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Spectrophotometric assay using *o*-phtaldialdehyde for the determination of transglutaminase activity on casein

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Abstract

In this work, the possibility of using a simple and quick method was tested for determining transglutaminase activity on casein using a spectrophotometric assay. The enzyme activity was estimated on the basis of the decrease of *o*-phthaldialdehyde reactive ε-amino groups of lysine following the formation of isopeptide bonds. The lysine residues involved in the formation of isopeptide bonds when the reaction reaches its plateau are equal to 0.126 μmol per mg of casein. This value results as equal to 0.205 μmol per mg of casein when *N*-carbobenzoxy-glutaminyl-glycine is added to the reaction medium as a small size acyl group donor. The electrophoretic analysis of the reaction products emphasised a different kinetic formation of casein polymers with the two substrate solutions used. This proposed method has resulted as accurate, with a mean coefficient of variation of 4.6%. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Transglutaminase; Casein; *o*-Phtaldialdehyde; Spectrophotometric assay

1. Introduction

The transglutaminases (E.C. 2.3.2 13, TGases) are acyltransferases that catalyse a transacylation reaction between the γ-carboxyamide group of a glutamine residue (Gln) present on a peptide (acyl group donor) and a primary amine (acyl group acceptor). The nucleophilic acceptor could be an aliphatic amine, a polyamine, or the lysine (Lys) ε-amino group (Folk & Chung, 1985). In such cases, the TGase action allows the formation of a carboxyamide bond between the donor and the acceptor of the acyl group according to the reaction pattern shown in Fig. 1. Furthermore, in the absence of amino groups, water acts as the acyl group acceptor and the result of the enzymatic action is the deamination of the Gln of the substrate protein (Mycek & Waelsch, 1960). Therefore, the TGase is capable of modifying the amino acid composition as well as the properties of the protein on which it reacts.

Bovine casein is known as a substrate of the TGase. The modification of bovine casein through the TGase has allowed for obtaining heat resistant protein gels (Nonaka, Sakamoto, Toiguchi, Kawajiri, Soeda, & Motoki, 1992), derivatives with a high emulsifying action (Dickinson & Yamamoto, 1996), deaminated derivatives with improved solubility in acidic conditions

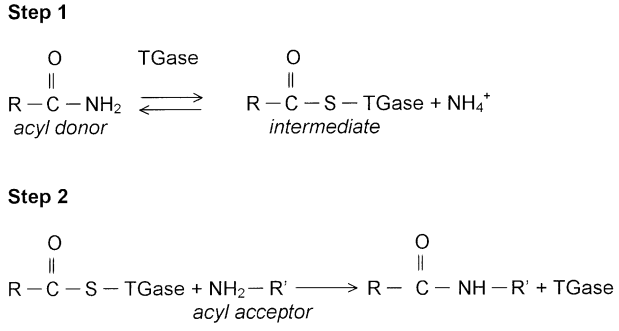

Fig. 1. Reaction scheme of the transglutaminase (TGase) catalysis. Step 1: formation of the intermediate acyl donor-enzyme and releasing of ammonia. Step 2: nucleophylic attack of the primary amino group acylic acceptor and releasing of the free enzyme.

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(Nonaka, Sawa, Matsuura, Motoki, & Nio, 1996) and derivatives enriched in essential amino acids (Bercovici, Gaertner, & Puigserver, 1987; Nonaka, Matsuura, & Motoki, 1996). Other than these, many other ideas for using TGase in the milk-dairy industry have been hypothesised and patented (Nielsen, 1995).

In this context, it would be useful to have simple and brief assays at hand in order to evaluate the TGase activity on the casein.

