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ORIGINAL ARTICLE

Transglutaminase-mediated modification of ovomucoid: effects on its trypsin inhibitory activity and antigenic properties

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Abstract Hen egg can cause food hypersensitivity in infants and young children, and ovomucoid is the most allergenic factor among proteins contained in egg white. Since proteinase treatment, a well-recognized strategy in reducing food allergenicity, is ineffective when applied to ovomucoid because of its ability to act as trypsin inhibitor, we investigated the possibility of reducing the ovomucoid antiprotease activity and antigenic properties by covalently modifying its structure. The present paper reports data showing the ability of the Gln115 residue of ovomucoid to act as an acyl donor substrate for the enzyme transglutaminase and, as a consequence, to give rise to a covalent monodansylcadaverine conjugate of the protein in the presence of both enzyme and the diamine dansylated derivative. Moreover, we demonstrated that the obtained structural modification of ovomucoid significantly reduced the capability of the protein to inhibit trypsin activity, also having impact on its anti-ovomucoid serum-binding properties.

Keywords Antigenic properties · Monodansylcadaverine · Ovomucoid · Transglutaminase · Trypsin inhibitory activity

Introduction

Egg is the food that most frequently causes hypersensitivity. Many egg proteins have been characterized, their primary amino acid sequences determined and the structures of the carbohydrate moieties established (Nisbet et al. 1981). Egg white, which is considered more allergenic than the yolk, contains 24 different glycoproteins as determined by crossed-immunoelectrophoresis (Langeland 1982). Among these, ovomucoid, which accounts for more than 10% of the total protein fraction of egg white proteins, is considered the most allergenic (Bernhisel-Broadbent et al. 1994; Urisu et al. 1997). Ovomucoid is a protein with a molecular mass of approximately 28 kDa and a p*I* of 4.1 (Tanabe et al. 2000) and consists of three structurally independent tandem domains (Kato et al. 1987). Each domain contains three intradomain disulfide bonds and behaves as a native globular protein. IgG- and IgE-binding reactivity to the third domain is significantly higher when compared with the first and second ones (Rupa and Mine 2006). Ovomucoid contains as much as 25% of carbohydrates (Bernhisel-Broadbent et al. 1994; Kato et al. 1987; Rupa and Mine 2006) present as oligosaccharides, bound to an asparagyl residue of the polypeptide chain (Montgomery and Wu 1963; Kato et al. 1987). However, it has been demonstrated that the carbohydrate moieties have no impact on ovomucoid IgE binding (Mine and Wei Zhang 2002). Currently, heat and proteinase treatments are well-known strategies for reducing the allergenicity of food, but such methods are ineffective when applied to ovomucoid because of its heat resistance and ability to act as a trypsin inhibitor (Lineweaver and Murray 1947; Lineweaver et al. 1949; Feeney et al. 1963; Kato et al. 1987; Kato and Matsuda 1997; Kojima et al. 1999). In fact, the three tandem domains of ovomucoid are homologous to the pancreatic secretory trypsin inhibitor (Kazal inhibitor) (Kato et al. 1987). It has been also determined that the trypsin binding site resides in the second domain (residues 65–130) and, in particular, that the major reactive site is localized at

