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In vitro cross-linking of gluten into high-molecular-weight polymers with transglutaminase

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From the amino acid composition of gluten proteins and the substrate specificity of transglutaminases (TGase) we concluded that gluten proteins can be favourable substrates for TGases due to their high glutamine content.

By use of sodium dodecyl sulfate polyacrylamide gelelectrophoresis it was demonstrated that from gluten-ES and gluten-TS high-molecular-weight proteins developed in the presence of Ca^{2+} and red blood cell lysate containing TGase. When ovalbumin or deamidated gluten were applied as substrates no high-molecular-weight products were formed. Upon spectrophotometric measurements we found that covalent cross-links (isopeptide bonds) formed under the effect of TGases presumably cause a change in the position of chromophore groups in the substrates. Absorption decrease was detected between 274–276 nm as a result in the case of gluten-TS and gluten-ES used as substrates for TGase. No such change occurred in ovalbumin and deamidated gluten, applied as controls, under the influence of TGase.

On the basis of our experiments it is postulated that the first step in gluten toxicity is presumably the binding of gluten to the intestine mucosa. In this binding the high transglutaminase activity in the intestines of coeliac patients and the high glutamine content of gluten may have an important role.

Transglutaminases (TGases, R-glutaminyl-peptide: amine gamma-glutamyltransferases, EC 2.3.2.13) are Ca^{2+}-dependent enzymes that catalyze the formation of intermolecular crosslinks – covalent isopeptide bonds, ε-(γ-glutamyl)lysine – between gamma-carboxyl amide groups of glutamines in polypeptide chains of certain proteins and ε-amino groups of lysines in another polypeptide chain releasing ammonia. The cross-links thus formed link protein molecules into rigid high-molecular-weight polymers that are insoluble in sodium dodecyl sulfate (SDS), urea and disulphide bridges reducing (2-mercaptoethanol, dithiothreitol etc.) solutions. Until recently production of the ε-(γ-glutamyl)lysine cross-links catalyzed by TGases was the only known physiological reaction. But nowadays

Abbreviations: EGTA, ethylene gylcol-bis-/2-aminoethyl ether/-N,N,N'N' tetraacetic acid; Glu, glutamic acid; Gln, glutamine; gluten-TS, supernatant fraction of gluten after treatment of gluten with Tris (to pH = 7.2–7.4); gluten-ES, supernatant fraction of gluten after treatment of gluten with EGTA (to pH = 7.2–7.4); PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; RBC, red blood cell; SDS, sodium dodecyl sulfate; Tris, Tris-/hydroxymethyl/-aminoethane.

there is unequivocal evidence that TGases catalyze the formation of cross-links between peptide-bound glutamines of proteins and amine groups of polyamines (spermidines, spermines), too [12]. Laki and Lórand were the first to report on the discovery of TGase (fibrin stabilizing factor, factor XIII) [8]. This enzyme, whose activity is thrombin and Ca^{2+}-dependent, can be found in the blood plasma, the placenta and the platelets. It plays an important role in the polymerization of fibrin monomers into stable fibrin network during clotting of normal plasma, in wound healing and the formation of atherosclerosis [11]. To date TGase has been demonstrated in several tissues including the liver [12], the epidermis [6, 23], the erythrocytes [19, 27], the seminal fluid [35], the kidney [7], the brain [25], the human jejunal mucosa [5].

Since only peptide-bound glutamine is the substrate of the enzyme (limited specificity is determined by the circumstances of the given glutamine), we thought that gluten and gliadin could be favourable substrates for TGase. According to our measurements [30, 31] and literary data [2, 36] gluten and gliadin contain 40–45% of glutaminyl residue (Glu + Gln) of which over 60% are in the form of amide residues (Gln). The latter, as mentioned above, is an absolute substrate requirement for TGase activity. Gliadin and gluten, however, exhibit only limited solubility on physiological pH, since they dissolve only in mild acids, alkalia or 70% of alcoholic solutions. Considering all these, we isolated fractions (gluten-TS, gluten-ES, v.s.: Methods) soluble in water and electrolyte solutions from gluten. Human red blood cell lysate and fibrogammin (factor XIII, Boehring Werke AG, Marburg) purified by us on Sephadex G-200 column and activated with thrombin were used as the source of TGase.