High molecular weight products formed when the TGase catalyses the cross-links formation on the substrate protein, can be pointed out by SDS PAGE (Kurth & Rogers, 1984) or by the determining of the variation of the rheological properties of the reaction mix (Faergemand, Murray, & Dickinson, 1997). These methods are extremely useful in evaluating if a protein is a TGase substrate, but they do not permit to quantify the enzyme activity in terms of amount of reaction product formed. The TGase mediated crosslinking of proteins produces ε-(γ-glutamyl)lysine isopeptide bonds. Detection of the isopeptide bonds is generally accomplished using exhaustive proteolysis of crosslinked proteins, derivatization of generated aminoacid mixture and purification by chromatographic techniques (Miller & Johnson, 1999; Siefring, Apostel, Velasco, & Lorand, 1978). The enzymatic activity can be measured also on the basis of the amount of ammonia released by the acyl group acceptor by coupling the TGase reaction to the reaction catalysed by glutamate dehydrogenase (Day & Keillor, 1999; De Backer-Royer, Traoré, & Meunier, 1992). Glutamate dehydrogenase catalyses the reductive amination of α-ketoglutarate with concomitant oxidation of NADH to NAD^+ which is followed spectrophotometrically as an absorbency decrease at 340 nm. Also flow injection analysis (FIA) has been utilized for measuring ammonia release during TGase reaction (Faergemand & Qvist, 1997).

When the substrate protein acts also as an acyl group acceptor, the decrease of the ε-amino groups of the Lys can be used to evaluate the TGase activity.

In the presence of mercapoethanol the *o*-phthaldialdehyde (OPA) reacts with the primary amines, leading into a fluorescent adduct called 1-alkylthio-2alkylisoindole. By using the flourimetric methods primary amines and the peptides have been determined (Porter, Swaisgood, & Catignani, 1982). Oh, Catignani, and Swaisgood (1993) have used the same principle for determining TGase activity on the casein.

The OPA adduct heavily absorbs at 340 nm; this property has been used for determining, peptides and amino acids by using a spectrophotometer (Church, Swaisgood, Porte, & Catignani, 1983; Svedas, Galev, Borisov, & Berezin, 1980). In 1990, Vigo, Malec, Gomez, and Llosa suggested a spectrophotometric assay based on the use of OPA for estimating the decrease in the casein Lys after the Maillard reaction.

The object of this study is to evaluate TGase activity on casein by estimating the decrease in the Lys ε-amino groups using OPA in a spectrophotometric assay.

2. Material and methods

2.1. Enzyme

Transglutaminase from *Streptoverticillium* sp. was kindly supplied by Ajinomoto Europe Sales GMBH, Hamburg, Germany.

2.2. OPA reactive lysine determination

The OPA reagent was prepared as described by Goodno, Swaisgood, and Catignani (1981). The following compounds were diluted with water to 100 ml: 80 mg OPA (dissolved in 2 ml 95% ethanol); 50 ml 0.1 M sodium tetraborate buffer solution with pH 9.5; 5 ml 20% SDS; 0.2 ml of 2-mercaptoethanol. The OPA reagent was prepared immediately before use. Casein samples with a concentration ranging from 0.02 to 0.62 mg/ml were prepared in phosphate buffer 0.1 M pH 7.0. 0.05 ml of the sample were added to 2 ml of OPA reagent. This solution was briefly stirred and the absorbency at 340 nm was measured after a 2-min incubation at room temperature. A standard curve was obtained by using *N*-α-acetyl-lysine as a reference compound. Reference samples with a concentration ranging from 0.0049 to 0.036 μmol/ml were prepared in phosphate buffer pH 7.0 and the *N*-α-acetyl-lysine determination was performed as described above.

2.3. Determination of TGase activity

The enzyme activity was determined in a phosphate buffer solution pH 7.0 containing 2.4 mg/ml TGase and 7.2 mg/ml casein. In some experiments, the reaction solution also contained N-carbobenzoxy-glutaminyl-glycine (Z-Gln-Gly) 2.4 mg/ml. The reactions were carried out at 37 °C under stirring. At regular intervals 0.05 ml were taken from the reaction solution and added to 2 ml of OPA reagent. The sample absorbency at 340 nm was measured after a 2-min incubation period (A_{Tot}). During the same moment, 0.450 ml fractions were withdrawn from the reaction solution and acidified with 0.050 ml of 15% TCA. The acidified fractions were centrifuged at 12,000 rpm for 5 min and a proteic pellet and a clear supernatant were obtained. Supernatant (0.05 ml) was added to 2 ml of OPA reagent and the absorbency at 340 nm measured after 2 min (A_{Sn}). The absorbency caused by OPA reactive Lys on the casein during the TGase reaction was calculated as $A_{Tot}-A_{Sn}$. The $A_{Tot}-A_{Sn}$ values corresponding to consecutive TGase reaction times, were plotted against

the *N*-α-acetyl-lysine reference curve, giving μmoles OPA reactive Lys.

