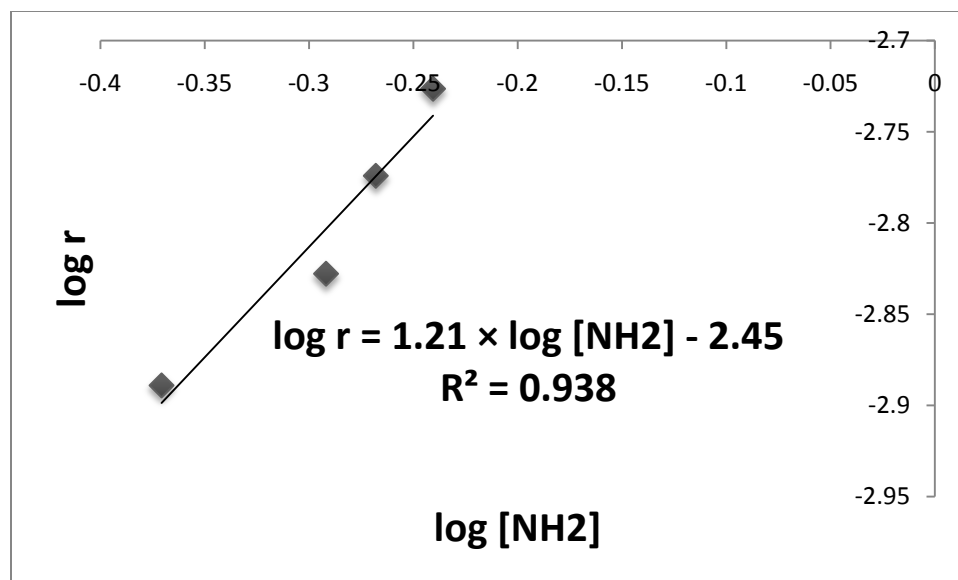
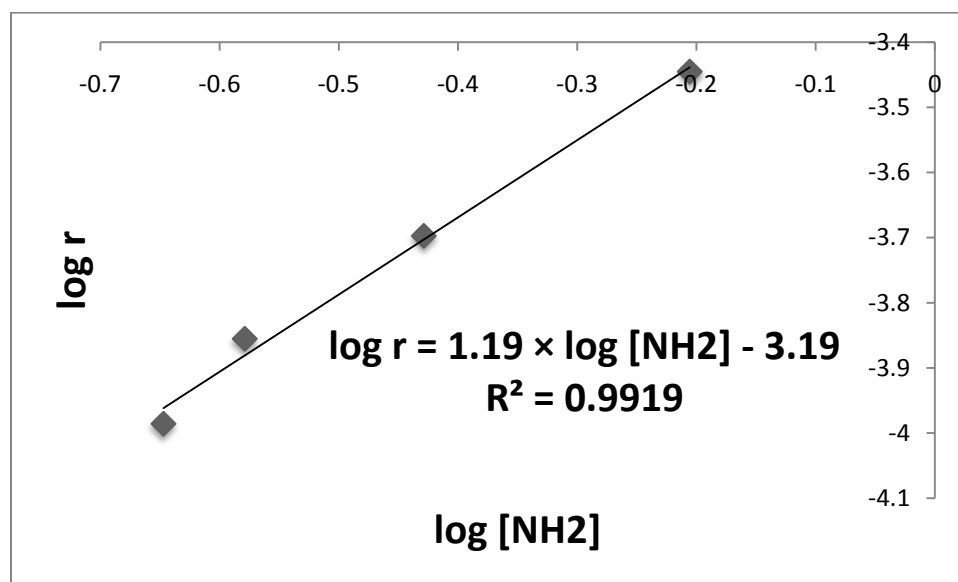Figure 18. Effect of reaction rate on amine group activity using 9% citric acid at pH 4.6 and