Several methods [16, 18, 19, 20, 25, 28] have been established to demonstrate TGases, measure their activities as well as to show the formation of cross-links occurring as a result of their activities in the presence of adequate substrates. During our experiments a method based on sodium dodecyl sulfate polyacrylamide-gel-electrophoresis (SDS-PAGE) was used. Besides we tried to view by spectrophotometry the changes taking place in the investigated substances through intermolecular cross-links produced under the influence of TGase. We thought that the position of chromophores (tyrosine, tryptophan, phenylalanine) would change in the investigated proteins due to the formation of cross-links, and as a result we would be able to detect absorption decrease or increase at the absorption maxima depending on the extent of enzyme activity. By the help of both methods, mentioned above, from gluten-TS and gluten-ES, formation of high-molecular-weight proteins were demonstrated in the presence of Ca^{2+} and TGase. Using ovalbumin or deamidated gluten no high-molecular-weight products were formed.

Further on the results of our experiments are described.

Materials and Methods

Isolation of human red blood cell (RBC) lysate containing transglutaminase (TGase). 5 ml of human blood taken freshly on heparin (100 NE heparin/ml blood) was centrifuged at 800 g, 4°C for 30 min. The RBC mass was then washed thrice, with a solution containing 133 mmol/l NaCl, 20mmol/l Tris-HCl, pH 7.4. The approximately 1 ml volume of RBC mass was kept with 15 ml of 20 mmol/l Tris-HCl buffer (pH 7.4) at room temperature for 1 hr, and centrifuged at 45000 g at 4 °C for 30 min. The precipitate was discarded, the supernatant containing TGAse was stored in 3 ml portions at −20 °C until next use. Under the described circumstances the enzyme is active for 4 weeks.

Isolation of gluten fractions soluble in water and electrolyte solutions from gluten. In order to make fractions we isolated gluten from bread by the method described in our previous paper [30]. Thereafter 7.2 ml ion-free-water and 0.2 ml 500 mmol/l Tris solution was added to 12 mg gluten protein dissolved in 3 ml 10 mmol/l acetic acid, and the mixture was shaken carefully. The Iris solution made the sample turbid and the pH to increase up to 7.2–7.4. Thereafter the sample was kept at 37 °C for 120 min and then centrifuged at 2000 g at 25 °C for 60 min. The clean supernatant was dialysed against 50-fold volume of 20 mmol/l Tris-HCl buffer (pH = 7.4) at 4 °C for 36 h while the buffer solution was changed twice. This supernatant fraction, subsequently gluten-TS, was stored at −20 °C until use. Gluten-ES preparation was isolated according to a previous paper [33].

Deamidation of gluten. 20 mg of gluten was heated at 100 °C in 8 ml 1 mol/l HCl for 45 min. After cooling, the mixture was dialysed against 2×1000 ml of ion-free-water at 4 °C for 24 h, then 1000 ml 20 mmol/l Tris-HCl buffer (pH = 7.4) for another 24 h. The dialysed deamidated gluten solution was stored at −20 °C [5].

Production of immune serum against, gluten, immune diffusion. The immune serum against gluten obtained from rabbit was produced by a method shown in a previous paper [33]. Immune diffusion experiments were performed on 1.5% agarose (Noble Difco) plate in phosphate buffered saline (PBS), pH 7.2. After transferring the immune serum and the sample material onto the plates, we incubated them at room temperature for 26–36 h. Thereafter the plates were washed with PBS solutions changed severalfold. After washing and drying, the plates were stained with 0.125% Coomassie Brilliant Blue (R-250) solution containing 1:9=cc. acetic acid: 50% methanol. The plates were destained with a differentiation solution containing 1:5:4=cc. acetic acid: methanol: water.

