Wheat γ Gliadin as Substrate for Bovine Plasma Factor XIII

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For the first time, a transglutaminase (EC 2.3.2.13) activity was investigated in water/solvent mixtures. This study highlighted that bovine plasma transglutaminase (factor XIII) was active in the presence of dioxane, acetonitrile, and ethanol up to 15%, 20%, and 20%, respectively. It allowed enzymatic modification of wheat prolamins. The transfer and hydrolytic activities of factor XIII were investigated on a purified γ 46 gliadin considered as a prolamin model substrate. In optimum conditions of pH and dioxane concentration, 15% of the glutaminyl residues present in γ gliadin were deamidated after 24 h of enzymatic treatment. The nonrepetitive region of the γ gliadin was preferentially modified by factor XIII. The transglutaminase was able to produce soluble polymeric complexes of gliadins, through intermolecular ϵ-(γ-glutamyl)lysyl cross-links. The two lysyl residues of γ gliadin were supposed to act as acyl acceptor sites.

INTRODUCTION

The increasing production of wheat gluten induced research to investigate new fields of utilization in not only food but also nonfood industries. For these purposes, these proteins should be chemically or enzymatically modified to improve their efficiency as functional agents, especially by increasing their poor solubility.

Chemical deamidation in mild conditions (acid concentration, temperature) was indeed shown to be a powerful tool to solubilize gluten proteins in water. This was explained by the negative charge increase resulting from the carboxylic group formation (Popineau et al., 1988). The authors have shown that, for the higher rate of deamidation (>20%), the acidic treatment generally induced a partial hydrolysis of the proteins but it was difficult to control the level of hydrolysis. From a general point of view, the use of enzymes for protein modification provides advantages including mostly the mild reaction conditions and the specificity of the reaction. In the case of food applications, enzymatic reactions may present advantages compared to the use of chemical reagents, which can lead by secondary reactions to certain toxic contaminants. For deamidation of glutaminyl residues, two types of enzyme have been reported, the peptidoglutaminases (EC 3.5.1.43) (Hamada, 1992) and the transglutaminases (EC 2.3.2.13). The present study focused on the transglutaminases, which alone are able to deamidate protein (Neidle et al., 1958; Mycek et al., 1959; Mycek and Waelsch, 1960), peptidoglutaminases being only active on low molecular mass substrates (<5000 Da).

Transglutaminases are mainly known to catalyze *in vivo* isopeptide bond formation [ϵ-(γ-glutamyl)lysyl bonds] between an acyl donor (glutaminyl residue) and an acyl acceptor (lysyl residue or primary amino groups) (Folk and Finlaysson, 1977; Folk, 1980). Without amino groups in the medium, water becomes the acyl acceptor, and the catalyzed reaction is the deamidation of glutaminyl residues. These enzymes are consequently able to improve nutritional and functional properties of food proteins by catalyzing either the cross-linking of proteins or the binding of amines as well as the deamidation of the glutaminyl residues (Mycek and Waelsch, 1960; Ikura et al., 1980, 1981; Motoki and Nio, 1983; Tanimoto and Kinsella, 1988).

Transglutaminases are widely distributed in various organs, tissues, and body fluids, such as liver, prostate, and epidermal tissue, hair follicles, and blood (Folk, 1980). They are distinguishable from each other to a large extent by their distribution in the body and their physicochemical properties. In blood, transglutaminase is known as coagulation factor and called factor XIII, fibrin-stabilizing factor, or fibrinoligase. Factor XIII is one of the best characterized transglutaminases, and its physiological role is well established. Factor XIII is a tetrameric proenzyme activated *in vivo* by thrombin (Schwartz et al., 1973; Tagaki and Doolittle, 1974; Takahashi et al., 1986; Ichinose and Davie, 1988). *In vitro*, it could also be activated by trypsin (Schwartz et al., 1973).

Bovine plasma factor XIII was chosen for the present study because it seemed to be well adapted for biotechnological uses. It may be available in relatively large amounts and can be stored over a long period as zymogen without significant loss of activity. The aim of this study was to evaluate the interest of this enzyme for modifying gluten proteins, in particular as an alternative tool to the acidic treatment for deamidation. Before technological applications were considered, it was necessary to basically investigate the conditions for an optimized utilization of this enzyme on such a particular substrate. Because of the poor solubility and reactivity of these wheat proteins in water, this study was especially focused on the activity of factor XIII in the presence of various solvents, which are known to increase the solubility of these proteins (Popineau and Pineau, 1985). To distinguish between the respective influence of solvents on the accessibility of wheat protein glutaminyl residues and the denaturation of factor XIII, the activity of the enzyme was also studied, in the various solvent conditions, on maleylated β casein. This protein is soluble in every medium and known to be one of the best substrates for factor XIII (Gorman and Folk, 1980). The γ gliadin was taken as a good acyl donor substrate for transglutaminases according to its respectively high and low contents in glutaminyl (102 glutaminyl residues/mol) and lysyl (2 lysyl residues/mol) residues (Popineau et al., 1990). It is essentially composed like most wheat prolamins of two large domains of about equal

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length: an N-terminal repetitive region, especially rich in proline and glutamine residues and without disulfide bridges, and a C-terminal nonrepetitive region that contained the two lysyl residues and all of the sulfhydryl groups (Shewry et al., 1986; Shewry and Tatham, 1990). The repetitive region is mainly composed of repeat sequences Pro-Gln-Gln-Pro-Phe-Pro-Gln and Gln-Gln (Sugiyama et al., 1986 ; Rafalski, 1986), which are very rich in glutamine residues and characterized by a high content in β turns (Tatham et al., 1990).