2.4. Gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to Schágger and von Jagow (1987), using a 4% spacer gel and a 5% running gel. Proteins were solubilized with a medium containing 4% SDS, 2% β-mercaptoethanol, 12% glycerol, 0.01% bromphenol blue, 50 mM Tris–HCI at pH 6.8. Standard proteins purchased from Sigma were used as a standard. Following electrophoresis, proteins were stained for 2 h in 0.2% Coomassie blue R-250 and 0.05% Coomassie blue G-250 in methanol:acetic acid:water (4:1:4, v/v/v), and excess dye was removed with acetic acid:methanol: water (1:4:5, v/v/v).

2.5. Statistical analysis

The data for the casein and the *N*-α-acetyl-lysine standard curves were analysed by a linear regression according to the least squares method.

TGase activity determinations were repeated at least five times and the mean data and experimental error values were recorded.

The variation coefficient of the OPA method was calculated based on 10 repetitions.

3. Results and discussion

The amount of primary OPA reactive amino groups on casein were measured. The relationship between the absorbency and casein concentration is linear in the concentration range pertaining to these experiments (Fig. 2). The amount of the OPA reactive amino groups was calculated as the ratio between the casein curve slope and the *N*-α-acetyl-lysine standard curve slope (Fig. 3) and resulted 0.64 μmol/mg casein. This value represents the sum of the α terminal amino groups and the Lys ε-amino groups. If we are to consider the amount and the average molecular weight of the different casein species present in the casein sample used, we can calculate 0.04 μmol/mg as the amount of α terminal amino groups; therefore 0.625 μmol/mg refers to the amount of ε-amino groups that could actually act as the acyl acceptor in the TGase reaction. According to Vigo and co-workers (1992) the absorbency caused by α terminal amino groups does not interfere with the application of OPA method since represents less than 10% of the total absorbency. An average lysine content of 8% by weight was estimated; and resulted as very similar to the data which has previously been reported (Mauron, 1977).

Casein has been used as a TGase substrate. Preliminary experiments were performed for assessing the stability of adducts from the OPA reactive amino groups in the reaction mixture and in the supernatant, which was resulted after acidic protein precipitation. Aliquots of the reaction mixture and of the supernatant were mixed with the OPA reagent and the absorption at 340 nm was measured along 15 min (Fig. 4). This value sharply increases, reaching the maximum after 2 min in both samples. After that, the OPA-reaction mixture

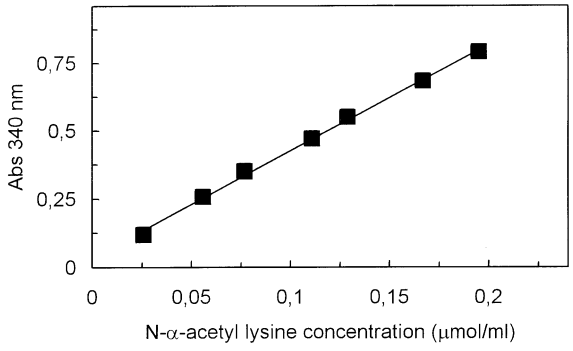

Fig. 3. *N*-α-acetyl-lysine standard curve. The relationship between the *N*-α-acetyl-lysine concentration and the increase in absorbency at 340 nm can be expressed by the following equation: $y = 3.9253x + 0.0337$; $R = 0.9977$.