Demonstration of high-molecular-weight proteins forming from water-soluble gluten fractions under the effect of TGase by SDS-PAGE. RBC hemolysate (33 µl, 13.4 mg per ml) containing TGase was incubated with gluten-TS (100 µl, 0.4 mg per ml) or gluten-ES (100 µl, 0.15 mg per ml) or crystallized ovalbumin (100 µl, 0.4 mg per ml) in the presence of 6–10 mmol/l $CaCl_2$ at 37 °C for 180 min at pH = 7.4. Control samples: 1. gluten-TS was incubated, as afore-mentioned, without $CaCl_2$; 2. with $CaCl_2$; 3. RBC hemolysate was incubated without $CaCl_2$; 4. with $CaCl_2$. After incubation, control samples 1 and 4, 2 and 3 were mixed. Then 50–50 µl incubation medium (0.01 mmol/l sodium phosphate buffer, pH = 7.1, 50 g/l SDS, 10 mg/l mercaptoethanol, 0.05 g/l bromphenol blue and 400 g/l glycerol) was added to all samples [29], and they were heated at 100 °C for 7 min. Incubated samples were quantitatively—under electrode buffer—transferred onto the top of preelectrophorized gels. Electrophoresis and the succeeding gel treatments (staining, destaining, etc.) were carried out according to Weber and Osborn [34] with the difference that the gels contained only 50 g/l acrylamide. To determine molecular mass, we used Serva bovine serum albumin

(M_r = 68000), purified human gamma-globulin (M_r = 50 000 and 23 000) and crystallized ovalbumin (M_r = 42 000) as standards. After washing, the gels were measured with a densitometer consisting of an Opton PM-2DL spectrophotometer connected with a recorder (Radelkis 814/1), on own-made gel tube and a gel sliding motor.

Demonstration of changes occurring in water-soluble gluten fractions under the influence of TGase with spectrophotometer. Preparation of assay and control samples was similar to that applied during SDS-PAGE, but 3-fold volume was measured from each component. Incubation time was 180 min, at 37 °C. Thereafter control samples 1 and 4, and 2 and 3 were mixed and diluted in 2.7 volume of 20 mmol/l Tris-HCl buffer, pH = 7.4 and absorption spectra were investigated with Specord M40 spectrophotometer. Spectra were taken up against 20 mmol/l Tris-HCl buffer. Investigation of assay samples was carried out as described above.

Results

Figure 1 illustrates the absorption spectra of gluten-TS, gluten-ES, deamidated gluten and ovalbumin used during experiments. The absorption maxima of gluten-TS, gluten-ES and deamidated gluten lie at 276 nm, that of ovalbumin lies at 278 nm. Gluten-ES, as shown in a previous study [33] on the basis of results obtained by immune disc electrophoresis, is related to gluten from immunological point of view. Accordingly we did not find any important differences in the amino acid composition of gluten and gluten-Es [33]. According to our experiments carried out on Ouchterlony plates, both gluten-ES—similarly to the facts described above—and gluten-TS exhibited 2 precipitation bands with the immune serum produced against gluten (Fig. 2). Deamidated gluten did not react to the immune serum.

Figure 3 shows the absorption spectrum of RBC hemolysate containing TGase. It can be seen that the hemolysate has absorption maxima at 275, 348, 405–420, 542 and 577 nm. When the RBC hemolysate containing TGase was incubated in the presence

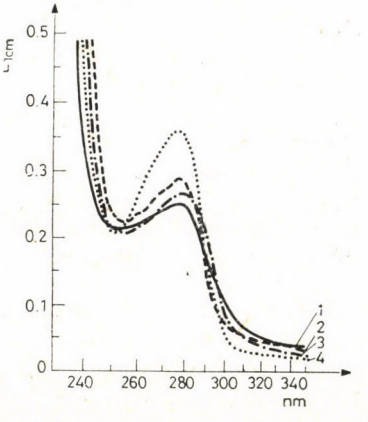

Fig. 1. Absorption spectra of (1) gluten-ES: ——, (2) gluten-TS: — — —, (3) ovalbumin: ·—·—·, (4) deamidated gluten: ·····