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the Arg89–Ala90 peptide bond (Matsuda et al. 1982; Kato et al. 1987). Trypsin inhibitors of protein origin are widely present in animal and plant foods and most of them have deleterious effects on animal nutrition. For example, trypsin inhibitors in raw soybean, the Bowman-Birk inhibitor and the Kunitz factor, cause pancreatic hypertrophy and hyperplasia in rats, mice, chickens and other experimental animals (Morgan et al. 1986; Kato and Matsuda 1997). Inactivation of the trypsin inhibitors is not easily accomplished by the usual physical processing of food, mostly because of their small molecular sizes and the occurrence of numerous disulfide bonds. Therefore, we found of interest the attempt to modify hen ovomucoid structure by the enzyme transglutaminase and, possibly, trypsin inhibitory activity as well as its antigenicity. Transglutaminases (protein-glutamine γ-glutamyl-transferase, EC 2.3.2.13, TGs) are enzymes widely distributed in nature and found in mammals, fish, plants and bacteria. They catalyze the acyl transfer reaction of γ-glutamyl residues, present in protein and peptide substrates (acyl donor or Q-donor), to an acyl acceptor substrate, resulting in a variety of different products, depending on the involved molecules (Folk and Finlayson 1977; Lorand and Graham 2003). The transamidation reaction occurs when the acyl acceptor is either the ε-amino group of an endoprotein lysine or a low molecular mass primary amine, thus generating ε-(γ-glutamyl)lysine crosslinks in the first case and protein–amine conjugates in the latter. Either water or alcohol molecules can replace the acyl acceptor substrate with their hydroxyl group, leading to deamidation (Shan et al. 2002; Aktories and Schmidt 2003) or esterification (Nemes et al. 1999) of the recognized glutamines, respectively. The most studied microbial TG was isolated from the culture medium of *Streptoverticillium* sp. S-8112 (Ando et al. 1989), which has been identified as a variant of *Streptoverticillium mobaraense* and also known as *Streptomyces mobaraensis* (Zotzel et al. 2003). In contrast to many other TGs, the microbial isoform (mTG) is Ca^{2+} independent and is remarkably stable over a wide range of temperatures and pHs. Such characteristics, including the higher reaction rate, the broad substrate specificity for the acyl donor and the low-cost mass production by traditional fermentation technology, make mTG particularly useful for industrial and biotechnological applications as a food-grade additive capable of improving many important features of different protein-based foods (Mariniello et al. 2007a, b). Previous investigations demonstrated that such an enzyme is able to crosslink several proteins of different origin including legume globulins, such as phaseolin (Mariniello et al. 2007a, b), 11S globulin from soybean and milk proteins, e.g., α-lactalbumin and β-lactoglobulin, as well as other albumins. mTG modification changes protein functional properties such as solubility, gelation and emulsion formation (Mariniello et al. 2007a, 2008) and in some cases also biological features such as their antigenicity and allergenicity (Watanabe et al. 1994; Babiker et al. 1998). In the present study, we demonstrate that ovomucoid acts as an effective acyl donor mTG substrate and that the mTG-mediated modification of ovomucoid reduces its capability of inhibiting the proteolytic activity of trypsin and affects also the protein antigenicity.

Materials and methods

Materials

Hen ovomucoid, bovine pancreatic trypsin, monodansylcadaverine (MDC) and *N*α-benzoyl-L-arginine *p*-nitroanilide (BAPNA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dithiothreitol (DTT) was from MP Biomedicals, Inc. (Illkich, France). Microbial TG (Activa WM), derived from the culture of *Streptoverticillium* spp., was supplied by Ajinomoto Co. (Japan). The enzyme was prepared by dissolving the commercial preparation (containing 1% of mTG and 99% of maltodextrins) in distilled water. The specific activity of the enzyme was 92 U/g of Activa WM. Estimation of enzymatic activity was carried out by a colorimetric hydroxamate test described by Folk and Chung (1985) and modified according to Pasternack (1998). Chemicals for electrophoresis were from Bio-Rad (Segrate, Milano, Italy). Anti-denatured ovomucoid sera obtained from rabbit were purchased from InCura srl (Cremona, Italy). All other chemicals were of analytical grade. Phaseolin was isolated from *Phaseolus vulgaris* beans by using the ascorbate-NaCl procedure described by Sun and Hall (1975). The procedure was modified according to Mariniello et al. (2007a). The purified protein was dissolved in distilled water at a concentration of 4 mg mL^{-1}.

Methods

mTG substrate assay

The occurrence of reactive acyl donor glutamine residue in the ovomucoid structure was assayed by a fluorimetric method (Folk and Chung 1985) based on the mTG-mediated incorporation of MDC as an acyl acceptor. Ovomucoid (100 µg) was preliminarily incubated at 100°C for 5 min in a water bath before being tested, and then the heat-treated protein was incubated for 24 h at 37°C in 100 µL of 80 mM Tris–HCl buffer, pH 7.5, with MDC (5 mM) in the presence of mTG (0.09 or 0.18 U). Control samples were prepared by incubating the assay mixtures in the absence of the enzyme. At the end of incubation, the