100 ℃

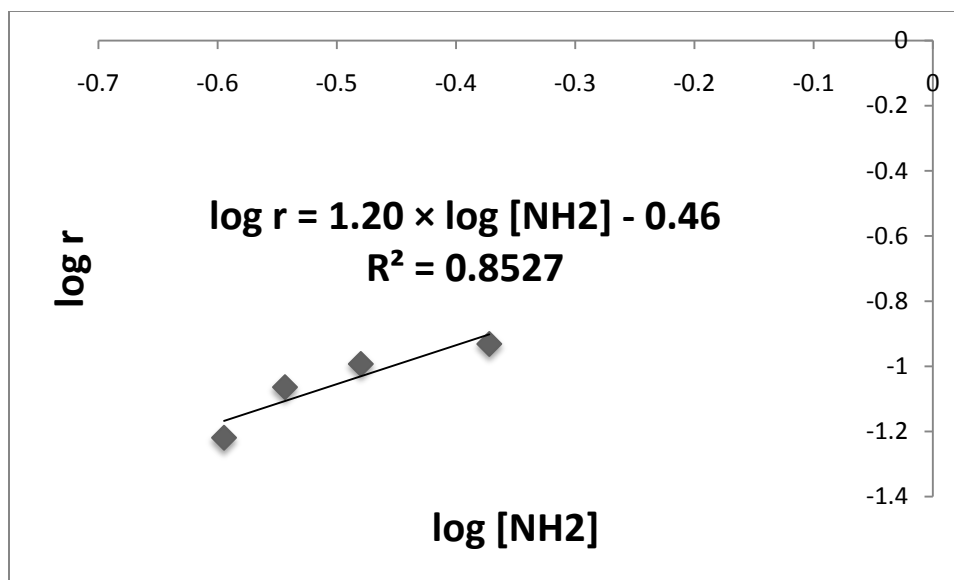Figure 19. Effect of reaction rate on amine group activity using 9% citric acid at pH 4.6 and

80 ℃

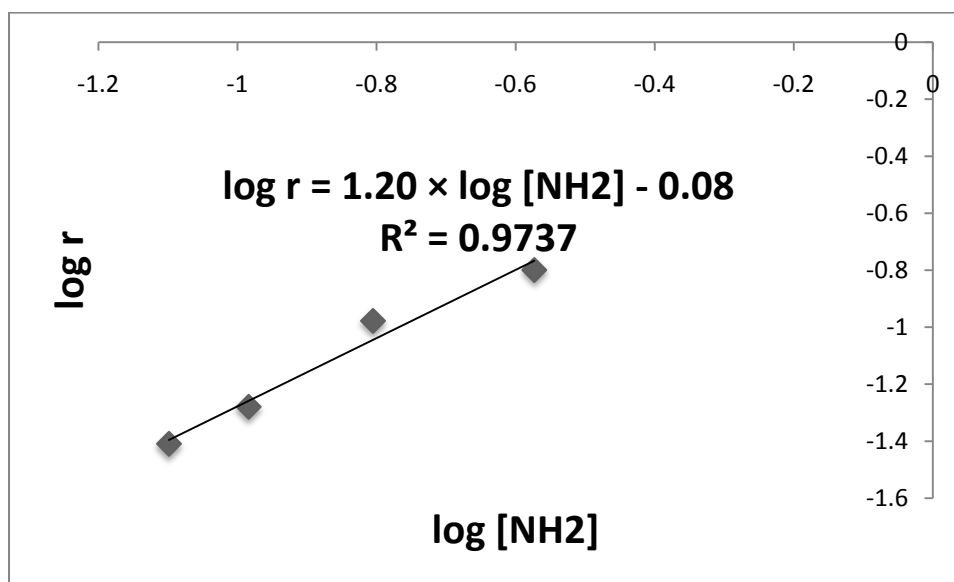Figure 20. Effect of reaction rate on amine group activity using 9% citric acid at pH 4.6 and

140 ℃

Figure 21. Effect of reaction rate on amine group activity using 9% citric acid at pH 4.6 and