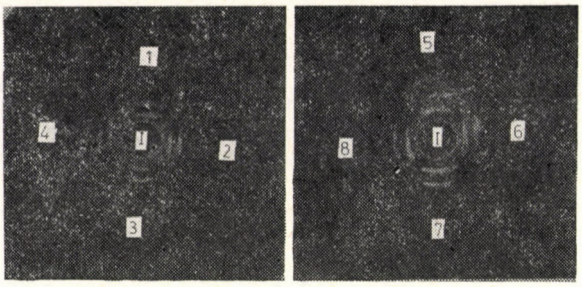

FIG. 2. Immune reaction of gluten-TS and gluten-ES with antigluten immune serum

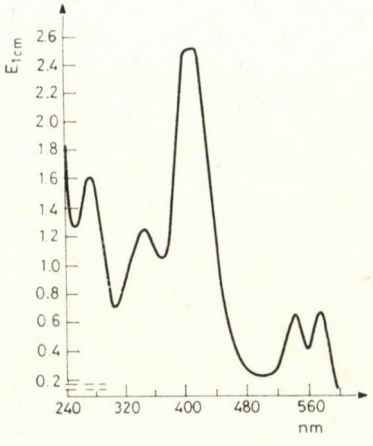

FIG. 3. Absorption spectra of red blood cell hemolysate

of 10 mmol/l $CaCl_2$ and gluten-TS at 37 °C for 180 min, approximately 10% absorption decrease was detectable at 276 nm compared with the corresponding control (Fig. 4). At 348, 405–420, 542 and 577 nm no significant changes occurred.

Using ovalbumin in the place of gluten-TS as substrate for TGase, we measured approximately 2–3% absorption increase at 276 nm compared with the corresponding control (Fig. 5). At 348, 405–420, 542 and 577 nm practically no absorption change was detected.

Spectrophotometrical investigations were also carried out using fibrogammine (factor XIII) and thrombin as follows. 400 μl (240 μg) fibrogammine purified by us on Sephadex G-200 column, 20 μl (25 μg) thrombin (topostasine) and 400 μl (160 μg) gluten-TS o ovalbumin, and deamidated glute were incubated in the presence of 10 mmol/l $CaCl_2$ at 37 °C for 60 min in 60 mmol/l NaCl and

20 mmol/l Tris-HCl buffer (pH = 7.4) solution. Control samples were prepared according to the principles shown in the "Method". We established that if gluten-TS was the substrate of TGase, the absorption decrease at 276–277 nm was 8.4% as compared to the respective control. If

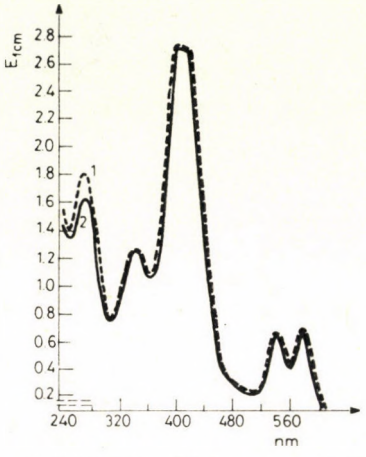

FIG. 4. Effect of red blood cell (RBC) hemolysate containing TGase on the absorption properties of gluten-TS. — — —: the composition of control samples was the same as in (2). The only difference was that gluten-TS and the RBC hemolysate in the presence of 10 mmol/l $CaCl_2$ were incubated separately. After incubation the two components were united, the Ca^{2+} concentration was set, diluted, etc. (2) ———: 300 μl of gluten-TS was incubated with 100 μl (1340 μg) RBC hemolysate containing TGase in the presence of 10 mmol/l $CaCl_2$ at 37 °C for 180 min. Then the 442 μl volume incubation medium was diluted with 2.7-fold volume 20 mmol/l Tris-HCl buffer (pH 7.4) and its absorption spectrum was immediately taken up against the latter buffer