samples were heated at 100°C for 5 min and then concentrated to a volume of about 10 μL in a Savant Speed Vac® Plus and, to prevent hydrophobic interactions between MDC and ovomucoid, incubated at room temperature for 1 h with 90 μL of a solution of 9 M urea, 2% (w/v) SDS and 40 mM DTT in 50 mM Tris–HCl buffer, pH 7.1. Hence, 28.5 μL of the sample buffer (15 mM Tris–HCl, pH 6.8 containing 0.5% (w/v) SDS, 2.5% (v/v) glycerol, 200 mM 2-β-mercaptoethanol and 0.003% (w/v) bromophenol blue) were added and the samples were boiled for 5 min. Aliquots of 10 μL were finally analyzed by 12% SDS-PAGE (Laemmli 1970) and the visualization of reaction products was achieved by Coomassie Brilliant Blue R-250 staining and, for protein-bound MDC, by ultraviolet (UV) illumination on a Fluor-S Multimager apparatus (Bio-Rad Richmond, USA). Bio-Rad Precision Protein Standards were used as molecular weight markers.

Purification of mTG-modified ovomucoid by high-performance liquid chromatography (HPLC)

For a preparative mTG-modified ovomucoid purification, 250 μg of heat-treated ovomucoid were incubated for 24 h at 37°C with mTG (0.45 U) in 80 mM Tris–HCl, pH 7.5, containing 5 mM MDC (final volume 250 μL). Control samples were prepared by incubating the assay mixtures in the absence of mTG. At the end of incubation, the samples were boiled for 5 min to inactivate the enzyme, cooled down in an ice bath and promptly loaded on to C18 Sep-Pak cartridge (Sep-Pak Classic, Waters) to separate proteins from the assay mixture. The cartridge was preconditioned with 10% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). After loading the reaction mixture, the cartridge was washed with 2 mL of the preconditioning solution. Hence, different concentrations of ACN (20, 40, 60%, respectively) containing 0.1% TFA were passed through the cartridge. The collected samples were evaporated to dryness and then dissolved in 400 μL of distilled water. Aliquots of each sample were analyzed by 12% SDS-PAGE. To separate mTG-modified ovomucoid from the unmodified counterpart, the ovomucoid-containing fraction, eluted from C18 Sep-Pak cartridge, was subjected to reverse-phase HPLC by using a Nucleosil C18 column (250 × 4 mm ID, Machery-Nagel, Düren). The column was heated at 40°C and equilibrated with 2.5 mM sodium acetate buffer, pH 5.6:ACN (90:10 v/v, flow rate 1.0 mL/min), and the proteins were eluted with the following procedure: 2 min after the sample injection, the percentage of ACN increased to 20% in 1 min; after 2 min, the percentage of ACN increased to 25% in 0.5 min, and then increased linearly to 50% in 10 min. The protein fractions were detected at 220 nm by using a UV/VIS diode-array detector (Beckman).

Trypsin assay

Phaseolin (100 μg) was incubated in 100 μL of 90 mM Tris–HCl buffer, pH 8.1, with 1 μg of bovine trypsin for 10 min at 37°C both in the absence and presence of different amounts (0.18 and 0.36 μg) of either ovomucoid or mTG-modified ovomucoid. Control samples were prepared by incubating the assay mixture in the absence of trypsin. At the end of incubation, the digestion reactions were stopped by adding 28.5 μL of sample buffer and heating the tubes at 100°C for 5 min before electrophoresis. Finally, 15 μL of each sample were analyzed by 12% SDS-PAGE followed by Coomassie staining of the gels. Densitometry analysis of protein bands was performed by using the software Quantity One (Bio-Rad, version 4.2.1).

ELISA

Antigenic properties of mTG-modified ovomucoid and of its natural counterpart were determined by indirect ELISA. Experiments were carried out four times. Ovomucoid was coated to a 96-well microtiter plate (Coster, Corning Incorporated, USA) in 0.1 M sodium carbonate buffer (pH 9.6) at a concentration of 0.5 μg mL^{-1} and incubated overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20 (PBST), then blocked with 3% bovine serum albumin (BSA) in PBS for 2 h at 37°C, washed three times with PBST and finally incubated with 100 μL of anti-denaturated ovomucoid sera diluted 1:25,000 for 90 min at 37°C. After incubation, the plate was washed again three times with PBST and, after addition of 100 μL of goat anti-rabbit diluted 1:7,500, the incubation was carried out at 37°C for a further 1 h. The plate was washed and finally developed with 100 μL of 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABST) solution (Southern Biotech, Birmingham, Alabama, USA). After 5 min, absorbance at 405 nm was read by the microplate reader (Bio-Rad).