MATERIALS AND METHODS

Preparation of Purified γ Gliadin. The γ 46 gliadin was purified from a crude gliadin fraction, extracted from wheat flour (var. Hardi) by water/ethanol (30/70 v/v) according to the Popineau and Pineau (1985) method.

Total Gliadin Solubility in Dioxane. Gliadins were solubilized in water/dioxane mixtures at different solvent concentrations. The protein concentration in the clear supernatant samples were determined using the Kjeldahl procedure.

Preparation of the Polypeptides Corresponding to γ 44 Gliadin Repetitive and Nonrepetitive Regions. These polypeptides were prepared from the limited digestion of γ 44 gliadin by chymotrypsin. γ 44 gliadin was chosen instead of γ 46, first, because of the previous studies carried out on this protein by Popineau et al. (1990), which described the method for preparing the two types of polypeptides, and, second, because the sequences of γ 44 and γ 46 are very similar.

Maleylation of β Casein and γ 46 Gliadin. Amino groups of both proteins were maleylated according to by the Klapper and Klotz (1972) method. The proportion of acylated lysyl residues, determined by the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979), was around 95–98% for β casein as well as for γ 46 gliadin.

Factor XIII Purification. The bovine plasma was kindly supplied by Blood Groups Laboratory of the Institut National de Recherche Agronomique, Jouy-en-Josas, France. Factor XIII was purified from bovine plasma and frozen at −20 °C, according to the slightly modified Lorand and Gotoh (1970) method. Purification factor and yield were, respectively, 1700 and 15%. All of the purification procedure was monitored at 4 °C, and the buffers were added with 1.0 mM benzamidine and 1.0 mM EDTA. The plasma (12 L) was precipitated by ammonium sulfate at 20% saturation and centrifuged at 2400g for 30 min. The resulting pellet was solubilized in 0.15 M KCl (1200 mL) and the pH adjusted to 5.4 using 1.0 N acetic acid. The factor XIII and fibrinogen were again precipitated by ammonium sulfate at 16% saturation and recovered by centrifugation. The precipitate was then resolubilized in 50 mM Tris-HCl buffer, pH 7.5 (600 mL), containing 0.1 M NaCl, and the resulting solution heated at 56 °C for 3 min in the presence of 5 mM DTT to remove the fibrinogen by coagulation. After the suspension was cooled at 5 °C in an ice bath, the solution was centrifuged at 8000g for 30 min. The supernatant was again precipitated with 36% saturation of ammonium sulfate; the precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.5 (buffer A, 120 mL), and dialyzed against the same buffer for 48 h. The dialyzed enzymatic extract was loaded onto a DEAE Fast Flow column (5.5 × 6.5 cm) equilibrated in buffer A. The nonadsorbed proteins were eluted by washing the column with 300 mL of buffer A. The factor XIII was recovered by using a linear gradient 0–0.25 M NaCl in buffer A at a flow rate of 150 mL/h. The active fractions were eluted between 0.05 and 0.12 M NaCl, pooled, and concentrated by ammonium sulfate precipitation at 40% saturation. The resulting pellet was solubilized in 50 mM Tris-HCl buffer, pH 7.5/0.15 M NaCl and the ammonium sulfate eliminated by dialysis of the enzymatic solution against the same buffer. This enzymatic preparation was stored at 5 °C for 4 months without loss of activity. It was verified to be free of proteolytic activity.

Factor XIII Proteolytic Activation. The factor XIII was purified as a zymogen. For analytical purposes, it was activated by thrombin (3.75 NIH units of thrombin for 60 μL of enzyme solution at pH 7.5, 37 °C, for 10 min). If larger amounts were needed, the proteolytic cleavage was carried out by trypsin, immobilized on CNBr-activated Sepharose 4B [0.4 mL of immobilized trypsin (4000 BAEE units)/mL of factor XIII, 12 min at 37 °C, hand shaking]. Activated factor XIII was recovered by filtration of the gel suspension through a 0.22-μm Millipore filter.

Determination of Factor XIII Activity. The enzyme activity was measured by the amount of radiolabeled putrescine incorporated into casein according to the slightly modified Lorand et al. (1972) method.

After thrombin activation, 1 mg of N,N′-dimethylated casein, 36 mM putrescine including 200 nCi of [^{14}C]putrescine, and 7 mM $CaCl_2$ (final concentrations) were added to the enzymatic preparation up to a final volume of 150 μL of 0.25 M Tris-HCl, pH 7.5. The enzymatic reaction was carried out at 37 °C for 2 h. After incubation, the proportion of [^{14}C]putrescine incorporated into casein was counted on the reaction mixture (50 μL) (Larré et al., 1992). One unit of enzymatic activity was defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of putrescine (mg of N,N′-dimethylated casein)$^{-1}$ min^{-1} at 37 °C.