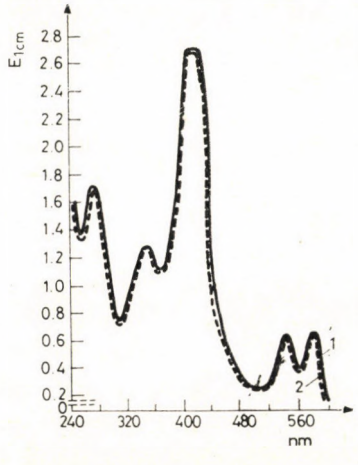

FIG. 5. Effect of red blood cell hemolysate containing TGase on the absorption properties of ovalbumin. The procedure and the incubation conditions, etc. were the same as shown in Fig. 4., but in this experiment ovalbumin was used in the place of gluten-TS. ——— ovalbumin, (2) — — — control

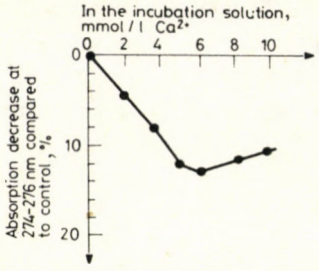

FIG. 6. Effect of red blood cell hemolysate containing TGase on the absorption properties of gluten-TS at different final Ca^{2+} concentrations. The procedure, the incubation conditions, etc. were the same as shown in Fig. 4., but in this series of experiments Ca^{2+} final concentrations were variable in the incubation solution. Absorption changes occurring at higher Ca^{2+} concentrations were denoted in the percentage of the absorption measured in the incubation solution without Ca^{2+}

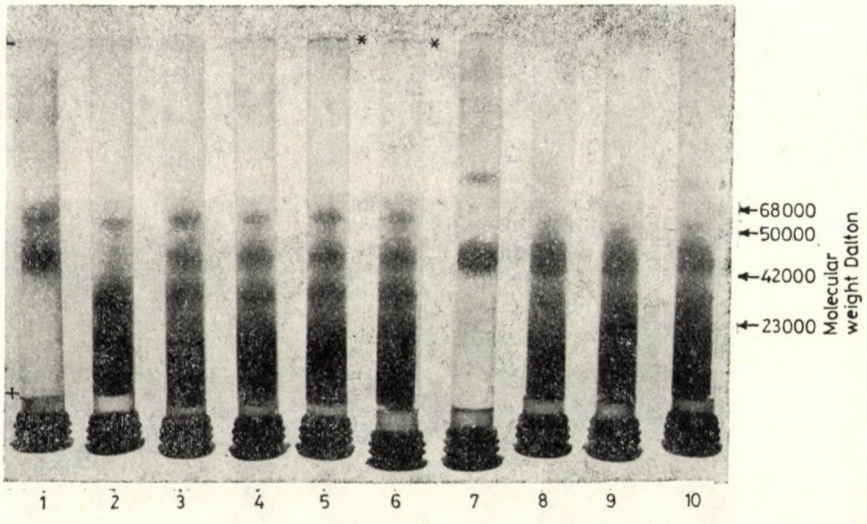

FIG. 7. Demonstration of high-molecular-weight components formed from gluten-TS under the effect of RBC hemolysate containing TGase in the presence of 6 mmol/l Ca^{2+} by SDS-PAGE. In control experiments in the place of gluten-TS crystallized ovalbumin was applied. Gel 1: 40 μg gluten incubated at 37 °C for 180 min, then dissolved in incubation medium (see Methods). Gel 2: 450 μg RBC hemolysate containing TGase, further on as at gel 1. Gel 3: 40 μg gluten-TS in the presence of 6 mmol/l Ca^{2+} and 450 μg RBC hemolysate containing TGase, respectively, incubated separately without Ca^{2+} at 37 °C for 180 min. After incubation the two incubation solutions were mixed then Ca^{2+} concentration was completed to 6 mmol/l and the mixture was dissolved in incubation medium. Gel 4: 450 μg RBC hemolysate containing TGase in the presence of 6 mmol/l Ca^{2+}, and 40 μg gluten-TS without Ca^{2+} was incubated separately at 37 °C for 180 min. After incubation the two solutions were mixed and further on as at gel 3. Gels 5 and 6: 40 μg gluten-TS in the presence of 6 mmol/l Ca^{2+} was incubated with 450 μg RBC hemolysate containing TGase at 37 °C for 180 min and then dissolved in incubation medium. Gel 7: 40 μg of ovalbumin, further on as at gel 1. Gels 8 and 9: the same as gels 3 and 4, but in the place of gluten-TS ovalbumin was employed. Gel 10: the same as gels 5 and 6, but ovalbumin was used instead of gluten-TS. Materials dissolved in incubation medium in the case of every gel were heated at 100 °C for 7 min then SDS polyacrylamide gel electrophoresis was carried out