Determination of the inhibition constant (K_i) toward trypsin

K_i values of both unmodified and mTG-modified ovomucoid toward trypsin were determined by Dixon plot after a proteolytic assay carried out using different concentrations of BAPNA as substrate. In particular, 0.4 μg of bovine trypsin was added to 1 mL of 90 mM Tris–HCl buffer, pH 8.1, containing 3.3 mM $CaCl_2$ and increasing concentrations of unmodified or mTG-modified ovomucoid (from 1.0 to 10 nM), in the presence of 0.125 and 0.25 mM of BAPNA. The reaction was carried out in cuvettes of 1 cm light-path at 37°C and the formation of the product (*p*-nitroanilide) was monitored following the increase in

absorbance at 410 nm using a Lambda 25 spectrophotometer (Perkin-Elmer) against a blank containing the assay mixture without trypsin. The velocity of the reaction was obtained from the slope of the linear portion of the curve obtained plotting absorbance versus time. The experiment was performed three times for each data point and the average and standard deviations were calculated.

Protein determination

Protein determination was carried out by the Bio-Rad Protein Assay (Bio-Rad), using BSA as standard (Bradford 1976).

Statistical analysis

Microsoft Excel-2007 was used for all statistical analyses. The data were subjected to the analyses of variance and the means were compared using Student's *t* test. Differences were considered to be significant at $P < 0.05$.

Results

Ovomucoid acts as substrate for mTG

Hen ovomucoid molecule possesses one glutamine residue (Gln115) that appears well exposed on the protein three-dimensional structure. With the aim of evaluating the ability of such residue to act as an acyl donor substrate of mTG, the incorporation of the fluorescent marker MDC into its polypeptide chain was first investigated (Fig. 1). Heat-treated ovomucoid was incubated both in the absence (lanes 1a and 1b) and in the presence of different concentrations of mTG (lanes 2a–2b and 3a–3b). In panel A, the presence of a fluorescent band with an electrophoertic mobility between 25 and 37 kDa is evident, which clearly indicates the covalent incorporation of MDC into ovomucoid obtained in the presence of mTG. The detection of an extra fluorescent band of about 14 kDa corresponding to lysozyme, a well known contaminant of the partially purified ovomucoid preparation, confirms that also lysozyme acts as acyl donor mTG substrate as previously described by Lim et al. (1998).

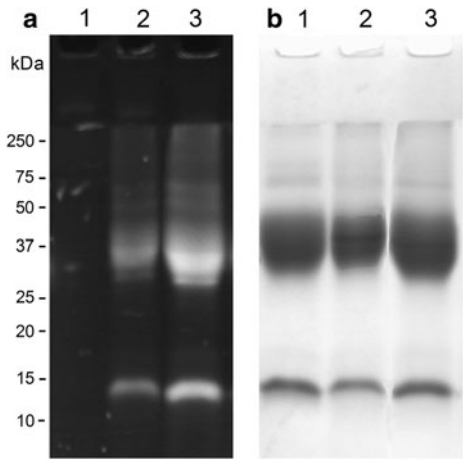

Fig. 1 mTG-dependent incorporation of MDC into ovomucoid. Ovomucoid (100 µg), after a preliminary heat treatment at 100°C for 5 min, was incubated for 2 h at 37°C in 100 µL of 80 mM Tris–HCl buffer, pH 7.5, with MDC (5 mM) in the presence of either 0.09 U (*lane 2*) or 0.18 U of mTG (*lane 3*). The control was simultaneously carried out incubating ovomucoid with MDC in the absence of mTG (*lane 1*). Aliquots of 10 µL were analyzed by 12% SDS-PAGE and the protein bands visualized by UV illumination (**a**) and Coomassie staining (**b**) of the gel. Further experimental details are given in the text