Stability of Factor XIII Activity. The stability of factor XIII was studied by incubating the enzyme in 0.25 M Tris-HCl buffer, pH 7.0/7 mM $CaCl_2$/10mM DTT, at 37 °C, in water and in water/dioxane (90/10, 85/15, and 80/20) (v/v) mixtures. From time to time, up to 24 h, aliquots were sampled and the remaining activity was then monitored by measuring the incorporation of [^{14}C]putrescine into β-maleylated casein (0.4 mg) in 250 mM Tris-HCl buffer, pH 7, containing 7 mM $CaCl_2$ and 10 mM DTT, after 30 min of reaction at 37 °C. The enzyme concentrations were 3.6 (7 units) and 1.56 μM (3 units) in the incubation and in the activity test media, respectively.

Chemical Deamidation of Gliadin. Deamidation was carried out by 0.1 N HCl (70 °C) protein treatment according to the method of Popineau et al. (1988). The degree of deamidation was determined by the measurement of supplementary carboxylic acid groups appearing in the modified protein. A 4-h chemical treatment led to 25% deamidated gliadin.

Enzymatic Modification of the Protein Substrate. Enzymatic reaction was carried out by incubating 0.4 mg of protein substrate (β casein or γ 46 gliadin) and 3 units/mg of substrate of factor XIIIa activated by immobilized trypsin. The reaction was monitored in various media but in the presence of 7 mM $CaCl_2$ and 10 mM DTT. It was stopped by adding 30 mM EDTA (final concentration). Ionic strength and pH conditions are specified in the figure captions.

Two-Substrate Conditions. Proteins were modified in the presence of a low molecular weight amine molecule as a second substrate. Protein reactivity for amine incorporation was determined by the amount of [^{14}C]putrescine incorporated into the protein. Putrescine concentration was the same as in the activity test.

Single-Substrate Conditions. Without amine in the medium, the whole transfer activity of factor XIII (deamidation plus cross-linking) was followed by the ammonia concentration, determined using glutamate dehydrogenase (EC 1.4.1.2.) according to the method of Kun and Kearney (1974). The total amount of ammonia released was corrected from the enzyme autodeamidation (factor XIII incubated without substrate) and from chemical deamidation of gliadin, occurring specially in extreme pH conditions (all reagents incubated without factor XIII). These blank values were generally very low compared to the experimental ones.

Degree of Deamidation. To distinguish glutaminyl residues involved in amide groups hydrolysis and in cross-linking, the increase of free carboxylic acid groups in proteins, which resulted from the glutaminyl residue deamidation, was measured, the asparagyl residues not being substrates of factor XIII. The carboxylic acid groups in proteins were determined according to the method of Hoare and Koshland (1967). This technique involved the activation of the carboxyl group by a water-soluble carbodiimide and the subsequent reaction of the activated carboxyl group with a nucleophile such as glycinamide. The chemically modified proteins were dialyzed against water and freeze-dried. Further, they were vacuum hydrolyzed in 6 N HCl for 24 h at 110 °C for amino acid analysis. After derivatization by PITC, amino acid compositions were determined by RP-HPLC

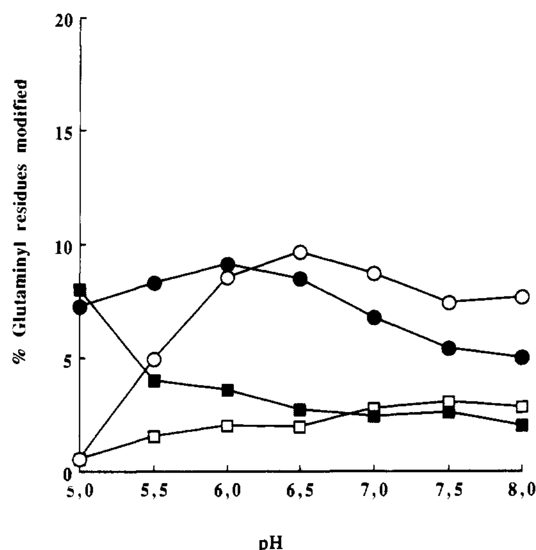

Figure 1. Influence of pH on glutaminyl residue reactivity of native and maleylated γ 46 gliadin. Experimental conditions: 100 mM Tris–acetate, pH 5–5.5, or 100 mM Tris-HCl, pH 6–8.5, containing 7 mM $CaCl_2$, 10 mM DTT, 2.5 mg/mL protein substrate, 36 mM putrescine including 200 nCi of [^{14}C]putrescine (two-substrate conditions), and 3 units/mg factor XIII a at 37 °C. The glutaminyl residues reactivity was monitored in single-substrate (solid symbols) and two-substrate (open symbols) conditions by, respectively, measurement of total ammonia released and putrescine incorporation after 2 h of incubation at 37 °C. (□) Native γ 46 gliadin; (○) 100% maleylated γ 46 gliadin.

on a C_{18} column (Picotag-Waters), according to the Bidlingmeyer et al. (1987) method. The free carboxylic acid groups of the protein were determined as the difference between the numbers of glycine residues detected in the enzymatically treated protein and in the native one.

Gel Filtration. Gel filtration was performed on a Sephacryl S400 column ($^1/_{30}$ cm) equilibrated with 12.5 mM borate buffer, pH 8.5, and 0.1% SDS. Samples were eluted with the same solvent at 0.2 mL/min. Proteins were detected at 280 nm.