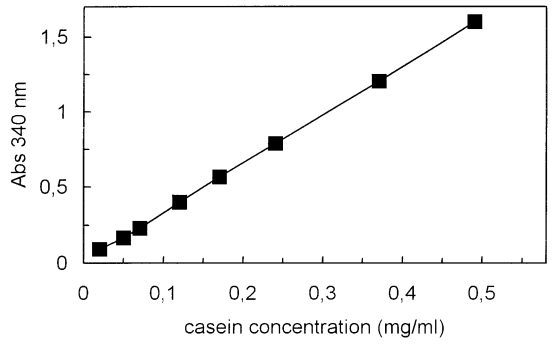

Fig. 2. Relationship between absorbency value at 340 nm (Abs 340 nm) and casein concentration (mg/ml). The relationship is described by the following equation: $3.2292x + 0.0142$; $R = 0.9998$.

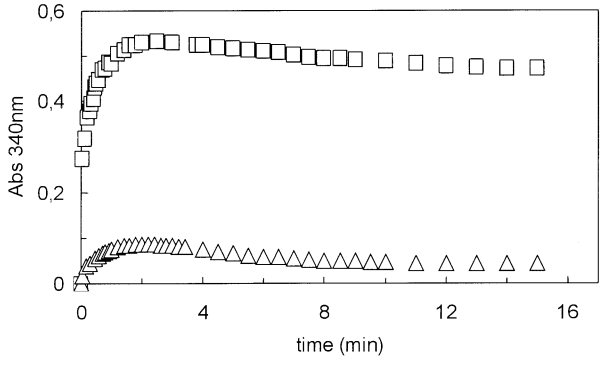

Fig. 4. Absorbency of the adducts formed by OPA reactive groups of the reaction mix (□) and of the supernatant (△) at consecutive reaction times. The data refer to a casein solution reacted with TGase for 2 h.

sample absorption slowly decreases, and a loss as equal as the 11% of the initial value is found after 15 min. A faster absorption decrease was found for the OPA-supernatant sample with a loss of 51% in 15 min. All the subsequent absorption measurements were performed after exactly 2 min of reaction between samples and OPA reagent.

At regular intervals during the TGase reaction, OPA reactive amino groups were measured in the reaction mixture (A_{Tot}) and in the supernatant (A_{Sn}; Fig. 5). The A_{Tot} value did not change significantly during the TGase reaction, whereas the A_{Sn} value slowly increased during the reaction time. Ammonia is released from the Lys in the reaction mixture just after the isopeptide bond formation has occurred. OPA also forms a fluorescent adduct even when it reacts with ammonia (Goyal, Rains, & Huffaker, 1988) and it was hypothesised that this complex absorbs also at an analytical wavelength (Panasiuk, Amarowicz, Kostyra, & Sijtsma, 1998). Since one ammonia molecule is released for each Lys residue used, it may be said that the total absorbency (A_{Tot}) is caused both by the free lysine residues in the casein as well as the ammonia that is released in the solution while the reaction is taking place; therefore the A_{Tot} value does not change. In the supernatant, on the other hand, proteins are not present and the A_{Sn} absorbency is due to the presence of ammonia alone which concentration raises as the TGase continues to react on the casein. By subtracting the A_{Sn} value from the A_{Tot} value, we can emphasise the TGase action on the casein in terms of a decrease in free primary amino groups.

The TGase activity on the casein was calculated by subtracting the residual amount of OPA reactive amino groups, after a few reaction times, from the amount of OPA reactive groups initially present on the casein, using the following formula:

$$\frac{[(A_{\mathrm{Tot}i} - A_{\mathrm{S}ni})/a] - [(A_{\mathrm{Tot}x} - A_{\mathrm{S}nx})/a]}{b} = \mu\text{mol boundLys/mg casein}$$

where: $A_{\mathrm{Tot}i}$ = value of the reaction mix absorbency at zero time; $A_{\mathrm{Sn}i}$ = value of the supernatant absorbency at zero time; $A_{\mathrm{Tot}x}$ = value of the reaction mix absorbency after a given time x of reaction; $A_{\mathrm{Sn}x}$ = value of the supernatant absorbency after a given time x of reaction; $a = 3.9253$ (A/μmol), the slope value of the *N*-α-acetyl-lysine reference curve; and b = amount of casein (mg) in the OPA reagent.