170 ℃

3.2.5 Activation energy

As shown in Figure 22, the natural logarithm of k_2 (ln k_2) has an inverse linear relationship with temperature at pH 4.6 ($\ln k_2 = -13.441 \times \dfrac{1}{T} + 30.685$, $R^2 = 0.9737$). Because $-\dfrac{E_a}{R} = -13.441$, where R is the molar gas constant, the average E_a of 111.7 kJ mol^{-1} was obtained. This value was larger than that obtained in the wet state reaction, indicating more energy was required for the dry state reaction to occur. Although less energy was needed for the wet state reaction to take place, the crosslinking degree of the wet state crosslinking is low. To form intermolecular crosslinkages in aqueous conditions was more difficult than in the dry states because the distance between two protein molecules was larger. Therefore, the crosslinking degree of the wet state reaction is relatively lower than that of dry state reaction.
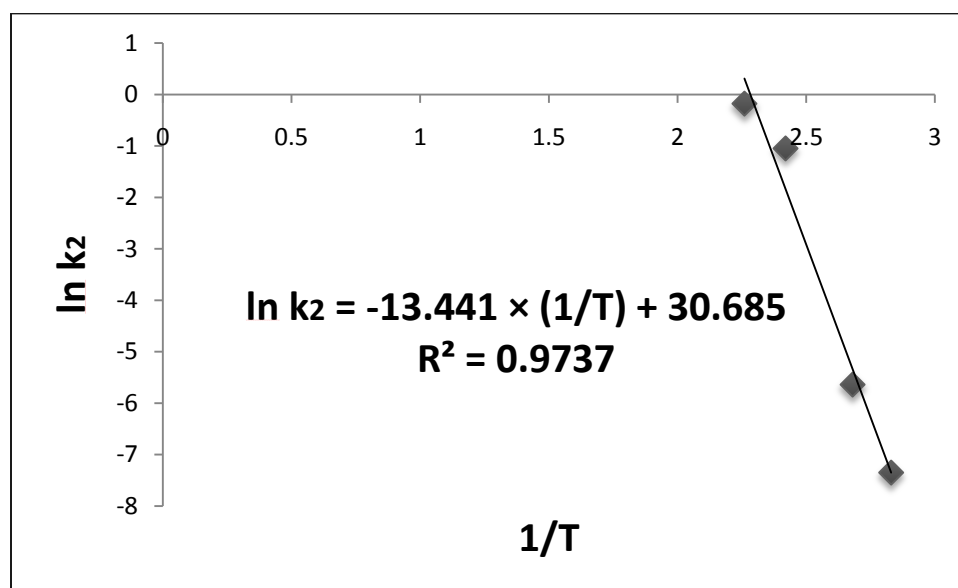

$$\text{ln } k_2 = -13.441 \times (1/T) + 30.685$$
$$R^2 = 0.9737$$

Figure 22. Effect of the inverse of temperature (1/T) on ln k_2 studied using 9% citric acid at temperatures from 80 to 170 ℃ at pH 4.6

3.3 Relationship between tensile properties and crosslinking degree

Figure 23 shows the relationship between the breaking tenacity and the portion of carboxyl groups participated in the reaction. The crosslinking degree is represented by the amount of the carboxyl groups in each citric acid molecule participated in the reaction. Gliadin molecular chains are short without crosslinking. Breakage of the films is mainly caused by the sliding between the polymer molecules. When the amount of the carboxyl groups in each citric acid molecule participated in the reaction equals 1.0, one side reaction between citric acid molecules and gliadin polymer chains would be the major form of the reaction and no crosslinking had taken place. However, the breaking tenacity has already obtained 50% increase at the crosslinking degree of 1.0 when compared to non-crosslinked gliadin samples. The strength increase mainly caused by the ionic strength from salt linkages between free carboxyl groups and hydroxyl/amine groups on the acylated gliadin, forming physical interactions and attractive forces between the polymer chains. When the amount of the carboxyl groups in each citric acid molecule participated in the reaction increased above 1.0, crosslinking had taken place. A linear increase in tensile strength up to 15.6 MPa was obtained when the crosslinking degree was less than 1.2. This is because crosslinking interconnects the gliadin molecules. The molecules can slide from each other but can not be separate completely because of the crosslinkages. Therefore, the force during the tensile test can be shared evenly by neighboring molecules, leading to an increase in tensile strength. When the crosslinking degree further increases, the excess crosslinking limits the mobility of the gliadin molecules and reduces the load that can be shared by neighboring molecules during the tensile testing, leading to a linear decrease in tensile strength when the amount of the carboxyl groups in each citric acid molecule participated in the reaction was larger than 1.2.