RESULTS AND DISCUSSION

γ 46 Gliadin: Substrate of Factor XIII. The reactivity of glutaminyl residues of native γ 46 gliadin was measured with and without amine in the medium, at different pH values, in 0.1 M Tris-HCl buffer. Only 2.7% of glutaminyl residues were modified at pH 7, the optimal pH for factor XIII activity, in single- or two-substrate conditions. The poor reactivity of the γ gliadin could be explained by the poor solubility of this protein in this medium. Without amine in the medium and despite the lower factor XIII activity below pH 6.5, the number of reactive residues was increased in acidic conditions, which greatly enhanced prolamin solubility (Figure 1). It was 3.5 times higher at pH 5 than at pH 7. In the presence of putrescine in the medium, the better accessibility of glutaminyl residues at pH 5 did not balance the poor reactivity of this amine in the acidic pH range. This was due to the low proportion of the un-ionized form of this amine in these pH conditions, since only the unprotonated amine is known to be a substrate for transglutaminases (Mycek and Waelsch, 1960).

The fact that wheat protein solubility was a limiting parameter in these reactions was confirmed by the higher reactivity of maleylated gliadin. Maleylation enhanced protein solubility in aqueous buffer. In the case of 100% acylated gliadin, around 9% of glutaminyl residues were reactive with or without amines in the medium at pH 6–6.5 (Figure 1). Considering the satisfying solubility of maleylated gliadin, these values could correspond to an

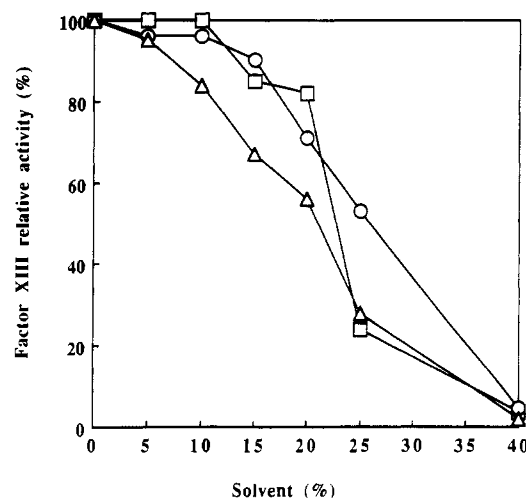

Figure 2. Influence of organic solvents on factor XIIIa activity. The activity was followed as the extent of glutaminyl residues modified for maleylated β casein. One hundred percent activity corresponded to 26.6% of glutaminyl residues modified in the absence of solvent. Glutaminyl residue reactivity was determined by putrescine incorporation after 2 h of incubation at 37 °C. Experimental conditions: 250 mM Tris-HCl, pH 7, containing 7 mM $CaCl_2$, 10 mM DTT, 2.5 mg/mL protein substrate, 36 mM putrescine including 200 nCi of [^{14}C]putrescine, and 3 units/mg factor XIIIa. (□) Water/acetonitrile; (△) water/dioxane; (○) water/ethanol mixtures.

optimal reactivity for gliadin. According to these preliminary results, the reaction conditions were investigated to increase the reactivity of the native protein, by performing the enzymatic reaction in water/solvent mixtures, which are better solvents for gliadin (Popineau and Godon, 1978).

Modification of β Casein and γ Gliadin in the Presence of Solvents. Factor XIII activity was investigated on gliadin in the presence of acetonitrile, ethanol, and dioxane up to 40%. Because β-maleylated casein is a good substrate for the enzyme and was soluble in all media tested, its rate of modification by the enzyme, in the various conditions tested, could be considered as representative of the degree of enzyme denaturation. The enzymatic reaction was performed on native gliadin and maleylated β casein, in two-substrate conditions, for a 2-h incubation at 37 °C. The reactivities of the two proteins were then compared.

Bovine plasma factor XIII still showed 70–80% of its efficiency in binding putrescine to β casein in water/solvent mixtures ranging from 0% to 20% of acetonitrile or ethanol and from 0% to 15% of dioxane (Figure 2). However, above a solvent concentration of 20%, a dramatic decrease in factor XIII activity was observed. Complete enzyme inactivation was reached above 40% in every case. This explained the low reactivity of gliadins in these conditions, despite their higher solubility compared to lower solvent concentrations. For gliadin (Figure 3A), the putrescine binding was maximal in water/ethanol (80/20 v/v), water/acetonitrile (85/15 v/v), and water/dioxane (90/10 v/v): 10%, 12%, and 12% of glutaminyl residues were reactive, respectively. These maxima correspond to an optimum compromise between the increasing substrate solubility (Figure 3B) and the decreasing enzyme activity (Figure 2).

These results showed that factor XIII can be used in water/solvent mixtures for improving the modification of gliadins. Despite the interest of ethanol for applications in the food industry, dioxane was kept in this basic study because it is very convenient for laboratory uses: proteins