Purification of mTG-modified ovomucoid

To isolate mTG-modified ovomucoid from its preparation reaction mixture, 250 µg of ovomucoid incubated with MDC in the absence and presence of mTG, as described in "Materials and methods" were loaded preliminarily onto a pretreated Sep-Pak C18 cartridge. The samples, eluted at different ACN concentrations in the presence of 0.1% TFA, were evaporated to dryness and then dissolved in distilled water. Aliquots of these samples were analyzed by SDS-PAGE and the occurrence of ovomucoid was assessed in the fraction eluted at 40% ACN (data not shown). The purification of ovomucoid-MDC derivative from the unmodified counterpart was, therefore, carried out by subjecting to HPLC (reverse-phase column) the fraction eluted from the Sep-Pak C18 cartridge with 40% ACN. The HPLC elution pattern of this sample (Fig. 2) shows the partial conversion of ovomucoid (P1) into its MDC derivative (P2), which was separated from the unmodified form by its higher hydrophobicity due to the covalent linkage of MDC to Gln115-ovomucoid residue. In fact, comparing the elution profile of ovomucoid incubated in the presence of both MDC and mTG (Fig. 2b) to the one recorded with the protein sample incubated without the enzyme (Fig. 2a), an additional peak (P2) more retained by the column and eluted after 10.7 min with higher ACN concentration was detected. SDS-PAGE (12%) analysis (Fig. 2c) confirmed the occurrence of unmodified ovomucoid form into the peak eluted at 7.14 min (P1) and the occurrence of ovomucoid-MDC derivative into the peak eluted at 10.7 min (P2) (Fig. 2c).

Decreased inhibitory activity of trypsin by mTG-modified ovomucoid

The ovomucoid-MDC derivative, isolated by HPLC, was tested in proteolytic assays to evaluate its capability to inhibit bovine pancreatic trypsin compared to the