Figure 24 shows the relationship between the breaking elongation and the amount of the carboxyl groups in each citric acid molecule participated in the reaction. The elongation first increased to 1.8%. However, excessive cross-linking or over-cross-linking decreases the mobility of the polymer chains and restricts the movement of the molecules, leading to reduced elongation.
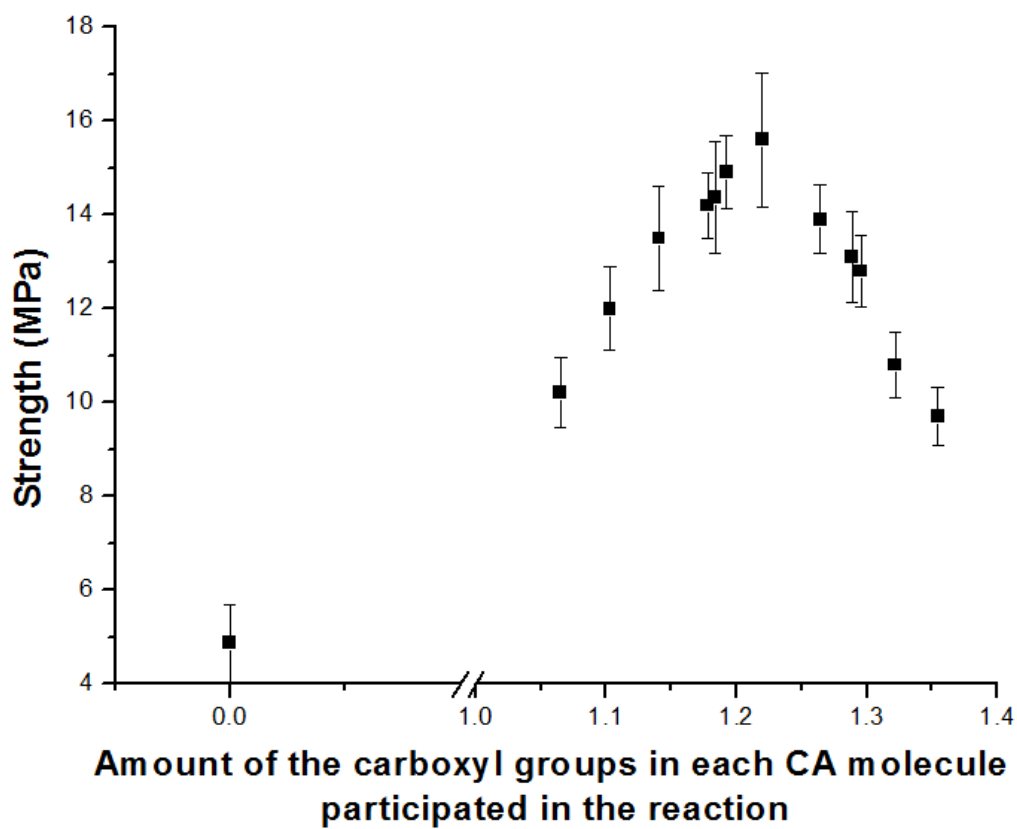

Figure 23. Relationship between the breaking tenacity and the amount of the carboxyl groups in each citric acid molecule participated in the reaction
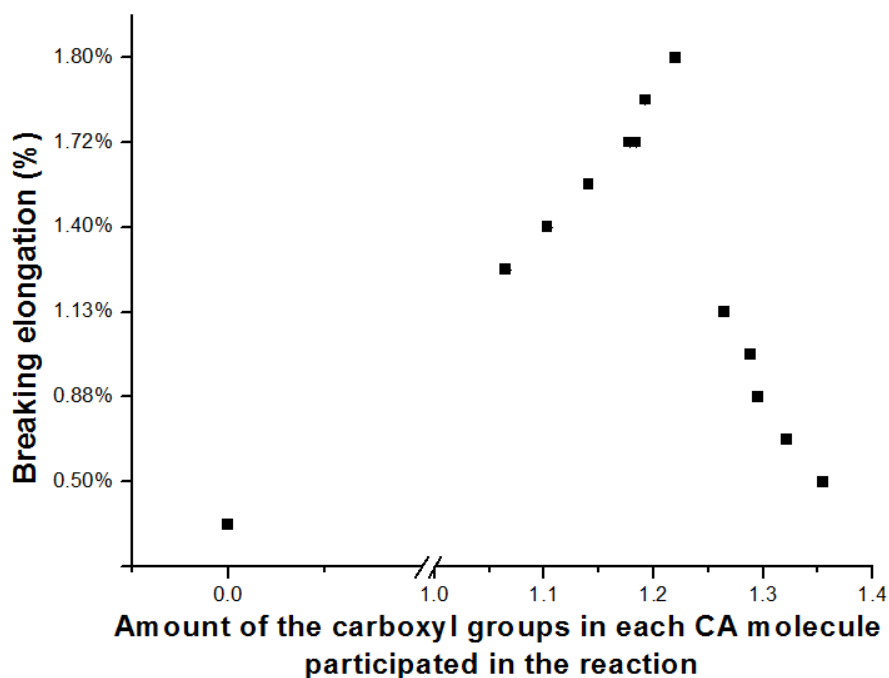
Figure 24. Relationship between the breaking elongation and the amount of the carboxyl groups

in each citric acid molecule participated in the reaction

3.4 SDS Electrophoresis

Figure 25 depicts the changes in the molecular weight of the gliadin proteins before and after

crosslinking for different reaction conditions from SDS electrophoresis. As seen from Lanes 2,