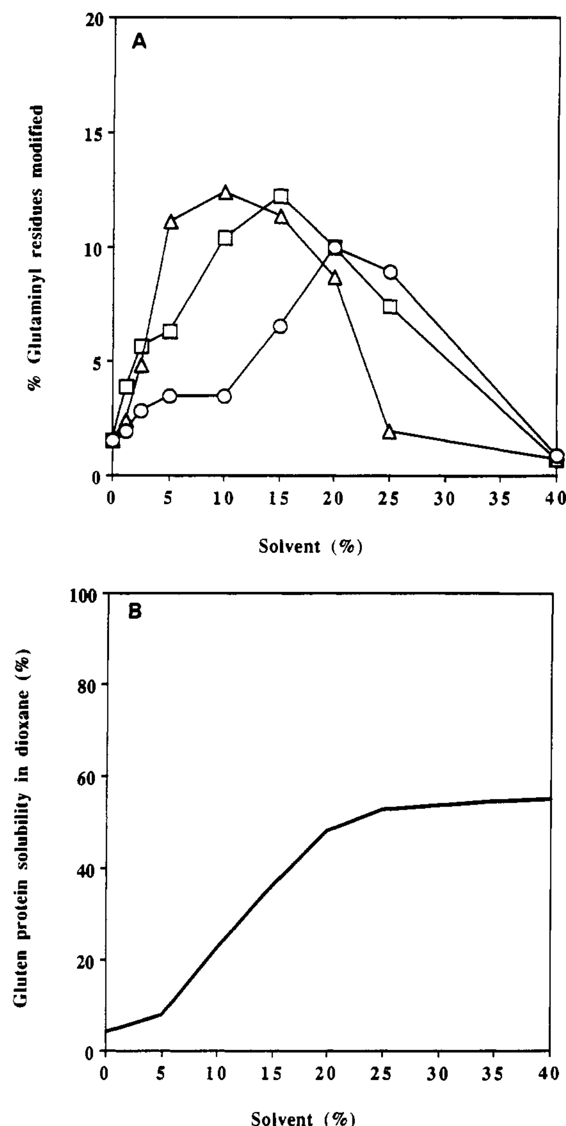

Figure 3. Modification of native γ 46 gliadin in the presence of organic solvents. (A) Extent of glutaminyl residues modified by factor XIIIa. Glutaminyl residue reactivity was determined by putrescine incorporation after 2 h of incubation at 37 °C. Experimental conditions: 250 mM Tris-HCl, pH 7, containing 7 mM $CaCl_2$, 10 mM DTT, 2.5 mg/mL protein substrate, 36 mM putrescine including 200 nCi of [^{14}C]putrescine, and 3 units/mg factor XIIIa. (□) Water/acetonitrile; (△) water/dioxane; (○) water/ethanol mixtures. (B) Gluten protein solubility in dioxane/water mixtures (—).

are very soluble in dioxane, and the solution can be freeze-dried without dialysis.

Factor XIII a Stability in the Presence of Dioxane. The stability of factor XIII was examined after the enzyme was incubated over an extended period of time in water/dioxane mixtures at pH 7 and 37 °C (Figure 4). In water, the activity of the enzyme was very stable for 8 h but then dramatically decreased. The stability of factor XIII activity was increased in presence of 10–15% dioxane but decreased for higher concentrations of solvent. According to Dordick (1989) and Stevenson and Storer (1991), a decrease in the dielectric constant of the medium, in the presence of solvents, by strengthening the hydrogen and electrostatic bonds in the proteins, contributed to the improved stability of the enzyme. On the other hand, higher concentrations of organic solvents, miscible in water, could reduce the enzyme activity by disrupting hydrophobic interactions and also by stripping away the layers of water molecules necessary for preserving the enzyme

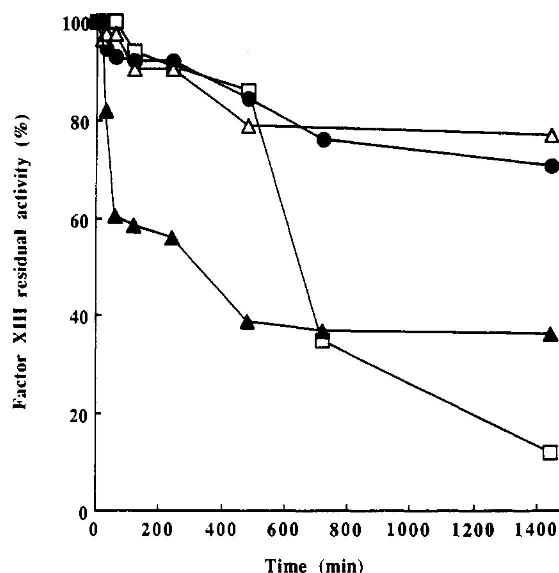

Figure 4. Stability of factor XIIIa activity in the presence of dioxane. Residual activity is expressed in percent of initial activity. Incubation medium: 250 mM Tris-HCl, pH 7, containing 7 mM $CaCl_2$, 10 mM DTT, 37 °C; [factor XIIIa] = 3.9 μM. Test medium: An aliquot (60 μL) of incubation medium was mixed with 90 μL of 250 mM Tris-HCl, pH 7, 7 mM $CaCl_2$, 10 mM DTT, 36 mM putrescine including 200 nCi of [^{14}C]-putrescine, and 2.5 mg/mL protein substrate and incubated for 30 min at 37 °C. The concentration of the enzyme in the test medium ([factor XIIIa]) was 1.56 μM; factor XIII/substrate = 3 units/mg. (□) Water. Water/dioxane: (●) 90/10 v/v; (△) 85/15 v/v; (▲) 80/20 v/v).

stability (Dordick, 1989). This could explain the decrease in factor XIII activity in the presence of dioxane concentrations above 15%, which is a rather highly polar solvent.

Optimization of Gliadin Modification in Presence of Dioxane. The effect of pH in water/dioxane mixtures was investigated to optimize γ gliadin modification (Figure 5). It was studied at medium salt concentration (0.1 M) to limit gliadin aggregation and for 2 h of incubation at 37 °C.