The data regarding the TGase activity on the casein at consecutive reaction times are recorded in Fig. 6 and shows that the enzymatic reaction reaches its plateau after 60 min with 0.126 μmol of Lys involved in the cross-links formation. This value represents 22% of the total Lys residues. This same figure also shows the TGase activity trend in the presence of the dipeptide Z–Gln–Gly as acyl group donor. In this case, when the reaction reaches its plateau, 0.205 μmol of Lys result as being involved in the formation of the isopeptide bond. This amount equals 33% of the total Lys residues. In both cases, Lys and Gln react in a sub-stoichiometric relationship; different factors are responsible for this phenomenon. As a matter of fact, not all residues of Lys and Gln which may be available for the TGase can actually be substrate for this enzyme, since the amino acid residues that surround them strongly influence their reactivity (Christensen, Sorensen, Hojrup, Torben, Petersen, & Rasmussen, 1996; Grootjans, Groenen, & de Jong, 1995). Furthermore, we need to consider the steric hindrance problems which are due to the dimensions of the protein substrate. In fact, only the Gln and Lys residues present in polypeptide regions that are closely adjacent, on the same, or on two different protein molecules can be cross-linked. When we add the Z–Gln–Gly to the reaction mix, steric hindrance is partly overcome thanks to the small size of this acyl group donor, and the amount of Lys involved in the cross-link formation increases.

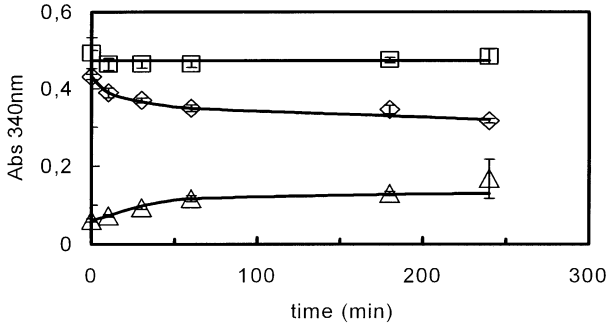

Fig. 5. Absorbency trend of the reaction mix (A_{Tot}, □), of the supernatant obtained after acid precipitation (A_{Sn}, △), and of the value A_{Tot}–A_{Sn} (◇) at consecutive reaction times. The data given are the average of at least 10 experiments in which casein solutions were used as TGase substrate.

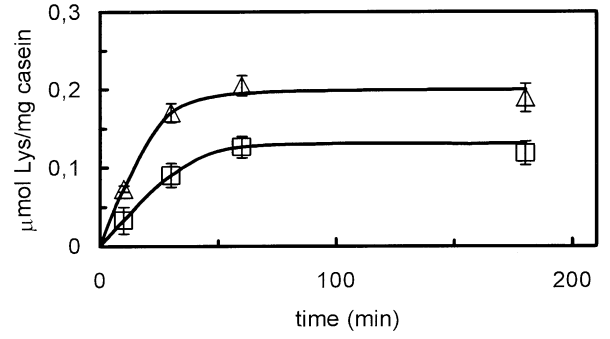

Fig. 6. Amount of lysine involved in the carboxyamid bond formation per mg of casein (μmol Lys/mg casein) at consecutive reaction times. The data given are the average of at least 10 experiments in which casein solutions (◆) and casein with Z–Gln–Gly (◇) were used as TGase substrate.