noncrosslinked gliadin powders have most strong bands in the region of 35-50 kDa. Both

crosslinked gliadin samples reacted at two different conditions show higher molecular weight

bands as Lane 3 and Lane 4. As seen from Lane 3, gliadin powders wet crosslinked with 0.9 M

citric acid at 50 ℃ for 4 h have darker bands in the 62–100 kDa regions, which are hardly seen

in the noncrosslinked gliadin samples. Although the same amount of proteins was used for each

lane, gliadin films dry crosslinked with 9% citric acid at 140 ℃ for 4 h (lanes 4) have fewer and

less intense bands in the region of 35-50 kDa when compared to lanes 2 and 3. However, more

and stronger bands have been seen in the 62-188kDa regions from Lane 4, indicating the lower

weight proteins have been cross-linked and become higher molecular weight proteins, and higher

crosslinking degree has been achieved in the dry state reaction than in the wet state.
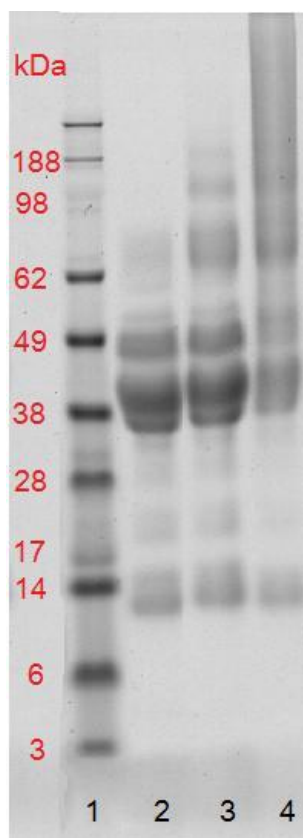

Figure 25. SDS-PAGE of molecular weight standards (lane 1), noncrosslinked gliadin powder

(lane 2), gliadin powders wet crosslinked with 0.9 M citric acid at 50 ℃ for 4 h (lane 3), and

gliadin films dry crosslinked with 9% citric acid at 140 ℃ for 4 h (lane 4)

3.5 Conclusions

In this study, both of the amine and carboxyl groups' change during the acylation process

under different reaction conditions were analyzed by titration, showing that the alkaline

catalyzed acylation reaction between gliadin and citric acid does occurred. Furthermore, SDS-

Page showed an increase in molecular weight after both wet and dry state crosslinking, proving

that citric acid does crosslink with the amine groups of gliadin. According to the increasing

reaction extent when increasing the temperature, a conclusion was made that the crosslinking of gliadin using citric acid was an endothermic reaction.

Although the wet state crosslinking eliminated the need for phosphorous-containing catalysts or high temperatures for carboxylic acid crosslinking of plant proteins, its crosslinking degree was relatively low and the reaction efficiency was not high. This was possibly because the reaction took place in an aqueous atmosphere; to form intermolecular crosslinkages in aqueous conditions was more difficult than in the dry states because the distance between two protein molecules was larger. The highest portion of carboxyl groups participated in the reaction achieved 0.393 when crosslinked with 1.9M citric acid solution at pH 8.0 and 50 ℃. The reaction between citric acid and gliadin proteins was found to be of pseudo-1.2-order, with alkali acting as a catalyst to the reaction. An average E_a of 81.8 kJ mol^{-1} was obtained from various temperatures at pH 8.0.

In order to improve the crosslinking degree of the reaction, the dry state crosslinking with alkaline was studied. The highest portion of carboxyl groups participated in the reaction achieved 0.490 when crosslinked with 9% citric acid at pH 4.6 and 170 ℃. It means 1.5 of the three carboxyl groups in a single citric acid molecule had reacted with the amine groups and the gliadin was crosslinked. The crosslinking degrees obtained as a result was much higher than those obtained during the wet state crosslinking. The reaction between citric acid and gliadin proteins was found to be of pseudo-1.2-order, with alkali acting as a catalyst to the reaction. An average E_a of 111.7 kJ mol^{-1} was obtained using various temperatures at pH 4.6.

The relationship between tensile properties and crosslinking degree was studied. The tensile strength showed a linear increase up to 15.6 MPa when the amount of the carboxyl groups in each citric acid molecule participated in the reaction was less than 1.2 and then decreased

because the excess crosslinking limits the mobility of the gliadin molecules. The elongation first increased to 1.8%, but excessive cross-linking or over-cross-linking decreases the mobility of the polymer chains and restricts the movement of the molecules, leading to reduced elongation.

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