In the presence of putrescine in the media (Figure 5B), the modification rates were maximal at acidic pH (pH 5.5) for low dioxane concentrations (5–10%) and at pH 6.5–7 for higher solvent concentrations (15–20%). In the optimal conditions (10% dioxane, pH 6) 16% of glutaminyl residues were reactive. For the higher solvent contents, pH had little effect on gliadin solubility but influenced the putrescine reactivity for the enzyme (Mycek and Waelsch, 1960). This explained the shift of the optimal pH of amine binding to gliadin toward the optimal pH for factor XIII activity (pH 6.5–7) (data not shown) rather than toward pH corresponding to the maximum gliadin solubility (acidic pH).

In single-substrate conditions (Figure 5A), amide group hydrolysis was favored at acidic pH and inhibited at basic pH. The optimum was obtained at pH 5 for 2.5% dioxane, and the reactivity of glutaminyl residues reached 12%. Acidic pH increased gliadin reactivity because of higher solubility in all solvent conditions. Furthermore, ammonia, known to be an acyl acceptor only in un-ionized form (Mycek and Waelsch, 1960), could inhibit hydrolysis reaction in basic pH range. It may explain the lower reactivity of glutaminyl residues at pH 6–7.

Moreover, when we considered the glutaminyl residue reactivity of native gliadin for two- and single-substrate conditions, it was higher in the presence of putrescine as second substrate. In the case of maleylated gliadin, no

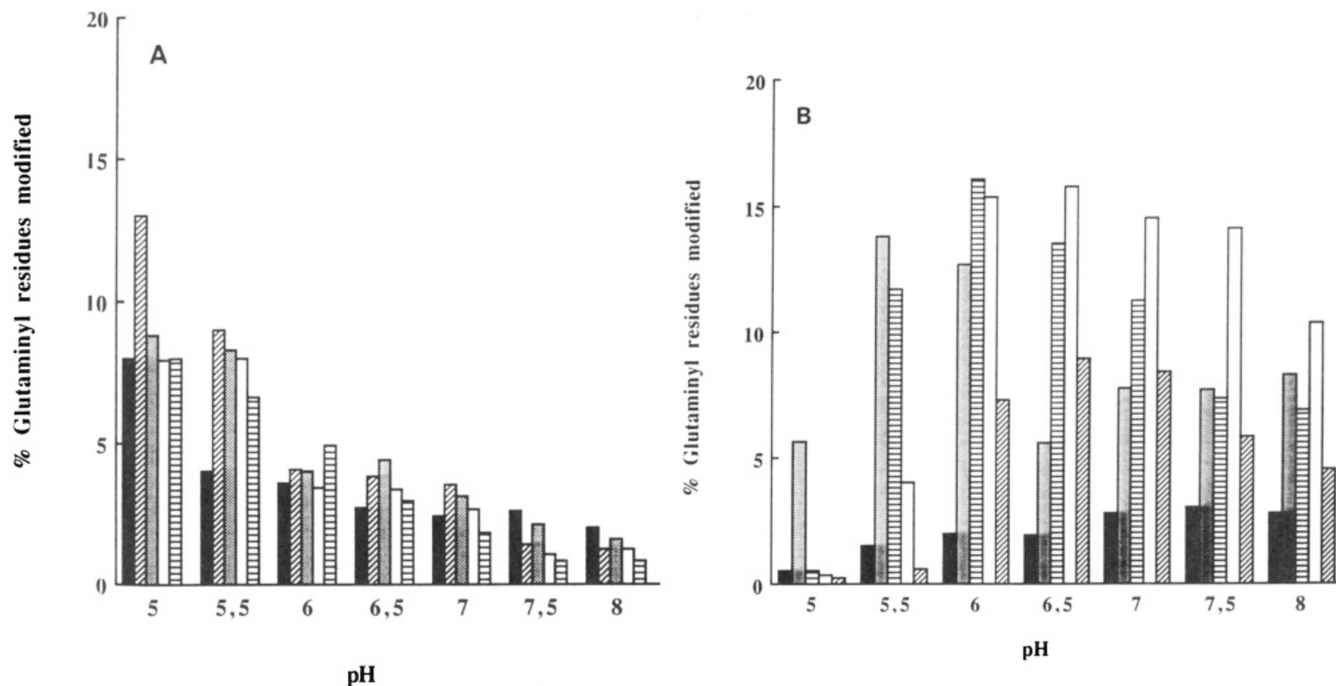

Figure 5. Influence of pH on modification of native γ 46 gliadin in water/dioxane mixtures. Glutaminyl residues reactivity measured in single-substrate (A) and two-substrate conditions (B) conditions after 2 h of incubation at 37 °C. Experimental conditions: 100 mM Tris-acetate, pH 5-5.5, 100 mM Tris-HCl, pH 6-8, containing 2.5 mg/mL gliadin, 3 units/mg factor XIIIa, 7 mM $CaCl_2$, 10 mM DTT (A); adding with 36 mM putrescine including 200 nCi of [^{14}C]putrescine (B). Dioxane concentrations (left to right for each set of bars): (A) 0%, 2.5%, 5%, 7.5%, 10%; (B) 0%, 5%, 10%, 15%, 20%.