ovalbumin is the substrate of TGase, the absorption increase at 278–279 nm is 2–3% compared with the respective control. When deamidated gluten is the substrate, 1–1.5% absorption increase can be detected at 276–277 nm. This must be explained by the fact that it is impossible for isopeptide-bonds to form under the effect of TGase due to deamidation of gamma-carboxyamide groups of gluten. In the case of ovalbumin, though peptide-bound glutamine (as substrate) is present cross-link formation is inhibited, because of the environments of the given glutamines.

Considering that TGase activity is Ca^{2+}-dependent we examined the effect of TGase upon the absorption properties of gluten-TS at different Ca^{2+} concentrations. In Fig. 6 it can be seen that absorption decrease, compared with absorption (100%) measured in the incubation solution without Ca^{2+} at 275–276 nm, was the greatest at 5–6 mmol/l Ca^{2+} concentration.

After finding the optimum of Ca^{2+} concentration necessary to TGase activity, we studied, by use of SDS-PAGE, if high-molecular-weight products were formed under the effect of TGase in the presence of proteins (gluten-TS, gluten-ES, ovalbumin) used as substrates compared with the respective controls.

In Fig. 7 using gluten-TS, a high-molecular-weight band (asterisk) de-

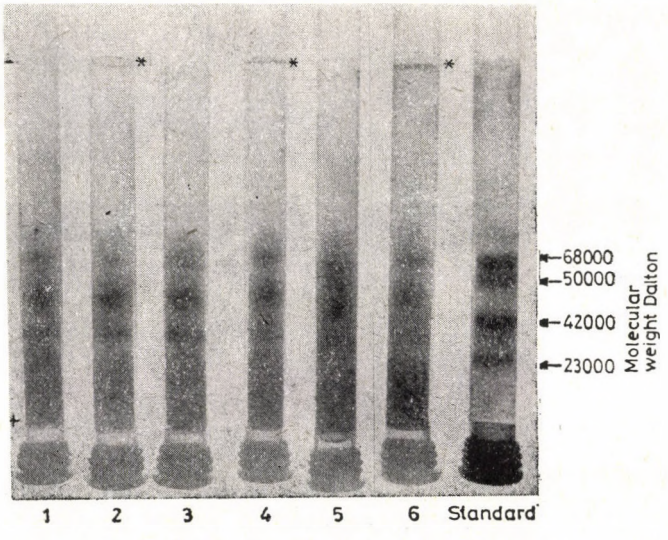

FIG. 8. Demonstration of high-molecular-weight components formed from gluten-TS under the effect of RBC hemolysate containing different amounts of TGase in the presence of 6 mmol/l Ca^{2+} by SDS-PAGE. The method and principle employed during the preparation of control incubation mixtures (1, 3, 5) were the same as at gel 4 in Fig. 7. Preparation of sample incubation mixtures was the same shown at gels 5., 6. in Fig. 7. In the case of every gel 40 μg of gluten-TS, 6 mmol/l final Ca^{2+} concentration, in gels 1 and 2: 117 μg, gels 3 and 4, 268 μg, and gels 5 and 6: 450 μg of RBC hemolysate containing TGase were employed