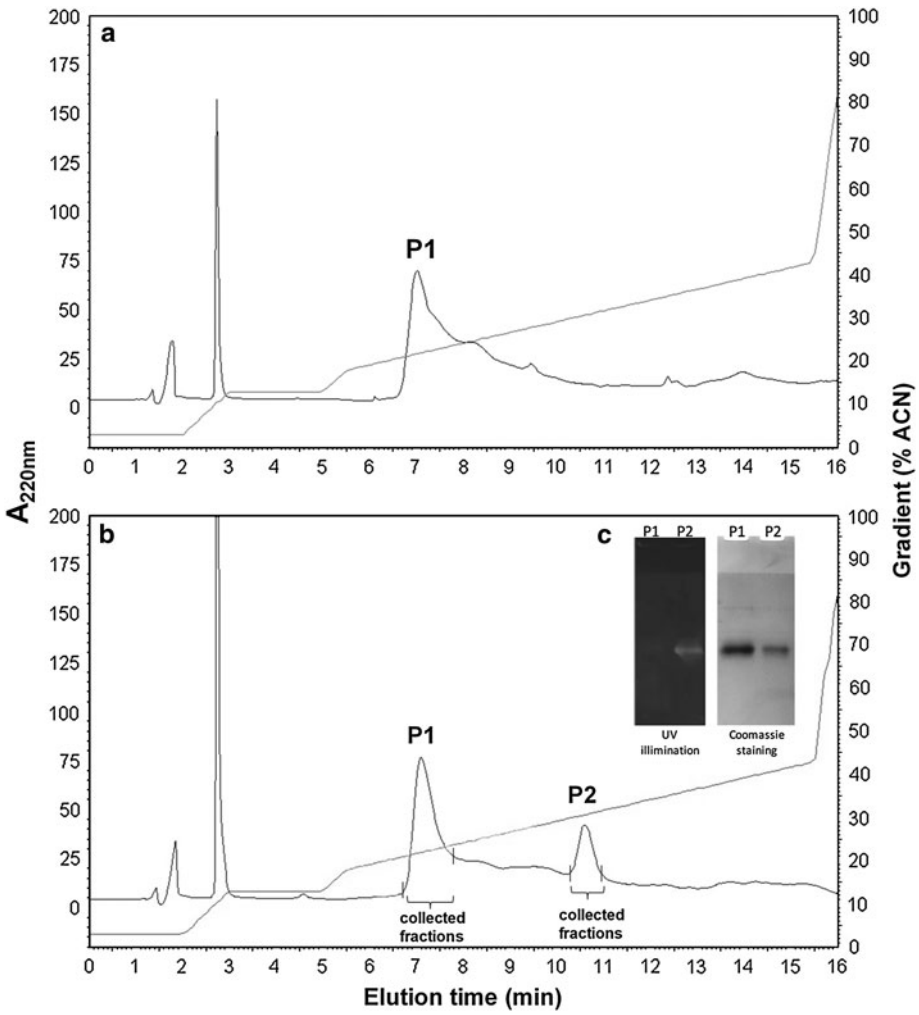

Fig. 2 HPLC separation on C18 column of mTG-modified ovomucoid from the unmodified protein. Ovomucoid samples were previously incubated with MDC either in the absence (**a**) or presence (**b**) of mTG. The two peaks shown in **b** eluted at 7.14 min (*P1*) and 10.7 min (*P2*) contained the unmodified (*P1*) and mTG-modified (*P2*) ovomucoid forms, respectively. SDS-PAGE (12%) of *P1* and *P2* is shown in **c**. The experimental details are described in the text

unmodified form. Assays were carried out by incubating phaseolin, a well-known trypsin substrate (Mariniello et al. 2007a; Deshpande and Nielsen 1987) with the protease over 10 min in the presence of different amounts of either mTG-modified or unmodified ovomucoid. The control sample was prepared by incubating the assay mixture in the absence of both trypsin and ovomucoid. At the end of incubation, an aliquot of the reaction mixture was analyzed by SDS-PAGE followed by Coomassie staining. As previously demonstrated (Mariniello et al. 2007a; Deshpande and Nielsen 1987), in vitro treatment of phaseolin with the proteolytic enzyme results in the generation of polypeptides with a molecular mass ranging from 20 to 25 kDa (Fig. 3, lane 1) that are resistant to further proteolysis (Mariniello et al. 2007a; Deshpande and Nielsen 1987; Sathe and Sze-tao 1997). When unmodified ovomucoid was added to the assay mixture at different concentrations, trypsin activity was inhibited, as evident by the decreased formation of the 20–25 kDa fragments (Fig. 3, lanes 2–3). A marked difference in the protein profile was observed when trypsin digestion of phaseolin was carried out in the presence of mTG-modified ovomucoid. In fact, the MDC derivative seems to be a much less effective inhibitor of the phaseolin breakdown (Fig. 3, lanes 4–5) with respect to the unmodified ovomucoid. This result was confirmed by the densitometry analysis showing that the percentage of intensity of undigested phaseolin after trypsin treatment was about 19 and 21% when 0.18 and 0.36 μg of mTG-treated ovomucoid were present, respectively, in the assay mixture (Fig. 3, lanes 4–5), whereas it was about 32 and 66%, respectively, when unmodified ovomucoid was tested at the same amounts (Fig. 3, lanes 2–3). Protease inhibitory activity by ovomucoid was also measured by using a different trypsin substrate, BAPNA, and the K_i value for both mTG-modified and unmodified ovomucoid was also determined. For this purpose, enzyme assays were performed at two different concentrations of trypsin substrate (0.125 and 0.25 mM) in the presence and absence of increasing concentrations of inhibitor, and K_i was obtained graphically by the Dixon plot. As it is described for many trypsin inhibitors, and in agreement with previously reported data for unmodified ovomucoid (Lineweaver and