Table I. Modification of Native γ 46 Gliadin by Factor XIII[a]

incubation, min	extent of deamidation,[b] %	total modified glutaminyl residues[c]
5	2	2
10	7	7
30	9	9
1440	15	18

[a] All assays were made in duplicate. [b] Determination of supplementary carboxylic acids groups according to the Hoare and Koshland (1967) method. [c] Determined by the amount of ammonia released according to the Kun and Kearney (1974) method. Experimental conditions: 100 mM Tris-HCl, pH 6, 2.5% dioxane, 7 mM $CaCl_2$, 10 mM DTT, 2.5 mg/mL gliadin, 3 units/mg factor XIIIa at 37 °C.

difference was observed. It suggests that the glutamyl-lysyl bond formation, inhibited by maleylation of the lysyl residues, is favored in the absence of putrescine and should decrease the accessibility of glutaminyl residues for the enzyme by leading to gliadin cross-linking. On the other hand, in the presence of an excess of putrescine, lysyl residue reactivity was probably lower.

Characterization of the Enzymatically Treated Gliadin. In the optimal conditions (2.5% dioxane, pH 5), the carboxylic groups formed by factor XIII treatment, evaluated by the Hoare and Koshland (1967) method, were 9% and 15% after reaction times of 30 min and 24 h, respectively. The numbers of reactive glutaminyl residues, measured by ammonia release, under the same conditions were 9% and 18%, respectively (Table I). The perfect agreement between the two measures obtained for the sample treated for 30 min proves that all of the reactive glutaminyl residues were deamidated. On the other hand, the discrepancy observed for the 24-h-treated sample suggested that above 3% the reactive glutaminyl residues were not transformed into glutamic acid but more probably involved in glutamyl-lysyl bonds. To verify this hypothesis, the modified gliadin samples were analyzed by gel filtration on Sephacryl S400 (Figure 6). The chromatography pattern showed an increase in the apparent molecular weight of the enzymatically treated proteins similar to that obtained on 25% chemically deamidated gliadin. Deamidation, by increasing the negative charges of the protein, led to a partial unfolding of the protein due to electrostatic repulsions and induced an increase of the hydrodynamic volume of the protein. In the case of 15% enzymatically deamidated gliadin, the chromatography pattern showed a large excluded peak which accounted for 33% of the chromatogram area and suggested formation of polymers of high molecular weight.

These results confirmed, moreover, the high reactivity of the two lysyl residues, located in the C-terminal nonrepetitive region of the protein, in the formation of glutamyl-lysyl cross-linking. These results were in agreement with those of Porta et al. (1990), who showed that the only lysyl residue of an A-gliadin was reactive for protein reticulation catalyzed by guinea pig liver transglutaminase.

To locate the region of the enzymatic deamidation sites, we studied the reactivity of the glutaminyl residues in N-terminal repetitive and C-terminal nonrepetitive regions of γ gliadin by treating the corresponding purified polypeptides. Table II shows that, after 2 h of reaction without reducing agent in the medium, the reactivities of the glutaminyl residues in the nonrepetitive (C2.2) and repetitive (C1.1) polypeptides were very similar (7-7.9%). When dithiothreitol (10 mM DTT) was added to the reaction mixtures, the number of modified glutaminyl residues in the nonrepetitive polypeptide C2.2 was drastically increased up to 19.7%, whereas it was only slightly modified in the repetitive polypeptide C1.1 (9.4%). The slight difference observed in the repetitive polypeptide with and without reducing agent was due only to the influence of DTT on factor XIII activity because this region did not contain disulfide bridges. The enzyme sensibility to the DTT could be estimated too by the low decrease in the glutaminyl residue reactivity of β casein from 29%, in