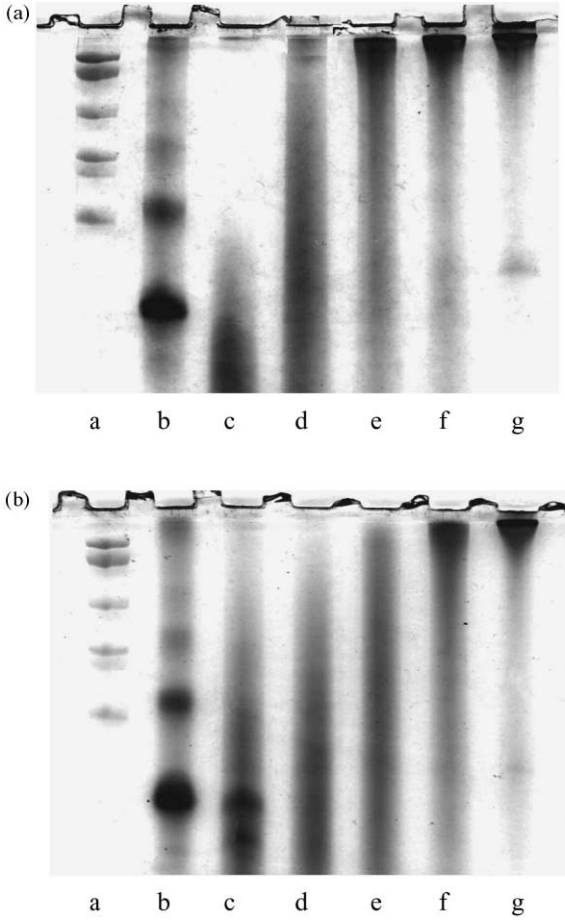

Fig. 7. (A) SDS PAGE on 5% acrylamide gel of reaction mixture TGase-casein at different incubation times. Lanes: (a) standard proteins (Pharmacia Biotech): myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic-dehydrogenase, 53 kDa; (b) casein solution; (c) reaction mix $t=0$; (d) reaction mix $t=10$ min; (e) reaction mix $t=30$ min; (f) reaction mix $t=60$ min; (g) reaction mix $t=240$ min. (B) SDS PAGE on 5% acrylamide gel of reaction mixture TGase-casein-Z–Gln–Gly at different incubation times. Lanes: (a) standard proteins (Pharmacia Biotech): myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; trasferrin, 76 kDa; glutamic-dehydrogenase, 53 kDa; (b) casein solution; (c) reaction mix $t=0$; (d) reaction mix $t=10$ min; (e) reaction mix $t=30$ min; (f) reaction mix $t=60$ min; (g) reaction mix $t=240$

The different nature of the TGase reaction products obtained either in the absence or in the presence of Z–Gln–Gly at consecutive incubation times, was studied through the electrophoretic analysis of these reaction mixes (Fig. 7A and B). When TGase has casein as an only substrate, proteic polymers of high molecular weight are quickly formed and are accumulated in the running-staking interface gel as the reaction time increases. After 30 min of reacting, proteic polymers with a molecular weight higher than 212 kDa are seen. When Z–Gln–Gly is also present in the reaction mix, these proteic polymers appear only after 1 h of reacting, since the likeliest pattern seems to be the formation of

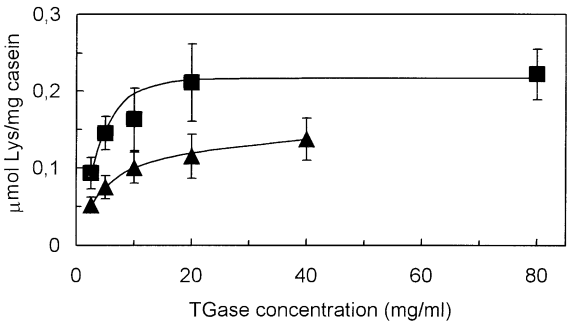

Fig. 8. Relationship between TGase activity (µmol Lys/ mg casein) and the enzyme concentration (TGase concentration, mg/ml). The recorded data are the average of five experiments in which different amounts of TGase were made to react for 30 min with solution of casein (▲) or casein and Z–Gln–Gly (■).

bonds casein Lys-dipeptide and *not* intermolecular casein cross-links.