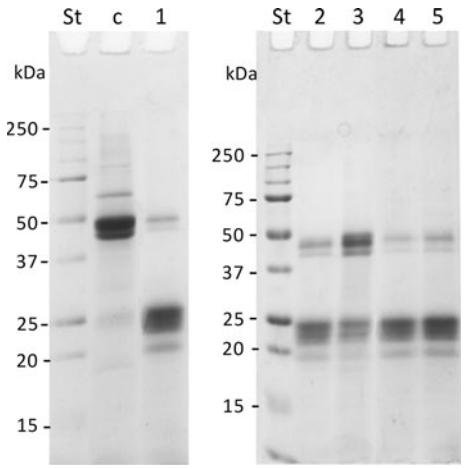

Fig. 3 Trypsin inhibition by unmodified and mTG-modified ovomucoid. Phaseolin (100 μg) was incubated with bovine trypsin (1 μg) for 10 min at 37°C in 100 μL of 90 mM Tris–HCl buffer pH 8.1 in the absence (*lane 1*) and presence of two different amounts of either unmodified (*lane 2* 0.18 μg, *lane 3* 0.36 μg) or mTG-modified (*lane 4* 0.18 μg, *lane 5* 0.36 μg) ovomucoid. *Lane c* (control) represents phaseolin incubated in the absence of both trypsin and ovomucoid. The reactions were stopped by sample buffer addition. The resulting samples were boiled for 5 min and 15 μL of each sample were finally analyzed by 12% SDS-PAGE. Proteins were visualized by Coomassie staining. Further experimental details are given in the text

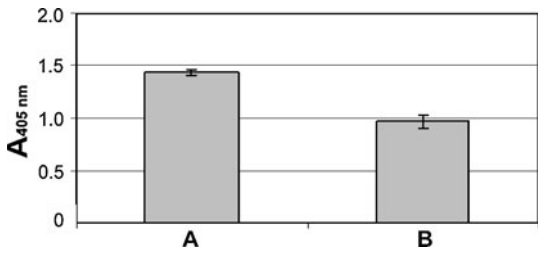

Fig. 4 Antigenic properties of unmodified (*A*) and mTG-modified (*B*) ovomucoid tested by ELISA. ELISA protocol, carried out by measuring the binding activity of anti-ovomucoid sera, is described in "Materials and methods". The results are expressed as mean values ± SD ($n = 4$)

Murray 1947; Lineweaver et al. 1949; Fraenkel-Conrat et al. 1949), the mechanism of inhibition was non-competitive for both ovomucoid forms. However, whereas unmodified ovomucoid was confirmed to be a powerful inhibitor with a K_i value of 0.78 ± 0.25 nM, our experimental data have shown that the mTG-catalyzed structural modification of the protein resulted in a significant reduction of its affinity for trypsin since its K_i value (6.07 ± 0.21 nM) increased by about eightfold.

Antigenic properties of mTG-modified ovomucoid

Besides being a bovine trypsin inhibitor, ovomucoid is also known as a major allergenic protein in hen egg white (Bernhisel-Broadbent et al. 1994; Urisu et al. 1997). We have, thus, evaluated the antigenic properties of the MDC derivative of ovomucoid in comparison with the untreated protein using rabbit anti-denatured ovomucoid sera. The performed ELISA test indicated that serum binding of mTG-modified ovomucoid was significantly lower when compared with the unmodified protein (Fig. 4).

Discussion

It is well known that the number of proteins acting as glutaminyl substrates for TG is limited, since both the primary and the three-dimensional polypeptide structures determine whether or not an endoprotein glutamine residue reacts as an acyl donor for the enzyme (Aeschlimann and Paulsson 1994). In the present study, the ability of hen ovomucoid Gln115, the only glutamine residue occurring in its polypeptide chain, to act as *Streptoverticillium* TG substrate was demonstrated by MDC incorporation into the protein. Although Gln115 is exposed on the surface of ovomucoid structure (Matsuda et al. 1982), the protein was not able to act as substrate of mTG when tested without heating treatment (data not shown). Thus, ovomucoid was thermally treated before its incubation in the presence of the enzyme to improve its flexibility and surface hydrophobicity (Kato and Takagi 1988; Mine et al. 1990; Matsumura et al. 1996). Under these experimental conditions, the protein is unfolded and, therefore, more susceptible to the enzyme binding with respect to its native form, as Lim et al. (1998) have already demonstrated by testing total protein mixture occurring in the hen egg white. Afterward, ovomucoid incubated in the absence and presence of mTG was isolated from the reaction mixture by Sep-Pak C18 cartridge also to get rid of lysozyme that was present as contaminant in the ovomucoid commercial preparation. The elution profile of ovomucoid incubated in the absence of mTG showed a single peak (P1) that eluted after 7.14 min. This peak appeared asymmetric, likely due to the high percentage of glycosylation of the ovomucoid molecule (Bernhisel-Broadbent et al. 1994; Kato et al. 1987; Rupa and Mine 2006; Yousif and Kan 2002). In fact, it is reported that ovomucoid, differently from other egg white proteins, contains as much as 25% of carbohydrates, present as oligosaccharides joined to the polypeptide chain by an asparagyl residue (Kato et al. 1987; Montgomery and Wu 1963). Conversely, the elution pattern of ovomucoid incubated in the presence of mTG showed the presence of a new peak (P2) with an increased hydrophobicity, eluted later (after 10.7 min) because of the covalent linkage of MDC to Gln115. The ovomucoid-MDC derivative, contained in P2, was collected and further tested, as well as unmodified ovomucoid occurring in P1. In particular, the