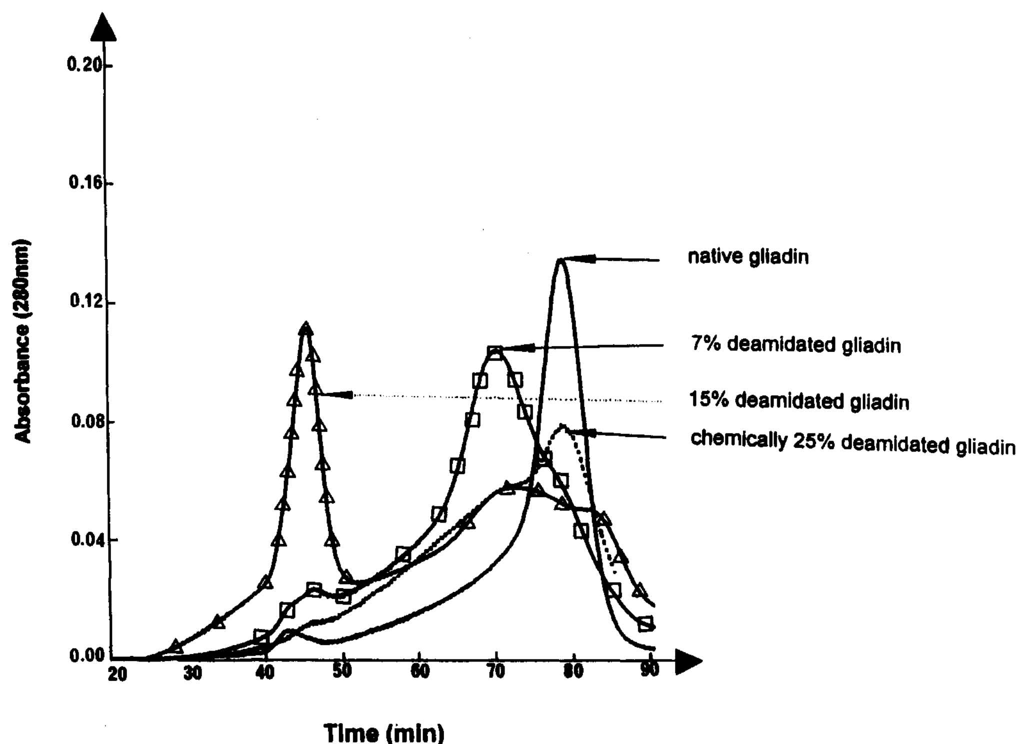

Figure 6. HPLC gel filtration patterns of native γ 46 gliadin and γ 46 gliadin treated by factor XIIIa. Experimental conditions: 100 mM Tris-HCl, pH 5, containing 2.5% dioxane, 7 mM $CaCl_2$, 10 mM DTT, 2.5 mg/mL native gliadin, and 3 units/mg factor XIIIa at 37 °C. Superimposition of HPLC elution patterns for untreated gliadin (—), factor XIIIa treated gliadin during 70 min (□) and (△) 24 h and chemical 25% deamidated gliadin (···).

Table II. Repetitive and Nonrepetitive Polypeptide Reactivity of γ Gliadin for Factor XIII

	repetitive polypeptide (C1.1)	nonrepetitive polypeptide (C2.2)	gliadin γ 46
total residues	167[a]	141[a]	272[b]
glutaminyl residues/mol	76[a]	33[a]	102[b]
glutaminyl residues modified			
10 mM DTT[c]			
glutaminyl residues/mol	6.8	6.5	16.3
% of total glutaminyl residues	9	19.7	16
without DTT[c]			
glutaminyl residues/mol	5.3	2.6	6.7
% of total glutaminyl residues	7	7.9	6.6

[a] According to the γ gliadin sequence published by Sugiyama et al. (1986) and the amino acid composition determined by Popineau et al. (1990). [b] Bollecker (1991). [c] Reactivity determined in the presence of putrescine as second substrate. Putrescine incorporation was measured after 2 h of incubation at 37 °C. Experimental conditions: 0.4 mg of protein or polypeptide substrate was mixed with 36 mM putrescine including 200 nCi of [^{14}C]putrescine and 3 units/mg factor XIIIa in 100 mM Tris-HCl, pH 6, containing 10% dioxane and 7 mM $CaCl_2$.

the presence of 10 mM DTT, to 24%, when no reducing agent was present in the medium, this last protein containing no disulfide bridges (Ribadeau-Dumas et al., 1972). The higher reactivity of the nonrepetitive polypeptide C2.2 in the presence of DTT cannot be explained by the influence of reductive condition on the enzyme activity. It may result from the reductive cleavage of the disulfide bridges, exclusively located in this part of the protein (Popineau et al., 1990). This disruption might increase the glutaminyl residue accessibility to factor XIII. Besides, the lower reactivity of glutaminyl residues in the repetitive polypeptide C1.1 compared to the nonrepetitive polypeptide C2.2, in the presence of DTT, was interpreted as due to the high amount of β turns and repeat sequences Gln-Gln and Pro-Gln-Gln-Pro-Phe-Pro-Gln in the repetitive polypeptide. These characteristics could limit the acyl donor accessibility for the enzyme. In particular, it is known that, in the Gln-Gln sequence, it is only the N-terminal glutaminyl residue which is substrate for transglutaminases (Gorman and Folk, 1980). Moreover, the modification of one glutaminyl residue can disturb the accessibility of the others for the enzyme by charge or steric effect. These structural and sequence characteristics also explained the great difference between the glutaminyl residue reactivity for the γ gliadin (16%) and the β casein (29%) in the presence of DTT.

Conclusion. For the first time, a transglutaminase activity was studied in water/solvent mixtures. This work brought to light that plasma factor XIII could work in the presence of organic solvents up to 10–15%; moreover, the stability of the enzyme is increased in water/dioxane mixture (85/15 v/v). These properties allowed the gliadin modification by factor XIII, because of the higher solubility of this substrate in presence of solvents.

For short enzymatic treatment (30 min), without amine in the medium, gliadin deamidation was essentially occurring, whereas longer time (24 h) led to protein cross-linking.

From a comparison of chemically and enzymatically deamidated proteins, it is clearly seen that the reaction products are very different. Chemically modified gliadins were partially hydrolyzed (Bollecker, 1991), while the formation of soluble polymers is catalyzed by enzymatic treatment. The repetitive region was preferentially deamidated by acid treatment (Bollecker, 1991), whereas factor XIII was mostly active on the nonrepetitive sequence. These differences should induce great variations of the functional properties, which remain to be studied in the case of the enzymatically deamidated protein; the properties of the soluble polymers should be of especially great interest.

Finally, bovine plasma factor XIII appeared to be more interesting than peptidoglutaminase isolated from *Bacillus circulans* to deamidate prolamines because the use of prior proteolysis was necessary to enhance deamidation by peptidoglutaminase (Hamada,1992). From this study, a procedure for modifying wheat protein could be proposed for application in which the enzymatic reaction has to be carried out in ethanol (20% v/v) at 37 °C. However, the limiting factor is the availability of the enzyme in large enough amounts. This point requires further study to prepare enriched enzymatic extract.

ABBREVIATIONS USED

BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; factor XIIIa, activated factor XIII; PITC, phenyl isothiocyanate; RP-HPLC, reversed-phase high-performance liquid chromatography.

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