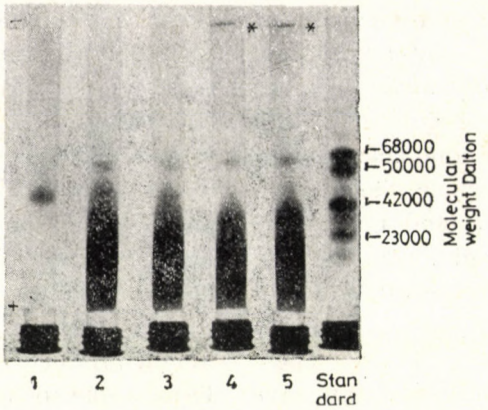

FIG. 9. Demonstration of high-molecular-weight components formed from gluten-ES under the effect of RBC hemolysate containing TGase in the presence of 6 mmol/l Ca^{2+} by SDS-PAGE. Gel 1.: 40 μg of gluten-ES was incubated at 37 °C for 180 min, then dissolved in incubation medium, etc. In the case of gels 2, 3, 4, 5 the preparation, incubation, electrophoresis, etc. were carried out as with the gels 3, 4, 5, 6 in Fig. 7. The only difference is that gluten-ES was used in the place of gluten-TS

veloped under the influence of RBC hemolysate TGase is visible on the parts of gels 5, 6 near the cathode. On control gels 3, 4 no such product is traceable. High-molecular-weight protein formation cannot be demonstrated in the case of gels 8–11, either, because ovalbumin was employed as substrate for TGase in the place of gluten-TS. According to the data obtained by densitometration of gels, the high-molecular-weight protein on gels 5, 6 mainly developed from the 48 000 and 40 000 molecular-weight components of gluten-TS. Moreover, this is obvious from the gel pictures if we compare pictures 3, 4 with 5, 6 in Fig. 7.

At a constant amount of gluten-TS and constant Ca^{2+} concentration increasing the amount of RBC hemolysate containing TGase, the amount of high-molecular-weight proteins increased, too. This can be seen very well in the places marked with asterisks in the gel pictures of Fig. 8. When we used gluten-ES instead of gluten-TS as substrate for TGase, the high-molecular-weight protein band (asterisk), as shown in Fig. 9, can also be demonstrated as compared to the respective controls (gel pictures 2, 3 Fig. 9), on the parts of gel pictures 4, 5 near the cathode. According to the data obtained by densitometration of gels, the high-molecular-weight protein visible in gel pictures 4, 5 developed from the 40 000 molecular-weight component of gluten-ES. This is also perceivable if we compare the components in gel pictures 2, 3 with those of 4, 5.

DISCUSSION

In our earlier papers we suggested that: 1. gluten and its fractions, having special amino-acid composition, contain protected sequences that can resist the hydrolytic effects of

proteases [29]; 2. they are capable of binding nucleosides and nucleotides, in which their phenylalanine content can play an important role [32]; 3. having high glutamic acid content (16–20% of the whole amino acid content), they can bind Ca^{2+} [33]; 4. owing to their high glutamine content (almost 30% of the whole amino-acid content) they can be suitable substrates for different TGases. These enzymes, as shown above, can have an important role in binding of gluten and its fractions to tissues by the production of isopeptide-bonds. The high glutamine content of gluten can also play a role in the formation of hydrogen bonds and forming high molecular-weight products [30].