The rate of enzyme catalysed reactions increases along with enzyme concentration if the substrate is not a limiting factor, that is to say it is present in a greater amount compared to the enzyme (saturating conditions). In non saturating conditions the rate of the product formation reaches its highest value and is no longer dependent on enzyme concentration. In order to check the dependency of the TGase activity on the enzyme concentration using the spectrophotometric method suggested, a few experiments have been conducted by incubating different amount of TGase with solutions of casein alone or casein and Z–Gln–Gly mix. The results recorded in Fig. 8 show that the product formation, given in terms of µmol of bounded Lys per mg of casein, depends on TGase concentration following the typical trend of enzyme-catalysed reactions. The amount of µmol of lysine involved in the isopeptide bond formation increases as the TGase concentration raises and tends to the plateau. Furthermore, the amount of bounded Lys is always higher in that reaction mix which contains Z–Gln–Gly and confirms that the reactivity of Lys is higher in the presence of the dipeptide.

The mean coefficient of variation for the OPA suggested method was calculated as 4.6%, based on data of 10 replicates of the sample TGase—casein reacted for 30 min. Therefore, this method results are reproducible for measuring the Lys residues decreasing caused by TGase activity.

4. Conclusion

Casein molecules modification via TGase shows great promise for practical application in the food industry and increasing interest is related to the development of simple and practical assays for measuring its activity. TGase action on proteic substrates leads to the production of isopeptide bonds and ammonia. Previous methods

proposed for determination of these reaction products must satisfy several criteria and require quite complicate procedures. Herein a simple spectrophotometric assay was proposed for following the progress of this enzymatic reaction on casein on the basis of the reduction of the free Lys residues. Amongst the methods available for free amino groups detection that one based on OPA reagent is reported to be one of the most advantageous because of the easiness of procedure, the rapidity and the small sample amounts required (Wallace & Fox, 1998). Moreover, the spectrophotometric detection of OPA adduct allows to avoid the effect of the fluorescence quenching from peptide bonds (Church et al., 1983) and can be performed with laboratory equipment readily available. This proposed method seems to be suitable for the routine analysis of TGase activity and its application to other food proteins can be envisaged.

References

Bercovici, D., Gaertner, H. F., & Puigserver, A. J. (1987). Transglutaminase-catalyzed incorporation of lysine oligomers into casein. *Journal of Agricultural and Food Chemistry*, *35*, 301–304.

Christensen, B. M., Sorensen, E. S., Hojrup, P., Torben, E., Petersen, T. E., & Rasmussen, L. K. (1996). Localization of potential transglutaminase cross-linking sites in bovine caseins. *Journal of Agricultural and Food Chemistry*, *44*, 1943–1947.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science*, *66*, 1219–1227.

Day, N., & Keillor, J. W. (1999). A continuos spectrophotometric linked enzyme assay for trasglutaminase activity. *Analytic Biochemistry*, *274*, 141–144.

De Baker-Royer, C., Traorè, F., & Meunier, J. C. (1992). Purification and properties of factor XIII From human placenta. *Journal of Biochemistry*, *24*, 91–97.

Dickinson, E., & Yamamoto, Y. (1996). Rhealogy of milk protein-gels and protein-stabilized emulsion gels cross-linked with transglutaminase. *Journal of Agricultural and Food Chemistry*, *44*, 1371–1377.

Faergemand, M., Murray, B. S., & Dickinson, E. (1997). Cross-linking of milk proteins with transglutaminase at the oil–water interface. *Journal of Agricultural and Food Chemistry*, *45*, 2514–2519.

Faergemand, M., & Qvist, K. B. (1997). Transglutaminase: effect on rheological properties, microstructure and permeability of set style acid skim milk gel. *Food Hydrocolloids*, *11*(3), 2887–2892.

Folk, J. E., & Chung, S. I. (1985). Transglutaminase. In A. Meister (Ed.), *Glutamate, glutamine, glutathione, and related compounds, methods in enzymology,* Vol.113 (pp. 359–375). New York: Academic Press.

Goodno, C. C., Swaisgood, H. E., & Catignani, G. L. (1981). A fluorimetric assay for available lysine in proteins. *Analytic Biochemistry*, *115*, 203–211.