two purified molecular forms of the protein were analyzed for their trypsin inhibitory activity and antigenic properties. The results obtained by incubating the unmodified and the mTG-modified ovomucoid with trypsin in the presence of phaseolin, a known trypsin substrate, have demonstrated that mTG treatment of the protein led to a marked decrease of its protease inhibitory activity, with an approximately eightfold increase in the K_i value exhibited by the MDC derivative of the ovomucoid with respect to the unmodified protein. It is well known that ovomucoid consists of three structurally independent tandem domains (Kato et al. 1987; Rupa and Mine 2006). Such domains are homologous to pancreatic secretory trypsin inhibitor (Kato et al. 1987), but only the second domain of the ovomucoid (residues 65–130) acts as a major bovine trypsin binding site with the direct involvement of the Arg89–Ala90 dipeptide (Kato et al. 1987; Kato and Matsuda 1997; Kojima et al. 1999). Therefore, the lower trypsin inhibitory activity of mTG-modified ovomucoid is probably determined by the covalent MDC binding to the unique glutamine residue (Gln115) occurring in the ovomucoid polypeptide chain. In fact, MDC linked to the second ovomucoid domain might represent a sterically cumbersome factor able to mask the ovomucoid trypsin binding site. Besides being an effective trypsin inhibitor, ovomucoid is a well-studied allergenic component of egg white (Bernhisel-Broadbent et al. 1994; Urisu et al. 1997; Rupa and Mine 2006; Mine and Wei Zhang 2002). Studies by Bernhisel-Broadbent et al. (1994) have shown that ovomucoid is the immunodominant protein in egg white. Previous reports have already demonstrated that mTG catalyzes crosslinking reactions of several food proteins from soy, meat, gluten, oat and milk (Mariniello et al. 2008), and some of them concluded that mTG treatment of some food based-products, such as wheat flour and soy proteins, leads to their decreased allergenicity (Watanabe et al. 1994; Babiker et al. 1998) as a consequence of the masking of IgE antibody binding sites by the mTG-catalyzed crosslinking. Therefore, in the present study we have also investigated the antigenicity properties of ovomucoid following the enzyme-mediated covalent binding of MDC to its Gln115 residue. It is worth noting that ovomucoid allergenicity (IgE) and antigenicity (IgG) are quite resistant to heat denaturation (Mine and Wei Zhang 2002; Kurisaki et al. 1981; Matsuda et al. 1985), thus suggesting that antibody binding is directed toward a linear epitope. Our results showed that mTG-catalyzed MDC binding led to a reduced ovomucoid antigenicity, as measured by ELISA with specific antisera. Within the whole primary structure of ovomucoid, eight IgG epitopes (5–11 amino acids in length) and nine IgE epitopes (5–16 amino acids in length) have been found (Mine and Wei Zhang 2002), whereas within the second domain only two IgG epitopes (amino acids 71–75 and 101–105) have been identified (Mine and Wei Zhang 2002). Such epitopes are quite close to the Gln residue and, thus, are likely protected by the MDC hindrance, which reduces the antigenicity properties of the molecule. In conclusion, our results strongly suggest that the mTG-catalyzed modification occurring around the ovomucoid trypsin reactive site significantly influences both its interactions with protease and IgG binding capability. However, further studies are required to investigate whether mTG might be applied as a biotechnological tool also to decrease ovomucoid allergenic responses and to prevent hen egg hypersensitivity.

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Conflict of interest The authors declare that they do not have conflict of interest.

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