Bruce and co-workers [5] demonstrated that TGase activity of biopsy samples taken from the intestines of coeliac patients were 3–4 times higher than that of the healthy. This led them to the conclusion that intestinal TGase might be important in gluten proteins binding to tissues. Bruce and co-workers [5] used gliadin preparation showing limited solubility in their experiments, on the one hand, and they did not identify the high-molecular-weight products formed from gliadin under the effect of TGase, on the other hand. So we tried to demonstrate and identify in vitro by SDS-PAGE the high-molecular-weight products developing from water and electrolyte-soluble gluten components under the effect of TGase. In our experiments we used erythrocyte TGase and thrombin activated TGase (factor XIII) as enzymes, and water and electrolyte-soluble gluten-ES, gluten-TS preparations of which amino acid content similar to gluten as substrates. Ovalbumin and deamidated gluten were applied as controls. We established that high-molecular-weight products developed both from gluten-ES and gluten-TS, but not from ovalbumin. Figures 7–9 also illustrate that the formation of high-molecular-weight products in the case of gluten-TS was due to the 40 000 and 48 000 molecular-weight components (Fig. 7 gel pictures 5, 6) while in the case of gluten-ES the 40 000 molecular-weight one (Fig. 9 gel pictures 4, 5). From literary data [4, 6, 19] it is known that in the case of different TGases (erythrocyte TGase, guinea pig liver TGase, blood plasma factor XIIIa, etc.) the substrate can be the enzyme itself or in the case of an unpurified enzyme, as e.g. erythrocyte lysate TGase, the accompanying protein. Our control experiments show that we do not have to reckon with this, because no high-molecular-weight products occur when erythrocyte lysate is incubated at 37 °C in the presence of Ca^{2+}, in spite of the fact that the enzyme gets active and accompanying proteins are present, too (v.s. Fig 7 gel picture 4).

Our spectrophotometrical measurements confirm our supposition that there is a change between 275–280 nm in the position of UV light absorbing chromophores (tyrosine, tryptophan, phenylalanine) due to the isopeptide bonds forming in the substrate under the effect of TGase. Absorption decreases (Fig. 4). This does not mean

that the number of the chromophores is reduced, but that the molecular structure of the gluten fraction undergoes a change, it presumably gets closer as a result of the formation of isopeptide bonds. Absorption decrease occurring under the effect of TGases arises from two changes. According to our measurements the absorption increase, as compared to the respective controls (fibrogammine + thrombin, 37 °C, 30 min, Ca^{2+} is added immediately before measurements), when fibrogammine (factor XIII) is activated with thrombin in the presence of Ca^{2+} (37 °C, 30 min). This absorption increase must be connected with the fact that in the presence of Ca^{2+} thrombin activated factor XIII dissociates into its subunits (catalytic a, catalytic b). That is followed by the opening of the catalytic centre. This opening (active centre) can increase the absorption measured at 276–278 nm. If active TGase and gluten-TS are incubated together in the presence of Ca^{2+}, isopeptide bonds are formed in the gluten-TS, which causes a reverse change, in the position of chromophores in the substrate. This manifests itself as an absorption decrease. In the case of ovalbumin and deamidated gluten substrates used as controls slight (1–3%) absorption increase can be detected under the effect of TGase (Fig. 5). According to literary data [4, 5] ovalbumin and deamidated gluten are 10–20-fold worse substrates for TGase (erythrocytes, the liver, etc.) than beta-casein or gliadin. The results of our experiments also demonstrate that water and electrolyte-soluble gluten fractions isolated by us are at least as good substrates for different TGase enzymes as dimethylcasein.

Transglutaminases, as mentioned above, are wide-spread enzymes. They are presumably present in the nucleus, membrane and cytoplasm of every cell. Their biological functions, roles with few exceptions (fibrin clot stabilization [11], postejaculatory clotting of seminal fluid [35], keratination [1]) remain to be determined. Findings of recent experiments suggest that TGases play an important role in receptor-bound endocytosis and the formation of receptorsignal [9, 10], cell division and differentiation [3, 13, 22], cell ageing [16, 19], manifestation of antigenicity and the covering of tumourspecific antigens [15, 17], regulation of cell proliferation [14, 24]. It is possible that the increased TGase activity of coeliac intestine mucosa reflects the proliferation of intestine mucosa cells [26]. The first step in gluten toxicity must be its binding to the intestine mucosa. In this the TGase may have an important role through forming isopeptide cross-links. These protease resistent isopeptide bonds can dissociate by the help of a newly discovered intracellular enzyme, gamma-glutamyl-amine cyclotransferase [21] after gluten gets into the cell.

Acknowledgments

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