Goyal, S. S., Rains, D. W., & Huffaker, R. C. (1988). Determination of ammonium ion by fluorimetry or spectrometry after on-line derivatization with *o*-phthalaldehyde. *Analytic Chemistry*, *60*, 175–179.

Grootjans, J. J., Groenen, P. J. T. A., & Jong, W. (1995). Substrate requirements for transglutaminases. Influence of the amine acid residue preceding the amine donor lysine in a native protein. *Journal of Biological Chemistry*, *270*, 22855–22858.

Kurth, L., & Rogers, P. J. (1984). Transglutaminase catalyzed cross-linking of myosin to soya protein, casein and gluten. *Journal of Food Science*, *49*, 573–577.

Mauron, J. (1977). General principles involved in measuring specific damage of food components during thermal processes. In T. Hoyem, & O. Kvale (Eds.), *Physical, chemical and biological changes in food caused by thermal processing,* Chapter 23 (pp. 328–359). London: Applied Science Publisher Limited.

Miller, M. L., & Johnson, G. V. W. (1999). Rapid, single-step procedure for the identification of transglutaminase-mediated isopeptide crosslinks in amino acid digests. *Journal of Chromatography B*, *732*, 65–72.

Mycek, M. J., & Waelsch, H. (1960). The enzymatic deamidation of proteins. *Journal of Biological Chemistry*, *235*(12), 3513–3517.

Nielsen, P. M. (1995). Reactions and potential industrial applications of transglutaminase. Review of Literature and Patents. *Food Biotechnology*, *9*(3), 119–156.

Nonaka, M., Matsuura, Y., & Motoki, M. (1996). Incorporation of lysine- and lysine dipeptides into α_{s1}- casein by Ca^{2+}-independent microbial transglutaminase. *Bioscience Biotechnology and Biochemistry*, *60*, 131–133.

Nonaka, M., Sakamoto, H., Toiguchi, S., Kawajiri, H., Soeda, T., & Motoki, M. (1992). Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. *Journal of Food Science*, *57*, 1214–1218.

Nonaka, M., Sawa, A., Matsuura, Y., Motoki, M., & Nio, N. (1996). Deamidation of several food proteins using free and immobilized Ca^{2+}-independent microbial transglutaminase. *Bioscience Biotechnology Biochemistry*, *60*, 532–533.

Oh, S., Catignani, L., & Swaisgood, H. E. (1993). Characteristics of an immobilized form of transglutaminase: a possible increase in substrate specificity by selective interaction with a protein spacer. *Journal of Agricultural and Food Chemistry*, *41*, 1337–1342.

Panasiuk, R., Amarowicz, R., Kostyra, H., & Sjjtsma, L. (1998). Determination of α-amino nitrogen in pea protein hydrolysates: a comparison of three analytical methods. *Analytical Food Chemistry*, *62*, 363–367.

Porter, D. H., Swaisgood, H. E., & Catignani, G. L. (1982). A rapid fluorimetric assay for measurement of peptidase activity. *Analytical Biochemistry*, *123*, 41–45.

Schágger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, *166*, 379–386.

Siefring, G. E., Apostel, A. B., Velasco, P. T., & Lorand, L. (1978). Enzymatic basis for the Ca^{++}-induced cross-linking of membrane proteins in intact human erythrocytes. *Biochemistry*, *17*, 2598–2601.

Svedas, V. J. K., Galaev, I. J., Borisov, I. L., & Berezin, I. V. (1980). The interaction of amino acids with *o*-phthaldialdehyde: a kinetic study and spectrophotometric assay of the reaction product. *Analytical Biochemistry*, 101–188.

Vigo, M. S., Malec, L. S., Gomez, R. G., & Llosa, R. A. (1992). Spectrophotometric assay using *o*-phthaldialdehyde for determination of reactive lysine in dairy products. *Journal of Agricultural & Food Chemistry*, *44*, 363–365.

Wallace, J. M., & Fox, P. F. (1998). Rapid spectophotometric and fluorimetric methods for monitoring nitrogenous compounds in cheese and cheese fractions: a review. *Food Chemistry*, *62*(2), 217–224.